MAGNESIUM ENRICHMENT OF SKIM MILK

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Abstract

Effective magnesium enrichment of dairy products depends on the amount of magnesium salt added and the nature of its distribution between the serum and micellar phases. Thus, this study firstly aimed to profile the magnesium distribution in skim milk as a function of pH (pH 5.50 to 7.20), preheating temperatures (20 to 80 ± 1 ^{o}C) and concentration of added magnesium chloride (0 to 40 mmol L^{-1}). The second aim was to investigate the rheological properties of magnesium-induced skim milk gels as a function of different concentrations of magnesium chloride (0 to 20 mmol L^{-1}) added to heated skim milk.

The total concentration of magnesium in skim milk and serum was determined using a complexometric titration (EDTA titration) and the EDTA results were validated by atomic absorption spectroscopy (AAS). The measurement of ionic magnesium (Mg^{2+}) in milk is of importance for understanding the bioavailability of magnesium-enriched dairy products. Hence, the concentration of ionic magnesium (Mg^{2+}) in the serum phase was measured using a novel magnesium fluorescence dye (Magnesium 510 probe). In all the samples, a reduction in the pH increased the total soluble magnesium and ionic magnesium (Mg^{2+}) concentrations in the serum phase, regardless of whether magnesium chloride (15 mmol L^{-1}) was added or not to skim milk at 20 ± 1 ^{o}C. At pH 5.50, more than 92% magnesium was found in the serum phase for both added (15 mmol L^{-1}) and no added magnesium chloride samples. The concentration of magnesium in the serum phase remained unchanged as the preheating temperature was increased from 20 to 80 ± 1^{o}C. The addition of magnesium chloride to skim milk reduced the milk pH and increased the ionic magnesium (Mg^{2+}) and total soluble magnesium concentration in the serum phase at 20 ± 1 ^{o}C. The pH values are important for new magnesium-enriched dairy products as the distribution of added magnesium chloride in the serum phase was different dependent on pH adjustment between the pH 6.50 and natural pH 6.70.

Rheological measurements using cone and plate geometry at constant strain showed that the addition of 5 mmol L^{-1} magnesium chloride induced the gelation of skim milk after 22 min of heating at 80 ^{o}C in the rheometer. The time and temperature for reaching the gelation in skim milk depended on preheating and concentration of added magnesium chloride. A higher concentration of added magnesium chloride achieved gelation at a lower temperature in the rheometer. With the magnesium-induced gels, *G*' values obtained were found to increase

with increasing concentrations of added magnesium chloride (0 to 20 mmol L^{-1}), increasing holding times (10 to 60 min) and increasing heating temperatures (70 to 80 ^{o}C). Compared with samples with 5 mmol L^{-1} magnesium chloride, those with 15 mmol L^{-1} magnesium chloride showed a more rapid increase in *G'*. The *G'* value obtained with 15 mmol L^{-1} magnesium chloride was 15.01 Pa at 80 ^{o}C during 10 min of holding and cooling to 20 ^{o}C rapidly increased the final *G*' value to 81.44 Pa. In contrast, the addition of 5 mmol L^{-1} magnesium chloride resulted in the formation of a weak gel with a final *G'* value of 6.87 Pa after cooling to 20 ^{o}C. The oscillation stress of milk gels also increased with increasing magnesium chloride concentration, heating temperatures and holding times in the rheometer. Preheating significantly ($P < 0.05$) affected the strength of magnesium-induced skim milk gels. The addition of 20 mmol L^{-1} magnesium chloride to preheated skim milk followed by heating at 85 ^{o}C then cooling to 20 ^{o}C formed strong skim milk gels. In parallel, samples with no added magnesium chloride did not undergo gelation. In conclusion, the distribution of magnesium was influenced by pH and magnesium concentration added and the strength of magnesium-induced gels was influenced by magnesium concentration added, preheating and the heating time and temperature in the rheometer. The technology of making magnesium-induced skim milk gels can be exploited commercially for the formation of non-fermented dairy products supplemented with magnesium.

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Conference poster presentation

1. **N. Begum**, H. E. Oh, M. Wong, *Effect of pH on Magnesium distribution in skim milk*, NZIFST conference, 3-5th July 2018, Hamilton, New Zealand.
2. H. E. Oh, **N. Begum**, M. Wong, *Magnesium-induced gelation of milk*, 3rd Food Structure and Functionality Forum Symposium and 3rd IDF Symposium on Microstructure of Dairy Products, 3–6 June 2018, Montreal, Canada (Ind. SCOPUS).

Symbols and Abbreviations

AAS	Atomic Absorption Spectroscopy
CCP	Colloidal Calcium Phosphate
Ca^{2+}	Ionic Calcium
DMT	Donnan Membrane Technique
EDTA	Ethylene Diamine Tetra Acetic acid
EDXRF	Energy Dispersive X-ray Fluorescence
FESEM	Field Emission Scanning Electron Microscope
GDL	Glucono-δ-lactone
IC	Ion Chromatography
ICP-AES	Inductively Coupled Plasma Atomic Emission Spectrometry
ICP-OES	Inductively Coupled Plasma Optical Emission Spectrometry
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
IOM	Institute of Medicine
ISE	Ion-selective Electrode
$MgCl_2$	Magnesium Chloride
MCP	Micellar Calcium Phosphate
Mg^{2+}	Ionic Magnesium
MSNF	Milk Solid Non-Fat
RDI	Reference Daily Intake
SAOS	Small Amplitude Oscillatory Shear
USDA	United States Department of Agriculture
WPNI	Whey Protein Nitrogen Index

Chapter 1

Introduction

1.1 Introduction

Magnesium plays a vital role in many biochemical processes (i.e. blood glucose control, nerve and muscle function, protein synthesis and blood pressure regulation) in humans (Zhang, Xun, Wang, Mao, & He, 2017; Kao et al., 1999). Bovine milk and dairy products constitute one of the main sources of dietary magnesium for humans (Cashman, 2011). Magnesium deficiency can cause a variety of problems, including gastrointestinal and renal losses, hypocalcaemia, cardiac and neurological disorders (Swaminathan, 2003). Despite its well-established nutritional value, magnesium is often reported as an under-consumed nutrient (Oh & Deeth, 2017). Thus, there is an opportunity to develop milk and dairy products as efficient vehicles for the delivery of supplementary dietary magnesium to overcome any problems of magnesium deficiency.

Mineral enrichment in milk is challenging because the application of almost any treatment is likely to change the properties of milk (Lewis, 2011). The addition of cations and heat treatment can destabilise the structure of casein micelles. The addition of magnesium salts to skim milk increases the magnesium concentration in milk which leads to a decrease in the pH, an increase in the ionic strength and can thus induce gelation (O'Mahony & Fox, 2013). Hence, understanding the magnesium-casein relationship in milk is important for developing magnesium-enriched dairy products.

Profiling total magnesium and ionic magnesium (Mg^{2+}) distribution between the micellar and serum phases is also important for designing magnesium-enriched dairy products and to understand the bioavailability of the products. Ionic magnesium (Mg^{2+}) is considered as the biologically active form, hence there is a considerable interest in its determination in milk and dairy products (Zhang, 2011).

1.2 Hypothesis

The hypotheses of this study are as follows:

- pH, preheating temperature and addition of different concentrations of magnesium chloride affect the distribution of magnesium between the serum and micellar phases of skim milk.
- Different heating temperatures (70 to 85 ^{o}C), in the rheometer, and different concentrations of magnesium chloride addition to skim milk affect the gelation properties of magnesium-induced milk gels.

1.3 Aim and objectives

The primary aim of this project was to profile the magnesium distribution in the micellar and serum phases of skim milk using simple, reliable and accurate methods and to understand the magnesium-protein relationship using a rheological approach. Based on this aim, the specific objectives of this study were:

1. To determine the total magnesium in skim milk and in the serum phase at different pH values, different preheating temperatures and at different concentrations of added magnesium chloride using the EDTA titration method and to validate the titration results using atomic absorption spectroscopy (AAS) technique;
2. To determine the content of ionic magnesium (Mg^{2+}) using a magnesium fluorescence dye (Magnesium 510 probe); and
3. To investigate the heat instability of milk and examine the gelation properties of magnesium-induced milk gels using a rheological approach.

Chapter 2

Literature Review

2.1 General introduction

Milk is a vital food in the human diet as it provides all the necessary nutrients such as proteins, carbohydrates, minerals and vitamins (Cashman, 2011). Minerals in milk are essential for the normal growth and development of infant and young children. Both calcium and magnesium are of critical importance for the growing infant (Luo et al., 2010). The mineral element's chemical form is important because it determines their absorption in the intestine and biological application (Greger, Smith, & Snedeker, 1981). Thus, the knowledge of the minerals present in milk samples and dairy products is of interest.

Milk minerals appear to be complex, dynamic and have strong interactions with proteins during processing of dairy products (de la Fuente, 1998). The technological properties and nutritional value of milk are both influenced by its mineral salts composition and distribution (McSweeney & Fox, 2009). Depending on the type of mineral ion, they are present in the serum phase or partially associated with casein molecules which form casein micelles (McMahon & Brown, 1984). Heating, cooling, acidification and the addition of mineral salts modify the physiochemical properties of milk and eventually, the salt equilibria is altered (Gaucheron, 2011). The distribution of salts especially calcium and magnesium between the serum and micellar phases has a great influence on the structure and stability of the casein micelle (De Kruif, Huppertz, Urban, & Petukhov, 2012; Holt, Carver, Ecroyd, & Thorn, 2013).

Ionic magnesium (Mg^{2+}) plays an essential role in dairy processing (Bijl, Van Valenberg, Huppertz, & Van Hooijdonk, 2013). Ionic magnesium (Mg^{2+}) is considered to be the biologically active form (Zhang, 2011). For fundamental research on the importance of magnesium in milk and for the development of dairy products enriched with magnesium, it is important to determine the milk mineral composition in terms of total and ionic magnesium (Mg^{2+}), its distribution behaviour between the serum and micellar phases and its association and interaction behavior with milk proteins.

Therefore, this literature review will cover dietary magnesium, milk composition, magnesium in bovine milk and its equilibria, factors that affect milk gelation and quantification methods of magnesium in milk.

2.2 Dietary magnesium

Magnesium is the fifth most abundant mineral in the human body that plays a major role in many biochemical processes (Zhang et al., 2017). It helps to maintain normal nerve and muscle function, supports a healthy immune system, keeps the heartbeat steady and helps bones remain strong (Kao et al., 1999). It also helps to regulate blood glucose levels and aids in the production of energy (Zhang et al., 2017). Deficiency of magnesium leads to nerve and muscle problems (such as muscle twitches, irritability and muscle weakness), low blood pressure, nausea, vomiting, diarrhea, dizziness and slurred speech. A number of chronic diseases, such as diabetics, hypertension, coronary heart disease and osteoporosis, have also been related to chronic low magnesium (Glasdam, Glasdam, & Peters, 2016).

Magnesium deficiency is a common problem with a prevalence of 10% hospitalised patients (Swaminathan, 2003). According to the USDA (2009), 57% of the US population may have an inadequate intake of magnesium. Based on RDI, the daily intake recommendation for magnesium is 320 to 420 mg for an adult (IOM, 1997). Though magnesium in milk and dairy products is one of the main sources of dietary magnesium, it contains about one third of the daily requirement based on one litre of milk (Oh & Deeth, 2017). Therefore, milk and dairy products can be used as a magnesium supplementation vehicle in order to overcome magnesium deficiency.

2.3 Constituents of bovine milk

The major components of bovine milk are water, milk fat and milk solid non-fat (MSNF), consisting of protein, lactose and minerals (Chandan, 1997). The gross composition of milk (w/w) is shown in Figure 2.1. The major constituents of milk vary more widely in individual cow's milk than in pooled market milk (Young, Hillers, & Freeman, 1986). Factors affecting the milk, such as breed of cow, intervals of milking, stages of milking, lactation period, season, feed, nutritional level, environmental temperature, health status, age, weather and gestation period, are known to cause variations in fat, protein, lactose and mineral levels in milk derived from individual cows (Jenness, Wong, Marth, & Keeney, 1988).

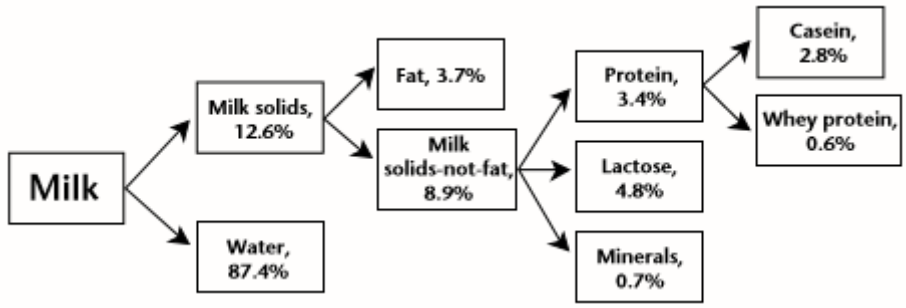

Figure 2.1. Gross composition of milk, showing major constituents (Chandan, 1997).

2.3.1 Mineral composition in bovine milk

Most of the minerals found in milk are considered essential for human nutrition (Zamberlin, Antunac, Havranek, & Samaržija, 2012). The typical composition of milk minerals is shown in Table 2.1. The major milk minerals are calcium, magnesium, sodium and potassium (Chandan, 1997).

Table 2.1: Mineral composition of bovine milk (Chandan, 1997).

Major mineral constituents	Concentration (mg mL^{-1} of whole milk)	Concentration range (mg mL^{-1})	Trace mineral constituents	Concentration range (µg mL^{-1} of whole milk)
Calcium	1.23	1.10 – 1.30	Iron	0.30 – 0.60
Magnesium	0.12	0.09 – 0.14	Zinc	2.00 – 6.00
Phosphorus (total)	0.95	0.90 – 1.00	Copper	0.10 – 0.60
Phosphorus (inorganic)	0.75	0.70 – 0.80	Manganese	0.02 – 0.05
Sodium	0.58	0.35 – 0.90	Iodine	0.02 – 0.06
Potassium	1.41	1.10 – 1.70	Fluoride	0.03 – 0.22
Chloride	0.99	0.90 – 1.10	Selenium	0 – 0.067
Sulphate	0.10	-	Cobalt	0 – 0.001
			Chromium	0.008 – 0.013
			Molybdenum	0.02 – 0.12
			Nickel	0 – 0.005
			Silicon	0.75 – 7.00
			Vanadium	0 – 0.03

2.3.2 Protein in bovine milk

Normal bovine milk contains 30 to 36 g L^{-1} of protein. Approximately 80% of milk proteins are classified as casein and the remaining are whey protein. Casein is further divided into α_{s1}, α_{s2}, β and Ƙ-casein fractions which represent approximately 37- 40%, 10%, 35% and 12 - 15% (w/w) of the total casein, respectively (Anema & Li, 2003). In contrast, the whey proteins are present in the serum phase as 20% of the total protein. The two main components of serum protein are α-lactalbumin and β-lactoglobulin and the rest are serum albumin, immunoglobulins, protease-peptones and a trace amount of enzymes (Walstra, 1999).

Different caseins have different amino sequences, different charge distribution and different tendencies to aggregate in the presence of divalent cations (Morr, 1985; Schein, 1990). All the caseins are phosphorylated and have a good capacity to bind calcium phosphate (Broyard & Gaucheron, 2015). The casein molecules can associate to form casein micelles by protein-protein interaction (hydrophobic, hydrogen and electrostatic binding) and by protein-mineral interactions (in the presence of calcium and phosphate) (Dalgleish & Corredig, 2012; Holt et al., 2013; Walstra, Geurts, & Wouters, 2005). The casein micelle contains on a dry weight basis 93% protein and 7% inorganic constituents. The main constituent of inorganic matter is calcium phosphate. Calcium phosphate exists in a colloidal form in the micelles bound to casein protein with magnesium, zinc and citrate, collectively referred to as colloidal calcium phosphate (CCP) (Aoki, Kawahara, Kako, & Imamura, 1987; Cashman, 2011; Oh & Deeth, 2017; Silva, Lopes, Nóbrega, Souza, & Nogueira, 2001). The close association of CCP with casein protects it from precipitation in the milk matrix (Walstra, 1999).

2.4 Casein micelle structure

Casein in milk exists as colloidal particles known as micelles, which are dispersed, irregular in shape and have a natural colloidal sponge-like structure (De Kruif et al., 2012) as shown in Figure 2.2. Casein micelles have an average diameter of 150 nm and a zeta potential about -20 mV. Each casein micelle contains a few hundred sub micelles (Phadungath, 2005).

Figure 2.2. Micrographs of an individual casein micelle captured by field emission scanning electron microscope (FESEM) (Dalgleish, Spagnuolo, & Goff, 2004).

The nature and structure of casein micelles have been extensively studied and different models have been proposed but due to the complex structure of casein micelles, the exact structure of casein micelles is still under research. Based on a variety of proposed models, the structures are described as (i) the sub-micelle model where the micelle is a cluster of submicelles connected with nanoclusters of calcium phosphate (Schmidt, 1980; Walstra, 1999) (Figure 2.3a). Walstra (1999) proposed a hybrid sub-micelle model and concluded that CCP is no longer placed at the periphery of the sub-micelles but within the sub-micelles (Figure 2.3b). The CCP then glues together the protein sub-micelles. (ii) the dual binding model (Horne, 2003), where caseins self-assemble to build micellar structures (Figure 2.3c). (iii) the nanocluster model is where it is proposed that CCP is dispersed as small 'cherry stones' in a homogeneous protein matrix (Figure 2.3d) (De Kruif & Holt, 2003). The nanocluster model is based on interactions between casein and calcium phosphate to hold the micelle together. κ-casein interacts weakly with CCP and thus remains on the surface of the micelle.

After the development of electron microscopy, x-ray and neutron scattering techniques, in recent years the researchers have suggested that the internal micelle structure is quite uniform with CCP nanoclusters rather than sub-micelles. More recent studies suggest that the casein micelle is composed of a highly hydrated sponge-like supramolecular structure where the casein molecule forms an interlocked lattice which is associated with calcium phosphate nanoclusters (Dalgleish & Corredig, 2012; McMahon & Oommen, 2008) (Figure 2.3e). Moreover, Dalgleish (2011) proposed a model where casein dense regions interact with nanoclusters of calcium phosphate (Figure 2.3f).

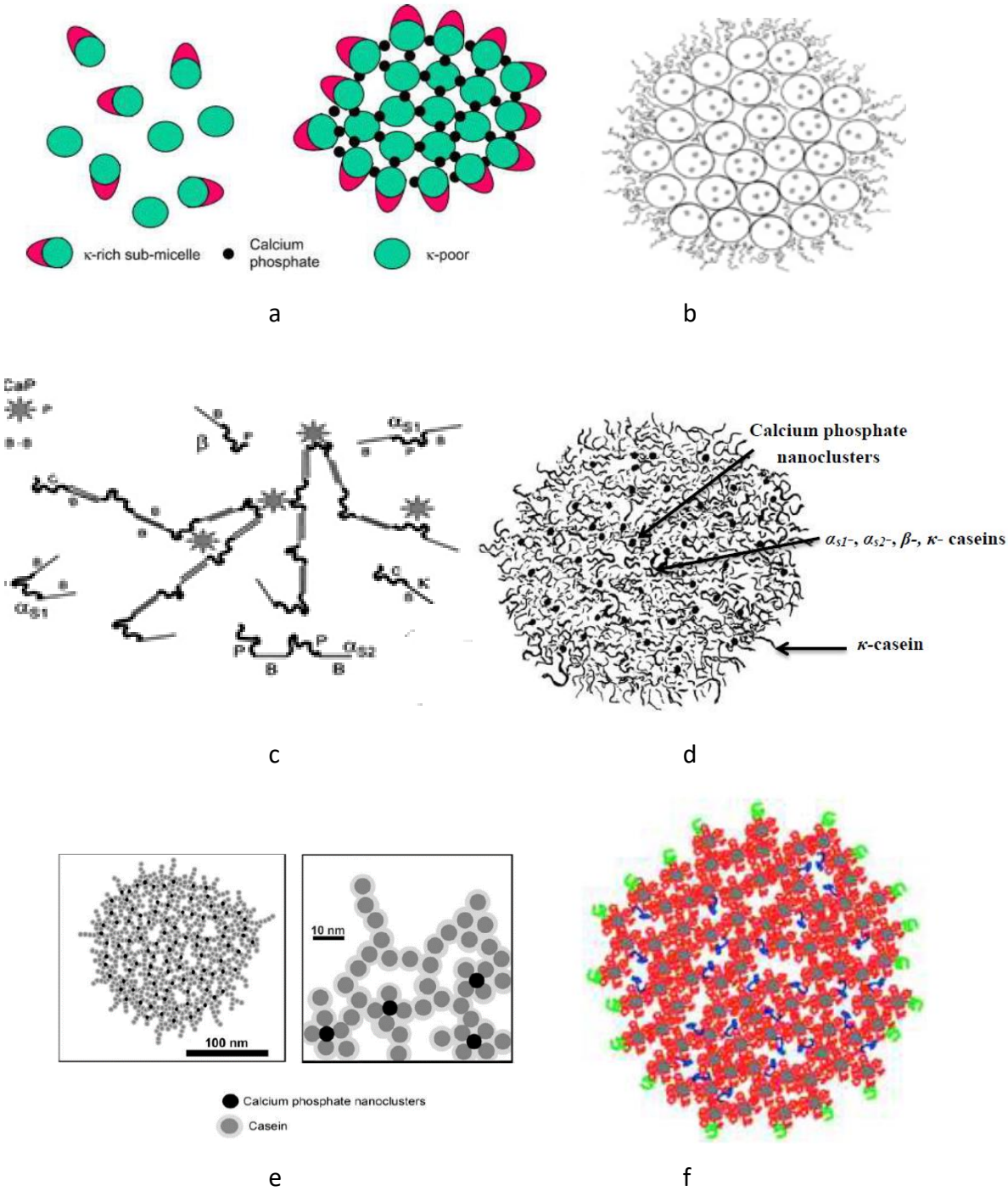

Figure 2.3. Various proposed models for the structure of casein micelle. Submicelle model of the casein micelle (a) (Schmidt, 1980) and (b) (Walstra, 1999). Dual binding model of the casein micelle (c) (Horne, 2003) and nanocluster model of the casein micelle (d) (De Kruif & Holt, 2003). The model proposed by McMahon and Oommen (2008) (e) and Dalgleish (2011) (f).

All the models confirm that κ-caseins are located at the surface of casein micelle, conferring a negative charge on the surface and stability in solution due to electrostatic repulsion and steric hindrance (Broyard & Gaucheron, 2015). In summary, in all the proposed models, the calcium phosphate nanocluster model is likely to be the most appropriate model based on electron microscopic and scattering experiments reported in the literature.

2.5 Mineral and protein interaction

The mineral fraction is a small fraction of the milk compared to other components like protein, lactose and fat. Minerals are present in concentrations ranging between 8 - 9 g L^{-1}, but their presence plays an important role in the structural organisation of casein micelles (Guetouache, Guessas, & Medjekal, 2014) and nutritional value (Bijl et al., 2013).

The majority of research and published literature on mineral interactions in milk is related to the presence of calcium. All the caseins, except κ-casein, are sensitive to calcium and tend to precipitate at the calcium concentrations present in milk (~1.23 mg mL^{-1}). Whereas, κ-casein is insensitive to calcium and κ-casein would stabilise other caseins against precipitation if present in adequate quantity (Walstra & Jenness, 1984). κ-casein is located at the exterior of the casein micelle and other calcium sensitive caseins are located in the interior of the micelle. κ-casein sticks out of the micelle and forms a hairy brush at the surface. The integrity of the casein micelle depends on cation interaction with proteins either in its colloidal form or by binding directly to casein. If the mineral is in a colloidal form it will become an integral part of the phosphate nanoclusters holding the casein micelles together (Holt et al., 1989).

2.6 Mineral equilibria in bovine milk

The mineral concentrations in the serum and micellar phases depend on multiple ion equilibria (Holt, Dalgleish, & Jenness, 1981). Table 2.2 presents the salt equilibria in bovine milk (Fox, Uniacke-Lowe, McSweeney, & O'Mahony, 2015).

Table 2.2: Distribution and form of salts in bovine milk (Fox et al., 2015).

Species	Concentration mg/l	Soluble %	Form	Colloidal %
Sodium	500	92	Completely ionized	8
Potassium	1,450	92	Completely ionized	8
Chloride	1,200	100	Completely ionized	
Sulphate	100	100	Completely ionized	
Phosphate	750	43	10 % bound to Ca and Mg	57
			51 % H_2PO^-	
			39 % HPO_4^{2-}	
Citrate	1,750	94	85 % bound to Ca and Mg	
			14 % $Citr^{3-}$	
			1 % $HCitr^{2-}$	
Calcium	1,200	34	35 % Ca^{2+}	66
			55 % bound to citrate	
			10 % bound to phosphate	
Magnesium	130	67	Similar to calcium	33

All the macro-elements (chlorides, phosphates and citrates of sodium, calcium and magnesium) in milk are distributed differently into diffusible (serum phase) and non-diffusible fractions (essentially casein micelles and micellar phase). In the micellar phase, they are bound to colloidal calcium phosphate or phosphoserine residues. In the serum phase, they are present as a soluble salts of citrate and phosphate or as free ions. Monovalent ions, such as sodium (Na^+), potassium (K^+) and chloride (Cl^-) are essentially diffusible and present in milk as free ions or in the ionic form (Gao, 2010). About one third of the calcium, half of the inorganic phosphate (phosphate which is associated to micellar calcium phosphate, calcium and free), two thirds of the magnesium and over 90% of citrate are present in the serum phase of milk (de la Fuente, Olano, & Juarez, 1997). However, the ionic species, such as calcium (Ca^{2+}), magnesium (Mg^{2+}), zinc (Zn^{2+}), iron (Fe^{3+}), carbonate (CO_3^{2-}), citrate ($C_6H_5O_7^{3-}$) and phosphate (PO_4^{3-}) ions, are distributed among the structural compartments of milk in a highly specific way, generating a complex chemical equilibrium (Crowley, Kelly, & O'Mahony, 2014). These cations exist in both micellar and serum phases, depending on the pH of the milk (Gao et al., 2009). Figure 2.4 shows the calcium equilibria between the serum and micellar phases of cows' milk.

Figure 2.4. Phosphate calcium equilibria in the presence of citrate ions between the serum and micellar phases in cows' milk at pH 6.75 (Gaucheron, 2011).

The mineral fraction in milk is differently distributed in the serum and micellar phases (Mekmene, Le Graët, & Gaucheron, 2009). In certain conditions, the protein and ions can shift from the micellar phase and move to the serum phase (Singh, 2004). Under different physicochemical conditions, the exchange of minerals, casein and water that can occur is presented in Figure 2.5.

Figure 2.5. Distribution of minerals, water and casein molecule as a function of different physicochemical conditions (Broyard & Gaucheron, 2015).

2.6.1 How the physicochemical condition in milk affects the mineral equilibria

The ion and mineral equilibria between the serum and micellar phases depend on various physicochemical conditions such as pH (acidification or alkalisation), thermal treatment (heating or cooling) and additions of different salts (De Kruif & Holt, 2003).

2.6.1.1 Acidification

Acidification is a common step in the processing of different dairy products such as cheese, fermented milk, yogurt and acid casein. Casein molecules are either precipitated by fast acidification or form a gel with slow acidification. During acidification when the pH decreases, different acid-basic groups (organic, inorganic phosphate, citrate and carboxylic residues) in milk bind to the proton added. This binding between the proton and the acid-basic groups depends on their pKa values. The affinities of the acid-basic groups to proton have been found to be higher than that of present calcium ions (Gaucheron, 2011). The anions associated with protons remain in the serum phase and this results in the increase of ionic strength in the serum phase.

Decreasing the pH lowers the stability of the casein micelle. During acidification the micellar calcium phosphate (MCP) is solubilised, κ-casein micellar tails are collapsed and therefore casein is released into the serum phase (Vasbinder & De Kruif, 2003). During acidification to pH 5.20, the calcium and inorganic phosphate are transferred into the serum phase, a subsequent decrease of pH to 3.50 leads to complete solubilisation of micellar calcium phosphate (Gaucheron, 2005). The aggregation of casein micelles occurs due to complex hydrophobic interactions, van der Waals and calcium-bridging (Lucey, Van Vliet, Grolle, Geurts, & Walstra, 1997).

2.6.1.2 Alkalisation

Alkalisation is not a common practice for the processing of milk in the dairy area. However, to understand the behavior of milk salt equilibria at high pH some research has been conducted with alkalisation (Anema & Li, 2003; Ozcan, Horne, & Lucey, 2015). It was observed that alkalisation of milk increases its viscosity and decreases its whiteness at pH 8.00 (Ozcan et al., 2015). The stability and structure of the casein micelle and salts fraction are changed due to the increase in pH. The pH increase induces modifications to the state of ionisation of inorganic phosphate present in the aqueous phase and of amino groups of proteins. The proposed mechanism for this change can be explained, as the pH increases, this induces the

modification of the ionisation state of the salts and amino groups of proteins. HPO_4^{2-} is changed to PO_4^{3-} which has a higher affinity towards calcium compared to HPO_4^{2-} and thus reacts with calcium (Jeantet, Croguennec, Schuck, & Brule, 2016). Consequently, the interaction between calcium and phosphoryl residues on the casein micelle shift the equilibrium to form PO_4^{3-} which reacts with calcium to form calcium phosphate salt. During alkalisation, the amount of calcium and inorganic phosphate in the serum phase was found to decrease, suggesting the transfer of salts to the micellar phase. As a consequence milk viscosity is increased and whiteness is decreased (Gaucheron, 2011).

2.6.1.3 Addition of salts

It is well established that the addition of salts to milk results in a decrease of the lightness of colour and an increase of viscosity due to increase in pH of milk (Gaucheron, 2010; Huppertz & Fox, 2006; Kaliappan & Lucey, 2011; Udabage, McKinnon, & Augustin, 2001). The micellar structure is lost with all of these physicochemical changes due to modifications to the salt equilibria (Philippe, Gaucheron, Le Graet, Michel, & Garem, 2003). For different added salts, the association constant influences their interaction with calcium and magnesium present in the milk (Vavrusova & Skibsted, 2014).

i) Addition of sodium chloride

Huppertz and Fox (2006) and Gualco (2010) have conducted research to understand the salt equilibria in milk after the addition of sodium chloride. Generally, NaCl changes the salt equilibria and as a result milk viscosity increases and the casein micelle becomes more hydrated (Huppertz & Fox, 2006). After NaCl addition of more than 0.10 mol L^{-1}, the pH of milk decreased (0.1 - 0.3 units) and the calcium concentration in the serum phase increased (Gaucheron, 2005). These changes occurred as a result of exchanges of divalent cations or protons which were attached directly to phosphoseryl residues of casein molecules by Na^+. Addition of NaCl increases the ionic strength which induces decrease in activity coefficients of the diffusible ions and consequently increases in the dissociation of the ion pairs. As a consequence of these exchanges, the hydration of casein micelles is increased (Gaucheron, 2011; Grufferty & Fox, 1985; Gualco, 2010).

ii) Addition of calcium salt

Calcium addition or enrichment is a common practice in the dairy sector to improve the nutritional status of the milk and to help solve some technological hurdles (e.g. sedimentation and mouthfeel) (Augustin & Williams, 2002; Ocak & Rajendram, 2013). Salt equilibria can alter differently depending on the nature of calcium salt added. If the added salts have very low solubility, then they do not affect the salt equilibria and casein micelle stability. If the added salts solubility is high like calcium chloride, the casein micelle physicochemical characteristics are modified strongly (Gaucheron, 2005). It was reported that after addition of solubilised calcium salt the casein micelle became less negative, hydration and heat stability were reduced, milk whiteness and turbidity increased, salt equilibria between the serum and micellar phases were altered (Huppertz, Fox, & Kelly, 2018). The added salt reacts with inorganic phosphate in the serum phase forming calcium phosphate by releasing protons which decreases the milk pH. As the serum phase is saturated in calcium phosphate salt, the newly formed calcium phosphate will shift towards the casein micelles (Gaucheron, 2011). The nature of the association of calcium salt with the casein micelle is not precisely understood. It was also reported that some part of added calcium (i.e. calcium chloride or calcium lactate) will remain in the serum phase as its ionic form and associated with citrate. Moreover, the counter ions of the added salt (chloride and lactate) will remain in the serum phase and increase the ionic strength. However, the intensity of this modification depends on the type and concentration of salt added and the subsequent change of pH after the addition of calcium salt.

iii) Addition of magnesium salt

It has long been known that added magnesium can cause casein precipitation (O'Mahony & Fox, 2013; Cuomo, Ceglie, & Lopez, 2011; Le Ray et al., 1998). Precipitation occurs more rapidly with calcium than with added magnesium (Oh & Deeth, 2017). Magnesium is naturally associated with protein in milk, added magnesium ions can interact with casein and induce gelation when milk is heated above 70 ^{o}C in much the same way as does calcium ions (Ramasubramanian, D'Arcy, Deeth, & Oh, 2014).

2.6.1.4 Effect of temperature

i) Heating

The common heat treatments in the dairy industry are pasteurisation, ultra-high temperature and sterilisation. Depending on heating intensity many bio-chemical reactions (e.g. denaturation of whey proteins, Millard reactions, proteolysis and destruction of some vitamins) can occur (Akkerman, 2014). Regarding the salt equilibria, dissociation of inorganic phosphate present in the serum phase increases when the milk temperature increases resulting in the formation of HPO_4^{2-} from $H_2PO_4^-$, which has higher affinity to calcium or magnesium than $H_2PO_4^-$ and consequently forms calcium or magnesium phosphate salts (Anema, 2009; de la Fuente et al., 1997). As the serum phase is supersaturated with calcium phosphate salt, the calcium and phosphate concentrations in the serum phase decrease during heating due to precipitation of calcium phosphate which is formed between the reaction of Ca^{2+} and HPO_4^{2-} present in the serum phase. However, the degree of this reduction in the serum phase depends on the intensity of the applied heat. If the heat treatment is less than 95 ^oC for a few min the alteration of the salt equilibria is reversible after cooling (Pouliot, Boulet, & Paquin, 1989). However, when milk was heated to 90 ^oC, a decrease in magnesium concentration in the serum phase was observed but was found to redistribute itself upon cooling (Abdulghani, Prakash, Ali, & Deeth, 2015). The distribution of calcium and magnesium is irreversible if the milk undergoes a severe heat treatment of more than 100 ^oC for several min (Gaucheron, 2011).

ii) Cooling

As opposed to the heating of milk, when milk is cooled, a decline in the dissociation of inorganic phosphate in the serum phase was observed and thus some HPO_4^{2-} was transformed back to $H_2PO_4^-$ (Gaucheron, 2011). At the same time, the concentration of calcium and phosphate increases in the serum phase as the solubility of calcium phosphate increases. Consequently, some micellar calcium phosphate (MCP) (10%) is transferred to the serum phase (Pierre & Brule, 1981). Moreover, β-casein is solubilised and casein micelles become more hydrated, however, these changes are reversible.

2.6.2 Magnesium in bovine milk and its equilibria

The mean concentration of magnesium in bovine milk was reported as 0.11 mg mL^{-1} (Oh & Deeth, 2017), but its concentration has been reported to present over a number of wide concentration ranges : 0.11 - 0.13 mg mL^{-1} (White & Davies, 1958); 0.08 - 0.27 mg mL^{-1} (Cerbulis & Farrell, 1976); 0.10 - 0.15 mg mL^{-1} (Gaucheron, 2005); 0.09 - 0.13 mg mL^{-1} (Fransson & Lönnerdal, 1983) and 0.08 - 0.13 mg mL^{-1} (Tsioulpas, Lewis, & Grandison, 2007). In colostrum, it is 2 - 3 times higher than in mature cows' milk and decreases within the first 1 - 3 days of lactation and remains relatively constant thereafter (Gaucheron, 2005). The concertation of magnesium in milk is not affected by the dietary intake of magnesium by the cow (Zamberlin et al., 2012). However, goat milk contains a similar amount of magnesium as cows milk, but ewes milk contains a higher amount of magnesium (de la Fuente et al., 1997). On the other hand, human milk contains the lowest amount of magnesium compared to these other three types of milk (Oh & Deeth, 2017).

Generally, two thirds of the magnesium is present in the serum phase and the remaining one third is in the micellar phase associated with the casein micelle (Gao, 2010). The distribution of magnesium and calcium in the different phases of skim milk is presented in Table 2.3.

Table 2.3: Distribution of magnesium and calcium in various phases of skim milk (Cashman, 2011; Oh & Deeth, 2017).

Milk phases	Magnesium	Calcium	Fraction with which associated	Magnesium	Calcium
	%			%	
Micellar	36	66	Casein	18	50
			Colloidal calcium phosphate	18	16
Serum	64	34	Magnesium or calcium citrate	40	23
			Magnesium or calcium phosphate	7	2
			Ionic or free	16	7
			Bound to α-lactalbumin	0	1.5

In bovine milk, about 98 to 100% of magnesium is found in the skim milk. Magnesium concentration is unaffected by fat removal; on a dry basis skim milk contains ~1300 mg kg^{-1} magnesium (Cashman, 2011). Of the magnesium present in skim milk, 63% is found in the

serum phase as free ions (16%) and complexes with citrate (40%) and phosphate (7%) (Zamberlin et al., 2012). The remaining part is found in the micellar phase bound to the casein micelle (Cashman, 2011). Of the magnesium present in the casein micelle, 50% of the magnesium is associated with CCP and the rest of 50% is bound to phosphoserine residues (Cashman, 2011). Dalgleish and Law (1989) suggested that magnesium may bind to non-phosphoserine binding sites on the caseins.

There are many published articles in which the partition of minerals in milk has been investigated and quantified in each phase (de la Fuente et al., 1997; Franzoi, Niero, Penasa, Cassandro, & De Marchi, 2018; Lin, Grandison, & Lewis, 2015; Silva et al., 2001). Silva et al. (2001) used trichloro acetic acid (TCA) and pepsin (PEP) to precipitate the caseins and found the magnesium content in the serum to be 0.11 mg mL^{-1} and 0.10 mg mL^{-1}, respectively, suggesting TCA and PEP acted on bovine milk casein micelle according to different mechanisms to release the magnesium. The addition of TCA leads to casein denaturation at its isoelectric point and PEP enzyme acts through cleavage of peptide bonds of the casein micelle. Franzoi et al. (2018) measured the minerals in the serum and micellar phases in bovine milk after rennet coagulation and found 1.13 mg mL^{-1} calcium, 0.10 mg mL^{-1} magnesium and 0.14 mg mL^{-1} potassium in the serum phase. Moreover, Dalgleish and Law (1989) reported that micellar magnesium decreased from 1.30 mmol L^{-1} at pH 6.70 to 0.20 mmol L^{-1} at pH 4.90.

The distribution of calcium, magnesium and phosphorous between the serum and micellar phases in goat and ewe milk has also been studied (de la Fuente et al., 1997). De la Fuente et al., (1997) reported that there was 32.80% calcium, 66.40% magnesium and 38.60% phosphorous present in the serum phase of goat's milk; the amounts were higher than that of ewe's milk (20.80%, 56.00% and 34.80%, respectively). In contrast, bovine milk serum phase contains 34.00% calcium, 67.00% magnesium and 43.00% phosphorus (Fox et al., 2015).

2.7 Ionic magnesium in bovine milk

Profiling ionic magnesium (Mg^{2+}) in milk is important to study because total magnesium in milk is nutritionally significant but the ionic magnesium (Mg^{2+}) has important for physiological implications (Zhang, 2011). Moreover, ionic calcium (Ca^{2+}) and ionic magnesium (Mg^{2+}) play important roles in the physicochemical properties of the casein micelle (Gao, 2010). The ionic calcium (Ca^{2+}) and ionic magnesium (Mg^{2+}) can influence the environment of the negatively charged casein micelle leading to an increase or decrease in its stability (Horne & Parker, 1981). A larger proportion of magnesium is present in its ionic form compared to calcium (Table 2.3), although the absolute concentration of ionic magnesium (Mg^{2+}) is much lower than ionic calcium (Ca^{2+}) (Oh & Deeth, 2017). Different models have been proposed to predict the theoretical concentration of ions and salts in the serum phase of milk and the proposed ionic magnesium (Mg^{2+}) concentration in the serum phase has been reported to be 1.14 mmol L^{-1} (Mekmene et al., 2009) and 0.81 mmol L^{-1} (Holt et al., 1981). On the other hand, according to Christianson, Jenness, and Coulter (1954) ionic magnesium (Mg^{2+}) in milk is 0.82 – 0.85 mmol L^{-1}. The calculated value (0.81 mmol L^{-1}) obtained by Holt et al. (1981) is now generally accepted (Oh & Deeth, 2017). Recently Bijl et al. (2013) and Gao (2010) used the Donan membrane technique to determine ionic magnesium (Mg^{2+}) in the serum phase and found 0.61 mmol L^{-1} and 0.58 mmol L^{-1}, respectively at natural pH, these values are lower than the calculated value by Holt et al. (1981).

2.8 Heat stability of milk protein

Heat treatment is a common step in the dairy industry. When milk is heated many physical and chemical changes occur such as pH shifts, ionic equilibria changes, lactose mutarotation, association of casein, whey protein aggregation or denaturation etc., all dependant on heating conditions (Klarenbeek, 1984). Caseins are unstable towards heating; mild heating does not affect the micelle, but they undergo some physicochemical changes when subjected to severe heating (O'Connell & Fox, 2003). pH-dependent dissociation of casein (κ-casein) from the casein micelle can occur due to heat treatment of milk. Temperature, duration of heating and the composition of milk are the factors that influence this dissociation (Anema & Li, 2003).

2.8.1 κ-Casein dissociation

A pH dependent dissociation of casein from the casein micelle can be caused by the heat treatment of milk. In milk at its natural composition, at pH 6.70 or below very little dissociation of κ-casein occurs regardless of the temperature and duration of heating (Anema, 1998). As the pH increased to pH ~7.10, a significant amount of κ-casein dissociates from the casein micelle (Anema & Klostermeyer, 1997). At 70 °C, all types of caseins are found in the serum, but the proportion of caseins is different from that found naturally in unheated milk (Anema & Li, 2003). Above 70 °C, the κ-casein level was found to increase in the serum whereas β-casein and α-casein decreased. Hence above 70 °C and at pH 7.10, the milk serum became rich in κ-casein (Anema & Klostermeyer, 1997; Anema & Li, 2003; Anema 1998), as shown in Figure 2.6.

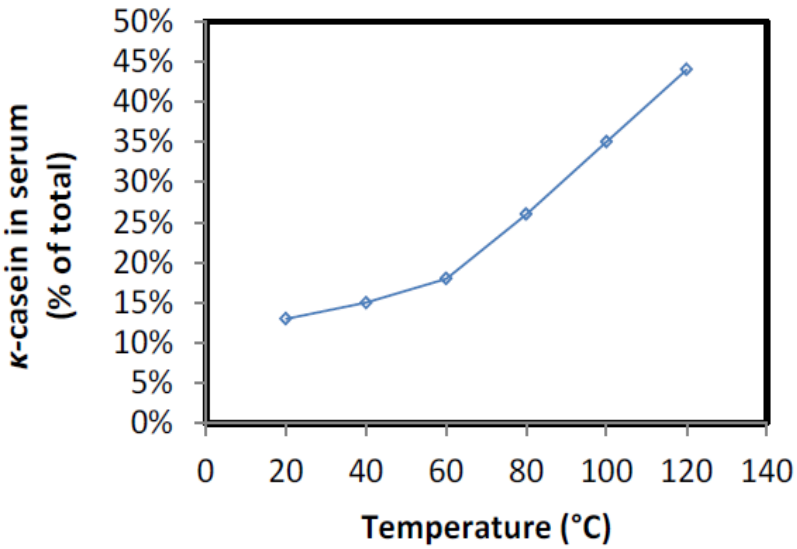

Figure 2.6. κ-casein in the serum phase found at different temperatures at pH 7.10 (Anema, 1998).

2.8.2 Whey protein denaturation

Depending on heating temperature and time, when milk is exposed to thermal processing, whey proteins undergo structural changes known as whey protein heat denaturation which leads to changes in the functional properties of whey protein (Klarenbeek, 1984). The quantitive distribution of denatured whey protein is presented in Figure 2.7 (Vasbinder, 2002). On increasing the heating temperature, the native whey protein decreases due to the formation of more whey protein aggregates (Vasbinder & De Kruif, 2003).

Heating β-lactoglobulin at natural pH causes the dimeric native protein to dissociate, partially unfold and aggregate via disulphide bonding. Heating above 65 °C, β-lactoglobulin structure changes due to irreversible reactions involving disulfide bonds present (Gaur, 2017; Havea, Carr, & Creamer, 2004; Lowe et al., 2004). Due to heating complexes are formed by aggregation of denatured whey proteins and complexes also form between β-lactoglobulin and κ-casein on the surface of the casein micelle by disulphide and hydrophobic interactions (Gaur, 2017).

Heating skim milk at 70 °C for 30 min denatures 29% of total whey protein while heating at 80 °C for 10 min denatures up to 90% of whey proteins (Agrawala, 1979). Vasbinder and De Kruif (2003) reported that the β-lactoglobulin is more heat susceptible than α-lactalbumin which is in agreement with other researchers (Klarenbeek, 1984; Lee & Hong, 2003; Oldfield, Singh, Taylor, & Pearce, 2000; Singh, 2004; Singh, Creamer, & Newstead, 1995). Immunoglobulin is denatured at 83 °C while α-lactalbumin was not affected because it is the most heat resistant fraction of the whey proteins (Yousif, 1991).

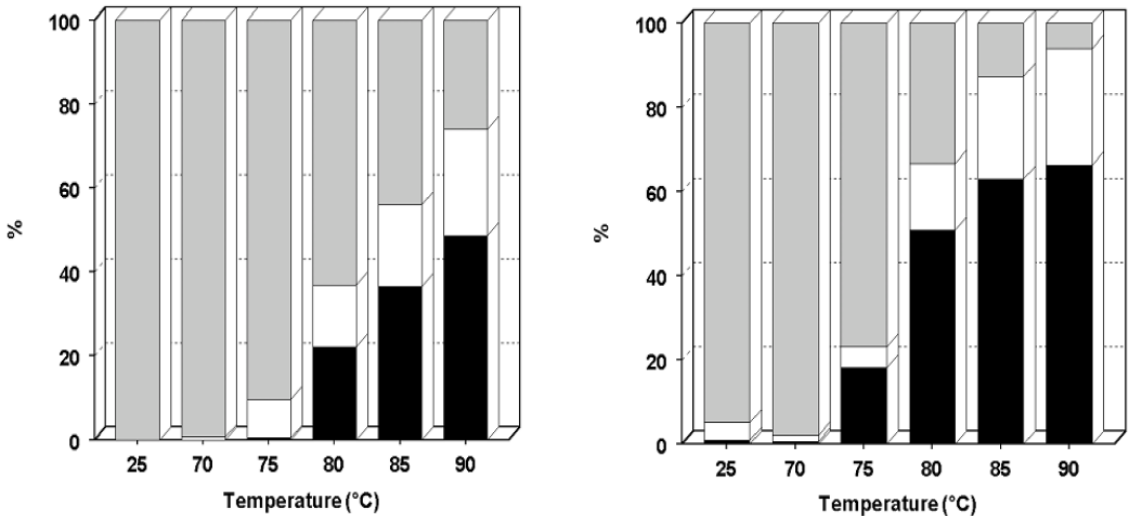

Figure 2.7. Percentage of denatured α-lactalbumin (left) and β-lactoglobulin (right) in milk as soluble whey protein aggregate (white bars) and associated with casein micelle (black bars) after 10 min of heating. The grey bars present native whey protein (Vasbinder, 2002).

2.8.3 Interaction of whey protein and casein micelle

Research has been carried out to understand the specific reaction mechanisms that occur upon heating of milk. During heating, β-lactoglobulin forms a complex with κ-casein on the micellar surface by thiol-disulphide exchange and hydrophobic interaction (Gaur, 2017). At

temperatures below 70 °C, the interaction between β-lactoglobulin and Ҡ-casein is mostly caused by hydrophobic interactions while at higher temperatures the interactions mostly occurred via disulphide bond interactions (Akkerman, 2014). The interaction between β-lactoglobulin and Ҡ-casein upon heating at 90 °C is presented in Figure 2.8 (Pesic et al., 2012).

Figure 2.8. Casein micelles and denatured whey proteins interaction in bovine milk after heat treatment at 90 °C for 10 min at natural milk pH 6.70 (Pesic et al., 2012).

Calcium salt addition affects β-lactoglobulin denaturation. In the presence of calcium and heating above 65 - 75 °C, β-lactoglobulin starts unfolding and reacts with Ҡ-casein (Lowe et al., 2004). Petit, Herbig, Moreau, and Delaplace (2011) claimed that calcium enhances β-lactoglobulin aggregation, by lowering the repulsion between the negatively charged β-lactoglobulin reactive species and bridging β-lactoglobulin proteins. pH also plays a predominant role on the interactions of the denatured whey protein with the casein micelles (Akkerman, 2014; Anema & Klostermeyer, 1997; Anema, Lee, Lowe, & Klostermeyer, 2004; Anema & Li, 2003). Heating milk at temperatures up to 100 °C, the percentage of denatured whey protein association with casein decreased from 80 to 30% when the pH increased from 6.50 to 6.70; at a higher pH, the association was even lower and decreased from 30 to 10% due to Ҡ-casein transferring into the serum (Anema & Li, 2003).

β- lactoglobulin and Ҡ-casein complexes are found both in the micellar phase associated with casein micelle and serum phase in heated milk. It was reported by Donato and Guyomarc'h (2009) that association may have occurred first followed by dissociation or dissociation occurs first followed by association. The micellar size can be increased by aggregation of the micelles and denatured β-lactoglobulin (Anema & Li, 2003) or with another micelle (Walstra et al.,

2005), this leads to micellar aggregation and thus a more stable micelle is formed. The reasons for micellar aggregation are stated below:

- The negative charge of κ-casein neutralised by a decrease in pH results in the lowering of the electrostatic repulsion between κ-casein molecules and thus κ-casein shrinks in diameter, hence their stabilising effect on the casein micelles is lost. When κ-casein molecules and casein micelles approach each other due to hydrophobic interaction the micelles easily aggregate (Walstra et al., 2005).

- When ionic Ca^{2+} in the serum phase increases, free Ca^{2+} around the micelle also increases. Free Ca^{2+} works as a bridge between two negatively charged amino acids on the casein micelle. They create a strong association between two micelles when multiple bridges are formed. During the heat treatment, calcium phosphate in the serum phase becomes less stable and moves towards the micellar phase (Walstra et al., 2005).

- Electrostatic and steric stability is reduced when κ-casein is dissociated from the micellar surface. κ-casein depleted micelles can aggregate through either hydrophobic interaction or calcium-mediated aggregation (Walstra et al., 2005). A high negative charge density of the phosphoserine clusters of α-casein and β-casein which previously were protected by κ-casein hair provides an ideal place for ionic Ca^{2+} bridge formation. Ca^{2+} mediated aggregation proceeds at a faster rate in κ-casein depleted micelles (Holt & Horne, 1996). Ionic magnesium (Mg^{2+}) has a similar effect on the casein in milk (O'Mahony, McSweeney, & Lucey, 2008).

2.9 Milk protein gelation

Milk protein gel formation is a topic of interest for its great economic importance (van Vliet, Lakemond, & Visschers, 2004). The gelation of milk due to protein-protein interaction results in a three-dimensional gel network (Siamand, Deeth, & Al-Saadi, 2014). A net negative charge and steric repulsion stabilise the casein micelle in milk which helps to prevent aggregation at its natural pH (Walstra et al., 2005). Heating, acidification and rennet enzyme addition are the main processes to achieve milk protein gelation (Havea et al., 2004; Horne, 2003; Phadungath, 2005; Sadeghi, Madadlou, Khosrowshahi, & Mohammadifar, 2014; van Vliet et al., 2004). For the gelation of dairy products from heated milk, both casein and whey protein play a major role.

2.9.1 Gelation mechanism

Casein gels can be formed; by enzyme action (enzyme-induced), by acidification (acid-induced) or by a combination of both. Whey protein gelation is the result of both physical and chemical interactions between whey protein molecules. If the gel formation occurs in unheated milk only casein contributes to gelation whereas for preheated milk then denatured whey proteins (β-lactoglobulin) become involved in gel formation (van Vliet et al., 2004). For enzyme-induce gelation preheating decreases gel formation but for acid-induced gelation preheating increases gel formation and the gel becomes firmer with less syneresis (Vasbinder, Rollema, & De Kruif, 2003). However, the addition of salts can also influence the gelation (Lin, Wong, Deeth, & Oh, 2018). The effect of additional ingredients depends on how they contribute to and interact with the gel architecture (Perreault, Morin, Pouliot, & Britten, 2017). Some common types of protein gel formation are discussed below:

2.9.1.1 Acid-induced gelation

Among all the types of protein gels, acid gels are the most common due to their formation in yogurt and related products (Lucey, Tamehana, Singh, & Munro, 2000). Acid-induced gelation forms upon acidification of milk by using bacterial cultures which convert lactose to lactic acid or by direct addition of acid such as citric acid, HCl or GDL (glucono-δ-lactone which is hydrolysed to gluconic acid), resulting in a reduction of pH and gelation occurs (Phadungath, 2005). Upon acidification, the casein charges are neutralised and a three-dimensional network forms and as a result the casein particles tend to aggregate. When the pH is reduced from pH 6.70 to 6.00, a small amount of CCP is solubilised, so the casein micelle structure remains relatively unchanged. At pH 6.00 to 5.00, CCP dissolves completely, however, this depends on both pH and temperature. At pHs lower than pH 5.00, at the isoelectric point (pH 4.60), casein aggregates (Lucey, 2017).

2.9.1.2 Enzyme-induced gelation

The addition of an enzyme (e.g. rennet) hydrolyses the C-terminal (the end of a protein or polypeptide, terminated by a free carboxyl group) of κ-casein consequently reducing the stability of the casein micelle, thus causing them to aggregate and an enzyme induced gel is formed (Horne, 2003). Gelation can occur with a lower addition of enzyme at reduced pH where acidification and renneting act synergistically and lead to stiffer gels (van Vliet et al., 2004). The gelation starts with casein micelle-controlled destabilisation through proteolysis

of Κ-casein that is located at the periphery of the micelle, as a result, the steric stabilisation is lost and attractive forces arise (van der Waals forces and hydrophobic interaction) which help with aggregation and coagulation (Holt et al., 2013; Phadungath, 2005; Horne, 2003; Anema, 2009; Dalgleish & Law, 1989).

2.9.1.3 Heat-induced gelation

Heat-induced whey protein gels occur when the denatured proteins aggregate creating three-dimensional structures that trap the water in the system (Havea et al., 2004). Whey protein destabilisation can be achieved by adding a chemical, heating, cooling, enzymatic hydrolysis (partial) and hydrostatic pressure (van Vliet et al., 2004). The balance between the attractive and repulsive forces between the aggregate particles controls the network formation. Heating a protein results in unfolding of the molecule and exposing reactive sites. Then the unfolded protein molecules interact by forming disulphide bonds and hydrophobic/ionic association takes place, immobilising large amounts of water and the three-dimensional gel is formed (Schmidt, 1980; Xiong, 1992). There are many factors that affect the whey protein denaturation and aggregation including the type of protein, protein concentration, pH, the temperature of the heating and the addition of salts (Crowley et al., 2014; Tsioulpas et al., 2007).

2.9.1.4 Salts-induced gelation

During the formation of milk protein gels, significant attention has been given to calcium or magnesium in milk as they contribute to the stabilisation of casein micelle. Heat treatment of calcium or magnesium-added milk results in increases in micellar calcium or magnesium and the concomitant decrease of pH leading to salts-induced gelation (Omoarukhe, On-Nom, Grandison, & Lewis, 2010; Ramasubramanian, Restuccia, & Deeth, 2008). Moreover, during heat treatment, CCP is solubilised and protein tends to lose its native structure and starts to aggregate. When milk is heated in the presence of additional calcium or magnesium ions, milk proteins became unstable, they alter the dynamic equilibrium and the formation of milk gelation occurs due to the hydrophobic interactions between proteins. The added salts decrease the electrostatic repulsion between the protein molecules and form salt bridges between negatively charged proteins. Ramasubramanian et al. (2008) suggested that the addition of soluble calcium salts increases the ionic calcium and thus reduces the milk pH

(Equation 1). Milk can be thickened, gelled or coagulate even at mild heating with the addition of calcium chloride due to calcium protein linkage (Ramasubramanian et al., 2008).

$$3\ Ca^{2+} + 2\ HPO_4^- \Leftrightarrow Ca_3PO_4 + 2H^+ \quad (1)$$

However, the gelation properties of reconstituted milk protein concentrate are strongly affected not only by the amount of soluble calcium but also by soluble casein present (Sandra & Corredig, 2013).

2.9.2 Factors affecting gelation

2.9.2.1 Effect of temperature and pH

Temperature and pH affect the calcium and phosphorus (and other minerals) equilibrium between the serum and the micellar phases in milk. Subsequently, this can affect the final structure, texture and functionally of dairy products (Udabage, McKinnon, & Augustin, 2000). During acidification when CCP is solubilised from the casein micelle, resulting in calcium release in the serum phase which then influences the final structure of the gel. When CCP is solubilised, a significant amount of calcium remains in the micelle by attaching to the carboxylic group of glutamate and aspartate that may participate in the structure of gels by forming a bridge between two negative casein molecules (Holt, 1995). Moreover, when the milk temperature is raised (higher than 40 ^{o}C), calcium and phosphorus shift from the serum phase to the micellar phase and form heat-induced gels (Holt, 1995).

For both concentrated and unconcentrated milk, heat stability depends on pH (Singh, 2004). Heated milk forms firmer gels than unheated milk on slow acidification (Anema et al., 2004). If the temperature is increased during acidification, gels are formed at markedly higher pH (Anema, 2008; Horne, 2003). Increasing the temperature of the milk, the gelation rate increased but this did not affect the final elastic modulus of the whey protein aggregate gel (Kharlamova, Chassenieux, & Nicolai, 2018).

Preheating the milk increased the gel strength of calcium-induced gels compared to non-preheated milk samples (Ramasubramanian et al., 2014). Depending on the nature of preheat treatment applied, the microstructure of the coagulum differs, and the hardness, adhesiveness and protein content of the coagulum are affected by heat treatment and pH.

Preheat treatment plays a role on the yield of calcium-induced coagulum; preheating the milk at 90 ^{o}C for 10 min, maximum coagulation occurred and resulted in an increase in hardness and storage modulus of the coagulum due to complete denaturation of whey protein (Ramasubramanian, Webb, D'Arcy, & Deeth, 2013).

Regarding the gelation properties of acid-induced gels, heat treatment of milk significantly increases the textural qualities of milk products, makes the gel firmer and reduces syneresis (Lindkvist, 2012). On the other hand, severe heat treatment increased rennet coagulation time and formed weaker gels (Anema, Lee, & Klostermeyer, 2007). In particular, the heat stability of milk was low at pHs below about pH 6.50 (Ji, Lee, & Anema, 2016). Aggregation of proteins occurs as the isoelectric point of casein (pH < 4.90) is approached and maximum gel firmness occurs around pH 4.60. Generally, a slower rate of acidification results in slightly higher gel firmness (Lucey, 2017).

2.9.2.2 Effect of protein concentration

Milk stability decreased as the milk solids or protein concentration was increased (Ji et al., 2016; Anema, 2009). The concentrated milk formed a heat-induced gel at an elevated temperature at natural pH. If milk protein concentration is increased or whey protein is added before heating or acidification, gelation occurs at even higher pH and the firmer gels are formed (Anema, 2009).

2.9.2.3 Effect of salts addition

Milk gelation by the addition of calcium salt has been studied extensively (Koutina, Knudsen, Andersen, and Skibsted 2014; Koutina, Christensen, Bakman, Andersen, & Skibsted, 2016; Lin et al., 2018; Ramasubramanian, D'Arcy, & Deeth, 2012; Ramasubramanian et al., 2014; Ramasubramanian et al., 2013). Gels similar to yogurt type were formed at a pH close to that of natural milk by the addition of calcium chloride (Ramasubramanian et al., 2014) and glucono-δ-lactone (GDL) (Lucey et al., 1997) to milk. Ramasubramanian et al. (2012) reported the coagulation of milk by the addition of 20 to 200 mmol L^{-1} calcium chloride at 70 ^{o}C. They found that calcium addition less than 20 mmol L^{-1} did not cause milk coagulation at 70 ^{o}C. The rheological results suggest that the gel strength increased with increasing the addition of calcium chloride from 10 to 20 mmol L^{-1} on heating at 70 ^{o}C, resulting in the increase in final *G*' and breaking stress. If the concentration of calcium addition increased more than 20 mmol

L^{-1}, the coagulated milk released whey (Ramasubramanian et al., 2012). However, the gel properties are affected by the types of calcium salt added. Different association constants for different salts influence the gelation properties of milk (Lin et al., 2018). Ramasubramanian et al. (2008) reported that gel strength of yogurt increased with 2 mmol L^{-1} of calcium chloride addition, but the gel strength decreased further on the addition of ionic calcium up to 13.5 mmol L^{-1}. Addition of 49.8 mmol L^{-1} nonionic calcium improved the firmness and viscosity without affecting the gel smoothness. Moreover, Ramasubramanian et al. (2013) concluded that the calcium-milk coagulum has different characteristics in texture, microstructure and composition from an acid-induced coagulum.

2.10 Monitoring gelation

To monitor the gelation there are several techniques in recent reviews (Klandar, Lagaude, & Chevalier-Lucia, 2007; Lucey et al., 2000). Ultrasonic spectroscopy and diffusing wave spectroscopy are two promising techniques for the study of milk gels (Alexander & Corredig, 2014). Comparing between ultrasonic spectroscopy and traditional rheological methods, Wang, Bulca, and Kulozik (2007) found that ultrasonic spectroscopy was able to measure enzymatic hydrolysis and aggregation process, but this method was not sensitive for gel formation detection. Whereas, traditional rheological methods were not able to detect enzymatic hydrolysis processes but very suitable to characterise the formation of a gel network.

Rheological properties can be characterised by dynamic low amplitude oscillations to determine both viscous and elastic components. In general, the rheological measurement of milk gels is conducted using very small strains ($\leq$ 1%) and the oscillating strain rate ($\leq$ 0.1 Hz) to avoid gel destruction (Phadungath, 2005). The measurement is accomplished in the linear viscoelastic range where strain is proportional to applied stress (Lucey et al., 2017; Phadungath, 2005). The elastic or storage modulus (G') is a measure of energy stored per oscillation cycle (Equation 2), the viscous or loss modulus (G'') represents the measurement of energy dissipated as heat per cycle (Equation 3) and the loss tangent (tan δ) is the ratio of the loss modulus to the storage modulus (Equation 4). These parameters are presented as follows:

$$G' = \left(\frac{\sigma_0}{\gamma_0}\right)\cos\delta \quad (2)$$

$$G'' = \left(\frac{\sigma_0}{\gamma_0}\right)\sin\delta \quad (3)$$

$$\tan\delta = \frac{G''}{G'} \quad (4)$$

(Where σ_0 is the amplitude of shear stress, γ_0 is the amplitude of the strain and δ is the phase angle)

2.11 Methods of analysing magnesium in milk

To control the functional behavior of dairy products during manufacturing, it is often useful to determine the total concentrations of minerals or profile the distribution of ions and salts in the serum and micellar phases (de la Fuente, Belloque, & Juárez, 2004). The determination of total minerals in milk and milk-based products is particularly difficult to perform directly because they are present in complex matrices (Khan et al., 2014). It is important to use the correct analytical methods and correct preparation procedures for the sample for precise measurement of these salts and ions in a milk system. Therefore, sample preparation is an important step in the whole analytical procedure.

2.11.1 Sample preparation

2.11.1.1 Serum phase separation

Four physical separation methods to obtain serum phase are used: dialysis, ultrafiltration, ultracentrifugation and rennet coagulation followed by the recovery of whey. During the separation of free ions by one of these techniques, it is crucial to work at a controlled pH and temperature since mineral equilibrium is very dependent on these physicochemical parameters (Gaucheron, 2010). For dialysis and ultrafiltration, a 10,000 Da membrane is recommended to avoid transfer of small molecules and for ultracentrifugation 80,000 × g for 2 h is suitable for whey separation. Different sample preparation processes have also been used to date for the detection of minerals in milk and dairy products, such as dry ashing, wet digestion and microwave digestion (Martino, Sánchez, & Medel, 2000; Luo et al., 2010; Gaucheron, 2010).

i) Ashing

It is useful to eliminate the organic matter (proteins, fat and lactose) in milk. The organic matter may interfere with the determination of the minerals. Dry or wet ashing are possible ways to destroy this organic matter (Martino et al., 2000). Dry ashing is performed by heating the material between 400 - 800 ^{o}C for several hours. The dry ashes obtained can be used, after dissolution in dilute hydrochloric or nitric acid solution, for the determination of one or several specific mineral ions. Wet ashing requires a concentrated acid mixture of nitric, sulfuric and perchloric acid (Gaucheron, 2010).

ii) Digestion

Digestion leads to a destruction of organic matter by oxidation with various mixtures of pure nitric, sulfuric and perchloric acid. The sample is placed in a digestion vessel with acid and hydrogen peroxide. The digester is sealed tightly and heated on the electric hot plate between 90 - 120 ^{o}C. For microwave digestion, microwave energy is used to achieve combustion. Microwave-based acid digestion is well-established and becoming popular because of the rapid speed, lower acid consumption and high digestion efficiency (Luo et al., 2010).

Iii) Acid extraction

Total mineral content can be obtained after an acid-extraction using nitric, sulfuric, chlorohydric or trichloroacetic acid. At pH 3.00, all milk proteins and high molecular mass peptides are precipitated and all the minerals transfer to the serum phase. After acidification, the precipitated form is filtered off and the serum solution is used for the determination of minerals (Gaucheron, 2010).

2.11.2 Quantification of total magnesium

There are several methods employed for measuring the concentration of total magnesium in milk and milk serum. Atomic absorption spectroscopy (AAS), inductively coupled plasma spectrometry (ICP), x-ray fluorescence (XRF), spectrophotometer and ion chromatography etc. are used as instrumental methods. Complexometric titration is used as a wet chemistry method for total magnesium quantification in milk.

2.11.2.1 Atomic spectroscopic method

The atomic spectroscopic method is a simple, rapid and an accurate technique that has been widely applied for the determination of calcium, magnesium, sodium and potassium in biological samples, milk and dairy products (Gaucheron, 2010). The different available atomic spectroscopic methods are flame atomic absorption spectroscopy method (FAAS), graphite furnace atomic absorption spectroscopy (GFAAS), inductively coupled plasma atomic emission spectrometry (ICP-AES) and inductively coupled plasma mass spectrometry (ICP-MS) (Luo et al., 2010; Noël, Carl, Vastel, & Guérin, 2008; Rodrıguez, Alaejos, & Romero, 2001; Gaucheron, 2010). The traditional FAAS (flame atomic absorption spectroscopy) requires longer detection time for multi-element detection. Essential and trace elements in human milk have been determined by modified FAAS where two multi-element hollow cathode lamps (HCLs) were used instead of single-element HCLs (Luo et al., 2010). Inductively coupled plasma atomic emission spectrometry (ICP-AES) has been extensively used for the determination of a wide range of metals such as Ca, Mg, Cu, Zn, Al, Ba, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Pt, Sr and Fe in milk, biological fluids and food products (Bakircioglu, Topraksever, Yurtsever, Kizildere, & Kurtulus, 2018; Nóbrega, Gélinas, Krushevska, & Barnes, 1997; Silva et al., 2001). Silva et al. (2001) used inductively coupled plasma-optical emission spectrometry (ICP-OES) for the determination of protein-bound fraction of calcium in bovine milk. They used 10% trichloroacetic acid (TCA) and 5% pepsin solution to precipitate the proteins out of bovine milk samples prior to determination. Martino et al. (2000) analysed essential and toxic elements in milk whey using a double focusing inductively coupled plasma mass spectrometry (ICP-MS). Before the measurement, sample preparation was carried out by lyophilisation and ashing. Rahil-Khazen, Henriksen, Bolann, and Ulvik (2000) concluded that inductively coupled plasma atomic emission spectrometry can be used for multi-elemental analysis of trace elements in human serum.

Atomic spectroscopic method (AAS and ICP-MS or ICP-AES) has become accepted as the most powerful analytical tool for the simultaneous determination of trace elements due to its extreme sensitivity, selectivity and capability of multi-elemental analysis (Rahil-Khazen et al., 2000; Silva et al., 2001; Bakircioglu et al., 2018; Yoshinaga, 1996). Moreover, the advantage of the method is that sample preparation is simple. Sample preparation by a digestion step helps to eliminate or minimise the risk of contamination and any loss of analytes. This method

is simple and fast but expensive and requires a digestion process for the milk prior to analysis. Although it provides high accuracy, the high cost for ICP and rigid operating protocols greatly limits its use in small laboratories.

2.11.2.2 Ion chromatography

Ion chromatography (IC) is another technique often used to measure inorganic anions and cations, transition metals, organic acids, amines, phenols, surfactants and sugars based on their conductivity and fluorescence (Weiss, 2016). It is mostly used for the analysis of inorganic ions in foods and beverages. Cataldi, Angelotti, D'erchia, Altieri, and Di Renzo (2003) used ion chromatographic separation and determination of sodium, potassium, magnesium and calcium in milk and whey protein. Cataldi et al. (2003) found identification and quantification of this method were interference free and very sensitive with simple sample preparation. Rahimi-Yazdi, Ferrer, and Corredig (2010) used high-performance non-suppressed ion chromatography to avoid citrate interference for total calcium determination as well as the calcium present in the colloidal and salt state in milk. However, to measure minerals in milk, samples need to undergo preparation steps to change all the mineral forms in milk to free ions because the measurement is based on M^{2+} conductance. This technique is not suitable for the rapid detection of minerals in a milk sample.

2.11.2.3 Energy dispersive x-ray fluorescence (EDXRF)

X-ray fluorescence (XRF) is a technique for rapid and simultaneous multi-element analysis (Matsunami et al., 2010). Although XRF analysis has a great potential for direct analysis of solid samples without any chemical treatment, it has not yet become widespread in the dairy industry (Pashkova, 2009). Some researchers used this technique to measure the concentrations of minerals, anions and element contents in human serum, human milk, bovine milk and dairy products (Ekinci, Ekinci, Polat, & Budak, 2005; Pashkova, 2009; Perring & Andrey, 2003; Rinaldoni, Campderrós, Pérez Padilla, Perino, & Fernández, 2009). Ekinci et al. (2005) and Pashkova (2009) used this method along with other analytical techniques with high accuracy and versatility such as inductively coupled plasma spectrometry (ICP) and atomic absorption spectroscopy (AAS) to validate their findings.

Energy dispersive x-ray fluorescence (EDXRF) determines the relative concentration of each of the elements present in the sample. It requires other highly sensitive methods such as AAS

and ICP to support the obtained data. XRF is very suitable for a solid sample for rapid and direct measurement and considered to be a time and cost saving method. This method can be used for a liquid sample with a suitable sample preparation method prior to analysis. However, this method is not suitable for small companies due to the high capital cost involved.

Atomic absorption spectroscopy is commonly used as an instrumental method for quantification of magnesium or other minerals in milk and dairy products (Udabage et al., 2000; Khan et al., 2014; Bakircioglu et al., 2018; Luo et al., 2010). This method is accurate, simple and fast compared to other instrumental methods such as ion chromatography and energy dispersive x-ray fluorescence methods (Luo et al., 2010)

2.11.2.4 Complexometric titration

Complexometric titration with EDTA is a well-known method for measuring calcium and magnesium in liquid and powdered milk. EDTA is commonly used as a chelator and forms strong 1:1 complex with most of the heavy metal ions (Tandy et al., 2004). Direct complexometric and improved complexometric titration for the determination of calcium and magnesium in milk and dairy products has been studied (Kindstedt & Kosikowski, 1985; Ntailianas & Whitney, 1963). In the case of dairy products containing a mixture of calcium and magnesium, the magnesium concentration is determined as the difference between two titrations; one that measures calcium only and the other titration determines calcium and magnesium together (Gaucheron, 2010). Some comparative studies have also been carried out for the determination of metals in milk and biological fluids. Aljerf and Mashlah (2017) and Hussain, Nazir, Shafique and Salman (2010) compared EDTA complexometric titration method with flame atomic absorption spectroscopy (FAAS) method for the determination of calcium and magnesium in biological fluids and milk and found that EDTA is a good chelating agent for mineral ion detection.

EDTA is most extensively used for calcium determination in milk. However, magnesium and zinc can also be determined. This method suffers from being time-consuming and less accurate (Hussain et al., 2010). However, reference methods such as ICP-MS, ICP-OES or AAS are used to validate the results.

2.11.3 Quantification of ionic magnesium (Mg^{2+})

For measuring free ion concentration, traditional methods such as cation exchange resin technique (Christianson et al., 1954), Donnan membrane technique, ion-selective electrode (ISE) and fluorescence dye have been used (Gao, 2010; Lin, Lewis, & Grandison, 2006; Silanikove, Shapiro, & Shamay, 2003; Tsioulpas et al., 2007). Ionic magnesium (Mg^{2+}) determination using a cation exchange resin is time-consuming and less accurate because the determination is influenced by the competition between ionic calcium (Ca^{2+}) and ionic magnesium (Mg^{2+}) (Gao et al., 2009).

2.11.3.1 Ion-selective electrodes

Electrochemical methods are widely used in the analytical chemistry field because, they are simple, fast and low cost. Ion-selective electrodes (ISE) have been widely used to measure the activity of Na^+, K^+, Cl^-, Ca^{2+}, Mg^{2+} and Li^+ in biological fluids (Dimeski, Badrick, & St John, 2010). Ranjith (1995) used an ISE system that had been designed for measuring ionic calcium in the blood and later adapted it for milk. After that, the calcium ion-selective electrodes (Ca-ISE) have been used to rapidly determine free Ca^{2+} activity in milk (Lin et al., 2006; Silanikove et al., 2003; Tsioulpas et al., 2007). Ca-ISE can be used to detect free ion activity. If the ion is present in a complex form, total ion concentrations cannot be directly assessed (Neville, Zhang, & Allen, 1995). To overcome this limitation, Gemene and Bakker (2009) used calcium ion-selective electrodes integrated in a flash chrono potentiometric transducer for the direct measurement of total calcium. Compared to other analytical techniques ion-selective electrodes are inexpensive, simple to use, fast and can be used with a wide range of concentrations. However, data can be less accurate with poor calibration and electrode contamination (Gao, 2010).

Filos and Okorodudu (1995) evaluated a magnesium ISE based on AVL 988-4 Na/Mg/Ca/pH analyser (AVL Scientific, Roswell, Germany) for ionic magnesium (Mg^{2+}) determination in blood and found the accuracy of ionic magnesium (Mg^{2+}) measurement was difficult to assess because of the lack of a reference method and primary standards. Magnesium ion-selective electrodes have been used for clinical purposes but do not appear to have been used for milk systems yet. Dimeski et al. (2010) concluded that magnesium ion-selective electrodes selectivity and specificity in terms of Ca^{2+} were not ideal and the interference of Ca^{2+} varies between electrodes from different suppliers. However, another method, Donnan membrane

technique (DMT) has been used for the determination of ionic magnesium (Mg^{2+}) in a milk system and found 0.61 mmol L^{-1} of ionic magnesium (Mg^{2+}) present in milk serum (Gao et al., 2009).

2.11.3.2 Spectrophotometric method

Spectrophotometry is a method of choice for measuring magnesium in biological samples (Ryan & Barbour, 1998). Several dyes such as calmagite, magon and methylthymol blue have been used routinely in laboratories to measure magnesium in alkaline conditions with detection by absorbance at 550 - 600 nm (Barbour & Davidson, 1988; Elin, 1991; McCoy, Maclaren, & Gudat, 1983; Ryan & Barbour, 1998). Using these dyes magnesium can be overestimated due to complex formation with calcium in milk like systems (Bazydlo, Needham, & Harris, 2014). Recently some magnesium fluorescence probes have been used for the intracellular Mg^{2+} detection in biological samples (Komatsu et al., 2004). A company Ursa Bioscience (Maryland, USA) offers a magnesium probe (Magnesium 510 probe) and has been claimed that it is selective for the determination of ionic magnesium (Mg^{2+}) in milk systems (Ursa BioScience, 2017) However, there are no studies reported of its use for the milk system.

2.11.4 Quantification of micellar magnesium

The micellar pellet can be obtained after ultracentrifugation, but it is not easy to solubilise into solution again. So, the concentration of minerals associated with casein micelles can be found by subtracting the concentration remaining in the serum phase from the total concentration (Gaucheron, 2010).

2.12 Conclusions

Milk contains approximately 0.11 mg mL^{-1} magnesium and one of the major sources of dietary magnesium. It is clear that magnesium has a significant health impact on human. Magnesium in milk, both in technical and biological aspects warrants more investigation for designing magnesium-enriched milk and dairy products.

Magnesium exists in a dynamic equilibrium between the serum and micellar phases in milk. The dynamic magnesium equilibria in milk is altered by applying most of the treatments used in the dairy industry such as heating, cooling, acidification and addition of salts. pH, the temperature of milk and concentrations of added salts play an important role on the distribution of magnesium between the serum and micellar phases. Profiling the magnesium distribution is important as its absorption in the intestine and in a biological application depends on its chemical form.

For determining total magnesium using the EDTA method is inexpensive, simple and easy but suffers from overestimation. The results obtained from the EDTA method can be validated by the atomic absorption spectroscopy (AAS) method. AAS method is more economical than other instrumental methods such as ion chromatography. Ionic magnesium (Mg^{2+}) determination using a cation exchange resin, Donnan membrane technique are time-consuming. Ion-selective electrodes are selective, but they are not free of interferences. The selectivity and specificity of magnesium ion-selective electrodes over calcium are less and change over time and require proper calibration of electrodes. On the other hand, magnesium fluorescence dye (Magnesium 510 probe) shows high sensitivity and selectivity and is suitable for milk systems.

Magnesium in milk plays an important role in protein stability. Milk protein gels can be formed by application of heat, acidification and addition of salts. Heat processing of mineral added milk is challenging as adding soluble salts increases the ionic strength of milk and reduces the milk pH, both of which lead to the coagulation of milk during heat processing. Salts-induced gels tend to form when milk is heated at 70 ^{o}C with the addition of salts and gel strength increases as the salts concertation is increased. The nature of preheat treatment also influences the strength of salts-induced gels.

Milk gels are viscoelastic materials. In order to observe the gel formation process and to evaluate the elasticity and viscosity of the gels, a dynamic non-destructive technique such as small amplitude oscillatory rheology is suitable compared to an ultrasonic spectroscopy method. The acid-induced and heat-induced gelation have been studied for many years. However, salts-induced gels have not been studied until recently. Magnesium-induced gelation has not been studied in detail which deserves further investigation for a better understanding of the role of magnesium on milk gelation.

Chapter 3

Materials and Methodology

3.1 Introduction

This research was carried out to study the effect of magnesium chloride addition to skim milk at different pH values and temperatures. The magnesium distribution between the serum and micellar phases was determined and the gelation process after magnesium chloride addition was monitored using a rheological method. The serum was separated from skim milk using a micro-centrifugation process. The quantification of total magnesium in skim milk and serum was performed by a complexometric titration. Atomic absorption spectroscopy was also used to validate the results of the complexometric titration. Ionic magnesium (Mg^{2+}) was determined using the spectrophotometric method.

3.2 Materials

Low heat skim milk powder was supplied by Fonterra Co-operative Group, New Zealand. The skim milk specification was reported as; protein 32.63% (w/w); fat 0.92% (w/w); moisture 3.76% (w/w); whey protein nitrogen index (WPNI) 6.3% (w/w) and protein in milk solids non-fat (MSNF) 34.23% (w/w). Patton-Reeder (indicator grade, Sigma-Aldrich, India) and Eriochrome Black T (EBT) (indicator grade, Sigma-Aldrich, USA) were used in this study as indicators for complexometric titration. The fluorescence dye (Magnesium 510 probe) was obtained from Ursa BioScience (Maryland, USA) (Ursa BioScience, 2017) and dichloromethane (CH_2Cl_2) (HPLC grade, Fisher Chemical, UK) was used to reconstitute the dye. All other reagents $MgCl_2.6H_2O$ (analytical grade, Sigma-Aldrich, Switzerland), NaOH (analytical reagent grade, Fisher Chemical, UK), NH_4OH (ACS reagent grade, Sigma-Aldrich, USA), HCl (analytical grade, Fisher Chemical, UK), ethanol (analytical reagent grade, Lab Serve-Thermo Fisher Scientific, New Zealand) and EDTA (ACS reagent grade, Sigma-Aldrich, USA) were used in this study.

3.3 Experimental procedures

3.3.1 Preparation of reconstituted skim milk samples

Reconstituted skim milk samples of 12% solids (w/w) were prepared fresh for each batch of experiment using skim milk powder with distilled water at 20 ± 1 ^{o}C. Sodium azide (0.02% w/v) (research grade, SERVA, Germany) was added as a preservative. Skim milk powder was weighed and the required amount of water was added. Then the skim milk solution was stirred for 3 h with a magnetic stirrer. The skim milk solution left for at least 10 h at 20 ± 1 ^{o}C to complete the hydration of protein before use. The skim milk solution was stored at 20 ± 1 ^{o}C and no samples which were older than 72 h were analysed, ensuring fresh samples were used consistently for this experiment.

3.3.2 Preparation of magnesium chloride stock solution

Magnesium chloride stock solution of 1 mol L^{-1} was prepared with distilled water in which 102.67 g of magnesium chloride ($MgCl_2$. $6H_2O$, 99%) was added into a 500 mL volumetric flask and made up with distilled water to the mark. One mole of magnesium chloride dissociates to one mole of magnesium ion (Mg^{2+}).

3.3.3 Preparation of magnesium chloride added skim milk samples

Milk solids in the reconstituted skim milk were adjusted to 10% (w/w) after the addition of magnesium chloride stock solution which was added to achieve final magnesium chloride concentrations from 0 to 40 mmol L^{-1}.

3.3.4 Preparation of EDTA and indicator solutions

An EDTA solution of 0.10 mol L^{-1} was prepared by dissolving 37.22 g of EDTA with distilled water and made up to one litre. The solution was further diluted to prepare 0.01 mol L^{-1} EDTA solution. Patton-Reeder indicator (0.5% w/v) was prepared in 0.10 mol L^{-1} NaOH. Eriochrome Black T (EBT) indicator was prepared by dissolving one gram of EBT indicator in 50% ethanol in a 100 mL volumetric flask. The indicators were prepared fresh on the day of testing.

3.3.5 Preparation of reconstituted fluorescence dye and miscellaneous solutions

A stock solution of dye (Magnesium 510 probe) was prepared by adding 50 µL of CH_2Cl_2 into the vial containing the dye. Then the concentration of the dye became 4.4 mmol L^{-1} (4400 µmol L^{-1}).

HCl (0.10 mol L^{-1} and 2 mol L^{-1}), NaOH (0.10 mol L^{-1} and 8 mol L^{-1}) and NH_3 solution (2 mol L^{-1}) were prepared by taking the required amount of reagent in distilled water using volumetric flasks.

3.3.6 Separation of skim milk serum from reconstituted skim milk

A benchtop micro-centrifuge (Himac, Alphatech CT 15RE, Hitachi, Japan) was used to separate milk serum from reconstituted skim milk. Micro-centrifugation was carried out at 21,500 × *g* for 90 min at 20 °C. After 90 min, the sediment (micellar) and serum phases were separated by decanting the serum into 60 mL polystyrene (PS) yellow cap container. The serum was then subjected to the magnesium quantification.

3.3.7 pH adjustment of skim milk samples

The pH of the reconstituted skim milk and its serum were measured and adjusted using a pH meter (Sartorius, basic pH meter PB-20). The pH probe was calibrated prior to measurements using pH 4.00 and pH 7.00 buffer solutions (Lab serve-Biolab, Australia). All the calibrations and measurement were carried out at 20 ± 1 °C. The pH (original pH of milk is 6.70) of the skim milk solutions was adjusted from pH 5.50 to 7.20 with HCl or NaOH and then micro-centrifugation was performed to recover the serum.

3.3.8 Preheat treatment of skim milk samples

For analysis of magnesium distribution, the reconstituted skim milk at 12% solids (w/w) was heated in a water bath at 30 to 80 ± 1 °C. For the rheological study, the reconstituted skim milk at 12% solids (w/w) was heated in a water bath at 90 ± 1 °C. For both situations, the milk samples were placed in 50 mL test tubes and then placed in a hot water bath. The temperature of the samples reached the required temperature (30 to 90 ± 1 °C) within 2 to 5 min. The samples were held at the required temperature for 10 min and then cooled down

to 20 ± 1 ^{o}C within 5 min in an ice water bath. Milk solids were then adjusted to 10% solids (w/w) by adding distilled water or magnesium chloride stock solution to achieve the final magnesium chloride concentration at 0 mmol L^{-1} or 15 mmol L^{-1} (for magnesium distribution analysis) and 0 to 20 mmol L^{-1} (for rheological analysis). Following this, the pH was measured and the samples were then subjected to micro-centrifugation to recover the serum for analysing magnesium distribution or the rheometer for rheological analysis.

3.3.9 Different concentrations of magnesium chloride addition

Different amounts of the magnesium chloride stock solution were added into 12% solids (w/w) skim milk solution and milk solids were adjusted to 10% solids (w/w), at 20 ± 1 ^{o}C to achieve magnesium chloride concentrations ranging from 0 to 40 mmol L^{-1}. Two sets of samples were prepared; the pH of the first set of samples after addition of magnesium chloride was checked and the samples were referred to as unadjusted pH (original pH after magnesium chloride added) samples. The pH of the second set of samples was adjusted to pH 6.70 or 6.50, these samples were referred to as adjusted pH samples.

A schematic flowchart for profiling the magnesium distribution under different conditions such as varying the pH, the addition of different concentrations of magnesium chloride or with heat treatment of skim milk at a different temperature is shown in Figure 3.1.

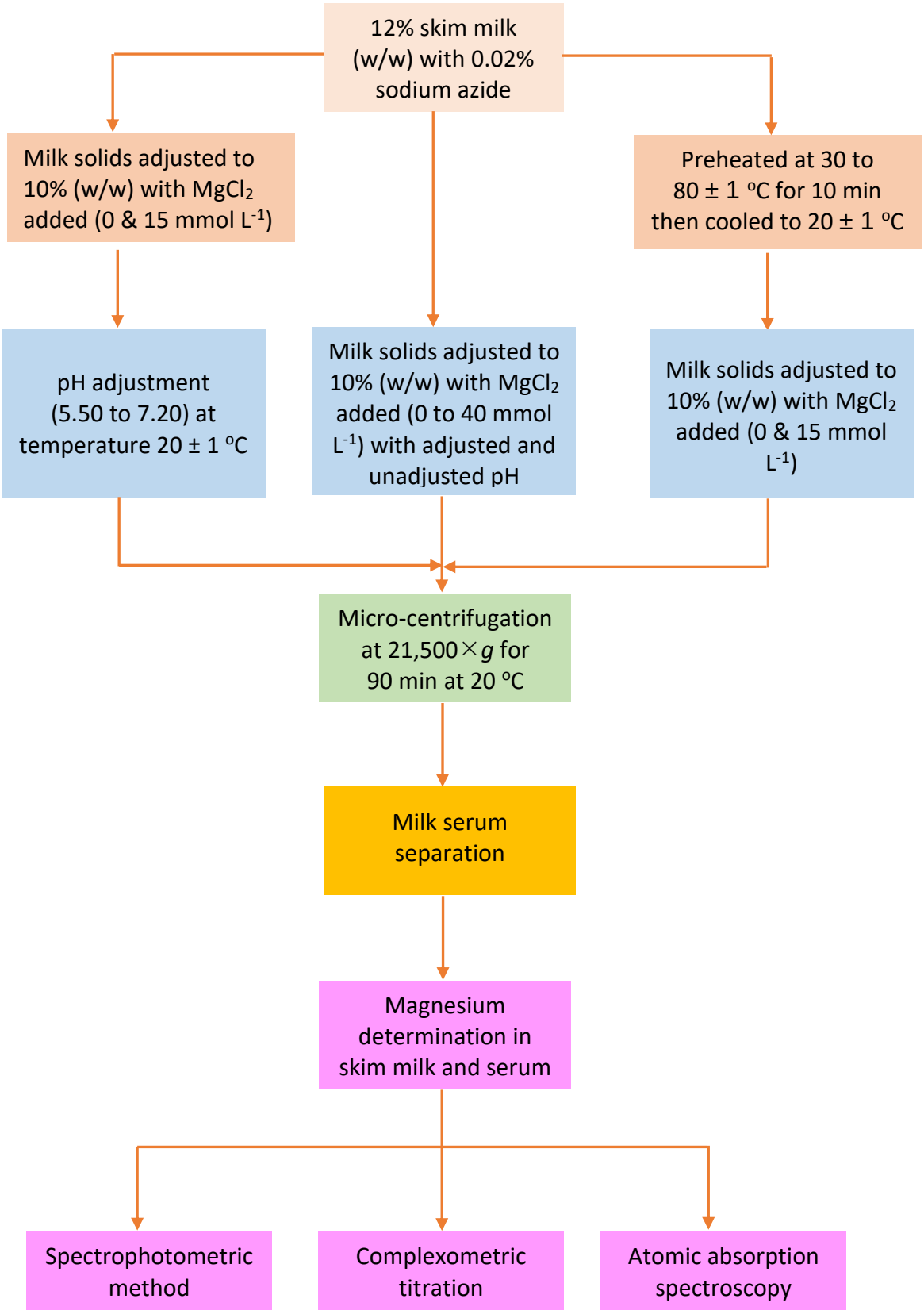

Figure 3.1. Schematic diagram of magnesium quantification in skim milk and milk serum.

3.3.10 Complexometric titration method for determination of total magnesium

The magnesium concentration in skim milk and serum was determined using a modified EDTA titration method described by Jiménez, Gil, Corvillo, and Díez (1988). One millilitre of magnesium chloride standard solution at each concentration (ranging from 0 to 80 mmol L^{-1}), reconstitute skim milk or skim milk serum samples were placed in a conical flask and diluted to 50 mL with distilled water and then titration was carried out.

(i) Calibration curve: A series of magnesium chloride solutions ranging from 0 to 80 mmol L^{-1} were prepared to provide a standard curve. The standard solution's pH was adjusted to pH 10.50 with ammonia solution (Jiménez et al., 1988). Two drops of EBT indicator were added to the samples and the samples were then titrated with 0.01 mol L^{-1} EDTA until the sample colour changed from pink to blue. A calibration curve was constructed and used to determine the unknown concentration of magnesium in skim milk and serum.

(ii) Measurement of calcium: The pH of diluted skim milk or serum solutions were adjusted to pH 12.50 with 8 mol L^{-1} NaOH and 0.10 mol L^{-1} NaOH. The samples were allowed to stand for 5 min with occasional swirling to allow any magnesium present to precipitate (Lin et al., 2018). Two drops of Patton-Reeder were added and mixed thoroughly giving the pink colour. The solutions were then titrated with EDTA (0.01 mol L^{-1}) until the pink color changed to blue.

(iii) Measurement of total calcium and magnesium: The pH of diluted skim milk or serum solutions were adjusted to pH 10.50 with concentrated ammonia and 2 mol L^{-1} ammonia solution. Two drops of Erio chrome black T (EBT) indicator was added and the solutions were then titrated with EDTA (0.01 mol L^{-1}) until the pink colour changed to blue.

(iv) Measurement of magnesium: Magnesium in skim milk and serum samples were determined indirectly by subtracting the EDTA volume needed for the titration of calcium alone from the titration volume for total calcium and magnesium content. The concentration of magnesium in skim milk and serum was determined from the titration. The concentration of magnesium in the sediment was calculated as the difference between the concentrations of magnesium in skim milk from that in the serum.

3.3.11 Spectrophotometric method for determination of ionic magnesium (Mg^{2+})

(i) Magnesium standard solution for calibration*:* Magnesium chloride (1 mmol L^{-1}) solution was prepared by taking 0.02 g of magnesium chloride and making this up to 100 mL with distilled water. The solution was then further diluted to prepare 1 to 7 µmol L^{-1} magnesium chloride solutions to produce a standard curve.

(ii) Ionic magnesium (Mg^{2+}) determination by fluorescence dye: Fluorescence response was measured using the fluorescence spectrometer (FLUOstar Optima, Alphatech, Auckland, New Zealand) where the excitation wavelength was 355 nm and the emission wavelength was 510 nm. Fluorescence dye, 0.4 µL aliquots, were added to 236 µL of magnesium chloride standard solution and skim milk serum samples (dilution was performed when required). The samples (236 µL) were pipetted into a black titrate plate with 96 wells of 300 µL volume of each well. The 96 well plate (Greinerbio-one, Germany) was placed into a plate reader and the fluorescence intensity was recorded. The fluorescence intensity of the samples was compared to a calibration curve and magnesium ion (Mg^{2+}) concentration was determined.

3.3.12 Atomic spectroscopic method for determination of total magnesium

An atomic absorption spectrometer (Xplor AA - dual, GBC Scientific, Australia) at Institute of Fundamental Sciences, Massey University, Palmerston North, New Zealand was used for total magnesium quantification in skim milk and skim milk serum samples. A series of standard solutions (0 to 6 mg mL^{-1}) were prepared and a standard curve was constructed. For the skim milk sample, 0.10 mL was taken into an Eppendorf centrifuge tube and made up to 10 mL with 5000 mg L^{-1} lanthanum chloride ($LaCl_3$) (analytical grade, Sigma-Aldrich, USA) solution. The solution was centrifuged for 6 min at 2000 rpm and the supernatant was then taken for the measurement. For serum samples, the determination was carried out directly or with dilution where necessary.

3.3.13 Preparation of magnesium-induced skim milk gels

Magnesium-induced skim milk gels were prepared with preheated and non-preheated skim milk samples in a water bath and in a rheometer with the addition of different concentrations

of magnesium chloride (0 to 40 mmol L^{-1}). The overall experimental flowchart for magnesium-induced gel preparation is shown in Figure 3.2.

3.3.14 Preparation of magnesium-induced skim milk gels in a water bath

For the preparation of milk gel in a water bath, both preheated and non-preheated skim milk samples (10% w/w solids), containing the different concentrations of magnesium chloride (ranging from 0 to 40 mmol L^{-1}) were placed in 50 mL test tubes and then placed in a hot water bath. Within 3 to 5 min, the temperature of the samples was reached the required temperature (50 to 85 ± 1 ^{o}C). The samples were held in a water bath at required temperatures with varying holding times from 10 to 60 min. The samples were then cooled to room temperature (20 ± 1 ^{o}C) within 5 min in an ice water bath. The gelation time and temperature of the skim milk in the test tubes were then monitored and observed.

3.3.15 Preparation and analysis of magnesium-induced skim milk gels in the rheometer

Rheological analysis of magnesium-induced skim milk samples was carried out with a rheometer (AR 550, TA Instruments, Delaware, USA). The analysis was performed using a stainless-steel cone and plate geometry with cone angle 2^{o} and diameter 40 mm. The truncation gap was 200 µm to monitor the gelation process and the properties of skim milk gels. A small aliquot of the skim milk samples (10% w/w solids) with varying magnesium chloride addition (0 to 20 mmol L^{-1}) was loaded on to the plate then the cone was lowered on to the skim milk samples.

The outer surface of the sample on the plate was covered with a thin layer of canola oil and an evaporation trap was used to prevent drying out of the samples during heating. The samples were first heated from 20 to 80 ^{o}C at 5 ^{o}C increments per min and then held at 80 ^{o}C for 10 to 60 min and cooled to 20 ^{o}C at 5 ^{o}C decrements per min. To monitor the gelation process (*G*' and *G*" changes), a temperature sweep and time sweep was carried out at a constant strain of 0.05% and a constant frequency of 0.1 Hz. The gel was then subjected to a frequency sweep from 0.01 to 10 Hz at 20 ^{o}C at a constant strain of 0.05%, followed by strain sweep from 0.10 to 300% to measure the oscillation stress on the sample at 20 ^{o}C at a constant 0.1 Hz frequency.

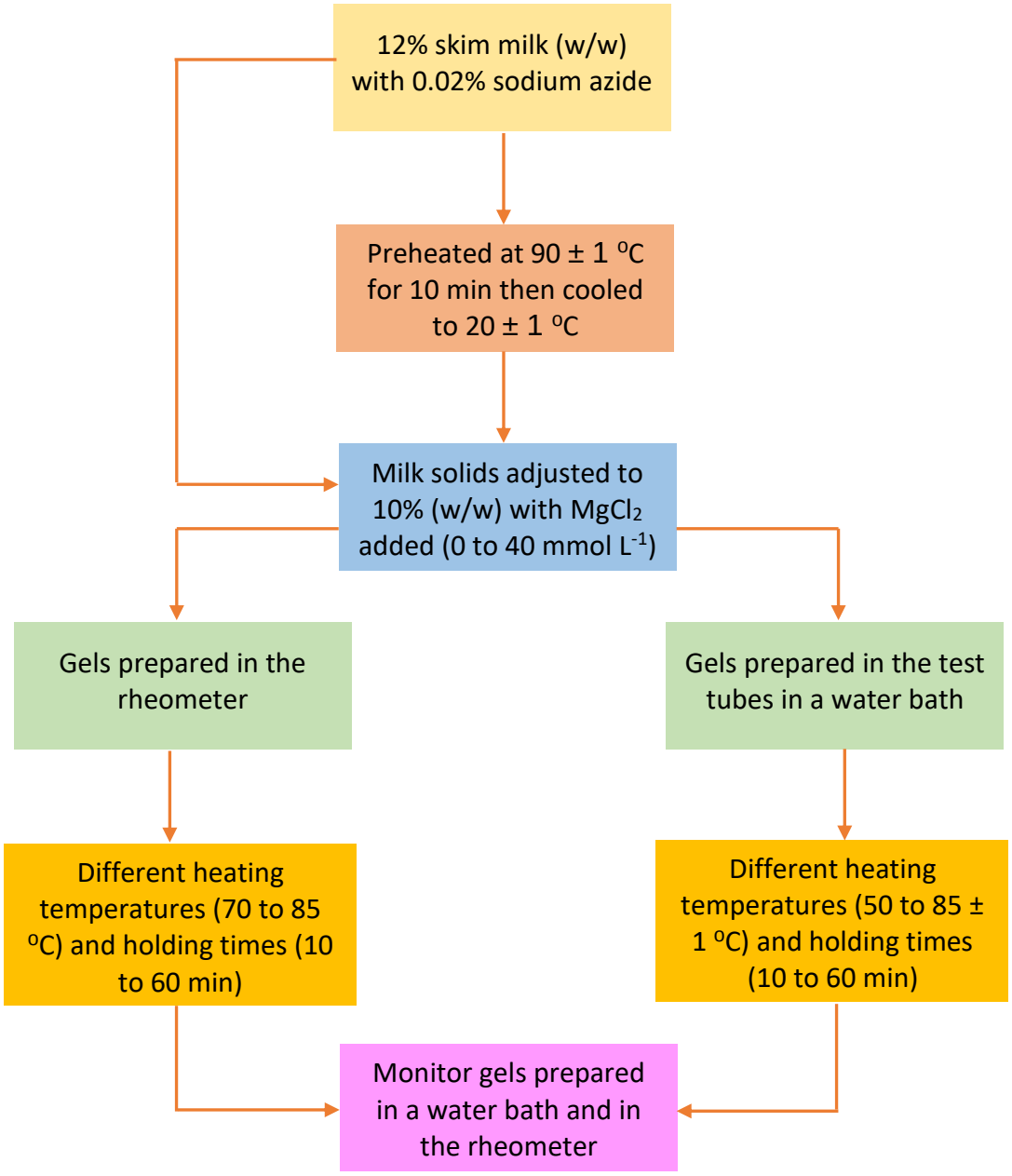

Figure 3.2. Flowchart for the preparation of skim milk gels with added magnesium chloride in a water bath and in the rheometer.

3.4 Statistical analysis

All the experiments in this study were performed in duplicates. Each experiment was carried out with two replicates. Statistical analysis was performed by one way ANOVA at the 95% significance level using Minitab 18 statistical software package (Minitab Inc. Chicago). The comparisons were performed using the Tukey Pairwise Comparisons method presented in detail in Appendix 9.1.

Chapter 4

Distribution and Quantification of Magnesium in Skim Milk

4.1 General introduction

Salts in milk and milk products play an important role in the stability of protein present and on the nutritional value of these products. They exist in a dynamic equilibrium between the serum and micellar phases and their equilibria exhibit notable shifts toward the micellar or serum phases as a function of pH, temperature, casein concentration and other parameters such as the addition of other salts. Thus, the supplementation of milk with soluble calcium or magnesium salt is challenging because this supplementation modifies the ionic environment and destabilises the milk proteins. Free divalent cations, particularly Ca^{2+} and Mg^{2+}, significantly influence the surrounding environment of negatively charged casein micelles and thus enhance or reduce the repelling force between them. A number of studies have been published to identify the role of free divalent cations during production of dairy products due to their importance in dairy products (Koutina, Knudsen, & Skibsted, 2015; Koutina et al., 2016; Ramasubramanian et al., 2013). Magnesium in milk and milk products is a major contributor of dietary magnesium, but much less is known about its technical functionality in dairy products because the role of magnesium in bovine milk has not been studied in detail (Oh & Deeth, 2017). It is suggested that magnesium salts with the greatest aqueous solubility resulted in the highest bioavailability of magnesium (Lindberg, Zobitz, Poindexter, & Pak, 1990). Thus, profiling magnesium distribution is important for understanding the bioavailability of magnesium-enriched dairy products. In this study, a fundamental analysis was performed to investigate the effect of pH, preheating temperatures and the addition of magnesium chloride to skim milk on the magnesium distribution between the serum and micellar phases. The concentration of total magnesium in milk and serum was measured using EDTA titration and the result was validated using the atomic absorption spectroscopy (AAS) technique. The amount of magnesium in the sediment (micellar) was calculated from the difference between the total magnesium concentrations in milk and the serum phases. Ionic magnesium (Mg^{2+}) has a significant effect on milk stability. Limited studies have been performed on detailed investigations of ionic magnesium (Mg^{2+}) in milk under natural or

modified conditions (Gao et al., 2009). Thus, this study attempted to determine ionic magnesium (Mg^{2+}) in milk samples using a magnesium fluorescence dye (Magnesium 510 probe).

4.2 Magnesium ion and milk pH

The effect of the addition of magnesium chloride on the pH and ionic magnesium (Mg^{2+}) concentration of skim milk is presented in Figure 4.1. The addition of magnesium chloride salt resulted in a significant reduction in the skim milk pH ($P < 0.05$) and an increase in the serum ionic magnesium (Mg^{2+}) concentration at 20 ± 1 °C ($P < 0.05$) for both preheated and non-preheated milk samples. The decrease in pH observed with the addition of soluble salts has been reported previously and it is the ion concentration that leads to the reduction of milk pH (Ramasubramanian et al., 2013). The addition of salts disrupts the dynamic ionic equilibrium existing in milk and thus causes a decrease in the pH. The added magnesium salt dissociates in solution to release magnesium ions and these ions may bind to the available citrate and phosphate ions in milk to form magnesium citrate and magnesium phosphate, respectively which disturbs the equilibria between citrate ions ($HCitrate^{2-}$ and $Citrate^{3-}$) and between phosphate ions (HPO_4^{2-} and $H_2PO_4^-$) in milk. In general, when $MgHPO_4$ forms from Mg^{2+} and HPO_4^{2-}, more $H_2PO_4^-$ ions will be converted to HPO_4^{2-} to restore the equilibrium, resulting in a concomitant release of H^+ ions and thus a reduction in the milk pH (Lin et al., 2018).

In addition, preheating of skim milk at 90 ± 1 °C for 10 min resulted in a slight decrease in pH compared to non-preheated skim milk samples (Table 4.1). For preheated skim milk samples without added magnesium chloride the pH reduction was not significant ($P > 0.05$) compared to samples with added magnesium chloride (15 mmol L^{-1}) where it was significant ($P < 0.05$) (Table 4.1). This reduction in pH on preheating was also found by Ramasubramanian et al. (2014). However, the decrease in pH due to preheating without the addition of magnesium chloride was very small and was noted in this study because the measurement was performed at 20 ± 1 °C within 10 min of preheating.

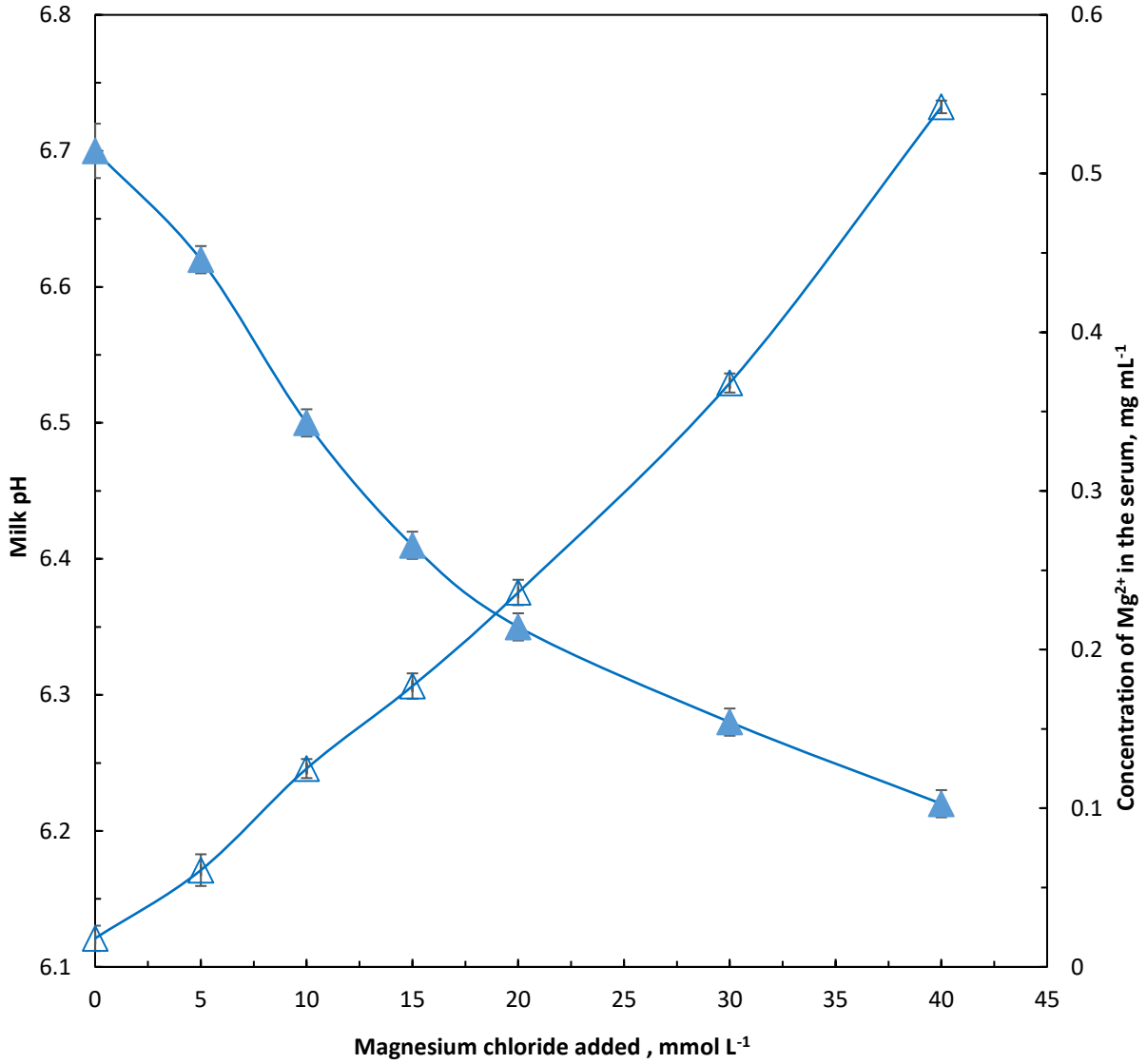

Figure 4.1. Effect of the concentration of added magnesium chloride on the pH and magnesium ion (Mg^{2+}) concentration of skim milk. The closed symbols indicate the milk pH and open symbols show the concentration of ionic magnesium (Mg^{2+}) in serum for non-preheated skim milk samples. The data points show the mean values ± standard deviations (n = 4).

In general, the pH decrease observed after heating without any addition of magnesium chloride disappeared after 24 h, at which point the pH returned to the original natural pH. Heating increases the dissociation of inorganic phosphate in the serum phase of milk resulting in the formation of calcium phosphate and magnesium phosphate and these can be reversed upon cooling (Zoon, 1988). However, the reduction of the pH obtained by heating and by the addition of magnesium chloride is irreversible indicating the existence of a magnesium equilibrium in the milk system (Abdulghani et al., 2015).

Table 4.1: pH of preheated and non-preheated skim milk samples at various concentrations of added magnesium chloride and 10 min after addition (n = 4). Different superscript letters (a, b, c, d, e, f, g) indicate significant difference across the magnesium chloride concentration added and different Greek letters (λ, ρ, ϕ, ω) indicate significant difference across the different preheating temperatures (95% confidence level).

Preheat temperature, °C	pH at 20 ± 1 °C						
	Concentration of added magnesium chloride (mmol L^{-1})						
	0	5	10	15	20	30	40
20	$6.70^{a,\lambda}$	6.62^{b}	6.50^{c}	$6.41^{d,\lambda}$	6.35^{e}	6.28^{f}	6.22^{g}
30	6.70^{λ}			$6.40^{\lambda,\rho}$			
45	6.70^{λ}			$6.40^{\lambda,\rho}$			
60	6.68^{λ}			$6.38^{\lambda,\rho,\phi}$			
70	6.68^{λ}			$6.36^{\rho,\phi,\omega}$			
80	6.68^{λ}			$6.35^{\phi,\omega}$			
90	$6.68^{a,\lambda}$	6.50^{b}	6.43^{c}	$6.34^{d,\omega}$	6.30^{e}	6.20^{f}	6.12^{g}

Pooled standard deviation 0.01

4.3 Quantification of magnesium in the serum and sediment (micellar) phases of skim milk

4.3.1 Effect of pH

The magnesium distribution at different pH values (pH 5.50 to 7.20) in natural skim milk and milk enriched with magnesium chloride (15 mmol L^{-1}) is presented in Table 4.2. The addition of 15 mmol L^{-1} magnesium chloride to the milk significantly decreased the pH in the natural milk pH from 6.70 to 6.41 ($P < 0.05$). After magnesium chloride addition, the milk pH was then adjusted to a value in the range of pH 5.50 to 7.20 (detail procedure is given in Section 3.3). The concentration of total magnesium was determined by EDTA titration and the AAS technique was used to validate the EDTA results. The EDTA method was unable to determine ionic magnesium (Mg^{2+}) in milk thus a fluorescence dye was used to determine ionic magnesium (Mg^{2+}) in milk. Hence, ionic magnesium (Mg^{2+}) was determined using a commercial fluorescence dye (Magnesium 510 probe) and results are also presented in Table 4.2.

Table 4.2: Concentration of total magnesium in milk, serum and sediment (calculated) at different pH values determined by EDTA titration and AAS methods and concentration of ionic magnesium (Mg^{2+}) in serum determined by Magnesium 510 probe method. Different superscript letters (a, b, c, d, e) indicate significant difference across different pH values, different Greek letters (λ, ρ) indicate significant difference across magnesium chloride added and different letters (x, y) indicate significant difference across EDTA and AAS determination methods (95% confidence level).

Conc. of added magnesium chloride (mmol L^{-1})	pH	Determination by EDTA titration				Determination by AAS method			Determination by Mg 510 probe	
		Total magnesium (mg mL^{-1})			% of magnesium	Total magnesium (mg mL^{-1})			Ionic magnesium (Mg^{2+}) (mg mL^{-1})	% of ionic Mg (Mg^{2+})
		skim milk	serum	sediment	serum	skim milk	serum	sediment	serum	serum
0	5.50	$0.135 \pm 0.011^{a,\lambda}$	$0.127 \pm 0.013^{a,\lambda}$	$0.008 \pm 0.002^{c,\lambda}$	94.07		-	-	$0.041 \pm 0.004^{a,\lambda}$	32.28
	5.70	$0.137 \pm 0.010^{a,\lambda}$	$0.121 \pm 0.012^{a,\lambda}$	$0.016 \pm 0.002^{b,c,\lambda}$	88.32	-	-	-	$0.035 \pm 0.004^{a,b,\lambda}$	28.92
	6.00	$0.136 \pm 0.013^{a,\lambda}$	$0.115 \pm 0.015^{a,\lambda,x}$	$0.021 \pm 0.007^{a,b,c,\lambda}$	84.55		$0.106 \pm 0.001^{a,x}$		$0.032 \pm 0.005^{a,b,\lambda}$	27.82
	6.20	$0.137 \pm 0.010^{a,\lambda}$	$0.112 \pm 0.011^{a,\lambda}$	$0.025 \pm 0.001^{a,b,c,\lambda}$	81.75	-	-	-	$0.030 \pm 0.008^{a,b,\lambda}$	26.78
	6.50	$0.137 \pm 0.011^{a,\lambda}$	$0.107 \pm 0.013^{a,\lambda}$	$0.030 \pm 0.003^{a,b,c,\lambda}$	78.10	-	-	-	$0.026 \pm 0.009^{a,b}$	24.30
	6.70*	$0.137 \pm 0.013^{a,\lambda,x}$	$0.100 \pm 0.010^{a,\lambda,x}$	$0.037 \pm 0.002^{a,b,\lambda,x.y}$	73.00	0.138 ± 0.001^{x}	$0.090 \pm 0.001^{b,\lambda,x}$	$0.048 \pm 0.001^{x,y}$	$0.018 \pm 0.007^{a,b,\lambda}$	18.00
	7.00	$0.136 \pm 0.011^{a,\lambda}$	$0.095 \pm 0.011^{a,\lambda}$	$0.041 \pm 0.003^{a,\lambda}$	69.85	-	-	-	$0.016 \pm 0.006^{a,b,\lambda}$	16.84
	7.20	$0.137 \pm 0.013^{a,\lambda}$	$0.087 \pm 0.010^{a,\lambda}$	$0.050 \pm 0.008^{c,\lambda}$	63.50	-	-	-	$0.014 \pm 0.007^{b,\lambda}$	16.09
15	5.50	$0.492 \pm 0.011^{a,\rho}$	$0.454 \pm 0.011^{a,\rho}$	$0.038 \pm 0.000^{e,\rho}$	92.27	-	-	-	$0.200 \pm 0.006^{a,\rho}$	44.05
	5.70	$0.490 \pm 0.010^{a,\rho}$	$0.448 \pm 0.013^{a,\rho}$	$0.042 \pm 0.003^{e,\rho}$	91.42	-	-	-	$0.183 \pm 0.007^{a,\rho}$	40.84
	6.00	$0.493 \pm 0.014^{a,\rho}$	$0.438 \pm 0.018^{a,\rho}$	$0.055 \pm 0.004^{d,e,\rho}$	88.84	-	-	-	$0.156 \pm 0.005^{b,\rho}$	35.61
	6.20	$0.493 \pm 0.010^{a,\rho}$	$0.427 \pm 0.011^{a,\rho}$	$0.066 \pm 0.001^{d,\rho}$	86.61	-	-	-	$0.147 \pm 0.004^{b,c,\rho}$	34.42
	6.50	$0.492 \pm 0.012^{a,\rho}$	$0.418 \pm 0.022^{a,b,\rho,x}$	$0.074 \pm 0.010^{c,d,\rho}$	84.96	-	$0.407 \pm 0.002^{a,x}$	-	$0.139 \pm 0.004^{b,c,d,\rho}$	33.25
	6.70	$0.492 \pm 0.011^{a,\rho}$	$0.397 \pm 0.015^{a,b,\rho,x}$	$0.095 \pm 0.004^{c,\rho}$	80.70	-	$0.390 \pm 0.004^{b,\lambda,x}$	-	$0.131 \pm 0.008^{c,d,\rho}$	33.00
	7.00	$0.490 \pm 0.010^{a,\rho}$	$0.359 \pm 0.021^{b,c,\rho}$	$0.131 \pm 0.011^{b,\rho}$	73.26	-	-	-	$0.116 \pm 0.008^{d,e,\rho}$	32.31
	7.20	$0.489 \pm 0.010^{a,\rho}$	$0.317 \pm 0.010^{c,\rho}$	$0.172 \pm 0.000^{a,\rho}$	64.82	-	-	-	$0.094 \pm 0.004^{e,\rho}$	29.65

- represents not detected, * represents natural milk pH, Conc. represents concentration, Mg represents magnesium

Percentage of serum magnesium was calculated based on total magnesium in milk

Percentage of ionic magnesium was calculated based on serum magnesium in milk

In all the samples (with and without added magnesium chloride), as the pH was decreased from 6.70 to 5.50, the total soluble magnesium and ionic magnesium (Mg^{2+}) in the serum phase increased. Specifically, ionic magnesium (Mg^{2+}) increased significantly for both added and no added magnesium chloride ($P < 0.05$), whereas the change in total soluble magnesium in the serum phase with no added magnesium chloride was not significant ($P > 0.05$). The concentration of total soluble magnesium in the serum phase increased by 5.10% as the pH decreased from pH 6.70 to 6.50, then only increased by 10.22% from 6.50 to pH 5.70 and then below pH 5.70 there was 5.75% increase. More than 90% magnesium (total magnesium) was found in the serum phase in both with and without added magnesium chloride samples. These results agreed with those reported previously for magnesium distribution in milk (Dalgleish & Law, 1989).

Using the AAS technique, the total soluble magnesium in the serum phase significantly ($P < 0.05$) increased as the pH decreased from pH 6.70 to pH 6.50 and from pH 6.70 to pH 6.00 for both added and no added magnesium chloride, respectively. For EDTA titration, the change in total soluble magnesium in the serum phase was significant (from pH 6.70 to pH 6.50) for magnesium added sample whereas for no added magnesium solution the change in total soluble magnesium in the serum phase was not significant (from pH 6.70 to pH 6.00). Comparing the EDTA titration method and the AAS technique, the total soluble magnesium concentration in the serum phase was not significantly different ($P > 0.05$) regardless of magnesium chloride was added or not. These findings indicate that complexometric titration using EDTA was a good method for the determination of total soluble magnesium in the serum phase of skim milk enriched with magnesium. However, for all the samples, the higher amount of magnesium was obtained using the EDTA method compared to the AAS technique. It has been reported that all types of casein were dissociated from the micelles during acidification from pH 6.70 to 4.60 at 20 ^{o}C (Anema & Klostermeyer, 1997). The higher liberation of casein from the micelle and the reduced binding of magnesium to casein at lower pH correlates to the increase in magnesium ions in milk serum observed during acidification in this study. The micelles in magnesium-enriched skim milk (15 mmol L^{-1}) contained more magnesium (0.038 ± 0.011 mg mL^{-1} at pH 5.50) than those in skim milk with no added magnesium (0.008 ± 0.013 mg mL^{-1} at pH 5.50), even though the percentage of total magnesium in the serum phase (92%) was lower in magnesium-enriched skim milk than in skim milk with no added magnesium chloride (94%). At pH values ranging from pH 6.70 to

5.50, skim milk enriched with magnesium chloride may result in a higher micellar association with magnesium than skim milk with no added magnesium chloride (Gastaldi, Pellegrini, Lagaude, & de la Fuente, 1994). The addition of magnesium chloride to skim milk results in the increases in all forms of magnesium (soluble, ionic and micellar) significantly compared to no added magnesium chloride ($P < 0.05$). At a natural pH with no added magnesium chloride, 73% of the magnesium in skim milk remains in the soluble form with 18% in the ionic magnesium (Mg^{2+}) form (Table 4.2), in agreement with a previous measurement obtained using the Donnan membrane technique (Gao et al., 2009).

During acidification, the magnesium equilibria between micellar and serum phases are disturbed and solubilisation of colloidal calcium phosphate (CCP) causes more magnesium to be transferred from the micelles in milk to the serum phase. As the CCP solubilised, the magnesium associated with CCP is also solubilised and transferred to the serum phase (Gaucheron, 2011). Calcium also showed the same behaviour: decreases in the pH increases the calcium ion (Ca^{2+}) concentration in the serum phase (Koutina et al., 2015).

During skim milk alkalisation (Table 4.2), the magnesium concentration in the serum phase decreased as the pH was increased from 6.70 to 7.20, regardless of whether magnesium chloride was added or not. Consequently, micellar magnesium increased at higher pH values. Jeantet et al. (2016) and Gaucheron (2011) suggested that during alkalisation, minerals transferred to the micellar phase. At the pH range of 6.70 to 7.20, the magnesium that remained bound to casein in the magnesium-enriched milk was greater than that found in milk with no added magnesium chloride. Magnesium in the serum phase was found as ionic (Mg^{2+}) and soluble magnesium form and in the micellar phase could be bound to casein either directly as Mg^{2+} or indirectly through salts, most likely phosphate, through an ester phosphate group (Dalgleish et al., 2004).

4.3.2 Effect of preheating temperature

The amounts of serum, micellar and ionic (Mg^{2+}) magnesium at different preheating temperatures determined by EDTA titration, AAS and Magnesium 510 probe methods are presented in Table 4.3. The concentration of total magnesium and ionic magnesium (Mg^{2+}) in the serum remained constant with increasing preheat temperature for both added (15 mmol L^{-1}) and no added magnesium chloride to skim milk.

Table 4.3: Concentration of total magnesium in milk, serum and sediment (calculated) at different preheating temperatures determined by EDTA titration and AAS methods and concentration of ionic magnesium (Mg^{2+}) in serum determined by Magnesium 510 probe method. Superscript letter (a) indicates significant difference across different pH values, different Greek letters (λ, ρ) indicate significant difference across magnesium chloride added and different letters (x, y) indicate significant difference across EDTA and AAS determination methods (95% confidence level).

Concentration of magnesium chloride added mmol L^{-1}	Temp. (°C)	Determination by EDTA titration			Determination by AAS method			Determination by Mg 510 probe
		Total magnesium (mg mL^{-1})						Ionic magnesium (Mg^{2+}) (mg mL^{-1})
		skim milk	serum	sediment	skim milk	serum	sediment	serum
0	20	$0.137 \pm 0.013^{\lambda,x}$	$0.100 \pm 0.010^{a,\lambda,x}$	$0.036 \pm 0.003^{a,x}$	0.138 ± 0.001^{x}	$0.090 \pm 0.001^{a,\lambda,x}$	0.048 ± 0.001^{x}	$0.018 \pm 0.007^{a,\lambda}$
	30		$0.099 \pm 0.012^{a,\lambda}$	0.039 ± 0.002^{a}	-	-	-	$0.020 \pm 0.004^{a,\lambda}$
	45		$0.098 \pm 0.010^{a,\lambda}$	0.040 ± 0.002^{a}	-	-	-	$0.019 \pm 0.007^{a,\lambda}$
	60		$0.100 \pm 0.011^{a,\lambda}$	0.038 ± 0.000^{a}	-	-	-	$0.018 \pm 0.005^{a,\lambda}$
	70		$0.098 \pm 0.012^{a,\lambda}$	0.036 ± 0.000^{a}	-	-	-	$0.020 \pm 0.008^{a,\lambda}$
	80		$0.098 \pm 0.010^{a,\lambda,x}$	0.040 ± 0.010^{a}	-	$0.090 \pm 0.001^{a,x}$	-	$0.019 \pm 0.005^{a,\lambda}$
15	20	$0.492 \pm 0.011^{\rho}$	$0.421 \pm 0.010^{a,\rho}$	0.071 ± 0.021^{a}	-	$0.411 \pm 0.002^{\rho,x}$	-	$0.145 \pm 0.008^{a,\rho}$
	30		$0.418 \pm 0.011^{a,\rho}$	0.074 ± 0.021^{a}	-	-	-	$0.148 \pm 0.007^{a,\rho}$
	45		$0.419 \pm 0.012^{a,\rho}$	0.073 ± 0.024^{a}	-	-	-	$0.147 \pm 0.009^{a,\rho}$
	60		$0.421 \pm 0.014^{a,\rho}$	0.071 ± 0.024^{a}	-	-	-	$0.152 \pm 0.007^{a,\rho}$
	70		$0.418 \pm 0.010^{a,\rho}$	0.074 ± 0.020^{a}	-	-		$0.148 \pm 0.005^{a,\rho}$
	80		$0.421 \pm 0.011^{a,\rho}$	0.071 ± 0.020^{a}	-		-	$0.146 \pm 0.006^{a,\rho}$

- represents not detected, Temp. represents temperature, Mg represents magnesium

This study showed that the distribution of magnesium between the serum and micellar phases was dependent on the pH (5.50 to 7.20) but not on the temperature of preheating 20 to 80 ± 1 ^{o}C). The constant concentration of magnesium in the serum phase during heating can be explained by the low solubility of CCP upon heating (Mekmene, Le Graët, & Gaucheron, 2010).

Koutina et al. (2014) found that the amount of calcium in the serum phase increased after heating at 90 ^{o}C regardless of whether calcium was added or not and Rose (1963) reported that Ca^{2+} in the serum phase increased with increasing temperature. On the other hand, Pouliot et al. (1989) reported a decrease occurred in the concentration of calcium and magnesium in the serum phase when milk was heated to 90 ^{o}C and it was found to redistribute itself upon cooling. Lewis (2011) concluded that heat treatment of milk reduced Ca^{2+} in the serum phase for short time heating and then Ca^{2+} concentration rapidly recovered upon cooling but did not reach its original value.

In contrast, Abdulghani et al. (2015) reported that heating followed by cooling has no effect on the distribution of magnesium between the serum and micellar phases, this is in agreement with this study. To better understand this phenomenon, further studies regarding the pH, ionic magnesium (Mg^{2+}) concentration and preheating temperatures are required.

4.3.3 Effect of magnesium chloride addition

The concentration of total, micellar, serum and ionic (Mg^{2+}) magnesium in skim milk samples after the addition of different concentrations of magnesium chloride between 0 to 40 mmol L^{-1} at different pH values (unadjusted and adjusted to pH 6.50 and 6.70) at 20 ± 1 ^{o}C was determined using EDTA titration, AAS and Magnesium 510 probe and the results are presented in Table 4.4. For all pH values (unadjusted and adjusted pH 6.50 and 6.70), the addition of magnesium chloride to skim milk between concentrations of 0 and 40 mmol L^{-1} resulted in a significant increase ($P < 0.05$) of the concentration of total, serum, micellar and ionic magnesium (Mg^{2+}) in skim milk.

In the samples in which pH was not adjusted, the addition of magnesium chloride (0 to 40 mmol L^{-1}) increased the concentration of micellar magnesium in the milk from 0.037 to 0.099 mg mL^{-1}, whereas the samples with an adjusted pH of 6.50 and 6.70 showed a greater increase

of micellar magnesium. The concentration of micellar magnesium increased from 0.030 to 0.115 mg mL^{-1} at pH 6.50 and from 0.037 to 0.120 mg mL^{-1} at pH 6.70 (Table 4.2 and Table 4.4).

In general, the addition of salts to milk affects the level of CCP, the ion activity, the casein concentration in the serum and micellar phases (Canabady-Rochelle et al., 2007). With the enrichment of milk with magnesium chloride (above 5 mmol L^{-1}), the concentration of ionic magnesium (Mg^{2+}) and serum total magnesium in the samples where pH was not adjusted was higher than that found in the samples with adjusted pH to 6.50 and 6.70, but not significantly higher ($P > 0.05$). For example, in the samples with 20 mmol L^{-1} added magnesium chloride with unadjusted pH (pH 6.35), the serum total magnesium concentration was 0.538 mg mL^{-1} whereas the concentrations obtained after the pH was adjusted to 6.50 and 6.70 were 0.524 mg mL^{-1} and 0.510 mg mL^{-1}, respectively. For total soluble magnesium in the serum phase, the difference between adjusted and unadjusted pH condition was not significant except for 40 mmol L^{-1} magnesium chloride addition. For ionic magnesium (Mg^{2+}) the difference was not significant up to 15 mmol L^{-1} magnesium chloride addition ($P > 0.05$).

Table 4.4: Concentration of total magnesium in milk, serum and sediment (calculated) at adjusted and unadjusted pH and different concentrations of added magnesium chloride determined by EDTA titration and AAS methods and concentration of ionic magnesium (Mg^{2+}) in serum determined by Mg 510 probe method. Different superscript letters (a, b, c, d, e, f, g) indicates significant difference across magnesium chloride addition, different Greek letters (λ, ρ, ϕ) indicate significant difference across magnesium chloride added and different letters (x, y) indicate significant difference across EDTA and AAS determination methods (95% confidence level).

Condition	Con. of added $MgCl_2$ mmol L^{-1}	pH	Determination by EDTA titration			Determination by AAS			Determination by probe
			Total magnesium (mg mL^{-1})						Ionic magnesium (Mg^{2+}) (mg mL^{-1})
			skim milk	serum	sediment	skim milk	serum	sediment	serum
Unadj pH	0	6.70	$0.137 \pm 0.013^{a,\lambda,x}$	$0.100 \pm 0.010^{a,\lambda}$	$0.037 \pm 0.003^{a,\lambda,x}$	0.138 ± 0.001^{x}	$0.090 \pm 0.001^{a,x}$	0.048 ± 0.001^{x}	$0.018 \pm 0.007^{a,\lambda}$
	5	6.62	$0.253 \pm 0.010^{b,\lambda}$	$0.205 \pm 0.014^{b,\lambda}$	$0.048 \pm 0.004^{a,b,\lambda}$	-	0.197 ± 0.001^{b}	-	$0.061 \pm 0.010^{b,\lambda}$
	10	6.50	$0.369 \pm 0.020^{c,\lambda}$	$0.311 \pm 0.010^{c,\lambda}$	$0.058 \pm 0.010^{b,c,\lambda}$	-	0.317 ± 0.001^{c}	-	$0.105 \pm 0.006^{c,\lambda}$
	15	6.41	$0.492 \pm 0.010^{d,\lambda}$	$0.421 \pm 0.014^{d,\lambda}$	$0.071 \pm 0.004^{c,d,\lambda}$	-	$0.411 \pm 0.002^{d,\lambda}$	-	$0.145 \pm 0.008^{d,\lambda}$
	20	6.35	$0.617 \pm 0.010^{e,\lambda}$	$0.538 \pm 0.012^{e,\lambda}$	$0.079 \pm 0.002^{d,e,\lambda}$	-	0.524 ± 0.004^{e}	-	$0.236 \pm 0.008^{e,\lambda}$
	30	6.28	$0.840 \pm 0.020^{f,\lambda}$	$0.749 \pm 0.021^{f,\lambda}$	$0.091 \pm 0.001^{e,f,\lambda}$	-	0.733 ± 0.002^{f}	-	$0.368 \pm 0.006^{f,\lambda}$
	40	6.22	$1.070 \pm 0.010^{g,\lambda}$	$0.971 \pm 0.015^{g,\lambda}$	$0.099 \pm 0.005^{g,\lambda}$	-	0.957 ± 0.005^{g}	-	$0.542 \pm 0.004^{g,\lambda}$
Adj pH	0	6.50	$0.137 \pm 0.010^{a,\lambda}$	$0.107 \pm 0.023^{a,\lambda}$	$0.030 \pm 0.013^{a,\lambda}$	-	-	-	$0.020 \pm 0.006^{a,\lambda}$
	5		$0.251 \pm 0.010^{b,\lambda}$	$0.210 \pm 0.018^{b,\lambda}$	$0.041 \pm 0.008^{a,b,\lambda}$	-	-	-	$0.064 \pm 0.004^{b,\lambda}$
	10		$0.365 \pm 0.020^{c,\lambda}$	$0.307 \pm 0.012^{c,\lambda}$	$0.058 \pm 0.008^{a,b,c,\lambda}$	-	-	-	$0.098 \pm 0.005^{c,\lambda}$
	15		$0.492 \pm 0.012^{d,\lambda}$	$0.418 \pm 0.022^{d,\lambda,x}$	$0.074 \pm 0.010^{b,c,d,\lambda}$	-	$0.407 \pm 0.002^{\lambda,x}$	-	$0.139 \pm 0.004^{d,\lambda}$
	20		$0.610 \pm 0.010^{e,\lambda}$	$0.524 \pm 0.018^{e,\lambda}$	$0.086 \pm 0.008^{c,d,e,\lambda,\rho}$	-	-	-	$0.209 \pm 0.004^{e,\rho}$
	30		$0.810 \pm 0.020^{f,\lambda}$	$0.714 \pm 0.018^{f,\lambda}$	$0.960 \pm 0.002^{d,e,\lambda}$	-	-	-	$0.322 \pm 0.006^{f,\rho}$
	40		$1.040 \pm 0.010^{g,\lambda}$	$0.925 \pm 0.019^{g,\lambda,\rho}$	$0.115 \pm 0.009^{e,\lambda}$	-	-	-	$0.495 \pm 0.008^{g,\rho}$
Adj pH	0	6.70	$0.137 \pm 0.013^{a,\lambda}$	$0.100 \pm 0.010^{a,\lambda}$	$0.037 \pm 0.003^{a,\lambda}$	-	-	-	$0.018 \pm 0.007^{a,\lambda}$
	5		$0.255 \pm 0.010^{b,\lambda}$	$0.200 \pm 0.010^{b,\lambda}$	$0.055 \pm 0.000^{b,\lambda}$	-	-	-	$0.057 \pm 0.006^{b,\lambda}$
	10		$0.367 \pm 0.020^{c,\lambda}$	$0.294 \pm 0.012^{c,\lambda}$	$0.073 \pm 0.008^{c,\lambda}$	-	-	-	$0.094 \pm 0.004^{c,\lambda}$
	15		$0.492 \pm 0.011^{d,\lambda}$	$0.397 \pm 0.015^{d,\lambda,x}$	$0.095 \pm 0.004^{d,\lambda}$	-	$0.390 \pm 0.004^{\rho,x}$	-	$0.131 \pm 0.008^{d,\lambda}$
	20		$0.612 \pm 0.010^{e,\lambda}$	$0.510 \pm 0.014^{e,\lambda}$	$0.102 \pm 0.014^{d,e,\rho}$	-	-	-	$0.193 \pm 0.005^{e,\rho}$
	30		$0.820 \pm 0.020^{f,\lambda}$	$0.705 \pm 0.016^{f,\lambda}$	$0.115 \pm 0.004^{e,f,\rho}$	-	-	-	$0.288 \pm 0.004^{f,\phi}$
	40		$1.020 \pm 0.010^{g,\lambda}$	$0.900 \pm 0.011^{g,\rho}$	$0.120 \pm 0.001^{f,\lambda}$	-	-	-	$0.460 \pm 0.007^{g,\phi}$

- represents not detected, Unadj and adj represent unadjusted and adjusted, respectively and con. represents concentration, Mg represents magnesium

For the AAS technique, the total soluble magnesium in the serum phase significantly increased as magnesium chloride was added ($P < 0.05$). Comparing the EDTA titration method and the AAS technique, the total soluble magnesium concentration in the serum phase was not significantly different ($P > 0.05$) for 15 mmol L^{-1} magnesium chloride sample.

The addition of magnesium chloride to skim milk resulted in a decrease in the pH and an increase in the ionic magnesium (Mg^{2+}) concentration, which subsequently increased the serum total magnesium concentration. Moreover, increasing the pH decreased the serum total magnesium concentration due to less solubilisation of CCP (Lewis, 2011). The addition of magnesium salt to milk increased the serum total and ionic magnesium (Mg^{2+}) with a concomitant increase of micellar magnesium concentration. It can be concluded that the amount of ionic magnesium (Mg^{2+}) increased with increasing in the amounts of added magnesium chloride, which suggested that a certain amount of added magnesium will remain as ionic magnesium (Mg^{2+}) in the serum phase and the remainder is bound in the serum phase with chloride, phosphate and citrate due to the protonation of phosphate and citrate during milk acidification, in much the same way as does calcium salt addition to milk (Koutina et al., 2015).

4.4 Conclusions

Acidification significantly influences the distribution of total soluble magnesium and ionic magnesium (Mg^{2+}) in milk. During acidification in the pH range 6.70 to 5.50, the total soluble magnesium and ionic magnesium (Mg^{2+}) in the serum phase increased. At pH 5.50, most of the magnesium remained in the serum phase. Addition of magnesium chloride to skim milk increases both ionic, total soluble magnesium and micellar magnesium concentration in skim milk. In contrast, heating followed by cooling had no effect on magnesium distribution in skim milk for both added and no added magnesium chloride skim milk samples. It can be concluded that amount of magnesium addition for magnesium-enriched dairy products influence the pH value of milk and the distribution of the magnesium between the serum and micellar phases and as a consequence may affect the bioavailability of magnesium-enriched dairy products.

Chapter 5

Magnesium-induced Gelation of Skim Milk

5.1 General introduction

The heat treatment of milk is a common industrial process, but the heat processing of milk samples with added minerals is always challenging. Milk protein gels can be formed by reducing the pH, applying heat, adding enzymes and minerals (Anema & Li, 2000; Bikker, Anema, Li, & Hill, 2000; Bohlin, Hegg, & Ljusberg-Wahren, 1984; Klandar et al., 2007). The addition of soluble salts, such as magnesium chloride, to milk increases the ionic magnesium (Mg^{2+}) concentration and decreases the milk pH, leading to the gelation of milk during processing. In many processes, such as sterilisation and UHT treatment, gelation or sedimentation should be avoided, whereas in the production of cheese and yogurt, decreasing the stability of milk is beneficial in achieving the texture desired in the end products.

The occurrence of gelation in heated milk with added calcium salt has been extensively reported (Koutina et al., 2016; Lin et al., 2018; Ramasubramanian et al., 2014), but studies on the heated skim milk with added magnesium are very limited. Magnesium-induced milk gels are viscoelastic materials and a rheological approach can be performed to investigate the formation of a gel network and differentiate the elasticity and flow properties of the formed gel (Phadungath, 2005). In this study, the physical changes that occurred during heating in the presence of added magnesium chloride (0 to 20 mmol L^{-1}) were monitored to investigate the thickening of skim milk or magnesium-induced gelation through rheological measurements.

5.2. Effect of holding time on gelation

The effects of holding time on gelation were evaluated by preparing the gel in the test tubes in a water bath and in the rheometer. For all the experiments skim milk samples were preheated at 90 ± 1 ^{o}C for 10 min then magnesium chloride was added. The sample preparation for this study and the conditions used in the water bath and rheometer are presented in Section 3.3.

5.2.1 Visual observations

Magnesium-induced skim milk gels were prepared in test tubes at 80 ± 1 °C with varying holding times from 10 to 60 min. Depending on the holding time, the skim milk became either thickened (magnesium-thickened skim milk) or formed a firm gel (magnesium-induced skim milk gel) at 80 ± 1 °C in the water bath, as shown in Figure 5.1. The visual observations revealed that a shorter holding time (10 min) yielded soft gels (very weak gels and when the test tube was inverted it broke), whereas a longer holding time (60 min) produced firmer gels (gelled well and when the test tube was inverted it did not break).

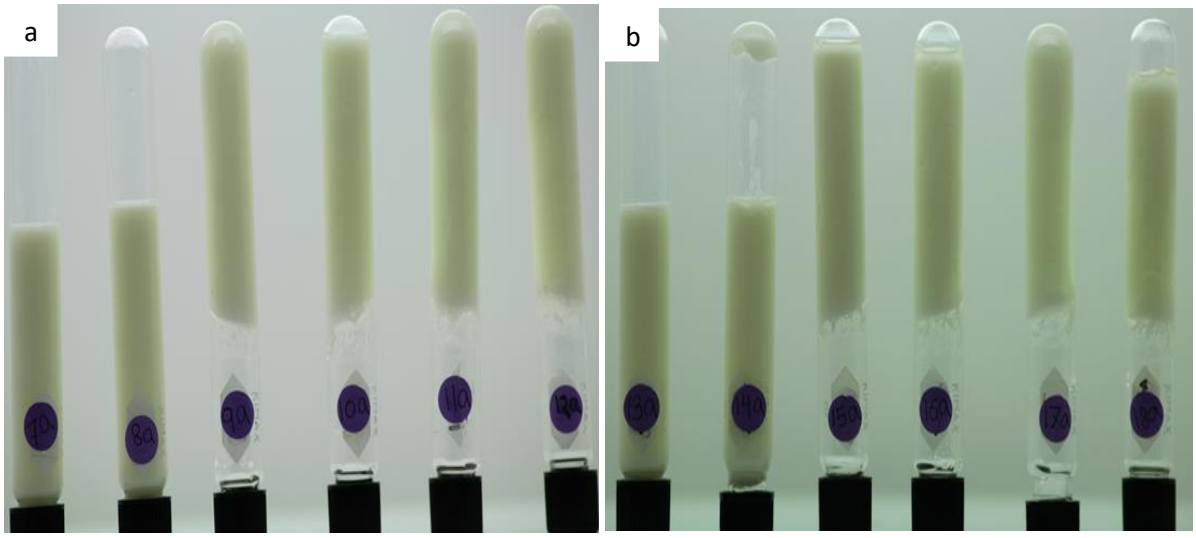

Figure 5.1. Magnesium-induced skim milk gels obtained after holding at 80 ± 1 °C for (a) 10 min and (b) 60 min. Skim milk samples were preheated at 90 ± 1 °C for 10 min then magnesium chloride was added to a final concentration of 5, 10 15, 20, 30 and 40 mmol L^{-1} (shown from left to right). Samples remaining at the top of the inverted test tube had gelled while more liquid samples dropped to the bottom of the inverted tube.

5.2.2 Rheological properties

For the rheological study of skim milk gelation, the changes in storage modulus (*G*') of magnesium-added skim milk samples were evaluated. The temperature and time sweeps were evaluated to estimate the moment at which gelation started and the firmness of the gel was closely predicted by measuring the *G*' value.

The storage modulus (*G*') of a magnesium-induced skim milk gel (with 15 mmol L^{-1} magnesium chloride) as a function of temperature is presented in Figure 5.2. The change in the storage

modulus (*G*') during heating from 20 to 80 °C, resulted in gelation during this heat treatment (Figure 5.2). For the sample shown, the *G*' value was 1.08 Pa as soon as the temperature reached 75 °C, as the *G*' was greater than 1 Pa, this indicated gelation (Ramasubramanian et al., 2014). The effect of holding time at 80 °C on the same magnesium chloride added skim milk sample was also investigated, the result is presented in Figure 5.3. The *G*' value of the sample was 2.58 Pa at 80 °C for 0.80 min of holding and increased significantly to 19.29 Pa ($P < 0.05$) as the temperature was maintained at 80 °C for 60 min.

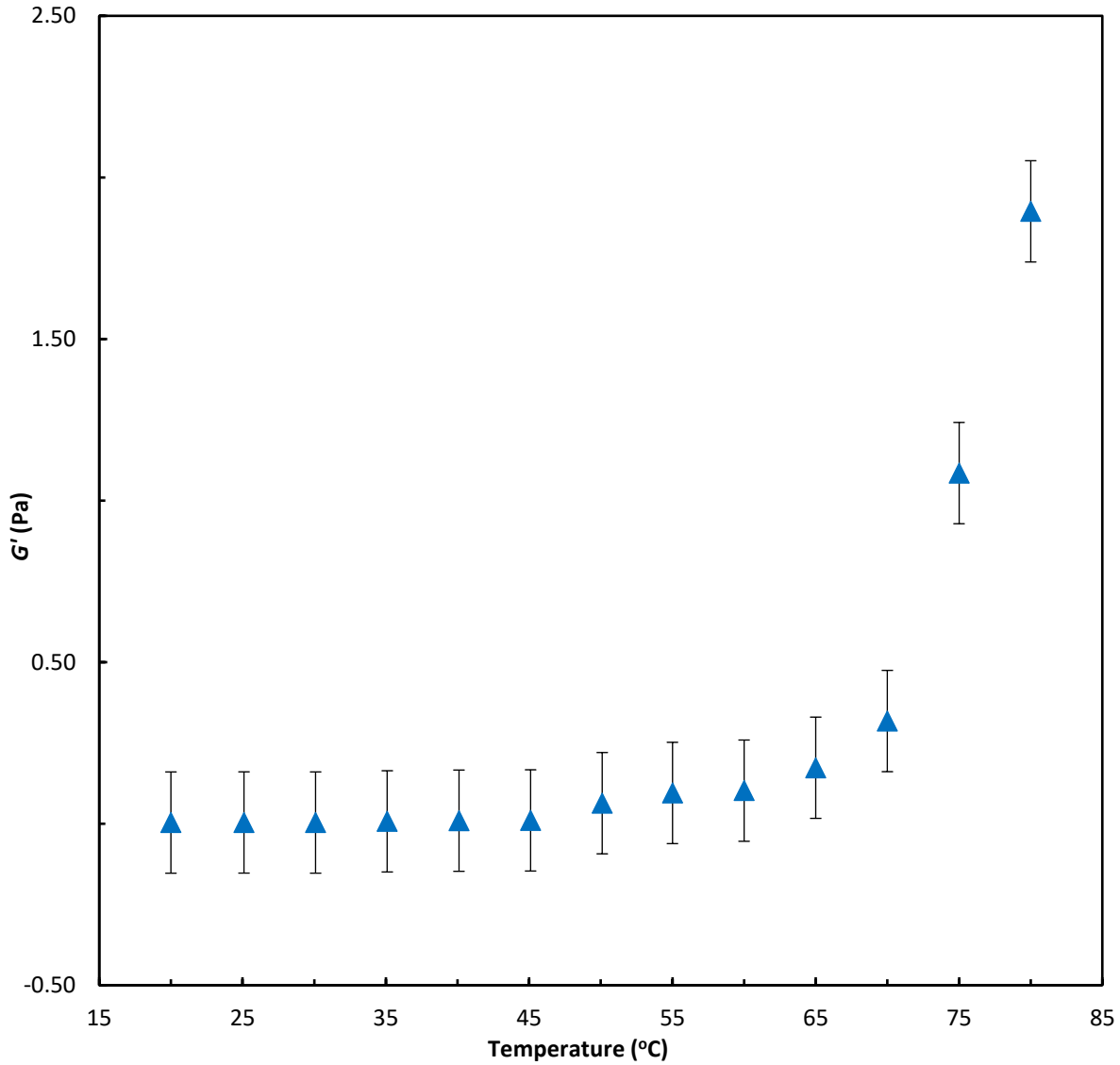

Figure 5.2. Storage modulus (*G*') of a magnesium-added skim milk sample with 15 mmol L^{-1} added magnesium chloride during heating from 20 to 80 °C. The data points show the mean values ± standard deviations (n = 4).

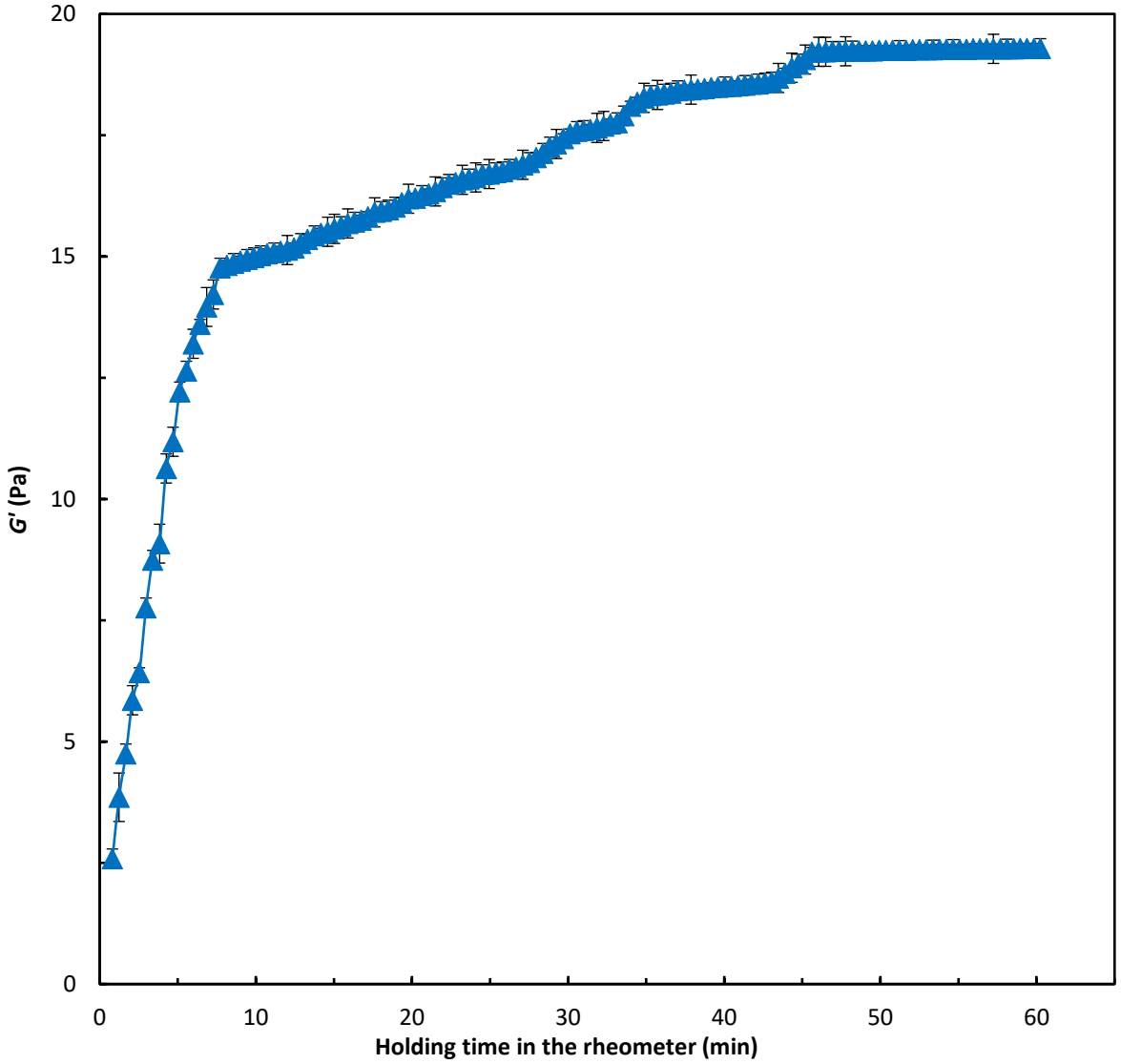

Figure 5.3. Storage modulus (*G*') of a magnesium-induced skim milk gel with 15 mmol L^{-1} added magnesium chloride as a function of holding time at 80 °C. The data points show the mean values ± standard deviations (n = 4).

As shown in Figure 5.3, during holding at 80 °C, the *G*' value increased significantly to 14.70 Pa within the first 7.7 min ($P < 0.05$). This indicated that the formation of a gel network was occurring during heating at 80 °C (Cura et al., 2009). The *G*' value continued to increase but at a slower rate from 7.7 to 45 min to a *G*' value from 14.70 to 19.06 Pa (significant, $P < 0.05$) and from 45 to 60 min the *G*' value increased to 29.29 Pa (not significant $P > 0.05$), this indicated that the gel was becoming firmer after 45 min of holding.

The changes in the *G*' of magnesium-induced skim milk gel while cooling from 80 to 20 °C for different holding times are shown in Figure 5.4 and the final *G*' at 20 °C for different holding times is shown in Figure 5.5. As shown in Figures 5.4 and 5.5, the *G*' value increased with decreasing temperature from 80 to 20 °C and increasing holding time. With the same concentration of added magnesium chloride (15 mmol L^{-1}), the highest final *G*' (116.20 Pa) was obtained with the longest holding time of 60 min, though this final *G*' was only 2 Pa more than obtained for a holding time of 45 min. Visual observations also showed the same results, higher the holding time the firmer the gel formed.

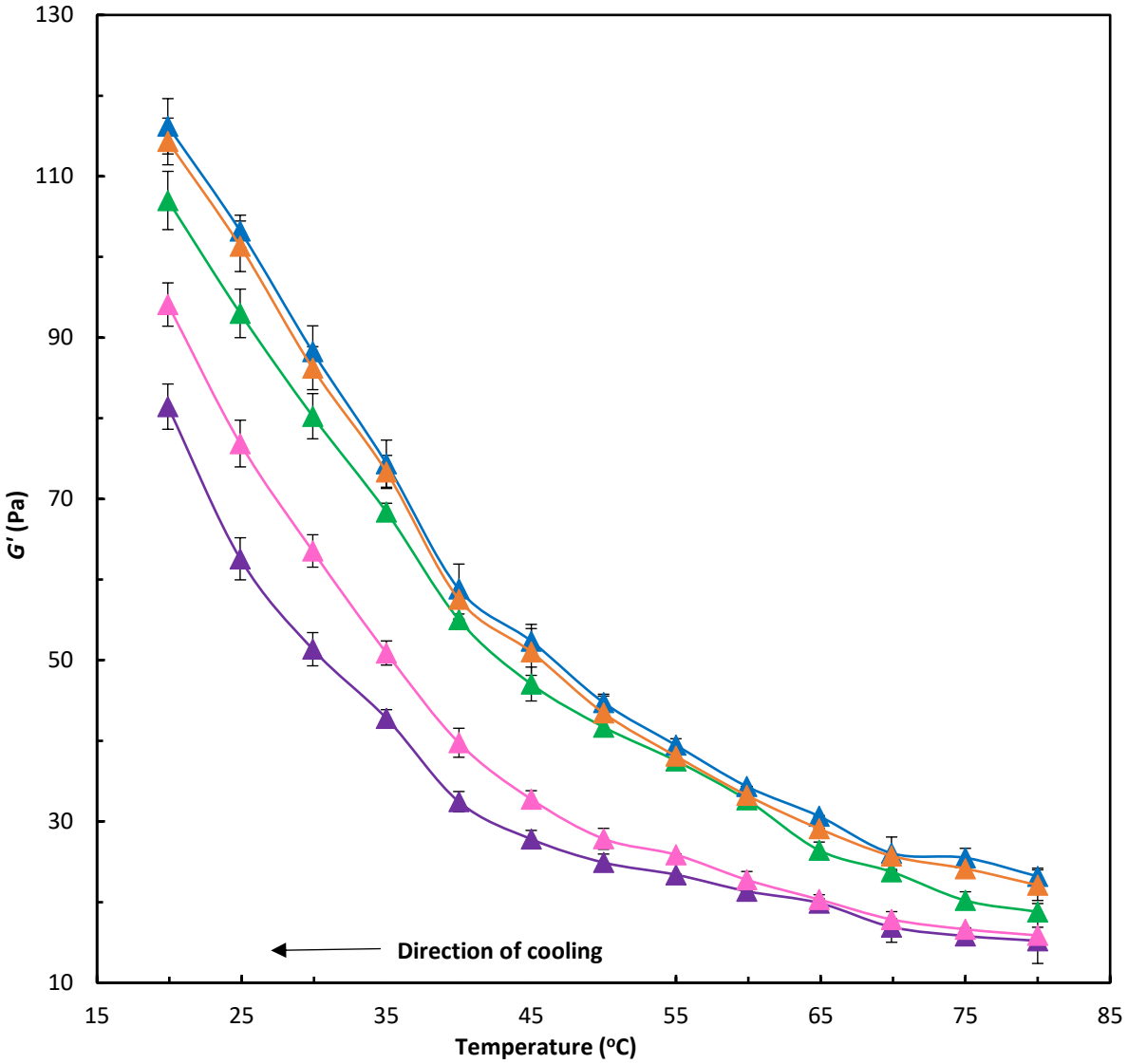

Figure 5.4. Storage modulus (*G*') of a magnesium-induced skim milk gel with 15 mmol L^{-1} added magnesium chloride as a function of temperature during cooling from 80 to 20 °C: holding at 80 °C for 10 min (▲), 20 min (▲), 30 min (▲), 45 min (▲) and 60 min (▲) in the rheometer. The data points show the mean values ± standard deviations (n = 4).

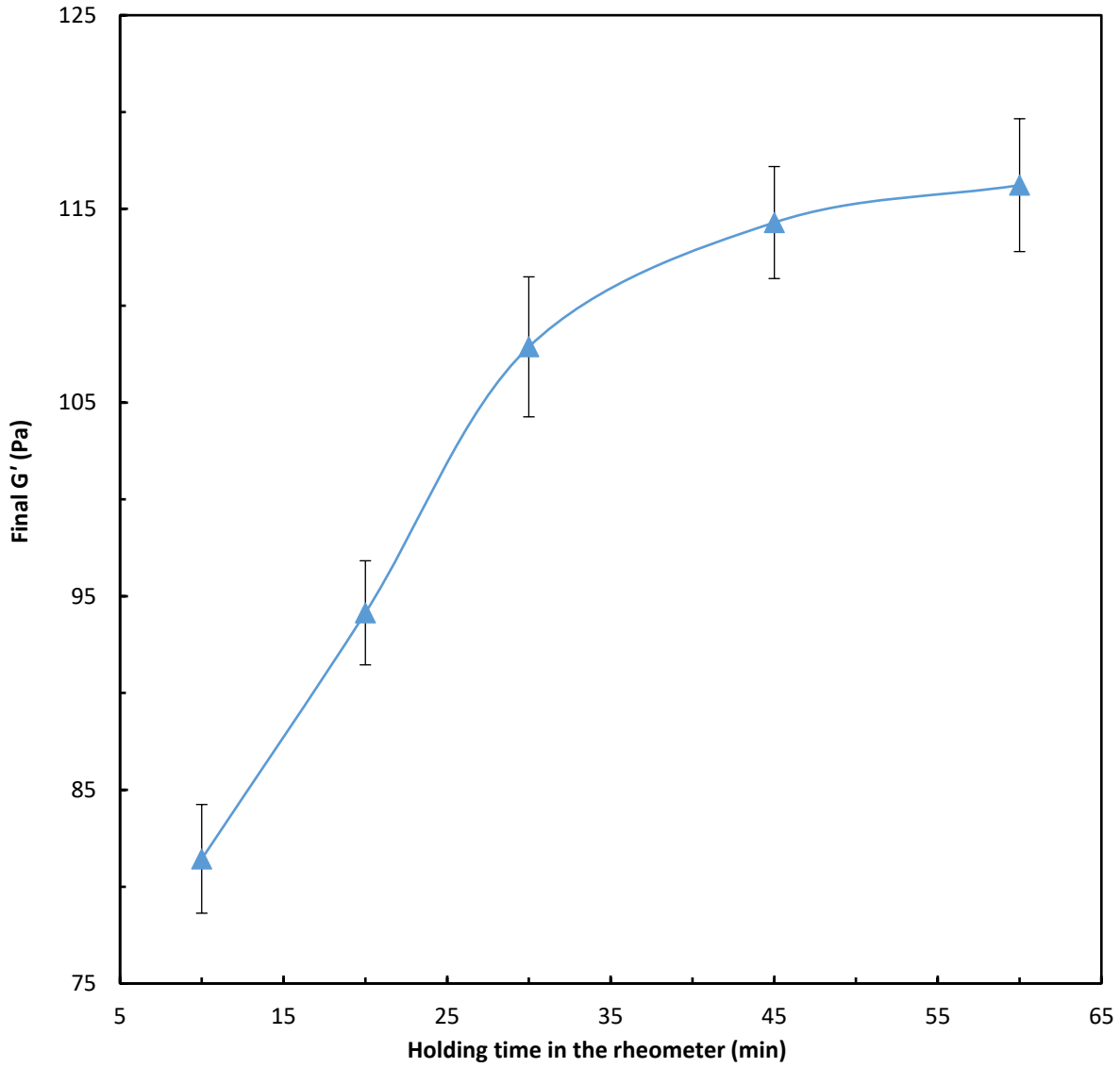

Figure 5.5. Effect of holding time on the final storage modulus (*G*') of magnesium-induced skim milk gels with 15 mmol L^{-1} added magnesium chloride at 20 °C. The data points show the mean values ± standard deviations (n = 4).

A gel network developed in magnesium-induced skim milk gel mainly during the holding phase. As the samples were cooled from 80 to 20 °C (Figure 5.4), the *G*' increased significantly for all the samples ($P < 0.05$). Other researchers found a similar increase in *G*' during the cooling of calcium-added skim milk gels: the *G*' values at 20 °C were higher than that at 70 °C (Lin et al., 2018; Ramasubramanian et al., 2014). During cooling, the *G*' increased due to the swelling of casein particles, which resulted in weak hydrophobic interactions and increased intermolecular bonds (Lee & Lucey, 2010). The observed increased in *G*' obtained for the magnesium-induced skim milk gel during cooling can be similarly explained.

The frequency and strain sweep results obtained for the magnesium-induced skim milk gels at different holding times are presented in Figures 5.6 and 5.7. The samples were heated to 80 °C in the rheometer, held for 10 to 60 min, cooled to 20 °C then the frequency and strain sweeps on samples were carried out. An increase in *G*' was observed with an increase in the holding time at constant frequency and an increase in *G*' was observed with an increase in the frequency from 0.01 to 10 Hz for the all the different holding times from 10 to 60 min (Figure 5.6).

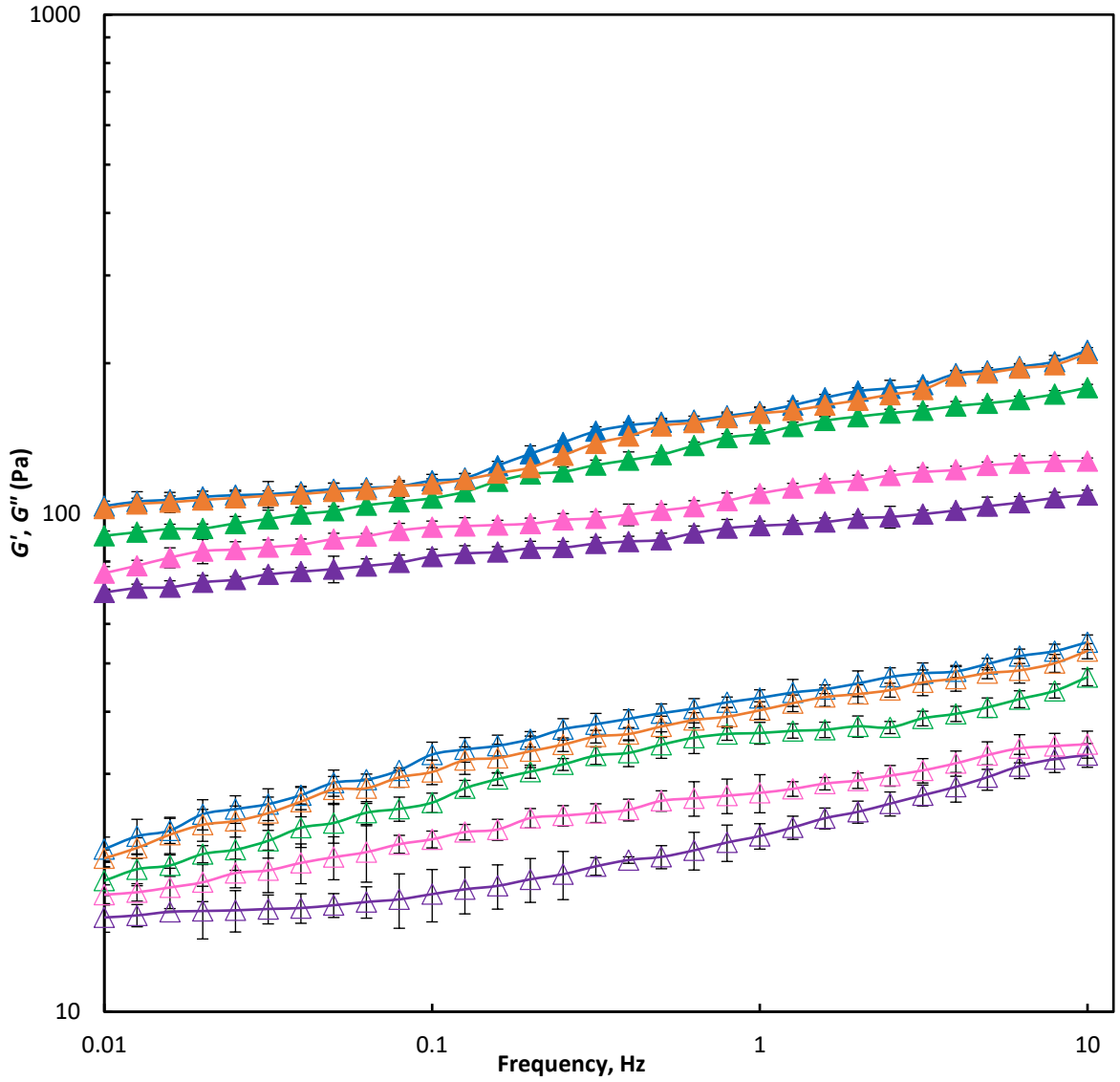

Figure 5.6. Storage (*G*') and loss (*G*") modulus of magnesium-induced skim milk gels with 15 mmol L^{-1} added magnesium chloride at 20 °C as a function of frequency: holding times of 10 min (▲, △), 20 min (▲, △), 30 min (▲, △), 45 min (▲, △) and 60 min (▲, △) in the rheometer. The closed symbols and the open symbols show the *G*' and *G*" values, respectively. The data points show the mean values ± standard deviations (n = 4).

The difference between *G*' and *G*" was less than one log (Figure 5.6) which indicated the formation of a gel due to weak interactions between milk proteins (Lapasin & Pricl, 1995). These differences remained less than one log for all the tested holding times, hence the gel structure in all the samples was maintained by weak interactions between the proteins.

The strain sweep test showed that as the % strain increased the breaking point of the gel was indicated by the maximum stress in Figure 5.7. After the gel broke the stress at higher % strain markedly decreased. Longer holding times were correlated with higher breaking stress.

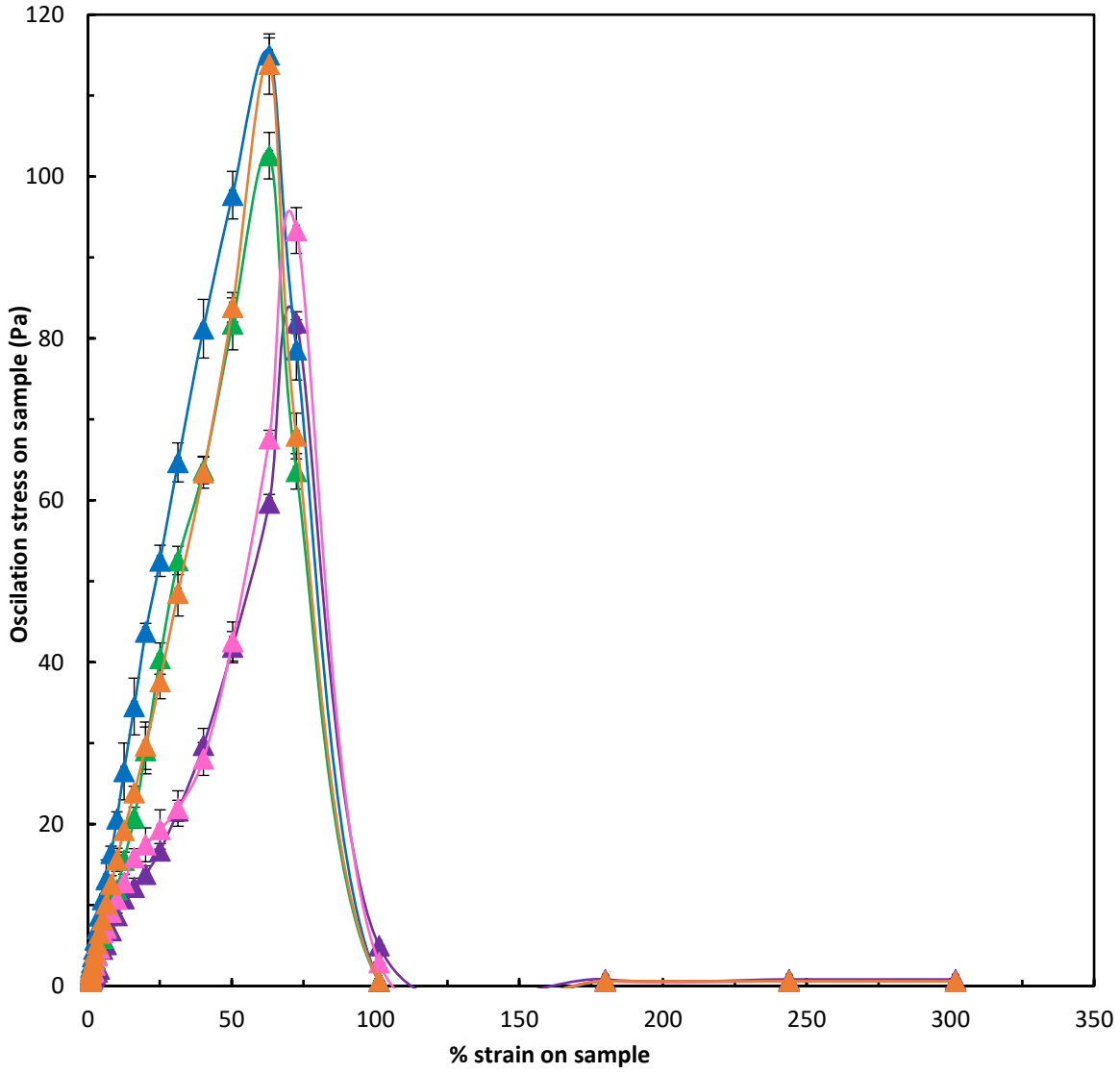

Figure 5.7. Oscillation stress of skim milk gels with 15 mmol L^{-1} added magnesium chloride as a function of strain at 20 °C: holding time of 10 min (▲), 20 min (▲), 30 min (▲), 45 min (▲) and 60 min (▲) in the rheometer. The data points show the mean values ± standard deviations (n = 4).

The structure of the gel network is linked by weak inter-particles interactions which can be destroyed by applied stress or strain (Phadungath, 2005). Small deformation is defined as a strain or change in dimension (e.g. ≤1%), which when applied does not disrupt the development of the network structure within the linear viscoelastic region. However, for all holding times, the breaking strain ranged from 63 to 72%. Specifically, a 60 min holding time resulted in a breaking strain of 63%, whereas a holding time of 10 min was correlated with a breaking strain of 72%, which indicated that a longer holding time yielded a firmer gel (Ramasubramanian et al., 2014).

5.3 Effect of magnesium chloride concentration on gelation

The effects of the concentration of added magnesium chloride were investigated by preparing the gels in the test tubes in a water bath and in the rheometer. Skim milk samples were preheated at 90 ± 1 ^{o}C for 10 min then magnesium chloride was added to a final concentration of 5, 10 15, 20, 30 and 40 mmol L^{-1}. The experimental conditions for this study are mentioned in Section 3.3.

5.3.1 Visual observations

An attempt was made to induce gelation of magnesium-added milk samples at 85 ± 1 ^{o}C in a water bath in the test tubes. Depending on the concentration of magnesium chloride added, the skim milk formed a soft gel or firm gel, as shown in Table 5.1.

Visual observations revealed that a firmer gel visually like yogurt was obtained with a minimum of 15 mmol L^{-1} added magnesium chloride after holding for 10 min. Similar findings have been reported by Ramasubramanian et al. (2014), who observed an increase in the viscosity of milk containing 5 mmol L^{-1} calcium after heating at 70 ^{o}C and gels were formed from 12.50 mmol L^{-1} calcium chloride addition.

Table 5.1: Observations of gelation in preheated samples heating to 85 ± 1 °C in a water bath, preparing the gels in the test tubes. The samples were cooled to 20 ± 1 °C and then observed. Milk samples were preheated at 90 ± 1 °C for 10 min and cooled to 20 ± 1 °C prior to addition of magnesium chloride.

Holding time, min	Concentration of added magnesium chloride, mmol L^{-1}	Observations
10	5	Thickened skim milk
	10	Soft gel
	15	Gel
	20	Gel
	30	Gel
	40	Gel
30	5	Soft gel
	10	Soft gel
	15	Gel
	20	Gel
	30	Gel
	40	Gel
60	5	Soft gel
	10	Gel
	15	Gel
	20	Gel
	30	Gel
	40	Gel

Soft gel = very weak gel when the test tube was inverted it broke; Gel = gelled well and when the test tube was inverted it did not break.

5.3.2 Rheological properties

The impact of magnesium chloride concentration on the rheological properties of the magnesium-induced skim milk gel is shown in Figures 5.8 to 5.13. Monitoring the effect of the addition of magnesium chloride on heating to 80 °C (Figure 5.8) revealed that some of the samples experienced gelation during heating from 20 to 80 °C.

Gelation during heating from 20 to 80 °C was observed in the samples with at least 15 mmol L^{-1} added magnesium chloride (Figure 5.8). The storage modulus of the samples with 15 mmol L^{-1} and 20 mmol L^{-1} added magnesium chloride reached 1 Pa as soon as the temperature reached 75 °C and 70 °C, respectively. The skim milk samples with 0 mmol L^{-1} and 5 mmol L^{-1} added magnesium chloride did not show any gelation, whereas the samples with 10 mmol L^{-1} and 12.50 mmol L^{-1} added magnesium chloride showed thickening as the *G*' increased at temperatures of at least 70 °C but the *G*' value at 80 °C was < 1 Pa.

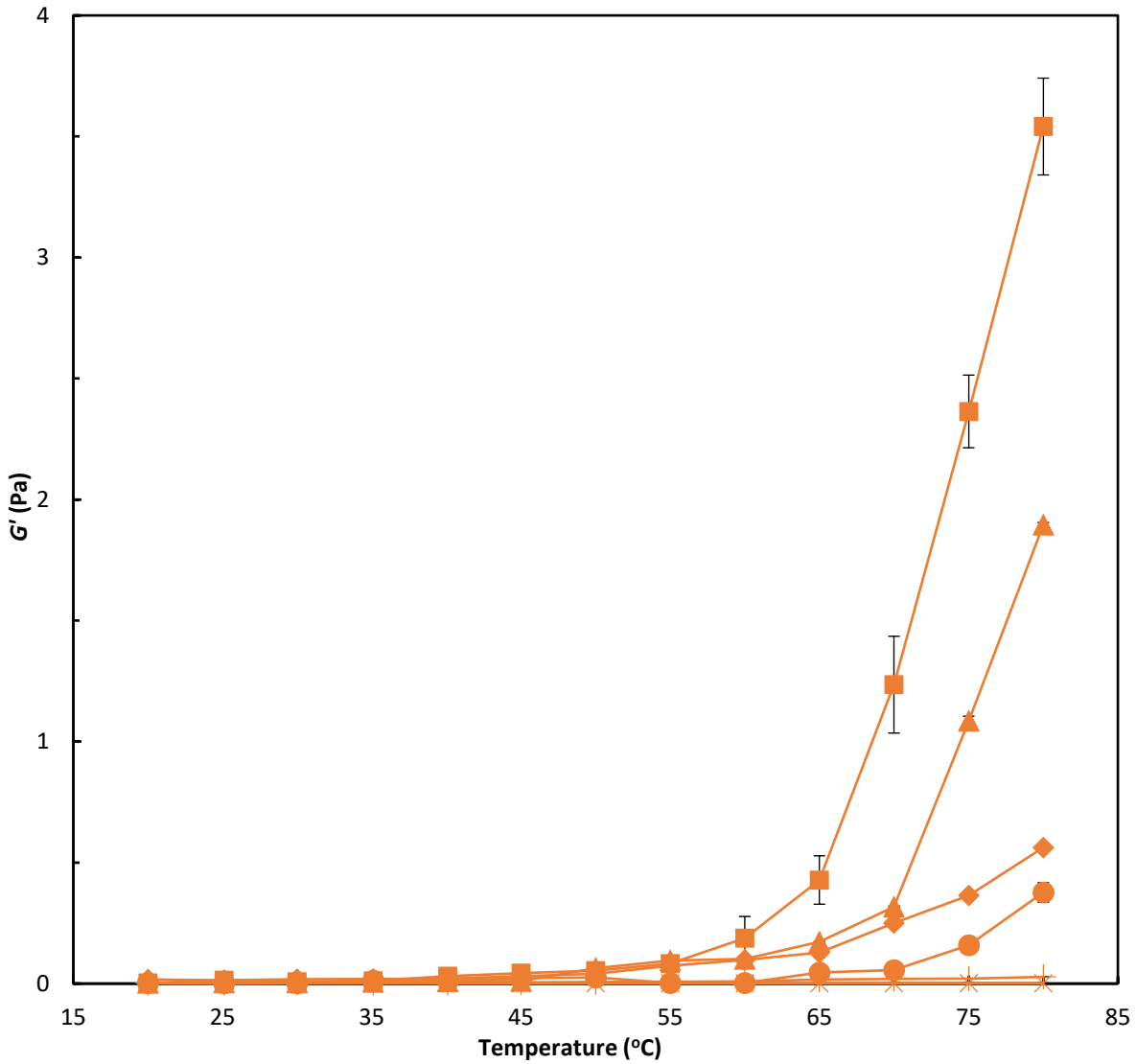

Figure 5.8. Storage modulus (*G*') of skim milk samples with different concentrations of added magnesium chloride during heating from 20 to 80 °C: 0 mmol L^{-1} (×), 5 mmol L^{-1} (+), 10 mmol L^{-1} (●), 12.50 mmol L^{-1} (◆), 15 mmol L^{-1} (▲) and 20 mmol L^{-1} (■) magnesium chloride added. The data points show the mean values ± standard deviations (n = 4).

The storage modulus *G*' of the samples with different concentrations of magnesium chloride addition as a function of the holding time (up to 45 min) is shown in Figure 5.9. For skim milk samples with ≥ 15 mmol L^{-1} added magnesium chloride, gelation occurred at temperatures below 80 °C, for skim milk samples with < 15 mmol L^{-1} added magnesium chloride resulted in a gel with *G*' > 1 Pa during holding at approximately 5 min at 80 °C.

The samples with 20 mmol L^{-1} added magnesium chloride formed a gel at 70 °C, which is a lower temperature than that required for gelation for the sample with 15 mmol L^{-1} added magnesium chloride (75 °C) (Figure 5.8). In contrast, the samples with 10 mmol L^{-1} and 12.50 mmol L^{-1} added magnesium chloride showed gelation during holding at 80 °C for 5 min and 2 min, with *G'* value of 1.32 Pa and 1.02 Pa, respectively (Figure 5.9).

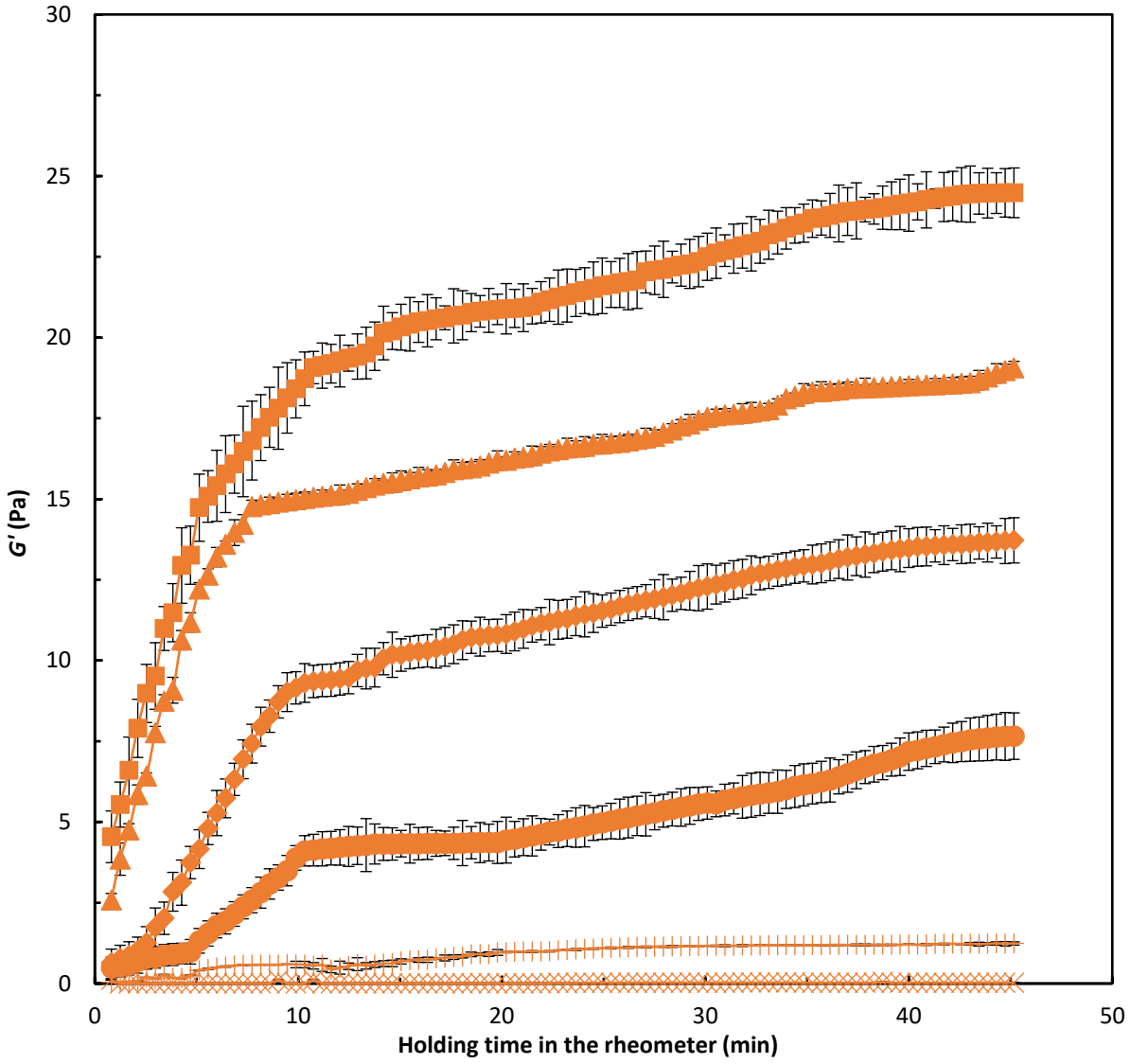

Figure 5.9. Storage modulus (*G'*) of skim milk samples with different concentrations of added magnesium chloride as a function of holding time in the rheometer at 80 °C: 0 mmol L^{-1} (×), 5 mmol L^{-1} (+), 10 mmol L^{-1} (●), 12.50 mmol L^{-1} (◆), 15 mmol L^{-1} (▲) and 20 mmol L^{-1} (■) added magnesium chloride. The data points show the mean values ± standard deviations (n = 4).

A significant increase in *G*' was clearly observed in these samples (10 mmol L^{-1} and 12.50 mmol L^{-1} added magnesium chloride) during the first 10 min of holding at 80 °C (4.10 Pa and 9.30 Pa, respectively) and the *G*' value increased at a slower rate to 7.66 Pa and 13.72 Pa, respectively, during the remainder of the 45 min holding period ($P < 0.05$). The samples with 15 mmol L^{-1} and 20 mmol L^{-1} added magnesium chloride had also exhibited a significant increase in the *G*' value (14.7 Pa) during the first 7.7 min and 5 min of holding, respectively and the *G*' value then increased slowly during the rest of the 45 min of holding time to yield the *G*' values of 19.06 Pa and 24.48 Pa, respectively ($P < 0.05$). For all the samples (10 mmol L^{-1}, 12.50 mmol L^{-1}, 15 mmol L^{-1} and 20 mmol L^{-1} magnesium chloride addition), the *G*' increased by more than 50% within the first 10 min of holding, indicating that the maximal gel network formed within the first 10 min.

A summary of the time and temperature of the gelation point of skim milk with different concentrations of magnesium chloride added evaluated is presented in Table 5.2. As shown in Table 5.2, the higher the concentration of magnesium chloride added led to more rapid gel formation dependent on temperature and holding time.

Table 5.2: Gelation time and temperature for the magnesium-added skim milk samples.

Concentration of added magnesium chloride, mmol L^{-1}	pH	Time and temperature to achieve a *G*' 1 Pa
0	6.68	No gelation
5	6.50	22 min/80 °C
10	6.43	5min/ 80 °C
12.50	6.38	2min/80 °C
15	6.34	At 75 °C
20	6.30	At 70 °C

- The heating process in the rheometer was as follows: the temperature was increased from 20 to 80 °C at a rate of 5 °C/min and then maintained at 80 °C for 45 min. The pH was measured after the addition of magnesium chloride to preheated skim milk. Preheating was conducted at 90 ± 1 °C for 10 min (mentioned in detail in Section 3.3).

The samples with added magnesium chloride (10 mmol L^{-1} and 20 mmol L^{-1}) corresponding to the pH values of pH 6.43 and pH 6.30, exhibited gelation at temperatures of 80 °C and 70 °C, respectively. In the absence of added magnesium chloride, no gelation was observed at

natural milk pH 6.70, even at a higher temperature of 80 ^{o}C and a longer holding time of 45 min (Figure 5.9). At this pH, no gel formed because the casein micelles have a high negative charge and the resulting repulsion forces prohibited the formation of gel networks (Koutina et al., 2016). As the concentration of added magnesium chloride was increased from 5 to 20 mmol L^{-1}, the concomitant pH was reduced from pH 6.50 to 6.30, dissociation of CCP occurred, more ionic magnesium (Mg^{2+}) was released in to the serum and casein started to form loosely entangled aggregates due to the formation of Mg^{2+} bridges and protein-protein hydrophobic interactions (Jeyarajah & Allen, 1994; O'Mahony & Fox, 2013). The increase in the amount of magnesium ions in the serum phase during magnesium addition (Table 4.4) indicated that ionic magnesium (Mg^{2+}) binds to phosphorus to form magnesium phosphate and it is postulated that the magnesium phosphate moves towards the micellar phase which induces the aggregation of casein. Although little magnesium is naturally associated with protein in milk, the added magnesium can interact with casein and induce gelation when milk is heated (O'Mahony & Fox, 2013). Magnesium-induced skim milk forms a gel network due to the formation of ionic magnesium (Mg^{2+}) bridges. The gels become stronger as the pH decreases with the addition of magnesium chloride because a higher amount of magnesium chloride dissociates to form Mg^{2+} which results in higher *G*' values (Koutina et al., 2016; Ramasubramanian et al., 2014).

The storage modulus (*G*') of magnesium-induced skim milk gels, with no pH adjustment, as a function of temperature during cooling from 80 to 20 ^{o}C is presented in Figure 5.10. At a constant holding time of 10 min, the *G*' value increased significantly with increases in the added magnesium chloride concentration ($P < 0.05$). The addition of magnesium chloride (0 to 20 mmol L^{-1}) to skim milk without adjusting the pH, increased the ionic magnesium (Mg^{2+}) concentration from 0.018 to 0.236 mg mL^{-1} (Table 4.4), which resulted in an increase in the *G*' value from 0 to 112 Pa (Figure 5.10).

A significant increase in *G*' observed ($P < 0.05$) when the magnesium-induced skim milk gels were cooled to 20 ^{o}C reflected an increase in the strength of the gel (Figure 5.10). The increase in *G*' during cooling was reported and explained in the previous section (Section 5.2). The skim milk samples with 5 mmol L^{-1} added magnesium chloride formed a very weak gel at the end of the cooling period, with a *G*' value of 6.87 Pa after 45 min of holding.

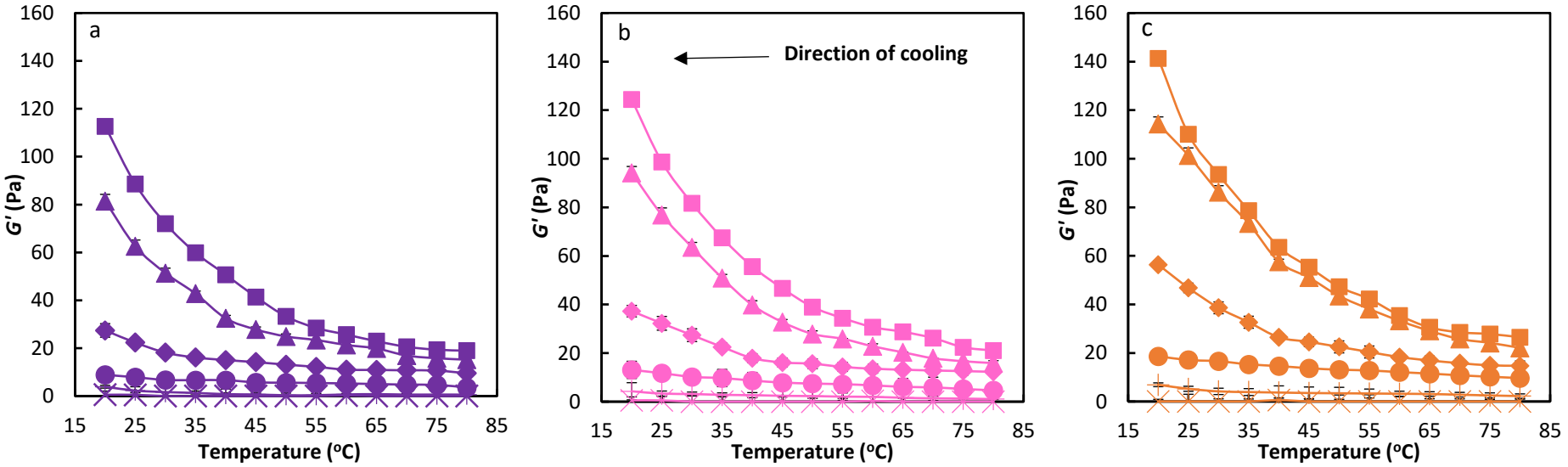

Figure 5.10. Storage modulus (*G'*) of magnesium-induced skim milk gels with different concentrations of added magnesium chloride, with no pH adjustment, as a function of temperature during cooling from 80 to 20 °C: holding for (a) 10 min (b) 20 min and (c) 45 min in the rheometer. 0 mmol L^{-1} (✕), 5 mmol L^{-1}(+), 10 mmol L^{-1} (●), 12.50 mmol L^{-1} (◆), 15 mmol L^{-1} (▲) and 20 mmol L^{-1} (■) added magnesium chloride. The data points show the mean values ± standard deviations (n = 4).

The *G'* value of the samples with 20 mmol L^{-1} added magnesium chloride was 112 Pa after 10 min of holding and reached 141 Pa with the holding time of 45 min (Figure 5.10a and Figure 5.10c). The strength of the gel increased with increasing holding time, possibly due to the swelling of casein micelles and the formation of the complete gel network over time.

The effect of the addition of magnesium chloride on the final storage modulus (*G'*) at 20 °C is presented in Figure 5.11. At higher concentrations of added magnesium chloride, the gel formed at lower temperatures and the rate of increase in the *G'* was observed to be faster on holding. Even after a long period of holding, the rate of *G'* increase was higher in the presence of a higher concentration of added magnesium chloride (Table 5.2 and Figure 5.11).

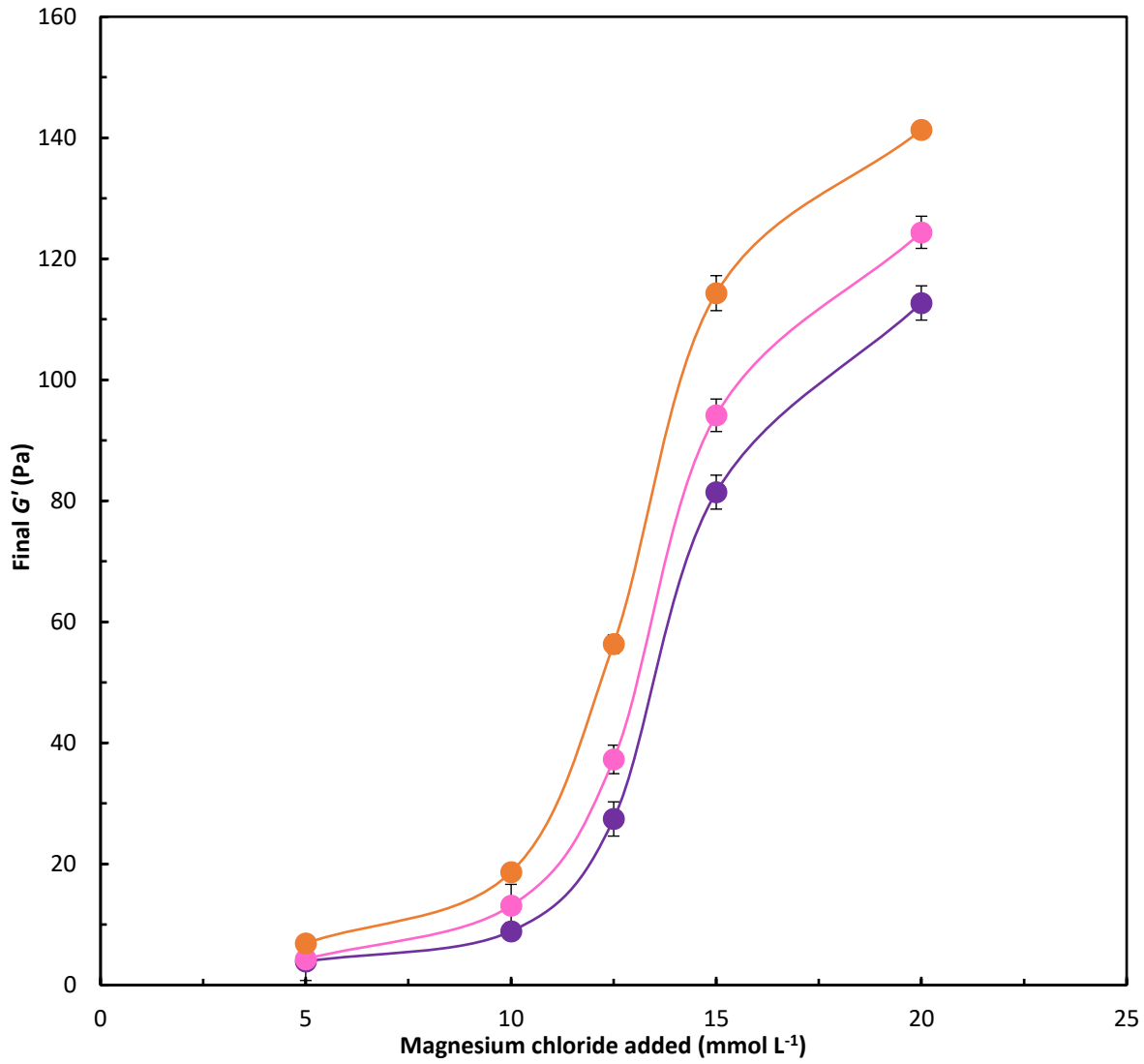

Figure 5.11. Effect of the addition of magnesium chloride on the final storage modulus (*G'*) at 20 °C: after holding time of 10 min (●), 20 min (●) and 45 min (●) at 80 °C in the rheometer. The data points show the mean values ± standard deviations (n = 4).

The difference of final *G*' at different holding times with same concentration of magnesium chloride increased with increasing holding time. These findings do not necessarily imply that the same network structure was obtained with all levels of magnesium chloride addition.

The building of a gel network was influenced by the concentration of added magnesium chloride, holding time and temperature, unfortunately, the type of network that forms in the presence of higher amounts of added magnesium chloride and at longer holding time is still unclear.

It is noticed from the visual observations that the skim milk sample with 5 mmol L^{-1} added magnesium chloride, heating at 85 ^{o}C showed a change in phase from liquid to thickened skim milk (Table 5.1) and increasing the concentration of added magnesium chloride resulted in complete gelation of the skim milk. When these conditions were tested in the rheometer for a 10 min holding time a final *G*' value for 5 mmol L^{-1} after cooling was 4.27 Pa and with 20 mmol L^{-1} the final *G*' was 112.70 Pa. The rheological properties of natural, thickened and gelled skim milk were co-currently evaluated using the rheometer and presented in Figures 5.2 to 5.11 which are in agreement with the visual observations. However, in this study, rheological tests were not performed with higher concentrations of added magnesium chloride (30 mmol L^{-1} and 40 mmol L^{-1}) but the visual observations revealed that a shorter holding time (10 min) and lower dose of magnesium chloride (10 mmol L^{-1}) yielded soft gels, whereas a longer holding time (60 min) and higher concentration of added magnesium chloride (40 mmol L^{-1}) produced firmer gels without any whey separation (syneresis) (Figure 5.1b). Hence, it appears that a longer holding time, a higher heating temperature and a higher concentration of magnesium chloride addition may tend to produce firmer gels. This finding is also in agreement with the results obtained for calcium-induced milk gels in a previous study by Ramasubramanian et al. (2014).

It has been reported that calcium plays an important role in the structural integrity and stability of milk and that the addition of calcium salts decreases the pH of the milk that contributes to the gelation of milk, but this does not constitute the main mechanism of gelation (Deeth & Lewis, 2015). The addition of calcium may result in a reduced zeta potential of the casein micelles, which is related to an increase in the number of cations and ionic calcium (Ca^{2+}). Although this indirect effect may have contributed to the destabilisation of milk protein, it is unlikely to be the sole cause of the observed gelation. Calcium-mediated

bridging can occur between two neighbouring casein micelles, where divalent Ca^{2+} bridges the phosphate or carboxylate group of β or α S_1 casein or carboxylate groups of κ-casein (Mellema, Leermakers, & De Kruif, 1999). The concept of cation-mediated bridging is in line with the effect of the ionic magnesium (Mg^{2+}) concentration in skim milk on the final *G*' values of skim milk gels presented in this study.

The findings from the strain and frequency sweeps obtained at different holding times 10 to 45 min with the addition of different concentrations of magnesium chloride are shown in Figures 5.12 and 5.13. As shown in Figure 5.12, an increase in the *G*' of the magnesium-induced skim milk gels was found with increasing frequency (0.01 to 10 Hz). The differences in *G*' and *G*" were higher for a longer holding time but the differences were always less than one log for all the holding times, which indicated that the structure of the gel was held together by weak interactions between the milk proteins (Lapasin & Pricl, 1995).

The strain sweep results showed that the stress required to break the gels increased with an increase in magnesium chloride concentration and holding time (Figure 5.13). At holding time of 10 min, the stress was 112.76 Pa whereas for 45 min holding, it was 124 Pa with 20 mmol L^{-1} magnesium chloride added, while the breaking strain was 63%. The breaking strains of the skim milk gels obtained with different holding times and magnesium concentrations ranged from 63 to 72%.

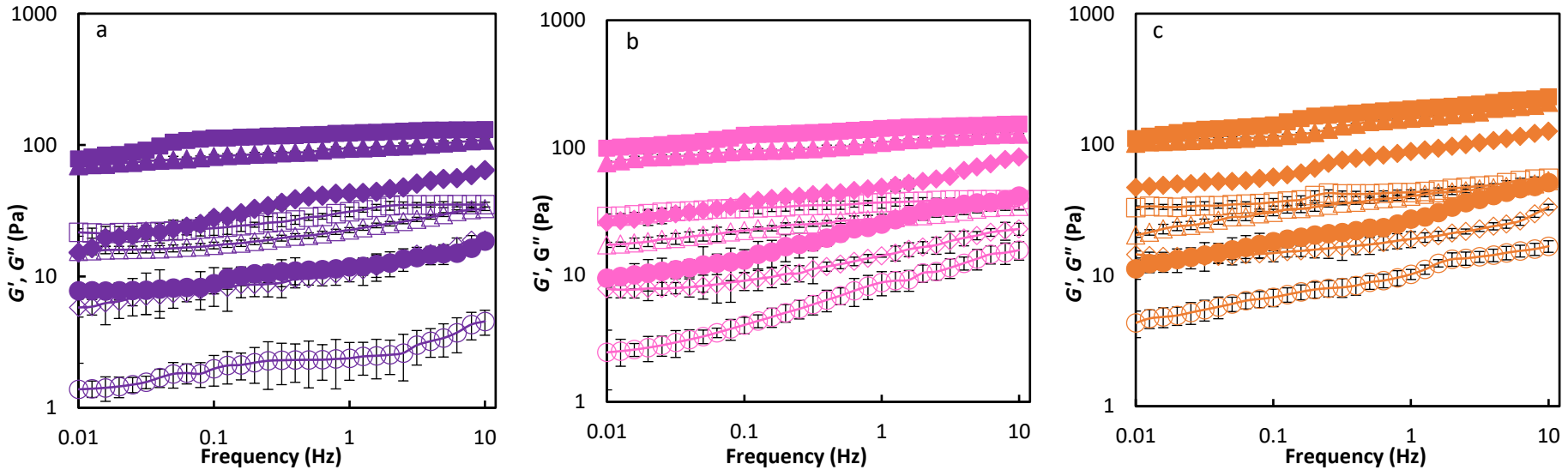

Figure 5.12. Storage (*G*') and loss (*G*") modulus of magnesium-induced skim milk gels with different concentrations of added magnesium chloride as a function of frequency at 20 °C: 10 mmol L^{-1} (●, ○), 12.50 mmol L^{-1} (◆,◇), 15 mmol L^{-1} (▲, △) and 20 mmol L^{-1} (■, □) added magnesium chloride and the holding time at 80 °C of (a) 10 min, (b) 20 min and (c) 45 min in the rheometer. The closed (●, ◆, ▲ and ■) and open symbols (○,◇, △ and □) show the *G*' and *G*" values, respectively. The data points show the mean values ± standard deviations (n = 4).

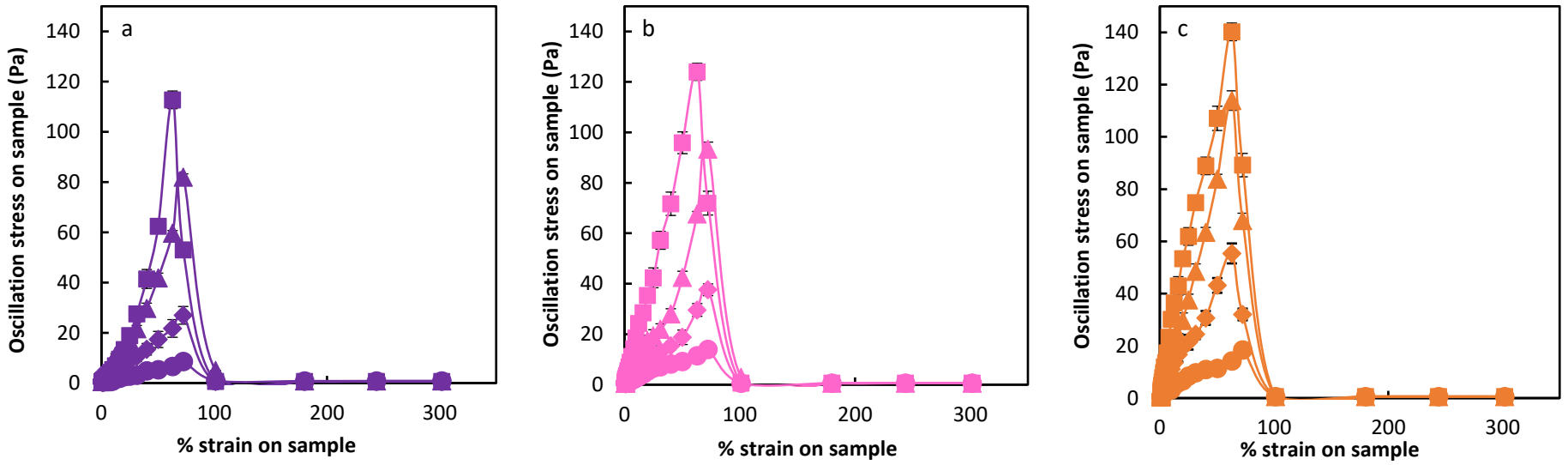

Figure 5.13. Oscillation stress of magnesium-induced skim milk gels as a function of strain in the presence of different concentrations of added magnesium chloride at 20 °C: 10 mmol L^{-1} (●), 12.50 mmol L^{-1} (◆), 15 mmol L^{-1} (▲) and 20 mmol L^{-1} (■) added magnesium chloride and holding time at 80 °C of (a) 10 min, (b) 20 min and (c) 45 min in the rheometer. The data points show the mean values ± standard deviations (n = 4).

5.4 Effect of preheating temperature on gelation

The effect of preheating was investigated by preparing the gels in the test tubes in a water bath and in the rheometer. For preheating, skim milk samples were preheated at 90 ± 1 ^{o}C for 10 min and cooled to 20 ± 1 ^{o}C prior to addition of magnesium chloride. The effect of different heating temperatures (50 to 85 ± 1 ^{o}C) in the water bath and (70 to 85 ^{o}C) in the rheometer was also investigated. The sample preparation procedure and experimental conditions used in this study for the rheometer and water bath are presented in Section 3.3.

5.4.1 Visual observations

Visual observations of magnesium-induced milk gels prepared in the test tubes are presented in Table 5.3 and Figure 5.14. The skim milk samples without added magnesium chloride did not show any gelation or thickening at heating temperatures in the range of (50 to 85 ±1 ^{o}C), regardless of preheating. With no preheating and at a low heating temperature (60 ±1 ^{o}C), gelation did not occur with samples with added magnesium chloride (5 to 40 mmol L^{-1}), even at high concentrations of magnesium chloride addition (40 mmol L^{-1}) (Table 5.3 and Figure 5.14a). In contrast, for the preheated samples, this heating temperature (60 ±1 ^{o}C) and magnesium concentrations added (5 to 40 mmol L^{-1}) resulted in thickened skim milk (for 20 mmol L^{-1} addition) or the formation of a soft gel (for 30 mmol L^{-1} addition) (Table 5.3 and Figure 5.14b). The addition of 5 to 10 mmol L^{-1} magnesium chloride to non-preheated skim milk samples did not lead to gelation with a heating temperature of 80 ±1 ^{o}C, but a noticeable increase in viscosity was observed (visual observations) (Table 5.3). The gels obtained from the non-preheated skim milk samples were not strong, whereas with the same amount of magnesium chloride addition and the same holding temperature, stronger gels resulted from the preheated samples. Moreover, non-preheated samples required longer holding times for gel formation compared with holding times required for preheated skim milk samples.

Table 5.3: Observations of gelation in preheated and non-preheated samples after holding for 10 min at different heating temperatures in a water bath. Milk samples were cooled to 20 ± 1 °C and then observed.

Heating temperature, °C	Type of sample	Concentration of added magnesium chloride, mmol L^{-1}						
		0	5	10	15	20	30	40
		Observations						
50	NP	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid
	P	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid
60	NP	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid
	P	Liquid	Liquid	Liquid	Liquid	Thickened	Soft gel	Soft gel
70	NP	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid
	P	Liquid	Liquid	Thickened	Soft gel	Soft gel	Gel	Gel
75	NP	Liquid	Liquid	Thickened	Thickened	Thickened	Thickened	Soft gel
	P	Liquid	Liquid	Soft gel	Soft gel	Gel	Gel	Gel
80	NP	Liquid	Liquid	Thickened	Soft gel	Soft gel	Soft gel	Gel
	P	Liquid	Thickened	Soft gel	Soft gel	Gel	Gel	Gel
85	NP	Liquid	liquid	Thickened	Soft gel	Soft gel	Soft gel	Gel
	P	Liquid	Thickened	Soft gel	Gel	Gel	Gel	Gel

P = preheated and NP = Non-preheated

Samples showed gelation at 85 °C with at least 15 mmol L^{-1} added magnesium chloride (Table 5.3 and Figure 5.14c). Gel formation was obtained with at least 10 mmol L^{-1} added magnesium chloride after 10 min of holding and a heating temperature of 75 °C (Table 5.3). Markedly less pronounced gelation was obtained with the non-preheated samples with 15 and 20 mmol L^{-1} added magnesium (visual observations) for a heating temperature of 85 °C. To induce the gelation in the presence of magnesium, a minimum amount of magnesium chloride was required and the amounts required differed between preheated and non-preheated samples and was also dependent on heating temperature.

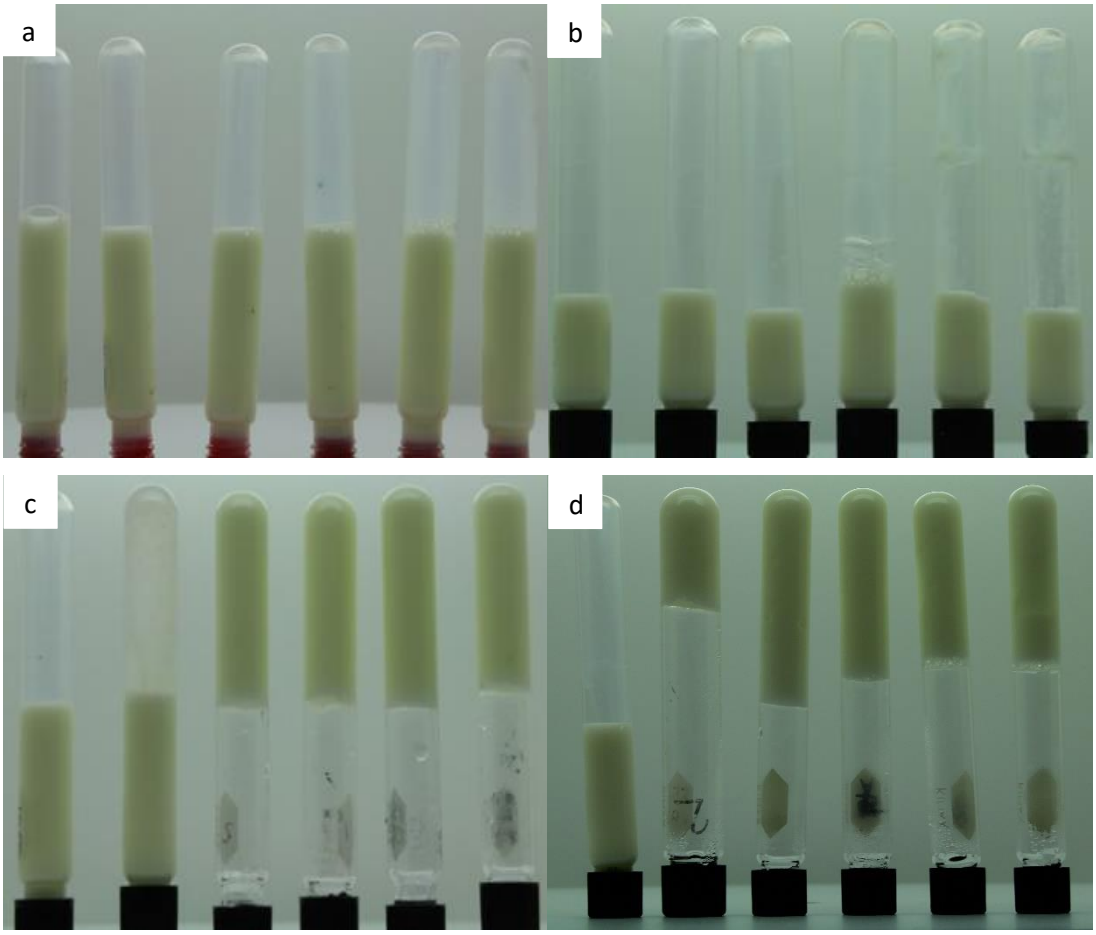

Figure 5.14. Magnesium-induced skim milk gels obtained after holding at 60 ± 1 °C (a and b) and 85 ± 1 °C (c and d) for 10 min. Magnesium chloride was added to a final concentration of 5, 10, 15, 20, 30 and 40 mmol L^{-1} (shown from left to right). Samples remaining at the top of the inverted test tube had gelled while more liquid samples dropped to the bottom of the inverted tube. (a) and (c) for non-preheated skim milk and (b) and (d) for preheated skim milk samples.

5.4.2 Rheological properties

To study the effect of heating temperature (70 °C, 75 °C, 80 °C and 85°C) in the rheometer, skim milk samples were heated to each temperature and held for a constant holding time. Gels were prepared by heating preheated and non-preheated skim milk to 70 °C, 75 °C, 80 °C and 85 °C with constant magnesium chloride concentration (20 mmol L^{-1}) and the effect of temperature on gel formation as a function of *G*' is shown in Figure 5.15. The *G*' value of the non-preheated and preheated skim milk gels was compared and the results revealed that the *G*' value of the magnesium-induced gel from the preheated samples was significantly higher ($P < 0.05$) than that obtained for the non-preheated skim milk gels (Figure 5.15).

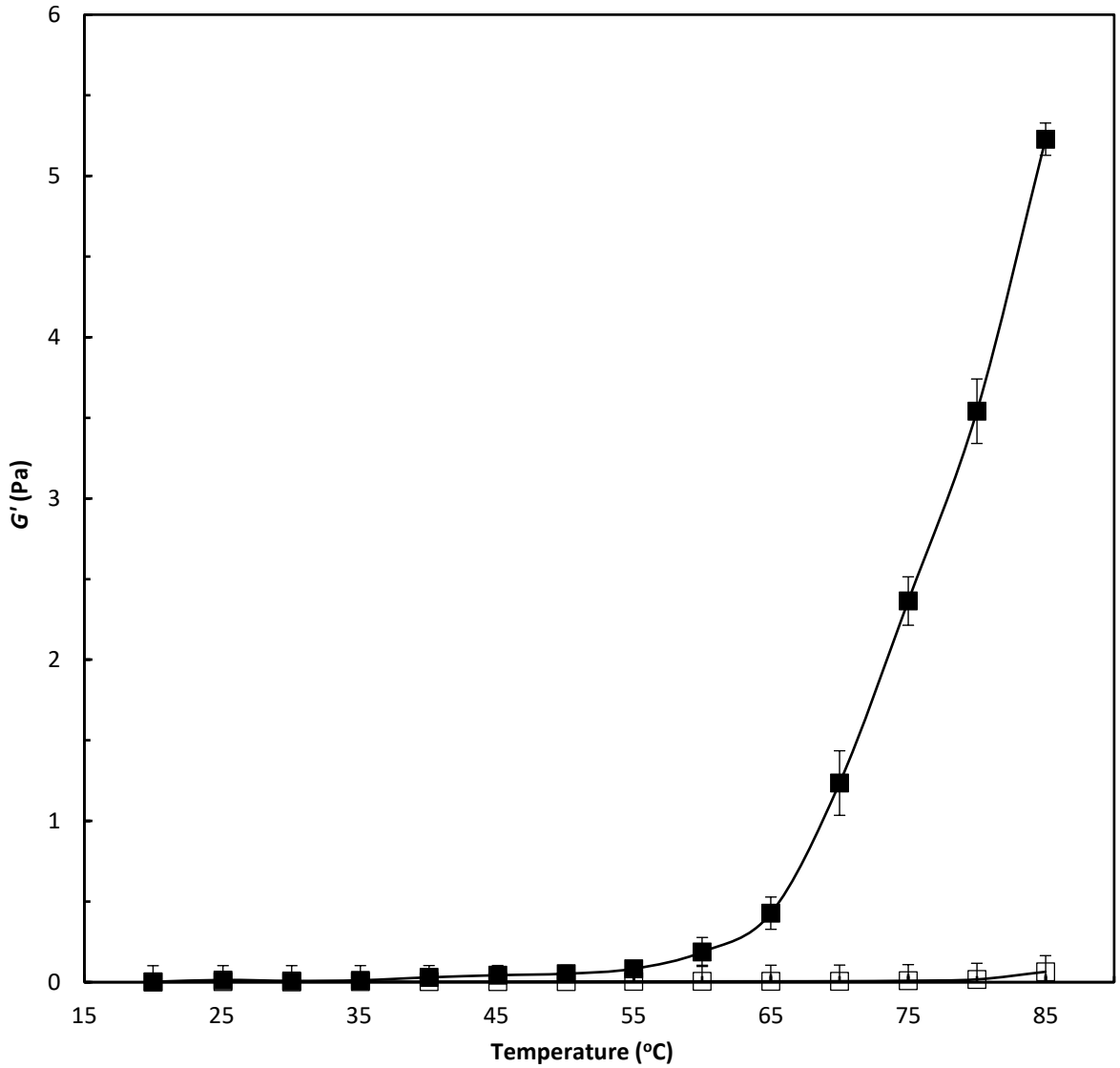

Figure 5.15. Storage modulus (*G*') of magnesium-induced skim milk samples with added magnesium chloride (20 mmol L^{-1}) during heating to 85 °C in the rheometer. The open symbols (□) refer to skim milk samples without preheating and the closed symbols (■) refer to skim milk samples that were preheated at 90 ± 1 °C for 10 min and cooled to 20 ± 1 °C prior to addition of magnesium chloride. The data points show the mean values ± standard deviations (n = 4).

The effects of holding time for preheated and non-preheated milk samples at different heating temperatures (70 °C, 75 °C, 80 °C and 85 °C) are presented in Figure 5.16. The non-preheated samples formed gels after 8 min and 10 min of holding at 85 °C and 80 °C, respectively but did not exhibit any gelation at 70 °C and 75 °C heating in the rheometer. In contrast, the gels formed from the preheated samples at all temperatures 70 to 85 °C and the *G'* values reached a maximum at a temperature of 85 °C after holding for 10 min (Figure 5.16).

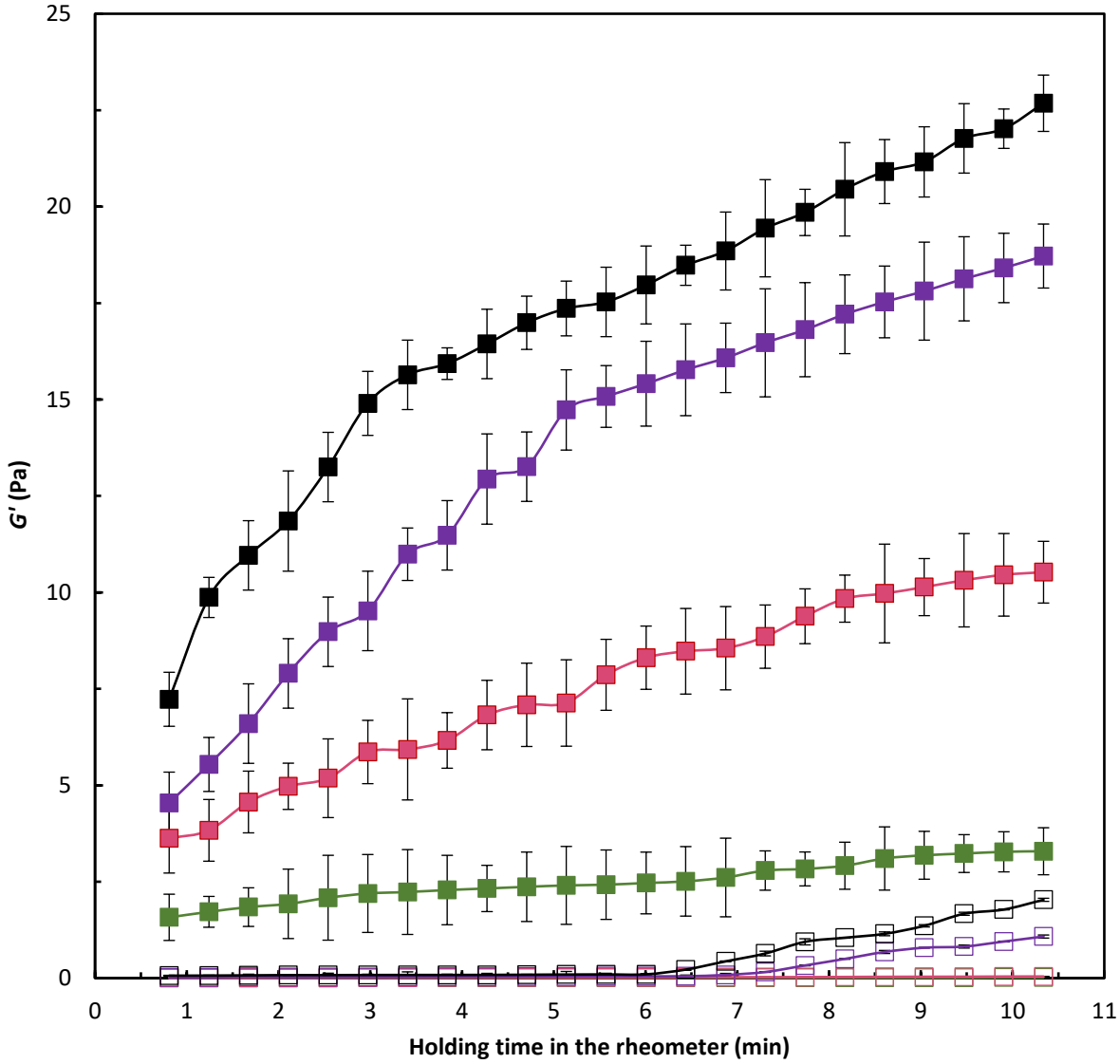

Figure 5.16. Storage modulus (*G'*) of magnesium-induced skim milk gels with magnesium chloride added (20 mmol L^{-1}) during holding for 10 min at different heating temperatures in the rheometer: 70 °C (■,□), 75 °C (■,□), 80 °C (■,□) and 85 °C (■,□). The open symbols and closed symbols refer to non-preheated and preheated skim milk samples, respectively. The data points show the mean values ± standard deviations (n = 4).

The storage modulus (*G'*) of magnesium-induced skim milk gels with magnesium chloride added (20 mmol L^{-1}) during cooling to 20 °C is presented in Figure 5.17. The *G'* values during cooling to 20 °C, increased significantly from 7.54 to 148.30 Pa and 0.01 to 24.36 Pa ($P < 0.05$) as the temperature was increased from 70 to 85 °C for preheated and non-preheated samples, respectively. The effect of holding temperature on final storage modulus (*G'*) at 20 °C obtained with preheated and non-preheated skim milk samples is shown in Figure 5.18. The final storage modulus (*G'*) of the preheated samples was significantly higher ($P < 0.05$) compared to non-preheated samples due to whey protein denaturation during the preheating treatment.

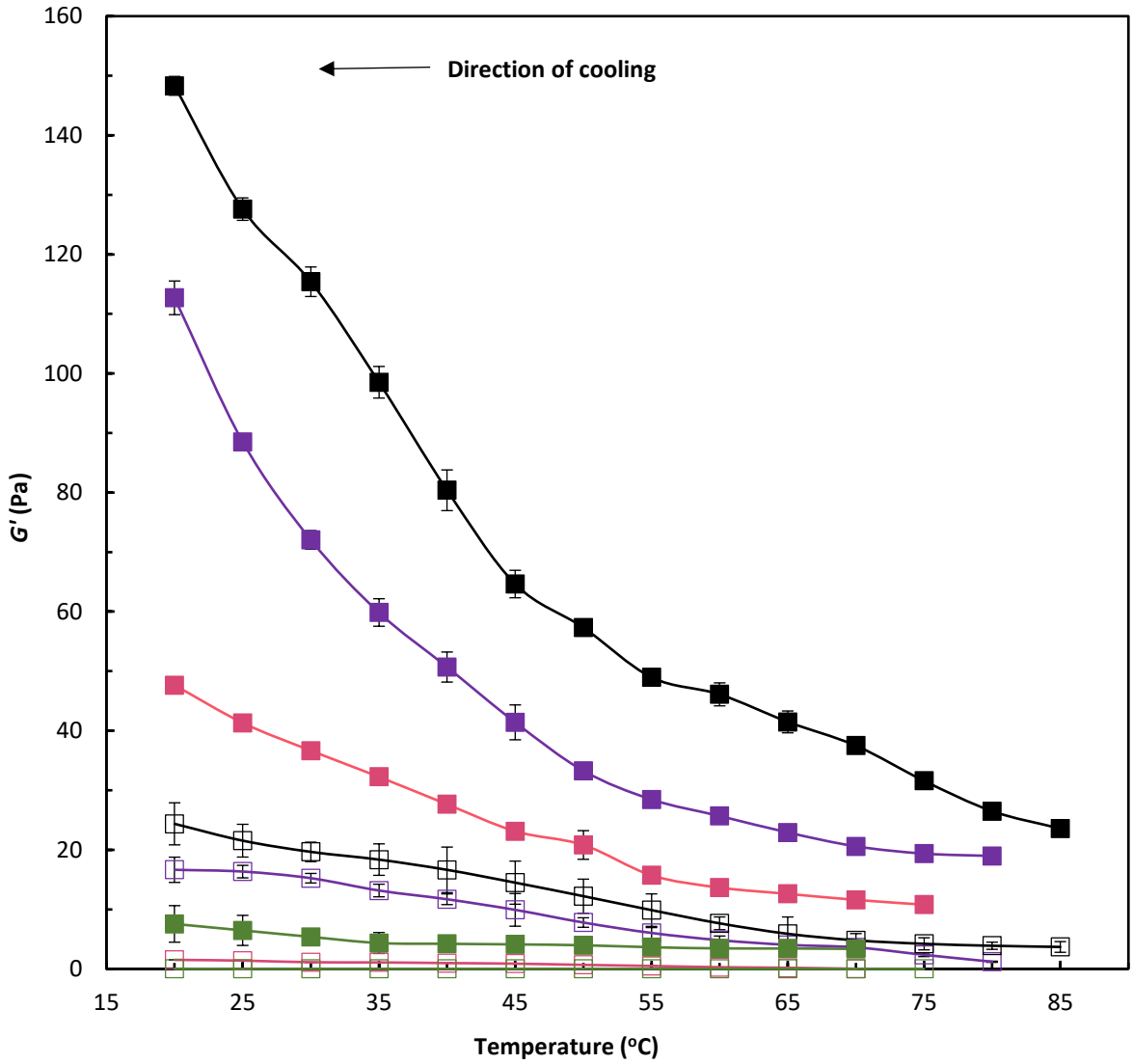

Figure 5.17. Storage modulus (*G'*) of magnesium-induced skim milk gels with magnesium chloride added (20 mmol L^{-1}) during cooling to 20 °C from 70 °C (■,□), 75 °C (■,□), 80 °C (■,□) and 85 °C (■,□). The open symbols and closed symbols refer to non-preheated and preheated skim milk samples, respectively. The data points show the mean values ± standard deviations (n = 4).

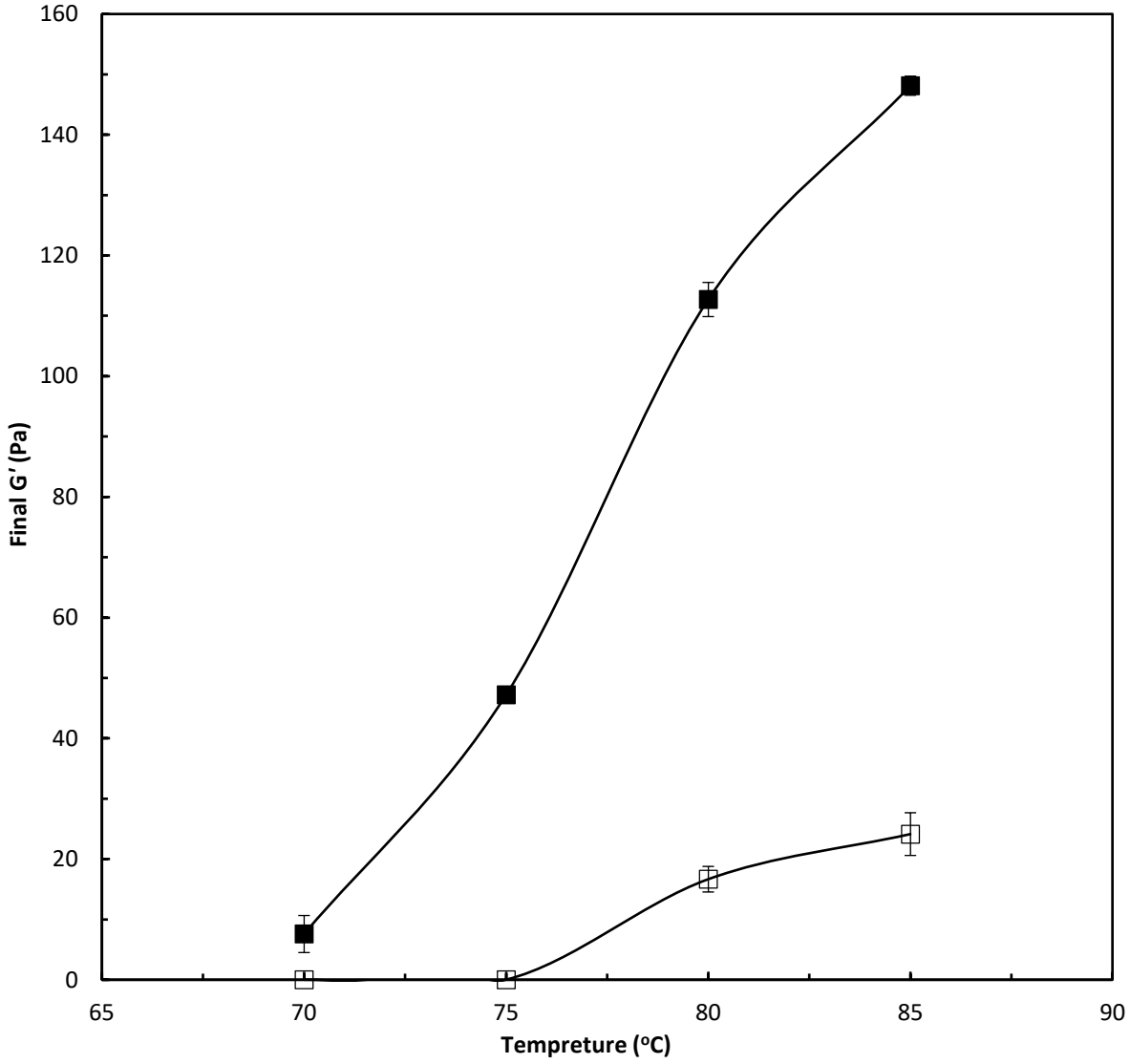

Figure 5.18. Effect of holding temperature on final storage modulus (*G'*) at 20 °C obtained with preheated and non-preheated skim milk samples, 10 min holding time and 20 mmol L^{-1} added magnesium chloride: □ non-preheated and ■ preheated skim milk samples. Milk samples were preheated at 90 ± 1 °C and cooled to 20 ± 1 °C prior to magnesium chloride addition. The data points show the mean values ± standard deviations (n = 4).

Both the preheating treatment and holding temperature had a significant effect on the formation of gels ($P < 0.05$). The results of this study demonstrate that temperature influenced gel formation but was not the main contributor because the preheated samples with no added magnesium salt showed no gelation even after the higher temperature of heating in the rheometer (Figure 5.10), the same result was found for the non-preheated samples. This finding was obtained because the high pH (6.70) of natural milk and low concentration of ionic magnesium Mg^{2+} (0.018 mg mL^{-1}) are not sufficient to cause

destabilisation of the skim milk. As discussed earlier, one of the factors that may contribute to the formation of skim milk gels is Mg^{2+} bridging between proteins.

It has been reported that during gel formation, the samples exhibit dense clusters of aggregated casein particles resulting from extensive particle rearrangement (Hahn, Krzeminski, Wille, Weiss, & Hinrichs, 2012; Takeuchi & Cunha, 2008). Moreover, it is known that the preheating increases the viscosity of milk, due to denaturation of whey proteins and the attachment of β-lactoglobulin to casein micelles, which increases the cross-linking of the resulting gel network (Lucey, Mishra, Hassan, & Johnson, 2005). The high gel strength (*G*') obtained in this study formed with the preheated skim milk samples can be explained as follows: during preheating at 90 ± 1 ^{o}C, denatured β-lactoglobulin and α-lactalbumin tend to aggregate to form a whey protein network and the resulting network strengthens the magnesium-induced milk gel by cross-linking the micelles during gelation at 80 ^{o}C (Pappas & Rothwell, 1991).

A comparative study of the frequency and strain sweep results for the gels obtained from the preheated and non-preheated skim milk samples was performed and the results are presented in Figures 5.19 and 5.20. As the gels were not formed with non-preheated samples at 70 ^{o}C and 75 ^{o}C holding temperatures in the rheometer, thus this data is not presented in Figure 5.19 and Figure 5.20. The *G*' and *G*'' difference between these samples indicated that the resulting gel network was weaker than that of the gels obtained from preheated samples (Figure 5.19). The effect of preheating was also evident in the physical characteristics of the resulting skim milk gels, such as with the oscillation stress observed. Strain was imposed and the resulting change in oscillation stress was observed and is shown in Figure 5.20. The resulting stress of the gels obtained from the preheated skim milk samples was notably higher than that of the gels obtained from the non-preheated skim milk samples. The preheating conditions affected the structure of the gel: a higher temperature was associated with a faster gelation rate, resulting in a significant increase in the oscillation stress compared with that of the gels obtained from the non-preheated samples ($P < 0.05$).

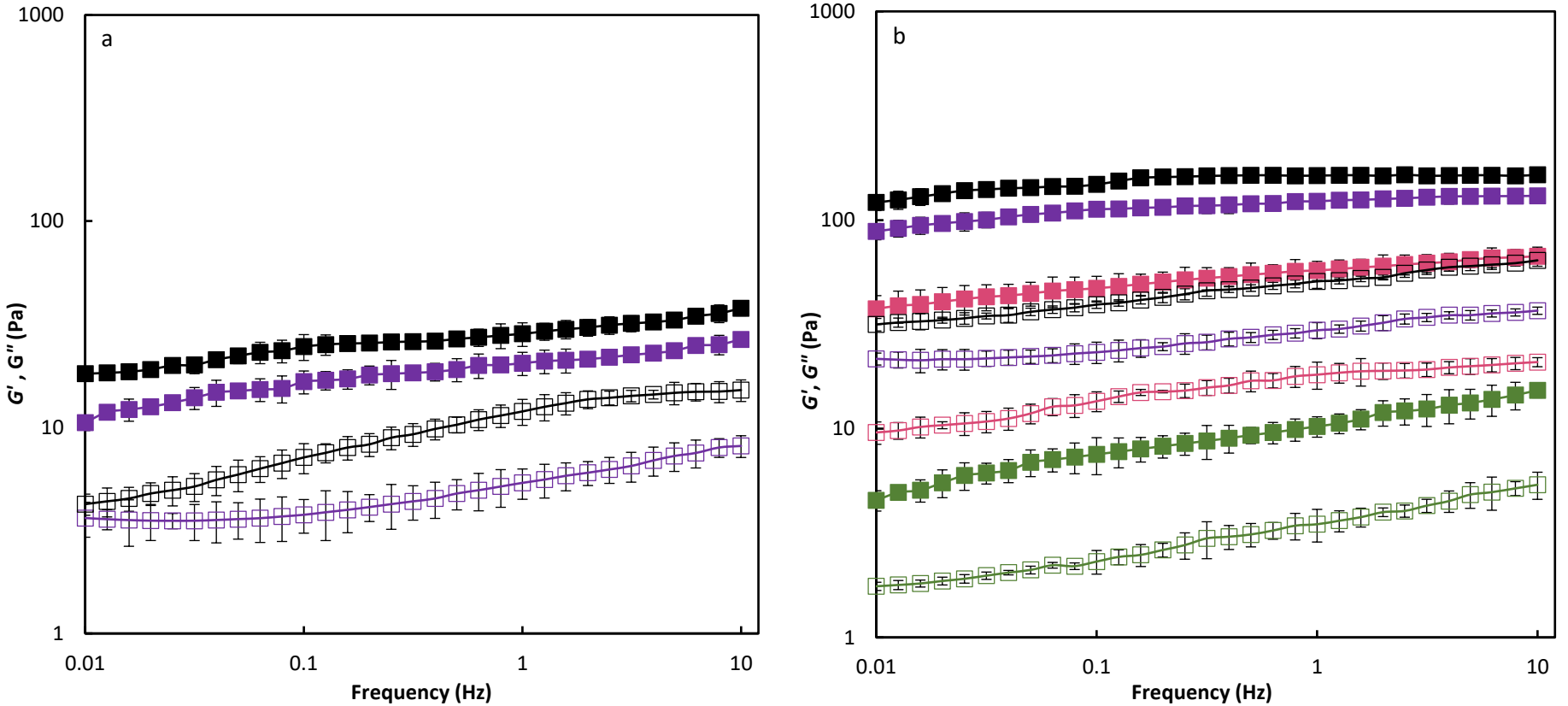

Figure 5.19. Storage (*G*') and loss (*G*") modulus of magnesium-induced skim milk gels with 20 mmol L^{-1} added magnesium chloride as a function of frequency at 20 °C: (a) non-preheated samples, (b) preheated samples and the samples were heated to temperatures at 70 °C (■,□), 75 °C (■,□), 80 °C (■,□) and 85 °C (■,□) in the rheometer and held for 10 min. The closed symbols represent the *G*' values and the open symbols present the *G*" values. The data points show the mean values ± standard deviations (n = 4).

The gels obtained from the non-preheated samples exhibited a breaking strain of 72%, whereas the breaking strain of the gels obtained from the preheated samples with the same concentration of added magnesium was 63%. The gels obtained from the preheated samples with a lower concentration of added magnesium (10 mmol L^{-1} and 12.50 mmol L^{-1}) showed the same breaking strain as the gels obtained from non-preheated samples with the higher concentrations of added magnesium (15 mmol L^{-1} and 20 mmol L^{-1}) (Figure 5.13 and Figure 5.20).

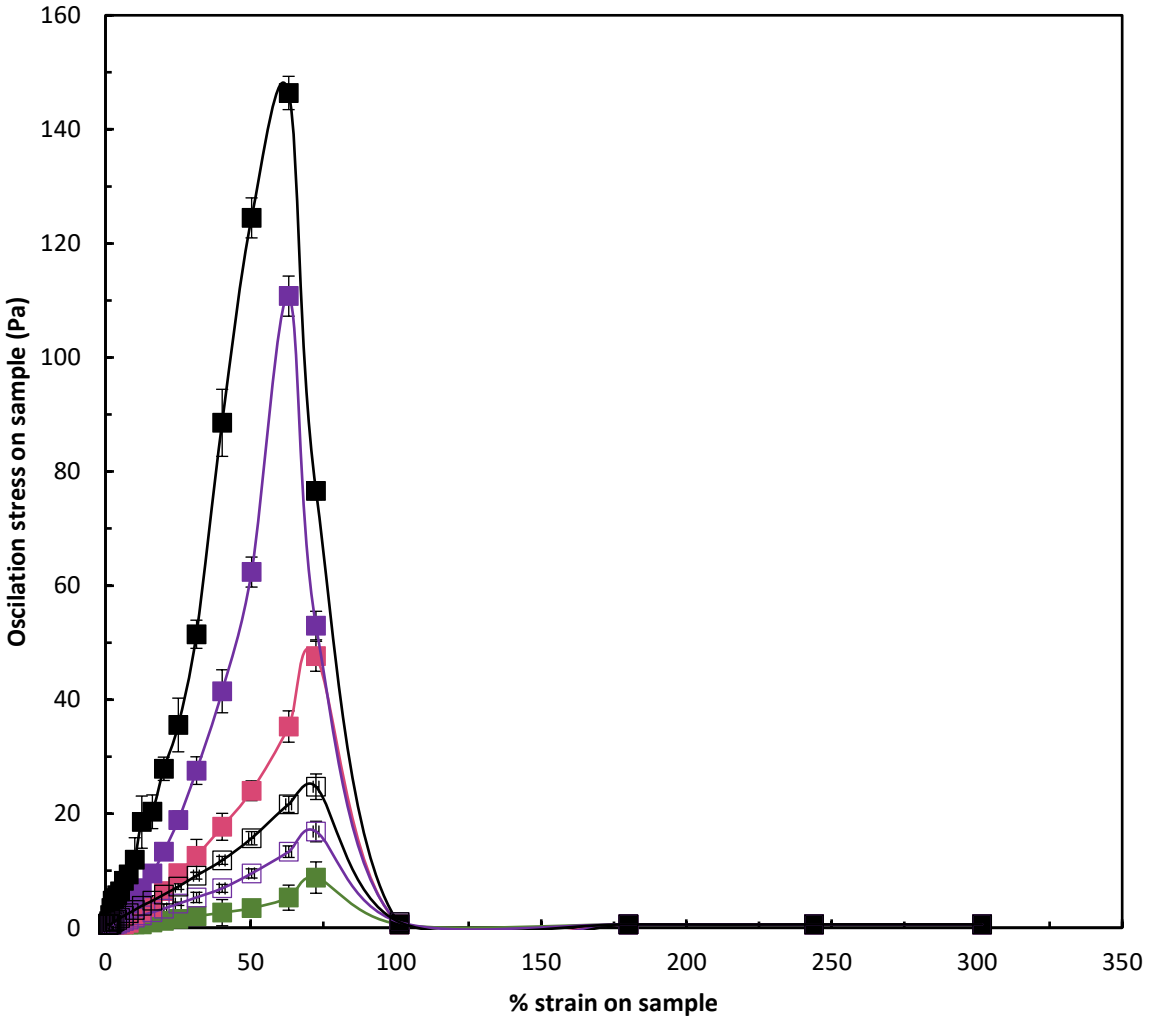

Figure 5.20. Oscillation stress of magnesium-induced skim milk gels as a function of strain in the presence of 20 mmol L^{-1} magnesium chloride at 20 °C: 70 °C (■), 75 °C (■), 80 °C (■,□) and 85 °C (■,□). The open symbols refer to the non-preheated and the closed symbols refer to preheated skim milk samples. The data points show the mean values ± standard deviations (n = 4).

5.5 Conclusions

It can be concluded from visual observations and rheometer results that added magnesium can cause gelation of skim milk when milk is heated to 70 ^{o}C. Heat treatment combined with magnesium chloride addition was found to provide a firm gel structure for skim milk. The amount of magnesium chloride required for gelation and gel firmness (*G*') varied depending on heating temperature and holding time in the rheometer. The final *G*' and breaking stress of skim milk gels increased as the magnesium concentration, heating temperature and holding time in the rheometer increased. Preheat treatment at 90 ^{o}C for 10 min influenced the structure development of magnesium-included skim milk gels and thus the strength of skim milk gels.

Chapter 6

Overall Discussion

6.1 Introduction

Wet chemistry (EDTA titration) and instrumental (atomic absorption spectroscopy) methods are commonly used for the measurement of total magnesium in milk. Thus, in this study, to profile the magnesium distribution between the serum and micellar phases, the total magnesium in skim milk and serum was determined using EDTA titration and AAS method was used as a reference method to validate the EDTA titration results (results from two methods are compared and discussed in Chapter 4). For the measurement of ionic magnesium in skim milk, the spectrophotometric method using magnesium fluorescence dye (Magnesium 510 probe) was used to determine ionic magnesium concentrations in the serum. Fluorometric measurement of ionic magnesium (Mg^{2+}) in milk using a Magnesium 510 probe was attempted for the first time and the results are presented in Chapter 4. To examine the properties of resulting magnesium-induced milk gels that can form a visual observation study and a rheological study using a controlled stress rheometer were performed and discussed in details in Chapter 5.

6.2 Distribution of magnesium between the serum and micellar phases

The distribution of magnesium between the serum and micellar phases in skim milk at different pH values and with different concentration of added magnesium chloride (Tables 4.2 to 4.4) was highly influenced by both factors, pH and magnesium chloride concentration added. When the pH was adjusted back to pH 6.50 (Table 4.4) this resulted in a reduction in the ionic magnesium (Mg^{2+}) concentration but this was not significant ($P > 0.05$). A relationship was found between pH and ionic magnesium (Mg^{2+}): a reduction in the pH leads to an associated increase in ionic magnesium (Mg^{2+}) in the serum phase. The addition of magnesium chloride to skim milk leads to the release of hydrogen ions due to a shift in the equilibrium in the milk; the added magnesium chloride reacts with inorganic phosphate in the serum phase forming magnesium phosphate by releasing hydrogen ions, which leads to a reduction in pH and an increase in ionic magnesium (Mg^{2+}) concentration in the serum phase

(O'Mahony & McSweeney, 2016). Attention needs to be paid to ionic magnesium (Mg^{2+}) due to the role of ionic magnesium (Mg^{2+}) in protein destabilisation.

6.3 Magnesium-induced skim milk gels

The impact of heat on skim milk with added magnesium chloride was evaluated and the results revealed that the thickening or gelation of skim milk obtained in the presence of added magnesium chloride. The gels were obtained in the samples at pH 6.43 with 10 mmol L^{-1} added magnesium chloride with heating to 80 ^{o}C, whereas gel strength increased with increasing concentrations of magnesium chloride added to skim milk. It has been reported that the viscosity of reconstituted skim milk increased with the addition of 5 mmol L^{-1} calcium chloride and heating to 70 ^{o}C for 10 min at pH 6.00, whereas gelation occurred with the addition of 20 mmol L^{-1} calcium after heating at 70 ^{o}C (McKinnon, Yap, Augustin, & Hemar, 2009). These observations support the findings obtained in the current study: increasing the amount of magnesium salt addition increases the gel strength.

The gels were obtained from preheated skim milk samples in the presence of lower concentrations of added magnesium chloride (or a higher pH), due to the denaturation of whey proteins during preheating at 90 ± 1 ^{o}C. The denatured whey proteins associated with the casein micelles, particularly κ-casein, have a higher isoelectric pH, as this pH is higher than that of casein, heated skim milk was likely to undergo gelation (Anema & Li, 2003). The gelation in the non-preheated samples occurred at a lower pH in the presence of added magnesium chloride and at a high heating temperature, which was required to induce Mg^{2+} bridging (Section 5.4). The final *G*' value of a gel formed with preheated skim milk was observed to be higher than that of non-preheated skim milk because the denatured whey proteins and casein micelles bind to each other and act as a bridge, increasing the strength and number of bonds between protein particles (Vasbinder & De Kruif, 2003; Oldfield et al, 2000; Petit et al., 2011).

6.4 Does magnesium distribution influence the magnesium-induced gelation?

It is well known that milk salts, particularly divalent cations and pH are important factors in the stability of milk during processing. Heating, the addition of magnesium chloride or acidification leads to an increase in ionic strength and thus induces gelation (O'Mahony & Fox,

2013). In particular, these factors influence the stability of milk proteins with respect to aggregation, gelation and precipitation (Holt & Horne, 1996; Vasbinder et al., 2003; Schmidt, 1981).

6.4.1 Effect of pH

Distribution results reveal that decreasing the pH increased the ionic magnesium in the serum phase (Table 4.2). Decreasing the pH increased the solubilisation of CCP and induced the dissolution of magnesium from the casein micelles (Anema, 2009; Anema & Klostermeyer, 1997; Dalgleish & Law, 1989) and these effects caused partial rearrangement of the inner structure of the casein micelles to help form the final gel network (Lucey, 2002). However, pH drop was not the main factor in the gelation of the magnesium-enriched skim milk samples, the concentration of magnesium ions and the heating temperature also contributed to the gelation process.

6.4.2 Effect of preheating temperature

It was found from distribution results that preheating temperature had no effect on the distribution of magnesium between the serum and micellar phases (Table 4.3). In contrast, preheating of skim milk and the heating temperature in the rheometer significantly affected the gel strength of magnesium-added skim milk samples (Figure 5.18). Gelation occurred upon heating because when magnesium-added milk is heated, magnesium moves towards the micellar phase, interacts with casein and induces casein aggregation (Pouliot et al., 1989; O'Mahony & Fox, 2013; Holt 1995).

In the presence of magnesium chloride, heating increased the cross-linking of micelles to enhance gel formation (Ramasubramanian et al., 2013). Moreover, the heating of skim milk samples induced the denaturation of whey proteins and their attachment to the surface of the casein micelles through the κ-casein. As a result, the denatured whey proteins became sensitive to aggregation due to a decrease in their net charge, which lead to less repulsion. The denatured whey proteins formed linkages to whey proteins already bound to casein micelles, which increased the number and strength of the protein bonds among particles and thus lead to the final gel network (Lucey et al., 1997; Vasbinder et al., 2003). The heated whey proteins were likely to be sensitive to aggregation in the presence of magnesium ions, causing

the formation of a firm gels network, as confirmed by the higher *G*' in Figure 5.8 for the skim milk samples with added magnesium chloride compared with that of samples with no magnesium chloride addition.

The magnesium-induced gelation of non-preheated milk was likely caused by an increase in micellar magnesium upon heating which initiates the casein aggregation and the formation of a gel network (Vasbinder & De Kruif, 2003). The formation of magnesium-induced skim milk gels from preheated skim milk may involve the following mechanisms: (1) the added magnesium to skim milk samples, increases the ionic magnesium (Mg^{2+}) concentration and this increase in ionic magnesium (Mg^{2+}) in skim milk samples may accelerate the aggregation of sensitive casein, (2) whey proteins undergo denaturation during preheating at 90 ^{o}C for 10 min and (3) the reduction in the skim milk pH obtained by the addition of magnesium decreases the electrostatic repulsion between micelles (Lin et al., 2018) and shifts the salt equilibria in the milk that lead to gelation. However, the main driving force for the interaction between proteins for gelation is hydrophobic interactions and gels obtained at a lower temperature are weaker due to weaker hydrophobic interactions (Phadungath, 2005).

6.4.3 Effect of magnesium chloride addition

The addition of magnesium chloride to skim milk decreased the concomitant pH (Table 4.1). For skim milk samples with added magnesium chloride at 10 and 20 mmol L^{-1}, the skim milk pH dropped to pH 6.43 and pH 6.30 and the temperature of gelation was 80 ^{o}C and 70 ^{o}C, respectively (Figure 5.8 and Table 5.2). Higher concentrations of added magnesium chloride decreased the gelation temperature because the addition of magnesium chloride to skim milk resulted in an increase in both ionic magnesium (Mg^{2+}) and micellar magnesium (Table 4.4) which induced casein aggregation and caused the magnesium-induced gelation by bridging between ionic magnesium (Mg^{2+}) and protein (O'Mahony & Fox, 2013). The enrichment of skim milk with calcium salt increased the calcium bridging between casein micelles by decreasing the net negative charge of the caseins molecules, which indicated that the addition of calcium salt to skim milk increased the amount of CCP in micelles and the number of cross-linking bridges between the phosphate groups of α-casein and β-casein on the surface of κ-casein (Bringe & Kinsella, 1991; Dalgleish & Law, 1989; Holt & Horne, 1996; Pesic

et al., 2012; Walstra et al., 2005). The addition of magnesium chloride appeared to have a similar effect.

It has also been reported that the addition of salts is known to make whey proteins more sensitive to denaturation and aggregation (Koutina et al., 2014; Pappas & Rothwell, 1991). Moreover, the enrichment of skim milk with salts generally swells the casein micelles, decreases the total protein concentration in the serum phase and decreases the zeta potential (Anema & Li, 2003; Huppertz & Fox, 2006; Kaliappan & Lucey, 2011; Koutina et al., 2016; Philippe et al., 2003). The added salts become associated with the micelles and leads to an increase in inorganic phosphate and citrate in the micelles (Philippe et al., 2003). Thus, the addition of salts to skim milk influences the mineral concentration in the serum phase and enhances the protein-protein interactions between casein micelles (Lowe et al., 2004; Petit et al., 2011), which is in agreement with this study (Table 4.4 and Figure 5.10).

The addition of magnesium chloride increased the ionic magnesium (Mg^{2+}) concentration as well as the concentration of micellar magnesium (Table 4.4); thus, it was not possible to judge which factor played a prominent role in gel formation. In a previous study of calcium-induced gelation, a relatively longer gelation time was observed for the samples with low calcium ion activity and a low micellar calcium phosphate concentration (Koutina et al., 2016). The gelation time for the samples with high calcium ion activity, low phosphorus and low micellar calcium concentrations were longer than that of the same calcium ion activity with high phosphorus and high micellar calcium concentrations. This finding revealed that not only calcium ion activity but also the amount of micellar calcium were important factors in the induction of gelation and the gelation time. Hence, it is possible not only ionic magnesium (Mg^{2+}) but also micellar calcium, magnesium and phosphorus contribute to the gelation process.

6.5 Comparison of calcium-induced (literature) and magnesium-induced gels (current study)

A comparison of magnesium-induced gels with calcium-induced gels obtained from reconstituted skim milk found that gelation occurs in the presence of 30 mmol L^{-1} added calcium after heating at 70 ^{o}C (Lin et al., 2018), whereas magnesium-induced gelation occurred with 20 mmol L^{-1} added magnesium chloride after heating at 70 ^{o}C (current study). The addition of the salts interrupted the dynamic ionic equilibria in milk. Upon heating, a

decrease occurred in the concentration of calcium and magnesium in the serum phase (Pouliot et al., 1989) but calcium and magnesium redistribute differently upon cooling (Holt 1995; Lewis, 2011, Abdulghani et al., 2015). The difference in redistribution behaviour of magnesium and calcium upon cooling is possibly the reason for the different concentration of salt needed for magnesium-induced gel (current study) and calcium-induced gel (Lin et al., 2018). The involvement of magnesium ions in magnesium-induced gelation deserves further research to evaluate the full mechanism of gelation.

A decreased gelation time was obtained with increasing concentrations of magnesium. A shorter gelation time indicated faster network formation with magnesium chloride addition though the temperature was higher (80 ^{o}C) than that of the calcium study (70 ^{o}C) (Lin et al., 2018). The comparison of the storage moduli clearly showed that the values found in this study were higher than those obtained in previous studies with calcium-induced gelation and this may be due to the different heating temperatures and holding times used (Lin et al., 2018). Moreover, Koutina et al. (2016) found that the skim milk samples with 30 mmol L^{-1} added calcium chloride reached gelation heating at 90 ^{o}C, reaching a value higher than 100 Pa during 60 min of holding at 90 ^{o}C, whereas Lin et al. (2018) found a storage modulus of 15 Pa with the same concentration of added calcium chloride at a lower temperature (70 ^{o}C). In contrast, in the present study, a storage modulus of 24 Pa and a final storage modulus of 141 Pa was obtained with 45 min of holding at 80 ^{o}C and 20 mmol L^{-1} added magnesium (Figure 5.9 and Figure 5.10). It can be concluded that the heating temperature in the rheometer affects the gel strength of magnesium-induced and calcium-induced gels.

6.6 Conclusions

Temperature, pH and the addition of magnesium chloride influence the distribution of magnesium as well as gelation properties of magnesium-induced milk gels. The mechanism of gel formation in the presence of added magnesium and the involvement of ionic magnesium (Mg^{2+}), casein and whey proteins in this process deserve further research. To produce a new type of yogurt, such as magnesium fortified dairy products at high pH, these types of gels should be studied in further detail.

Chapter 7

Conclusions and Recommendations

7.1 Conclusions

The aim of this study was to assess the magnesium distribution in skim milk and the effect of magnesium addition on skim milk gelation.

The addition of magnesium chloride (0 to 40 mmol L^{-1}) increased the amounts of the total soluble magnesium, micellar magnesium and ionic magnesium (Mg^{2+}) and resulted in a concomitant decrease in a pH from 6.70 to 6.22.

Decreasing the pH of the skim milk increased the total soluble magnesium and ionic magnesium (Mg^{2+}) in the serum phase.

Preheating of skim milk did not affect the distribution of magnesium between the serum and micellar phases but it contributed significantly on gelation.

pH was not the dominating factor in the formation of magnesium-induced gels as the gelation only occurred in the range of pH 6.20 – 6.12 when skim milk was heated above 60 ^{o}C.

Higher concentrations of magnesium chloride added to skim milk lead to firmer gels obtained.

Increased heating temperatures in the rheometer were associated with shorter gelation time, but this relationship depended on the concentration of magnesium chloride added.

pH and the addition of magnesium chloride to skim milk play a vital role on the distribution of magnesium between the serum and micellar phases, whereas heating time and temperature in the rheometer, preheating temperature and the concentration of added magnesium chloride contributed to the formation of magnesium-induced gels.

Therefore, the process of manufacturing magnesium-induced skim milk gels through heating at temperatures above 60 ^{o}C can be adapted to an industrial-level process for making yogurt like novel dairy products at high pH.

7.2 Recommendations for further research

Various conclusions were drawn based on the results obtained in this study. However, several questions arose and these can only be answered by further investigations that are beyond the scope of this study.

- In this study, heat treatment did not have any effect on magnesium distribution between the serum and micellar phases but increased the strength of the magnesium-induced gel. Thus, the effect of heating on magnesium distribution and the role of ionic magnesium (Mg^{2+}) during gel formation deserves further study to elucidate the full mechanism of heating.
- pH played a vital role on the distribution of magnesium and ionic magnesium (Mg^{2+}) but the effect of pH on gel formation was not assessed in this study. Thus, it would be interesting to investigate the effect of readjusting pH after the addition of magnesium chloride on the final *G'* of the formed skim milk gel and the contribution of pH to the magnesium-induced gelation.
- The addition of magnesium chloride to skim milk increased the strength of the resulting gel. In this study, the concentration of added magnesium chloride was limited to 20 mmol L^{-1} and it would be interesting to determine the maximum concentration of added magnesium on gel formation in terms of coagulation or syneresis.
- The scope of this study was limited to ionic magnesium (Mg^{2+}) but it would be interesting to determine the total ionic strength and the behaviour after the addition of magnesium chloride.
- The rheological behaviour of magnesium-induced gels was studied but the casein distribution between the serum and micellar phases after magnesium chloride added, the particle size of casein micelles and the mechanism of aggregation were not addressed in this study. Thus, it would be interesting to investigate the distribution of casein between the serum and micellar phases and the effects of distribution on the gel formation. The particle size of casein micelle can be measured to determine the effect of added magnesium chloride on the size distribution of casein micelle.
- The effect of preheating on the concentration of native whey protein in the samples with or without added magnesium chloride can also be investigated. The rheological study was conducted from 70 to 85 ^{o}C and thus, it would be useful to characterise the

rheology of the sample at lower temperatures (i.e., 60 to 65 ^{o}C) because skim milk thickening was observed at these temperatures in a water bath (visual observations).

- Other magnesium salts such as magnesium citrate, magnesium carbonate, magnesium lactate and magnesium gluconate could also be used to study the effect of heating in the presence of different salts.

Chapter 8

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Chapter 9

Appendix

9.1 ANOVA data

Section 4.2 Magnesium ion and milk pH

Page 46-48, Figure no 4.1 and Table 4.1

1. One-way ANOVA: Temperature 20 °C versus Concentration (non-preheated)

Factor Information

Factor	Levels	Values
Concentration	7	0, 5, 10, 15, 20, 30, 40

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Concentration	6	0.373200	0.062200	1244.00	0.000
Error	21	0.000350	0.000050		
Total	27	0.373550			

2. One-way ANOVA: Temperature 90 °C versus Concentration (preheated)

Factor Information

Factor	Levels	Values
Concentration	7	0, 5, 10, 15, 20, 30, 40

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Concentration	6	0.420943	0.070157	613.88	0.000
Error	21	0.000800	0.000114		
Total	27	0.421743			

3. One-way ANOVA: 15 mmol L^{-1} added magnesium chloride versus Temperature

Factor Information

Factor	Levels	Values
Temperature	7	20, 30, 45, 60, 70, 80, 90

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Temperature	6	0.010000	0.001667	15.56	0.001
Error	21	0.000750	0.000107		
Total	27	0.010750			

4. One-way ANOVA: 0 mmol L^{-1} added magnesium chloride versus Temperature

Factor Information

Factor	Levels	Values
Temperature	7	20, 30, 45, 60, 70, 80, 90

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Temperature	6	0.002143	0.000357	2.63	0.116
Error	21	0.000950	0.000136		
Total	27	0.003093			

Table 4.1 Page 48

5. One-way ANOVA: 0 mmol L^{-1} added magnesium chloride versus Condition

Factor Information

Factor	Levels	Values
Condition	2	Non-preheating, Preheating

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Condition	1	0.000400	0.000400	8.00	0.106
Error	6	0.000100	0.000050		
Total	7	0.000500			

6. One-way ANOVA: 5 mmol L^{-1} added magnesium chloride versus Condition

Factor Information

Factor	Levels	Values
Condition	2	Non-preheating, Preheating

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Condition	1	0.013225	0.013225	105.80	0.009
Error	6	0.000250	0.000125		
Total	7	0.013475			

7. One-way ANOVA: 10 mmol L^{-1} added magnesium chloride versus Condition

Factor Information

Factor	Levels	Values
Condition	2	Non-preheating, Preheating

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Condition	1	0.004900	0.004900	98.00	0.010
Error	6	0.000100	0.000050		
Total	7	0.005000			

8. One-way ANOVA: 15 mmol L^{-1} added magnesium chloride versus Condition

Factor Information

Factor	Levels	Values
Condition	2	Non-preheating, Preheating

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Condition	1	0.003600	0.003600	72.00	0.014
Error	6	0.000100	0.000050		
Total	7	0.003700			

9. One-way ANOVA: 20 mmol L^{-1} added magnesium chloride versus Condition

Factor Information

Factor	Levels	Values
Condition	2	Non-preheating, Preheating

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Condition	1	0.002500	0.002500	50.00	0.019
Error	6	0.000100	0.000050		
Total	7	0.002600			

10. One-way ANOVA: 30 mmol L^{-1} added magnesium chloride versus Condition

Factor Information

Factor	Levels	Values
Condition	2	Non-preheating, Preheating

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Condition	1	0.005625	0.005625	45.00	0.022
Error	6	0.000250	0.000125		
Total	7	0.005875			

11. One-way ANOVA: 40 mmol L^{-1} added magnesium chloride versus Condition

Factor Information

Factor	Levels	Values
Condition	2	Non-preheating, Preheating

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Condition	1	0.009025	0.009025	72.20	0.014
Error	6	0.000250	0.000125		
Total	7	0.009275			

Section 4.3.1 Effect of pH (page 48-51)

Table 4.2 (page 49)

1. One-way ANOVA: pH versus Magnesium chloride addition

Factor Information

Factor	Levels	Values
Addition, mmol L^{-1}	2	0, 15

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Addition, mmol L^{-1}	1	0.081225	0.081225	649.80	0.002
Error	6	0.000250	0.000125		
Total	7	0.081475			

2. One-way ANOVA: Total soluble magnesium in the serum versus pH (0 mmol L^{-1})

Factor Information

Factor	Levels	Values
pH	8	5.5, 5.7, 6.0, 6.2, 6.5, 6.7, 7.0, 7.2

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	7	0.002546	0.000364	2.48	0.113
Error	24	0.001172	0.000147		
Total	31	0.003718			

3. One-way ANOVA: Total soluble magnesium in the serum versus pH (15 mmol L^{-1})

Factor Information

Factor	Levels	Values
pH	8	5.5, 5.7, 6.0, 6.2, 6.5, 6.7, 7.0, 7.2

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	7	0.031751	0.004536	17.91	0.000
Error	24	0.002026	0.000253		
Total	31	0.033777			

4. One-way ANOVA: Ionic magnesium in the serum versus pH (0 mmol L^{-1})

Factor Information

Factor	Levels	Values
pH	8	5.5, 5.7, 6.0, 6.2, 6.5, 6.7, 7.0, 7.2

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	7	0.001328	0.000190	4.43	0.027
Error	24	0.000343	0.000043		
Total	31	0.001671			

5. One-way ANOVA: Ionic magnesium in the serum versus pH (15 mmol L^{-1})

Factor Information

Factor	Levels	Values
pH	8	5.5, 5.7, 6.0, 6.2, 6.5, 6.7, 7.0, 7.2

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	7	0.015922	0.002275	62.35	0.000
Error	24	0.000292	0.000036		
Total	31	0.016214			

6. One-way ANOVA: Total soluble magnesium in the serum versus Magnesium chloride added (pH 5.50)

Factor Information

Factor	Levels	Values
Level, mmol L^{-1}	2	0, 15

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Level, mmol L^{-1}	1	0.106929	0.106929	722.69	0.001
Error	6	0.000296	0.000148		
Total	7	0.107225			

7. One-way ANOVA: Total soluble magnesium in the serum versus Magnesium chloride added (pH 5.70)

Factor Information

Factor	Levels	Values
Level, mmol L^{-1}	2	0, 15

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Level, mmol L^{-1}	1	0.106929	0.106929	669.59	0.001
Error	6	0.000319	0.000160		
Total	7	0.107248			

8. One-way ANOVA: Total soluble magnesium in the serum versus Magnesium chloride added (pH 6.00)

Factor Information

Factor	Levels	Values
Level, mmol L^{-1}	2	0, 15

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Level, mmol L^{-1}	1	0.103684	0.103684	370.17	0.003
Error	6	0.000560	0.000280		
Total	7	0.104244			

9. One-way ANOVA: Total soluble magnesium in the serum versus Magnesium chloride added (pH 6.20)

Factor Information

Factor	Levels	Values
Level, mmol L^{-1}	2	0, 15

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Level, mmol L^{-1}	1	0.100489	0.100489	813.88	0.001
Error	6	0.000247	0.000123		
Total	7	0.100736			

10. One-way ANOVA: Total soluble magnesium in the serum versus Magnesium chloride added (pH 6.50)

Factor Information

Factor	Levels	Values
Level, mmol L^{-1}	2	0, 15

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Level, mmol L^{-1}	1	0.096721	0.096721	290.31	0.003
Error	6	0.000666	0.000333		
Total	7	0.097387			

11. One-way ANOVA: Total soluble magnesium in the serum versus Magnesium chloride added (pH 6.70)

Factor Information

Factor	Levels	Values
Level, mmol L^{-1}	2	0, 15

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Level, mmol L^{-1}	1	0.088209	0.088209	531.97	0.002
Error	6	0.000332	0.000166		
Total	7	0.088541			

12. One-way ANOVA: Total soluble magnesium in the serum versus Magnesium chloride added (pH 7.00)

Factor Information

Factor	Levels	Values
Level, mmol L^{-1}	2	0, 15

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Level, mmol L^{-1}	1	0.069696	0.069696	243.07	0.004
Error	6	0.000573	0.000287		
Total	7	0.070269			

13. One-way ANOVA: Total soluble magnesium in the serum versus Magnesium chloride added (pH 7.20)

Factor Information

Factor	Levels	Values
Level, mmol L^{-1}	2	0, 15

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Level, mmol L^{-1}	1	0.052900	0.052900	518.42	0.002
Error	6	0.000204	0.000102		
Total	7	0.053104			

14. One-way ANOVA: Ionic magnesium in the serum versus Magnesium chloride added (pH 5.50)

Factor Information

Factor	Levels	Values
Level, mmol L^{-1}	2	0, 15

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Level, mmol L^{-1}	1	0.025281	0.025281	952.90	0.001
Error	6	0.000053	0.000027		
Total	7	0.025334			

15. One-way ANOVA: Ionic magnesium in the serum versus Magnesium chloride added (pH 5.70)

Factor Information

Factor	Levels	Values
Level, mmol L^{-1}	2	0, 15

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Level, mmol L^{-1}	1	0.021904	0.021904	660.49	0.002
Error	6	0.000066	0.000033		
Total	7	0.021970			

16. One-way ANOVA: Ionic magnesium in the serum versus Magnesium chloride added (pH 6.00)

Factor Information

Factor	Levels	Values
Level, mmol L^{-1}	2	0, 15

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Level, mmol L^{-1}	1	0.015376	0.015376	602.74	0.002
Error	6	0.000051	0.000026		
Total	7	0.015427			

17. One-way ANOVA: Ionic magnesium in the serum versus Magnesium chloride added (pH 6.20)

Factor Information

Factor	Levels	Values
Level, mmol L^{-1}	2	0, 15

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Level, mmol L^{-1}	1	0.013689	0.013689	335.38	0.003
Error	6	0.000082	0.000041		
Total	7	0.013771			

18. One-way ANOVA: Ionic magnesium in the serum versus Magnesium chloride added (pH 6.50)

Factor Information

Factor	Levels	Values
Level, mmol L^{-1}	2	0, 15

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Level, mmol L^{-1}	1	0.012769	0.012769	258.01	0.004
Error	6	0.000099	0.000049		
Total	7	0.012868			

19. One-way ANOVA: Ionic magnesium in the serum versus Magnesium chloride added (pH 6.70)

Factor Information

Factor	Levels	Values
Level, mmol L^{-1}	2	0, 15

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Level, mmol L^{-1}	1	0.012769	0.012769	221.48	0.004
Error	6	0.000115	0.000058		
Total	7	0.012884			

20. One-way ANOVA: Ionic magnesium in the serum versus Magnesium chloride added (pH 7.00)

Factor Information

Factor	Levels	Values
Level, mmol L^{-1}	2	0, 15

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Level, mmol L^{-1}	1	0.010000	0.010000	196.00	0.005
Error	6	0.000102	0.000051		
Total	7	0.010102			

21. One-way ANOVA: Ionic magnesium in the serum versus Magnesium chloride added (pH 6.70)

Factor Information

Factor	Levels	Values
Level, mmol L^{-1}	2	0, 15

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Level, mmol L^{-1}	1	0.006889	0.006889	207.73	0.005
Error	6	0.000066	0.000033		
Total	7	0.006955			

Table 4.2 For AAS results, page 49

1. One-way ANOVA: Total soluble magnesium in the serum versus pH (0 mmol L^{-1}, AAS technique)

Factor Information

Factor	Levels	Values
pH AAS	2	6.2, 6.7

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH AAS	1	0.000256	0.000256	128.00	0.008
Error	6	0.000004	0.000002		
Total	7	0.000260			

2. One-way ANOVA: Total soluble magnesium in the serum versus pH (15 mmol L^{-1}, AAS technique)

Factor Information

Factor	Levels	Values
PH AAS	2	6.5, 6.7

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
PH AAS	1	0.000289	0.000289	22.23	0.042
Error	6	0.000026	0.000013		
Total	7	0.000315			

3. One-way ANOVA: Total soluble magnesium in the serum versus Magnesium chloride added (pH 6.70, AAS technique)

Factor Information

Factor	Levels	Values
Level, AAS	2	0, 15

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Level, AAS	1	0.090000	0.090000	9000.00	0.000
Error	6	0.000020	0.000010		
Total	7	0.090020			

4. One-way ANOVA: Total soluble magnesium in the serum versus Methods (pH 6.70, 0 mmol L^{-1})

Factor Information

Factor	Levels	Values
Method	2	AAS, EDTA

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Method	1	0.000100	0.000100	1.92	0.300
Error	6	0.000104	0.000052		
Total	7	0.000204			

5. One-way ANOVA: Total soluble magnesium in the serum versus Methods (pH 6.20, 0 mmol L^{-1})

Factor Information

Factor	Levels	Values
Method	2	AAS, EDTA

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Method	1	0.000016	0.000016	0.26	0.664
Error	6	0.000125	0.000063		
Total	7	0.000141			

6. One-way ANOVA: Total soluble magnesium in the serum versus Methods (pH 6.50, 15 mmol L^{-1})

Factor Information

Factor	Levels	Values
Method	2	AAS, EDTA

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Method	1	0.000121	0.000121	0.48	0.559
Error	6	0.000502	0.000251		
Total	7	0.000623			

7. One-way ANOVA: Total soluble magnesium in the serum versus Methods (pH 6.70, 15 mmol L^{-1})

Factor Information

Factor	Levels	Values
Method	2	AAS, EDTA

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Method	1	0.000049	0.000049	0.40	0.594
Error	6	0.000248	0.000124		
Total	7	0.000297			

Section 4.3.2. Effects of preheating temperature (page 51-53)

Table 4.3 (page 52)

1. One-way ANOVA: Total soluble magnesium in the serum versus Temperature (0 mmol L^{-1})

Factor Information

Factor	Levels	Values
Temperature	6	20, 30, 45, 60, 70, 80

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Temperature	5	0.000010	0.000002	0.02	1.000
Error	18	0.000739	0.000123		
Total	23	0.000749			

2. One-way ANOVA: Total soluble magnesium in the serum versus Temperature (15 mmol L^{-1})

Factor Information

Factor	Levels	Values
Temperature	6	20, 30, 45, 60, 70, 80

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Temperature	5	0.000026	0.000005	0.04	0.999
Error	18	0.000795	0.000132		
Total	23	0.000820			

3. One-way ANOVA: Ionic magnesium in the serum versus Temperature (0 mmol L^{-1})

Factor Information

Factor	Levels	Values
Temperature	6	20, 30, 45, 60, 70, 80

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Temperature	5	0.000008	0.000002	0.04	0.999
Error	18	0.000254	0.000042		
Total	23	0.000262			

4. One-way ANOVA: Ionic magnesium in the serum versus Temperature (15 mmol L^{-1})

Factor Information

Factor	Levels	Values
Temperature	6	20, 30, 45, 60, 70, 80

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Temperature	5	0.000064	0.000013	0.26	0.919
Error	18	0.000297	0.000049		
Total	23	0.000361			

5. One-way ANOVA: Preheating temperature (20 °C) versus Magnesium chloride concentration added (total soluble magnesium in the serum)

Factor Information

Factor	Levels	Values
Concentration, mmol L^{-1}	2	0.000, 15.000

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Concentration, mmol L^{-1}	1	0.103041	0.103041	915.92	0.001
Error	6	0.000225	0.000113		
Total	7	0.103266			

6. One-way ANOVA: Preheating temperature (30 °C) versus Magnesium chloride concentration added (total soluble magnesium in the serum)

Factor Information

Factor	Levels	Values
Concentration, mmol L^{-1}	2	0.000, 15.000

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Concentration, mmol L^{-1}	1	0.101761	0.101761	791.91	0.001
Error	6	0.000257	0.000129		
Total	7	0.102018			

7. One-way ANOVA: Preheating temperature (45 °C) versus Magnesium chloride concentration added (total soluble magnesium in the serum)

Factor Information

Factor	Levels	Values
Concentration, mmol L^{-1}	2	0.000, 15.000

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Concentration, mmol L^{-1}	1	0.103041	0.103041	801.88	0.001
Error	6	0.000257	0.000129		
Total	7	0.103298			

8. One-way ANOVA: Preheating temperature (60 °C) versus Magnesium chloride concentration added (total soluble magnesium in the serum)

Factor Information

Factor	Levels	Values
Concentration, mmol L^{-1}	2	0.000, 15.000

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Concentration, mmol L^{-1}	1	0.103362	0.103362	661.52	0.002
Error	6	0.000313	0.000156		
Total	7	0.103675			

9. One-way ANOVA: Preheating temperature (70 °C) versus Magnesium chloride concentration added (total soluble magnesium in the serum)

Factor Information

Factor	Levels	Values
Concentration, mmol L^{-1}	2	0.000, 15.000

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Concentration, mmol L^{-1}	1	0.102400	0.102400	796.89	0.001
Error	6	0.000257	0.000129		
Total	7	0.102657			

10. One-way ANOVA: Preheating temperature (80 °C) versus Magnesium chloride concentration added (total soluble magnesium in the serum)

Factor Information

Factor	Levels	Values
Concentration, mmol L^{-1}	2	0.000, 15.000

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Concentration, mmol L^{-1}	1	0.104329	0.104329	927.37	0.001
Error	6	0.000225	0.000113		

Total	7	0.104554

11. One-way ANOVA: Preheating temperature (20 °C) versus Magnesium chloride concentration added (ionic magnesium in the serum)

Factor Information

Factor	Levels	Values
Concentration, mmol L^{-1}	2	0.000, 15.000

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Concentration, mmol L^{-1}	1	0.016002	0.016002	289.63	0.003
Error	6	0.000110	0.000055		
Total	7	0.016113			

12. One-way ANOVA: Preheating temperature (30 °C) versus Magnesium chloride concentration added (ionic magnesium in the serum)

Factor Information

Factor	Levels	Values
Concentration, mmol L^{-1}	2	0.000, 15.000

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Concentration, mmol L^{-1}	1	0.016384	0.016384	481.88	0.002
Error	6	0.000068	0.000034		
Total	7	0.016452			

13. One-way ANOVA: Preheating temperature (45 °C) versus Magnesium chloride concentration added (ionic magnesium in the serum)

Factor Information

Factor	Levels	Values
Concentration, mmol L^{-1}	2	0.000, 15.000

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Concentration, mmol L^{-1}	1	0.016384	0.016384	268.59	0.004
Error	6	0.000122	0.000061		
Total	7	0.016506			

14. One-way ANOVA: Preheating temperature (60 °C) versus Magnesium chloride concentration added (ionic magnesium in the serum)

Factor Information

Factor	Levels	Values
Concentration, mmol L^{-1}	2	0.000, 15.000

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Concentration, mmol L^{-1}	1	0.017956	0.017956	437.95	0.002
Error	6	0.000082	0.000041		
Total	7	0.018038			

15. One-way ANOVA: Preheating temperature (70 °C) versus Magnesium chloride concentration added (ionic magnesium in the serum)

Factor Information

Factor	Levels	Values
Concentration, mmol L^{-1}	2	0.000, 15.000

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value

Concentration, mmol L^{-1}	1	0.016384	0.016384	315.08	0.003
Error	6	0.000104	0.000052		
Total	7	0.016488			

16. One-way ANOVA: Preheating temperature (80 °C) versus Magnesium chloride concentration added (ionic magnesium in the serum)

Factor Information

Factor	Levels	Values
Concentration, mmol L^{-1}	2	0.000, 15.000

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Concentration, mmol L^{-1}	1	0.016129	0.016129	504.03	0.002
Error	6	0.000064	0.000032		
Total	7	0.016193			

Section 4.3.3 Effect of magnesium chloride addition (page 53-56)

Table 4.4 (page 55)

1. One-way ANOVA: Total soluble magnesium in the serum versus Concentration of magnesium chloride added mmol L^{-1} (unadjusted pH)

Factor Information

Factor	Levels	Values
Concentration, mmol L^{-1}	7	0, 5, 10, 15, 20, 30, 40

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Concentration, mmol L^{-1}	6	1.30630	0.217717	1090.97	0.000
Error	21	0.00140	0.000200		
Total	27	1.30770			

2. One-way ANOVA: Total soluble magnesium in the serum versus Concentration of magnesium chloride added mmol L^{-1} (Adjusted pH 6.50)

Factor Information

Factor	Levels	Values
Concentration, mmol L^{-1}	7	0, 5, 10, 15, 20, 30, 40

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Concentration, mmol L^{-1}	6	1.21171	0.201952	1030.80	0.000
Error	21	0.00137	0.000196		
Total	27	1.21308			

3. One-way ANOVA: Total soluble magnesium in the serum versus Concentration of magnesium chloride added mmol L^{-1} (Adjusted pH 6.70)

Factor Information

Factor	Levels	Values
Concentration, mmol L^{-1}	7	0, 5, 10, 15, 20, 30, 40

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Concentration, mmol L^{-1}	6	1.17801	0.196335	968.96	0.000
Error	21	0.00142	0.000203		
Total	27	1.17943			

4. One-way ANOVA: Total soluble magnesium in the serum versus Concentration of magnesium chloride added mmol L^{-1} (Unadjusted pH) (AAS technique)

Factor Information

Factor	Levels	Values
Concentration, mmol L^{-1}	7	0, 5, 10, 15, 20, 30, 40

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Concentration, mmol L^{-1}	6	1.13654	0.189423	926.85	0.000
Error	21	0.00143	0.000204		
Total	27	1.13797			

5. One-way ANOVA: Total soluble magnesium in the serum versus Concentration of magnesium chloride added mmol L^{-1} (Adjusted pH 6.50) (AAS technique)

Factor Information

Factor	Levels	Values
Concentration, mmol L^{-1}	7	0, 5, 10, 15, 20, 30, 40

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Concentration, mmol L^{-1}	6	0.994174	0.165696	456.49	0.000
Error	21	0.002541	0.000363		
Total	27	0.996715			

6. One-way ANOVA: Total soluble magnesium in the serum versus Concentration of magnesium chloride added mmol L^{-1} (Adjusted pH 6.70) (AAS technique)

Factor Information

Factor	Levels	Values
Concentration, mmol L^{-1}	7	0, 5, 10, 15, 20, 30, 40

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Concentration, mmol L^{-1}	6	0.965987	0.160998	967.11	0.000
Error	21	0.001165	0.000166		
Total	27	0.967152			

7. One-way ANOVA: Ionic magnesium in the serum versus concentration of magnesium chloride added mmol L^{-1} (Unadjusted pH)

Factor Information

Factor	Levels	Values
Concentration, mmol L^{-1}	7	0, 5, 10, 15, 20, 30, 40

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Concentration, mmol L^{-1}	6	0.420351	0.070058	1316.72	0.000
Error	21	0.000372	0.000053		
Total	27	0.420723			

8. One-way ANOVA: Ionic magnesium in the serum versus Concentration of magnesium chloride added mmol L^{-1} (Adjusted pH 6.50)

Factor Information

Factor	Levels	Values
Concentration, mmol L^{-1}	7	0, 5, 10, 15, 20, 30, 40

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Concentration, mmol L^{-1}	6	0.333219	0.055537	1822.88	0.000
Error	21	0.000213	0.000030		

Total	27	0.333433

9. One-way ANOVA: Ionic magnesium in the serum versus Concentration of magnesium chloride added mmol L^{-1} (Adjusted pH 6.50)

Factor Information

Factor	Levels	Values
Concentration, mmol L^{-1}	7	0, 5, 10, 15, 20, 30, 40

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Concentration, mmol L^{-1}	6	0.282703	0.047117	1267.54	0.000
Error	21	0.000260	0.000037		
Total	27	0.282963			

10. One-way ANOVA: Total soluble magnesium versus pH (0 mmol L^{-1})

Factor Information

Factor	Levels	Values
pH	3	Adjusted 6.5, Adjusted 6.7, Unadjusted

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	2	0.000065	0.000033	0.13	0.881
Error	9	0.000744	0.000248		
Total	11	0.000809			

11. One-way ANOVA: Total soluble magnesium versus pH (5 mmol L^{-1})

Factor Information

Factor	Levels	Values
pH	3	Adjusted 6.5, Adjusted 6.7, Unadjusted

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	2	0.000100	0.000050	0.24	0.802
Error	9	0.000633	0.000211		
Total	11	0.000733			

12. One-way ANOVA: Total soluble magnesium versus pH (10 mmol L^{-1})

Factor Information

Factor	Levels	Values
pH	3	Adjusted 6.5, Adjusted 6.7, Unadjusted

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	2	0.000316	0.000158	1.20	0.415
Error	9	0.000396	0.000132		
Total	11	0.000712			

13. One-way ANOVA: Total soluble magnesium versus pH (15 mmol L^{-1})

Factor Information

Factor	Levels	Values
pH	3	Adjusted 6.5, Adjusted 6.7, Unadjusted

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	2	0.000684	0.000342	1.11	0.435
Error	9	0.000923	0.000308		
Total	11	0.001607			

14. One-way ANOVA: Total soluble magnesium versus pH (20 mmol L^{-1})

Factor Information

Factor	Levels	Values
pH	3	Adjusted 6.5, Adjusted 6.7, Unadjusted

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	2	0.000784	0.000392	1.74	0.316
Error	9	0.000678	0.000226		
Total	11	0.001462			

15. One-way ANOVA: Total soluble magnesium versus pH (30 mmol L^{-1})

Factor Information

Factor	Levels	Values
pH	3	Adjusted 6.5, Adjusted 6.7, Unadjusted

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	2	0.002161	0.001081	3.11	0.185
Error	9	0.001042	0.000347		
Total	11	0.003203			

16. One-way ANOVA: Total soluble magnesium versus pH (40 mmol L^{-1})

Factor Information

Factor	Levels	Values
pH	3	Adjusted 6.5, Adjusted 6.7, Unadjusted

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	2	0.005188	0.002594	10.79	0.043
Error	9	0.000721	0.000240		
Total	11	0.005909			

17. One-way ANOVA: Ionic magnesium versus pH (0 mmol L^{-1})

Factor Information

Factor	Levels	Values
pH	3	Adjusted 6.5, Adjusted 6.7, Unadjusted

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	2	0.000005	0.000003	0.06	0.944
Error	9	0.000137	0.000046		
Total	11	0.000142			

18. One-way ANOVA: Ionic magnesium versus pH (5 mmol L^{-1})

Factor Information

Factor	Levels	Values
pH	3	Adjusted 6.5, Adjusted 6.7, Unadjusted

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	2	0.000049	0.000025	0.48	0.661
Error	9	0.000155	0.000052		
Total	11	0.000204			

19. One-way ANOVA: Ionic magnesium versus pH (10 mmol L^{-1})

Factor Information

Factor	Levels	Values
pH	3	Adjusted 6.5, Adjusted 6.7, Unadjusted

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	2	0.000124	0.000062	2.37	0.242
Error	9	0.000079	0.000026		
Total	11	0.000203			

20. One-way ANOVA: Ionic magnesium versus pH (15 mmol L^{-1})

Factor Information

Factor	Levels	Values
pH	3	Adjusted 6.5, Adjusted 6.7, Unadjusted

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	2	0.000197	0.000099	2.01	0.279
Error	9	0.000147	0.000049		
Total	11	0.000344			

21. One-way ANOVA: Ionic magnesium versus pH (20 mmol L^{-1})

Factor Information

Factor	Levels	Values
pH	3	Adjusted 6.5, Adjusted 6.7, Unadjusted

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	2	0.001889	0.000945	26.45	0.012
Error	9	0.000107	0.000036		
Total	11	0.001996			

22. One-way ANOVA: Ionic magnesium versus pH (30 mmol L^{-1})

Factor Information

Factor	Levels	Values
pH	3	Adjusted 6.5, Adjusted 6.7, Unadjusted

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	2	0.006448	0.003224	107.71	0.002
Error	9	0.000090	0.000030		
Total	11	0.006538			

23. One-way ANOVA: Ionic magnesium versus pH (40 mmol L^{-1})

Factor Information

Factor	Levels	Values
pH	3	Adjusted 6.5, Adjusted 6.7, Unadjusted

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	2	0.006772	0.003386	77.17	0.003
Error	9	0.000132	0.000044		
Total	11	0.006904			

AAS and EDTA Comparison of methods
Table 4.4 (page 56)

1. One-way ANOVA: pH versus Methods (Unadjusted)

Factor Information

Factor	Levels	Values
Method	2	AAS, EDTA

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Method	1	0.000100	0.000100	0.98	0.427
Error	6	0.000204	0.000102		
Total	7	0.000304			

2. One-way ANOVA: pH versus Methods (Adjusted pH 6.50)

Factor Information

Factor	Levels	Values
Method	2	AAS, EDTA

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Method	1	0.000121	0.000121	0.48	0.559
Error	6	0.000502	0.000251		
Total	7	0.000623			

3. One-way ANOVA: pH versus Methods (Adjusted pH 6.70)

Factor Information

Factor	Levels	Values
Method	2	AAS, EDTA

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Method	1	0.000049	0.000049	0.40	0.594
Error	6	0.000248	0.000124		
Total	7	0.000297			

Rheology results
Section 5.2.1: Effect of holding time (page 57-65)
Figure 5.3 to 5.4 (page 60-61)

1. One-way ANOVA: *G*' versus Time (0.80 to 60 .25 min)

Factor Information

Factor	Levels	Values
Time, min	2	0.809, 60.256

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Time, min	1	279.057	279.057	6836.90	0.000
Error	6	0.082	0.041		
Total	7	279.139			

2. One-way ANOVA: *G*' versus Time (0.80 to 7.70 min)

Factor Information

Factor	Levels	Values
Time, min	2	0.809, 7.709

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value

Time, min	1	148.328	148.328	3634.04	0.000
Error	6	0.082	0.041		
Total	7	148.410			

3. One-way ANOVA: *G'* versus Time (7.70 to 45.19 min)

Factor Information

Factor	Levels	Values
Time, min	2	7.709, 45.193

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Time, min	1	18.4556	18.4556	278.25	0.004
Error	6	0.1327	0.0663		
Total	7	18.5883			

4. One-way ANOVA: *G'* versus Time (45.19 to 60 .25 min)

Factor Information

Factor	Levels	Values
Time, min	2	45.193, 60.256

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Time, min	1	0.05290	0.05290	0.80	0.466
Error	6	0.13265	0.06633		
Total	7	0.18555			

5. One-way ANOVA: *G'* versus Holding time

Factor Information

Factor	Levels	Values
Holding time, min	5	10, 20, 30, 45, 60

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Holding time, min	4	1739.13	434.782	45.01	0.000
Error	15	48.30	9.659		
Total	19	1787.42			

5.3 Effect of magnesium chloride addition (page 65-76)

Figure 5.9 to 5.10 (page 68-71)

1. One-way ANOVA: *G'* versus Time (5.12 to 10.31 min) (10 mmol L^{-1} magnesium chloride addition)

Factor Information

Factor	Levels	Values
Time, 10	2	5.127, 10.312

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Time, 10	1	7.7674	7.7674	41.68	0.023
Error	6	0.3728	0.1864		
Total	7	8.1401			

2. One-way ANOVA: *G'* versus Time (10.31 to 45.16 min) (10 mmol L^{-1} magnesium chloride addition)

Factor Information

Factor	Levels	Values
Time	2	10.312, 45.168

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value

Time	1	12.6167	12.6167	33.45	0.029
Error	6	0.7544	0.3772		
Total	7	13.3711			

3. One-way ANOVA: *G'* versus Time (2.10 to 10.31 min) (12.50 mmol L^{-1} magnesium chloride addition)

Factor Information

Factor	Levels	Values
Time	2	2.103, 10.312

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Time	1	68.5418	68.5418	254.39	0.004
Error	6	0.5389	0.2694		
Total	7	69.0807			

4. One-way ANOVA: *G'* versus Time (10.31 to 45.16 min) (12.50 mmol L^{-1} magnesium chloride addition)

Factor Information

Factor	Levels	Values
Time 12.5	2	10.312, 45.168

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Time 12.5	1	19.4922	19.4922	44.95	0.022
Error	6	0.8673	0.4337		
Total	7	20.3596			

5. One-way ANOVA: *G'* versus Time (0.81 to 7.71 min) (15 mmol L^{-1} magnesium chloride addition)

Factor Information

Factor	Levels	Values
Time 15	2	0.810, 7.719

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Time 15	1	148.328	148.328	3133.43	0.000
Error	6	0.095	0.047		
Total	7	148.423			

6. One-way ANOVA: *G'* versus Time (7.71 to 45.16 min) (15 mmol L^{-1} magnesium chloride addition)

Factor Information

Factor	Levels	Values
Time 15	2	7.719, 45.168

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Time 15	1	18.4556	18.4556	389.87	0.003
Error	6	0.0947	0.0473		
Total	7	18.5503			

7. One-way ANOVA: *G'* versus Time (0.81 to 5.12 min) (20 mmol L^{-1} magnesium chloride addition)

Factor Information

Factor	Levels	Values
Time 20	2	0.810, 5.127

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Time	1	103.795	103.795	118.17	0.008
Error	6	1.757	0.878		

Total	7	105.552

8. One-way ANOVA: *G*' versus Time (5.12 to 45.16 min) (20 mmol L^{-1} magnesium chloride addition)

Factor Information

Factor	Levels	Values
Time	2	5.127, 45.168

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Time	1	95.063	95.0625	111.27	0.009
Error	6	1.709	0.8543		
Total	7	96.771			

9. One-way ANOVA: *G*' versus Concentration (10 min holding)

Factor Information

Factor	Levels	Values
Concentration	5	5.0, 10.0, 12.5, 15.0, 20.0

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Concentration	4	448.012	112.003	413.33	0.000
Error	15	1.355	0.271		
Total	19	449.367			

10. One-way ANOVA: Final *G*' versus Concentration (10 min holding)

Factor Information

Factor	Levels	Values
Concentration	5	5.0, 10.0, 12.5, 15.0, 20.0

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Concentration	4	18396.0	4599.00	877.68	0.000
Error	15	26.2	5.24		
Total	19	18422.2			

11. One-way ANOVA: Final *G*' versus Concentration (20 min holding)

Factor Information

Factor	Levels	Values
Concentration	5	5.0, 10.0, 12.5, 15.0, 20.0

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Concentration	4	21977.7	5494.42	611.92	0.000
Error	15	44.9	8.98		
Total	19	22022.6			

12. One-way ANOVA: Final *G*' versus Concentration (45 min holding)

Factor Information

Factor	Levels	Values
Concentration	5	5.0, 10.0, 12.5, 15.0, 20.0

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Concentration	4	27647.0	6911.76	2377.31	0.000
Error	15	14.5	2.91		
Total	19	27661.6			

Section 5.4 Effect of preheating temperature on gelation (page 77-86)
Figure 5.15 to 5.20 (page 80-86)

1. One-way ANOVA: *G'* versus Holding temperature (non-preheated)

Factor Information

Factor	Levels	Values
Tem	4	70, 75, 80, 85

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Tem	3	5.55238	1.85079	2265.81	0.000
Error	12	0.00327	0.00082		
Total	15	5.55565			

2. One-way ANOVA: *G'* versus Holding temperature (preheated)

Factor Information

Factor	Levels	Values
Temperature	4	70, 75, 80, 85

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Tem	3	448.454	149.485	262.31	0.000
Error	12	2.279	0.570		
Total	15	450.733			

3. One-way ANOVA: Temperature in the rheometer (70 °C) versus Condition (preheated & non-preheated)

Factor Information

Factor	Levels	Values
condition	2	NP, Pre

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
condition	1	10.7065	10.7065	56.40	0.017
Error	6	0.3797	0.1898		
Total	7	11.0862			

4. One-way ANOVA: Temperature in the rheometer (75 °C) versus Condition (preheated & non-preheated)

Factor Information

Factor	Levels	Values
condition	2	NP, Pre

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
condition	1	109.826	109.826	336.34	0.003
Error	6	0.653	0.327		
Total	7	110.479			

5. One-way ANOVA: Temperature in the rheometer (80 °C) versus Condition (preheated & non-preheated)

Factor Information

Factor	Levels	Values
condition	2	Non-preheated, Preheated

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
condition	1	311.170	311.170	883.26	0.001
Error	6	0.705	0.352		
Total	7	311.874			

6. One-way ANOVA: Temperature in the rheometer (85 °C) versus Condition (preheated & non-preheated)

Factor Information

Factor	Levels	Values
condition	2	NP, Pre

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
condition	1	426.340	426.340	1563.38	0.001
Error	6	0.545	0.273		
Total	7	426.885			

7. One-way ANOVA: Final *G'* versus Temperature in the rheometer (non-preheated)

Factor Information

Factor	Levels	Values
Tem	4	70, 75, 80, 85

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Tem	3	898.82	299.606	70.56	0.001
Error	12	16.99	4.246		
Total	15	915.80			

8. One-way ANOVA: Final *G'* versus Temperature in the rheometer (non-preheated)

Factor Information

Factor	Levels	Values
Tem	4	70, 75, 80, 85

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Tem	3	24053.6	8017.87	1578.24	0.000
Error	12	20.3	5.08		
Total	15	24073.9			

Comparing Final *G'* of preheated and non-preheated samples

Figure 5.18 (page 83)

1. One-way ANOVA: Final *G'* versus Condition (for temperature 70 °C in the rheometer)

Factor Information

Factor	Levels	Values
condition FG	2	Non-preheated, Preheated

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
condition FG	1	56.742	56.742	12.05	0.074
Error	6	9.415	4.708		
Total	7	66.158			

2. One-way ANOVA: Final *G'* versus Condition (for temperature 75 °C in the rheometer)

Factor Information

Factor	Levels	Values
condition FG	2	Non-preheated, Preheated

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
condition FG	1	2268.71	2268.71	11464.63	0.000
Error	6	0.40	0.20		
Total	7	2269.11			

3. **One-way ANOVA: Final *G'* versus Condition (for temperature 80 °C in the rheometer)**

Factor Information

Factor	Levels	Values
condition FG	2	Non-preheated, Preheated

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
condition FG	1	8832.24	8832.24	2029.71	0.000
Error	6	8.70	4.35		
Total	7	8840.94			

4. One-way ANOVA: Final *G'* versus Condition (for temperature 85 °C in the rheometer)

Factor Information

Factor	Levels	Values
condition FG	2	Non-preheated, Preheated

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
condition FG	1	15361.1	15361.1	2061.29	0.000
Error	6	14.9	7.5		
Total	7	15376.0			

Comparing oscillation stress of preheated and non-preheated samples

Figure 5.20 (page 86)

1. One-way ANOVA: Oscillation stress versus Condition (temperature in the rheometer 80 °C)

Factor Information

Factor	Levels	Values
condition OS	2	Non-preheated, Preheated

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
condition OS	1	1303.69	1303.69	270.37	0.004
Error	6	9.64	4.82		
Total	7	1313.34			

2. One-way ANOVA: Oscillation stress versus Condition (temperature in the rheometer 85 °C)

Factor Information

Factor	Levels	Values
condition OS	2	Non-preheated, Preheated

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
condition OS	1	2690.16	2690.16	722.75	0.001
Error	6	7.44	3.72		
Total	7	2697.61			