A study of the physicochemical and rheological properties for zinc added skim milk

A Thesis Presented in Partial Fulfilment of the Requirements for the Degree of Master of Food Technology

Massey University, Auckland, New Zealand

Shuang Zhao

2021

Abstract

Zinc is present in bovine milk in low concentration (0.37 mg per 100g) but in this form is highly bioavailable. Addition of soluble zinc salts to bovine milk can increase the zinc content. However, changes in physicochemical and rheological properties in milk after addition of zinc salts has not been studied extensively. Thus, the aim of this study was to to investigate the physicochemical and rheological changes in skim milk after the addition of different zinc salts.

The skim milk pH, zinc and calcium distribution were monitored to determine the changes in physicochemical properties after zinc salts were added. The changes in the skim milk viscosity and storage modulus were monitored to study the changes in rheological properties. This research assessed the effect of added zinc salt concentration (0 - 40 mmol L^{-1}), type of zinc salt added (zinc acetate, zinc sulphate and zinc gluconate), pH, preheat treatment, and holding temperature (20°C to 80°C).

The addition of all three zinc salts lead to a significant decrease (p<0.05) in skim milk pH, and the order of final pH in the zinc+skim milk from lowest to highest at the same concentration level was zinc sulphate < zinc gluconate < zinc acetate. An increase in the serum and sediment (colloidal phase) zinc concentration was observed after zinc salt was added. As the zinc acetate concentration increased there was a corresponding decrease in the native calcium concentration in the collodial phase. When 40 mmol L^{-1} zinc acetate was added to skim milk, about 33% of the native calcium present in skim milk was released from the colloidal phase to the serum.

Rheological measurements using cone and plate geometry at constant strain showed that the addition of zinc salt could lead to an increase in zinc+skim milk viscosity and with further increase in the added zinc salt concentration, such as 22.5 mmol L^{-1} of zinc acetate, 30 mmol L^{-1} of zinc sulphate or zinc gluconate, the non-preheated skim milk started to form a gel at room temperature (20 ± 1°C). The increase in gel strength correlated with the colloidal phase zinc concentration; as a higher colloidal phase zinc concentration resulted in a stronger gel. Gelation was also observed for the zinc+skim milk which had its pH adjusted back to 6.73 ±

0.03 after pH drop with the added zinc, but the gel strength was significantly less than when the pH was not adjusted.

When skim milk samples were preheated, a higher final *G'* was achieved at the same concentration of zinc salt likely due to the participation of denatured whey proteins in the gel network. The addition of 15 mmol L^{-1} zinc acetate to preheated skim milk followed by heating at 80°C then cooling to 20°C formed strong skim milk gels with a final *G'* of 104.35 ± 0.94 Pa, while the non-preheated sample obtained a lower final *G'* of 98.82 ± 0.02 Pa. A higher holding temperature also resulted in higher final *G'* of gels. In conclusion, the final gel properties of a zinc+skim milk sample were influenced by zinc salt concentration added, type of zinc salt added, pH, preheat treatment and the temperature during gelation. These findings provide valuable information about the zinc-protein equilibrium in skim milk and offer an alternative method for texture modification in skim milk.

Acknowledgements

First and foremost, I would like to express my depth gratitude and thanks to my supervisors Professor Marie Wong and Dr Eustina Fraser, for their continuous support, guidance throughout the project. They have given me great instruction and encouragement in research topic selection, data collection and analysis, thesis writing, and even correcting the grammatical errors. The insightful comments on every draft from them, which provided me with many enlightening ideas and have inspired me to a great extent. Without their enlightening instruction, impressive kindness and patience, I could not have completed my project.

I would like to express my appreciation to all the faculty and staff in the School of Food and Advanced Technology, Massey University Auckland for their support and kind assistance throughout the period of my research work, especially Negah Nikanjam, Noorzahan Begum and Rachel Liu, for their help and support in all things related to laboratory use. Special thanks to Dr Marie-Anne Thelen and Erin Moffet, for their patience and training on MPAES. I would also like to thank Professor Al Nielson for his guidance and generosity on zinc chemistry.

I would like to thank the support, encouragement and enormous support from all my family members and friends throughout the duration of the project to allow me to achieve to the best of my abilities. I am grateful to my parents for the financial support provided and their unconditional love. Special thanks to Ge Tian, Mingyu Wang and Wen Wang, for the concern during the New Zealand lockdown periods and laughter we shared.

Last but not least, I wish to acknowledge everyone who has contributed to the completion of this thesis.

Table of Contents

List of Figures

List of Table

Chapter 1. Introduction

Zinc is an essential trace element required by the human body and plays an indispensable role in the growth and development of human, especially on children (Kahraman & Ustunol, 2012; Pabón & Lönnerdal, 1993). A severe zinc deficiency in childhood can lead to short stature or even dwarfism (Blakeborough *et al.*, 1983). Zinc promotes immune function, while malnutrition of the element will reduce resistance to infection (Gibson, 2012; Kahraman & Ustunol, 2012). Although the concentration of zinc in bovine milk is low (only about 3.3 mg/L milk), bovine milk can still be considered as a good source of zinc for humans, as 600 ml of milk or dairy products can provide approximately 20% of the recommended daily intake of zinc (Gaucheron, 2011a; Wong *et al.*, 1988).

The addition of mineral cations into milk or dairy product can increase the product's nutritive value, but also can change the mineral-protein equilibrium within the product (Tsioulpas *et al.*, 2007). Several studies have been reported on the native and added zinc states in milk (Blakeborough *et al*., 1983; Cousins & Smith, 1980; Harzer & Kauer, 1982; Parkash & Jenness, 1967; Singh *et al*., 1988; Singh *et al*., 1989a, 1989b; Sugiarto, 2004). The addition of zinc salts into skim milk can distrupt the mineral equilibrium which leads to a decrease in pH and an increase in ionic strength of the milk, furthermore, milk gelation can occur (Singh *et al.*, 1989a, 1989b). However, there is limited information on the effect of the addition of zinc salts on the rheological properties of skim milk.

The aim of this project was to determine the effect of the addition of different zinc salts to skim milk on the milk physicochemical and rheological properties.

The objectives of this study were to:

- Apply and validate analytical methods to monitor zinc in a milk system.
- Determine the effects of treatment conditions (preheating, holding temperature), zinc salt concentrations and pH on skim milk texture.
- Determine the effect of different types of zinc salts on the resulting rheological properties of the skim milk gel.

Chapter 2. Literature review

2.1. Bovine milk constituents

Milk is a white or slightly yellowish opaque colloidal liquid, secreted from the udder of a cow after calving (Wong *et al*., 1988). It is a food that people often eat in daily life. The pH of fresh milk is about 6.6 - 6.8, slightly lower than the pH of blood (McSweeney & Fox, 2013). Bovine milk is mainly composed of water, milk fat, milk protein, lactose and mineral. However, the composition of bovine milk is not fixed and will vary with several factors, including the breed of cow, environment, feeding method, lactation period, cow's age, season and processing methods (Council, 1988). Table 2.1 is an example of the proximate composition of mature whole bovine milk. It should be noted that ash does not exactly represent the salts in cow's milk because almost all organic salts are destroyed during ashing (Akinyele & Shokunbi, 2015). Most of the components in bovine milk do not exist as single molecules in the serum phase, which exist as large and complex associated structures. For example, lipids form larger spheres, and most casein exists as casein micelles (Damodaran & Parkin, 2017).

Table 2.1: Bovine milk constituents (Damodaran & Parkin, 2017).

Component	Percentage (%, w/w)
Water	85.4 - 87.7
Milk fat	3.7
Milk protein	3.4
Lactose	4.6
Ash	0.7

2.2. Bovine milk fat

There is approximately 3.7% fat in whole milk. Approximately 95% of the fat fraction is comprised of triacylglycerols, which are composed of glycerol and different lengths of fatty acids (chain length varying between 4 - 24 carbon atoms). Milk fat exists as small globules with size ranging from 0.1 - 20 mm and are dispersed in the milk serum, Figure 2.1 (Berton *et al.*, 2012). Milk is a thermal unstable emulsion, and the globules are the largest and least dense particles in the milk. Thus, after a long period of storage, the milk fat tends to float up to the milk surface and aggregate together to form a cream layer (Fox *et al.*, 1998). Homogenisation is one of the common approachs that have been applied to stabilise milk by

preventing creaming, which can break the large milk fat globules into small droplets with an average size of 0.16 mm, and this keeps the milk fat globules evenly distribute in the milk serum during storage (Berton *et al.*, 2012). With different milk fat contents; milk can be divided into whole milk (~ 3.25% fat), low-fat milk (~0.5% fat) and skim milk (<0.5% fat) (Wong *et al.*, 1988).

Figure 2.1: The milk fat globules (yellow) in milk (Berton *et al.*, 2012).

2.3. Bovine milk protein

Milk protein is a mixture of a number of different proteins, accounting for about 3.4 % (w/w) of whole milk (Wong *et al.*, 1988). Casein and whey proteins are the main proteins present in milk. Casein can be precipitated in milk at 20°C and at pH 4.6, while whey proteins are left in the serum phase (Damodaran & Parkin, 2017). Casein constitutes over 80% of the total milk protein, with the remaining being mostly whey proteins (Berton *et al.*, 2012; Damodaran & Parkin, 2017; Fox *et al.*, 1998; Jensen, 1995). The major protein percentages in mature bovine milk are listed in Table 2.2.

Table 2.2: Major protein comoposition in mature bovine milk (Damodaran & Parkin, 2017).

Protein		Percentage of total milk (wt%, Approx.)
Caseins	α_{s1} - Casein	34
	α_{s2} - Casein	8
	β - Casein	25
	κ - Casein	9
	λ - Casein	4
Whey Proteins	β - Lactoglobulin (β-lg)	9
	α - Lactalbumin (α-la)	4
	Proteose - peptones	4
	Serum albumin	1
	Immunoglobulins	2
Total		100

2.4. Casein

There are four major casein proteins in bovine milk which are α_{s1} - casein, α_{s2} - casein, β - casein and κ - casein. Table 2.3 lists the concentration of each casein in bovine milk. Casein is composed of amino acids as is whey protein. However, a unique feature of casein is the phosphorylation of the hydroxyl groups in serine through post-ribosomal modification which forms phosphoserine residues (Lyster, 1972; Varnam & Sutherland, 1994). The number of phosphoserine residues in each type of casein is listed in Table 2.3. The concentrated phosphoserine residue clusters have strong negative charges which is the polar end of the casein molecule (Damodaran & Parkin, 2017; Varnam & Sutherland, 1994). The parts of casein molecule which are away from the phosphoserine clusters are hydrophobic. The hydrophilic and hydrophobic residues give casein clear amphiphilic properties (Damodaran & Parkin, 2017).

Table 2.3: The concentration and number of phosphoserine residues for each type of casein in milk (Damodaran & Parkin, 2017; Varnam & Sutherland, 1994).

Fraction	Concentration (g/L milk)	Phosphoserine residues
α_{s1} - Casein	12 – 15	7 - 9
α_{s2} - Casein	3 – 4	10 - 13
β - Casein	9 – 11	5
κ - Casein	2 – 4	1[a]

[a]Only carbohydrate containing casein.

The caseins, α_{s1} -, α_{s2} - and β - casein have more phosphoserine residues and are considered as the calcium-sensitive caseins as the phosphoserine residues present in the polar domains can bind with calcium ions (Ca^{2+}) (Varnam & Sutherland, 1994). At the native milk pH (6.6 - 6.8), the phosphoserine residue clusters in the casein hydrophilic parts have a strong negative charge which can bind with the Ca^{2+} and cause the cluster to lose charge and dehydrate. Consequently, this affects the hydrophobic interactions and electrostatic repulsion equilibrium between calcium-sensitive caseins and results in calcium-sensitive casein precipitation (Wong *et al*., 1988). Kappa - casein is not a calcium-sensitive casein with only one phosphoserine residue (McSweeney & Fox, 2013). However, κ - casein also has hydrophobic and hydrophilic residues, which can associate with other calcium-sensitive caseins by protein-protein interactions and help to form a casein micelle and stabilise the calcium-sensitive caseins (Horne, 2006; Lyster, 1972; Phadungath, 2005). It has been indicated that a casein micelle is more stable when Ca^{2+} is present than when α_{s1} -, α_{s2} - and β - casein are alone (Lyster, 1972).

2.4.1. Casein micelle

Most caseins (~95%) in the milk exist in a complex form as casein micelles. According to Damodaran and Parkin (2017), the casein micelle has a net negative charge at native milk pH (6.6 - 6.8). Figure 2.2 shows a field-emission scanning electron micrograph of a casein micelle. The average diameter of the casein micelle is about 200 nm, but the size range can vary from 80 to 400 nm (Dalgleish, 2011). There are 92% - 94% protein in the casein micelles, composing of α_{s1} - casein, α_{s2} - casein, β - casein and κ - casein (Damodaran & Parkin, 2017; Phadungath, 2005). The approximate molar ratio of these four caseins in a casein micelle has been evaluated as 4:1:3.5:1.5, respectively (Dalgleish, 2011).

Figure 2.2: Field-emission scanning electron micrograph of a casein micelle. The particle is chemically bound to the substrate and is not metal coated before microscopy. The scale bar is 200 nm (Dalgleish, 2011).

The formation of casein micelles are primarily driven by hydrophobic interactions between individual caseins. The hydrophobic parts of the caseins are forced into the interior of a casein micelle and interacted with other non-polar parts. Concomitantly, hydrophilic parts move toward the surface of the casein molecule and associate with water (Damodaran & Parkin, 2017; Horne, 2006; Wong *et al.*, 1988; Phadungath, 2005). The associated water becomes more disordered (increased entropy) and increases the stability of the casein micelle (Wong *et al.*, 1988).

Electrostatic interactions between amino acid side chains and metal ions (mainly calcium ions) also increases the stability of the casein micelle structure. The negatively charged residues like phosphoserine residues and carboxylic acid residues can bind with metal ions and form a salt bridge between caseins, which contributes to the casein micelle stabilisation (Fox *et al.*, 1998). Calcium ions in the micellar phase do not only bind with the negative residues but also associate with phosphate and is present within the colloidal phase as colloidal calcium phosphate (CCP) (Wong *et al.*, 1988). CCP links the casein together to give them an open porous structure. Removal of CCP can lead to the disintegration of the casein micelle (Aoki *et al.*, 1987).

Although the protein-protein interactions and protein-ionic interactions in the casein micelle have been studied for many years. The casein micelle structure has not been fully understood. There are three widely accepted casein colloidal models: submicelle model, Holt model and the dual binding model (De Kruif & Holt, 2003; Horne, 2006; Phadungath, 2005).

2.4.1.1. Submicelle Model

In 1967, Morr proposed the submicelle model for casein (Phadungath, 2005). The main idea of this model is that casein colloidal particles are composed of many submicelle particles. Colloidal calcium phosphate (CCP) plays a key role in the formation of submicelle and colloidal particles (Horne, 2006). There are two kinds of submicelles that make up the casein micelle. One is the submicelle poor in κ - casein, which is concentrated in the interior of the casein micelle. Another is the submicelle rich in κ - casein, which is concentrated on the surface of the casein micelle (Figure 2.3). The C - terminal (the end of a protein or polypeptide, terminated by a free carboxyl group, negatively charged and hydrophilic) from κ - casein exists on the surface of the casein micelle and forms a hairy layer which ensures the stability of the colloidal particles (De Kruif & Holt, 2003). When the hairy layer is removed (treated with rennet) or collapsed (ethanol treatment), the stability of the colloidal particles is destroyed, then coagulation or precipitation will occur (Horne, 2006; Phadungath, 2005).

Figure 2.3: A schematic diagram of the submicelle model of the casein micelle (Horne, 2006).

2.4.1.2. Holt Model / Nanocluster Model

Later in 1992, Holt reported that casein micelles are entangled in a flexible casein network to form a nanocluster, Figure 2.4 is an attempt to portray the structure (De Kruif & Holt, 2003). In this structure, colloidal calcium phosphate nanoclusters stabilise the casein micelle. It combines with the phosphoserine residual clusters in calcium-sensitive casein and forms a complete internal structure (De Kruif & Holt, 2003; Horne, 2006). The surface of the casein micelle is the C-terminal region of κ - casein forming a hairy layer (Wong *et al*., 1988).

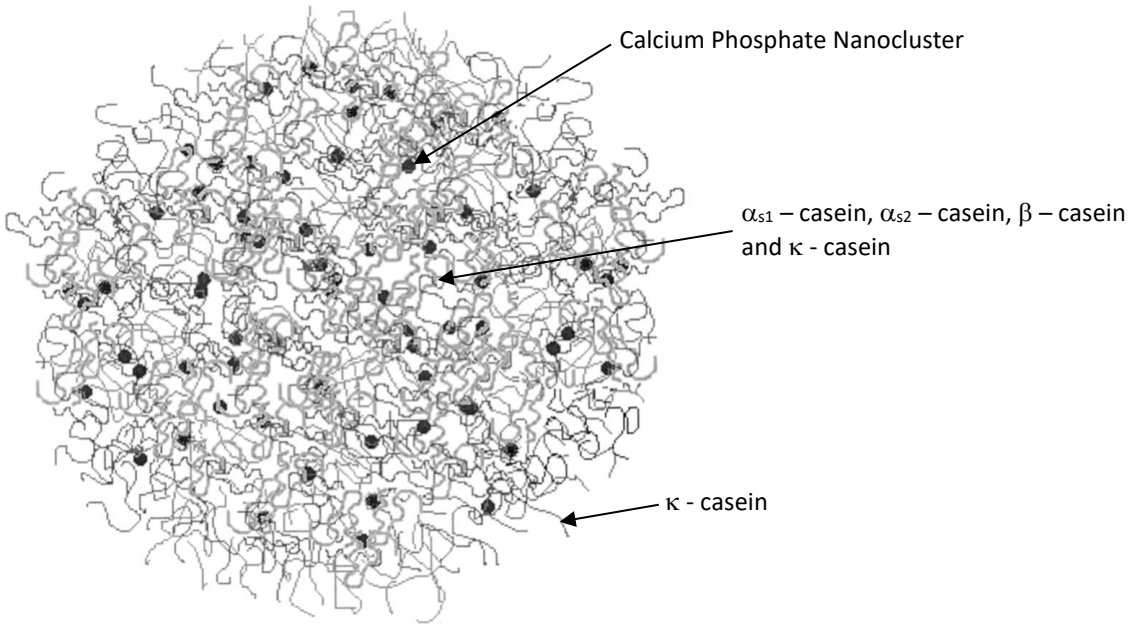


Figure 2.4: The nanocluster model of casein micelles (De Kruif & Holt, 2003).

2.4.1.3. Dual Binding Model

In 1998, the dual binding model from Horne suggested the casein association is a polymerisation driven by hydrophobic interactions (Horne, 1998; Phadungath, 2005). In the model, α_{s1} - casein, α_{s2} - casein and β - casein can associate either by hydrophobic interactions between the proteins or by crosslinking with CCP to form the core part of the casein micelle. Unlike other caseins, κ - casein does not have a phosphoserine cluster to bind with calcium ions or another hydrophobic chain to associate with other caseins (Horne, 1998, 2006;

Phadungath, 2005). Thus, κ - casein is like a propagation terminator which can stop the growth of the casein micelle and forms a hairy layer on the surface of the casein micelle (Damodaran & Parkin, 2017; Phadungath, 2005).

Figure 2.5: The dual binding model (Horne, 1998).

Since the micelle structure cannot be determined directly, the exact location of individual caseins has not been fully understood. Nevertheless, existing results have enough evidence to show that κ - casein is mainly located on the surface, and the α_{s1} - casein, α_{s2} - casein and β - casein are mostly in the casein micelle interior.

2.5. Whey protein

Whey proteins are highly water-soluble. After isoelectric (acidification) precipitation of casein, the liquid remaining contains water, lactose, whey or serum proteins and salts (Wong *et al.*, 1988; McSweeney & Fox, 2013). The whey protein contains α - lactalbumin, β - lactoglobulin, serum albumin and immunoglobulin (Damodaran & Parkin, 2017). Table 2.2 listed the percentage of each whey protein in the total protein present. Alpha - lactalbumin and β - lactoglobulin are the two main components which represents about 80% w/w of the total whey proteins (Wong *et al.*, 1988). Same as casein, whey proteins have a net negative charge in the native milk (Varnam & Sutherland, 1994). However, unlike casein, the order distribution of polarity and charged residues in whey proteins are uniform. Thus, whey proteins can extensively self-associate and keep most of the hydrophobic groups inside (Damodaran &

Parkin, 2017; Lyster, 1972). Figure 2.6 shows the protein structures for the three main whey proteins.

Figure 2.6: protein structure of the main whey proteins (Ortega-Requena & Rebouillat, 2015).

2.5.1. Whey protein heat stability and denaturation

The casein micelle is not sensitive to heat, it is stable at temperatures up to 140°C and the casein in raw milk will not denature under normal heat treatment of most milk processes (Varnam, & Sutherland, 1994; Damodaran & Parkin, 2017). Compared to casein, whey protein is more likely to denatured and aggregate during heating (Wong *et al.*, 1988). The order of heat stability for whey proteins from high to low is α - Lactalbumin (α - la) > β - Lactoglobulin (β - lg) > Bovine serum albumin > Immunoglobulin (Wong *et al.*, 1988). Table 2.4 lists the denaturation temperature for whey proteins at pH 6.0. The denaturation of whey protein happens in at least two steps. Initially, the native whey protein unfolds during heating and exposes the thiol groups that were originally hidden inside the native protein structure. Secondly, the unfolded thiol groups aggregate through the thiol-disulphide reactions, hydrophobic interactions or covalent binding with other proteins (Anema, 2008; Datta & Deeth, 2001; Fox *et al.*, 1998; Oldfield *et al.*, 1998). The aggregation for β - lg is mainly due to intermolecular disulphide linkages. Hydrophobic interactions, which account for approximately 40% of the aggregation, involve aggregation of α - la when the temperatures are below 85°C (Oldfield *et al.*, 1998). It should be noted that the process of whey protein denaturation by heating is very complicated, as aggregation not only happens with whey protein itself, but also with other proteins present. An example is that the unfolded

hydrophobic surface and the sulfhydryl groups on β - lg can interact with κ - casein and stabilise by the sulfhydryl-disulphide exchange reactions with the casein micelles (Anema, 2008; Damodaran & Parkin, 2017).

Table 2.4: Whey proteins denaturation temperature at pH 6.0 (Wong *et al*., 1988).

Protein	Denaturation temperature (°C)
α-Lactalbumin	62
β-Lactoglobulin	78
Immunoglobulin	72
Bovine serum albumin	64

2.6. Bovine milk salts and minerals

2.6.1. Salts and minerals concentration and distribution in bovine milk

The main salts in milk are chloride, phosphate, citrate, sulphate, calcium, potassium, sodium and magnesium (Damodaran & Parkin, 2017; Gaucheron, 2005; Wong *et al.*, 1988). The salts in milk can exist as free ions or ionic compounds in the aqueous phase or as colloidal particles. Colloidal salt particles participate in the structure of casein micelles (Gaucheron, 2011b). The concentration and distribution of the principal salts in bovine milk are listed in Table 2.5, and the concentration of trace elements in bovine milk are listed in Table 2.6.

Table 2.5: The concentration and distribution of the principal salts (Damodaran & Parkin, 2017; Fox *et al.*, 1998).

Component	Concentration		Colloidal	Serum
	mg/L	mmol/L	(Micellar) %	(Soluble) %
Calcium	1040 - 1280	26 - 32	69	31
Magnesium	100 - 150	4 - 6	47	53
Potassium	1210 - 1680	31 - 43	6	94
Sodium	350 - 600	17 - 28	5	95
Chloride	780 - 1200	22 - 34	5	95
Citrate	1320 - 2080	7 - 11	14	86
Total phosphorus	1800 - 2180	30 - 32	-	-
Inorganic phosphorus	930 - 100	19 - 23	53	47
Sulphate	~100	~1	0[a]	100[a]

[a] (Fox *et al.*, 1998)

Table 2.6: Trace elements in bovine milk (Wong *et al.*, 1988).

Element	Concentration (µg/L)	
	Range	Typical value
Aluminium	150 - 1000	500
Bromine	500 - 20,000	-
Chromium	5 - 80	15
Copper	10 - 200	75
Fluorine	70 - 220	-
Iodine	10 - 1000	-
Iron	100 - 1500	300
Lead	20 - 80	40
Manganese	20 - 100	50
Molybdenum	20 - 120	70
Selenium	4 - 1200	12
Strontium	40 - 500	170
Zinc	2000 - 5000	3300

-: not identified.

As can be seen from Table 2.5, most of the calcium is present in the micellar phase (69%) either associated with the casein by forming salt bridges or in the CCP (Damodaran & Parkin, 2017). The calcium and magnesium in milk serum are mainly in the form of salt complexes, including large amounts of calcium citrate and magnesium citrate, and small amounts of calcium phosphate. About 20 - 30% of the total calcium and magnesium exists as free divalent cations. In the native bovine milk, similarly, as a divalent cation, most of the zinc associates with the casein micelle and only a small portion of zinc is present in the serum phase and associated with citrate and phosphate ions (Blakeborough *et al*., 1983; Cousins & Smith, 1980; Parkash & Jenness, 1967; Singh *et al*., 1989a,1989b). Most citrate salt are also in the form of complexes, while most phosphates are in the form of $H_2PO_4^-$ and HPO_4^{2-}. Almost all monovalent ions such as sodium (Na^+), potassium (K^+) and chlorine exist as free ions (Damodaran & Parkin, 2017; Fox *et al.*, 1998; Jensen, 1995; Soliman, 2005).

2.7. Mineral-protein equilibria

The binding between the minerals and protein in milk is not static. They are in dynamic equilibria between the serum phase and micellar phase. These equilibria can easily be influenced by many factors like temperature, pH, the concentration of mineral salts and ionic

strength (Fox *et al.*, 1998; Mekmene *et al.*, 2009; Udabage *et al.*, 2000). Figure 2.7 shows the effect of the mineral, casein and water exchange under different physicochemical conditions. The impact of each of these factors will be described in the following sections.

Figure 2.7: Modification of salt equilibrium in different physicochemical conditions (Gaucheron, 2005).

2.7.1. Acidification

When lowering the milk pH, more and more protons present associate with the negatively charged groups in milk like phosphoserine residues. Thus, the salts, micellar calcium phosphate (MCP), small amounts of magnesium and citrate associated with casein micelles are dissolved and casein is released from the casein micelle (Gaucheron, 2005). At pH5.0, the micellar inorganic phosphates ($H_2PO_4^-$, HPO_4^{2-} and PO_4^{3-}) are completely dissolved into the serum phase, resulting in the completed loss of CCP and leading to the release of Ca^{2+} into serum phase (Fox *et al.*, 1998; Lucey, 2017). However, Gaucheron (2005) reported that not all of the calcium ions dissolve at pH 5.0, some calcium still associates with organic phosphate

i.e. phosphoserine residues of casein molecules. This modification of salt equilibrium by acidification is irreversible, once the structure of casein micelle is destroyed by the addition of hydronium ions in milk. An increase in pH cannot lead to reconstruction of the MCP and casein micelle structure at all. According to Singh *et al.* (1989b) at pH 4.6, more than 95% of the zinc, calcium and inorganic phosphate originally in the micellar phase is dissolved into the serum phase.

2.7.2. Alkalinisation

Milk alkalisation has the opposite effect on mineral equilibria than acidification (Ahmad *et al.*, 2009; Fox *et al.*, 1998). The explanation of the mineral equilibria during alkalinisation is presented in Figure 2.8. When NaOH is added, two of the dissociating Na^+ ions can replace one Ca^{2+} that originally associated with casein micelle and form a salt bridge between casein micelles (Figure 2.8,⑤) (Ahmad *et al.*, 2009). Addition of the complementary OH^- results in the increase of pH with neutralising H^+ ions in milk. To restore the equilibrium, the hydrogen ions are released from the casein micelle, and $H_2PO_4^-$, HPO_4^{2-} ions are converted to H^+ and PO_4^{3-} (Figure 2.8,②) (Damodaran & Parkin, 2017). Van Dijk (1991) reported that the PO_4^{3-} has a better affinity for Ca^{2+} than $H_2PO_4^-$ and HPO_4^{2-} ions and can interact with the calcium ions in the serum and micellar phase (Figure 2.8,③). Since the serum phase is supersaturated with calcium phosphate (Wong *et al.*, 1988), the newly formed calcium phosphate will percipitaed or shift into the micellar phase and becomes part of the MCP which results in a reduction in the serum calcium and inorganic phosphate concentration (Figure 2.8,④) (Ahmad *et al.*, 2009).

Figure 2.8: Proposed mechanism explaining the changes in the mineral equilibria during alkalinisation. ①: Ion distribution at pH 6.7. ②: Modification of Pi ionisation during alkalinisation. ③: Chelation of different calcium forms by triply ionised Pi. ④: Formation of new calcium phosphate salts. ⑤: Displacement of calcium by sodium (from NaOH) (Ahmad, *et al.*, 2009).

2.7.3. Effect of temperature

Calcium phosphate solubility has a negative linear corelation with temperature increasing (Anema, 2009). As can be seen in Figure 2.9, when heating from 20 to 80°C the calcium ion and inorganic phosphate concentration decreased in the serum phase from 8 to 5 mmol kg^{-1} and 10 to 8 mmol kg^{-1}, respectively, for the skim milk with 9.6% (w/w) total solids (Anema, 2009). Part of the calcium phosphate either shifts to the micellar phase or precipitates during heating (Fox *et al.*, 1998; Wong *et al.*, 1988). Anema (2009) and Pouliot *et al.* (1989a) reported the ratio of calcium ions to phosphate ions is close to 1:1 in the heat-precipitated calcium phosphate salt which is mainly $CaHPO_4$. During heating there is a transition from $H_2PO_4^-$ to H^+ and HPO_4^{2-} which lowers the milk pH (Anema, 2009). Huppertz (2016) reported that the increase of milk temperature can lead to a drop in pH, e.g. the milk pH decreased from about 6.8 to 6.3 when the milk temperature increased from 5°C to 80°C, but when the milk was held at 85°C for 40 minutes and then cooled to room temperature, the calcium in the micellar phase can move back into the serum (Pouliot *et al*., 1989b). However, if the milk was heated

to higher temperatures, like 120°C for 20 minutes, this resulted in an irreversible modification of the salt equilibrium (Gaucheron, 2005). This may be caused by the dephosphorylation of casein due to the high temperature(>120°C), as Huppertz (2016) pointed out there is about 55% dephosphorylation of caseins in unconcentrated milk when it was heated at 140°C for 20 min.

Figure 2.9: Changes in final calcium and final phosphate in the serum phase after 60 minute heating at a range of temperatures (20 to 80°C) for the 9.6% total solid (●), 19.2% total solid (○), 28.8% total solid (▼) and 38.4% total solid (▽) milk samples (Anema, 2009).

Many studies have reported that with the temperature increase, there was an increasing amount of casein present into the serum phase (Anema, 2009; Gaucheron, 2005; Huppertz, 2016). Heat-induced dissociation of α_s - casein and β - casein is improved with an increase in temperature and the maximum dissociation of casein occurs at 70°C. However, with a further increase in temperature, the concentration of the two casiens started decreasing in the serum phase (Anema, 2009). Unlike α_s - casein and β-casein, the dissociation of κ - casein has a positive linear corelation with the temperature increase, when the temperature was less than 100°C and pH value was greater than 6.5 (Anema, 2009). Singh and Fox (1985) reported at the higher temperature 140°C, the dissociation of micellar κ - casein was significantly increased when pH was greater than 6.7. Huppertz, (2016) mentioned the dissociation of κ - casein is more important in the heat-induced milk gelation than other caseins.

Compared to heating, cooling (from 20°C to 3°C) can increase the CCP solubility and increase the concentration of calcium and phosphate ions in the serum phase (Table 2.7). These changes are completely reversible when the milk is heated again (Gaucheron, 2005).

Table 2.7: Concentration of salts in bovine milk at 20°C and 3°C after analysis of permeate from dialysis (Fox *et al.*, 1998).

Constituent	Concentration (mg/L milk)	
	20°C	3°C
Total calcium	379	412
Ionized calcium	122	129
Magnesium	78	79
Inorganic phosphorus	318	326
Citrate (as citric acid)	1730	1750
Sodium	580	600
Potassium	1330	1330

2.7.4. Effect of adding chelating agents

Chelating agents like citrate, EDTA, oxalate, or cation-exchange resins have a high affinity for cations, like calcium ion. When a chelating agent is added into bovine milk, it binds with the calcium presented in the serum phase. Furthermore, the chelation decreases the serum calcium concentration. Consequently, more calcium would shift from the micellar phase to the serum phase to restore the equilibrium (Udabage *et al.*, 2000). In addition, the chelating agents not only associate with the cations in the serum phase, but also can displace them from the micellar phase. This results in a loss of structure of the casein micelle to some extent by the disruption of MCP (Gaucheron, 2011b).

2.7.5. Effect of adding divalent cations

The change of the mineral equilibrium in bovine milk when divalent cations are added depends on the type of cation, the amount added and the eventual pH of the milk. The common divalent cations added to the milk are calcium, magnesium and iron; with calcium being the most commonly added cation (Mekmene *et al.*, 2009). When adding calcium, the zeta potential, hydration and heat stability of the negatively charged casein micelle are all reduced (Gaucheron, 2011b; Udabage *et al.*, 2000). Addition of calcium also reduces the solubility of phosphate by forming colloidal calcium phosphate (Fox *et al.*, 1998). If calcium is

added to milk, it will react with $H_2PO_4^-$ to form $CaHPO_4$ and H^+ as shown in Equation 2-1. Therefore, more protons (H^+) are released, which further decreases the milk pH (Tsioulpas *et al.*, 2007).

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

Equation 2-1

2.7.6. Effect of adding sodium chloride

Grufferty and Fox (1985) reported the milk pH is decreased from 6.62 to 6.40 when they added NaCl up to 500 mmol L^{-1}. The decrease in pH was due to the partial replacement of H^+ by Na^+ on the caseins (Grufferty, & Fox, 1985; Huppertz & 2006). Adding 0 - 600 mmol L^{-1} NaCl can significantly increase the calcium concentration in the serum phase, but no significant change on the serum phosphate concentration was reported (Huppertz & Fox, 2006). When the sodium concentration increases, the calcium bound with organic phosphate in the micellar phase is partially exchanged by the added sodium (Gaucheron, 2005). Adding NaCl also increases the ionic strength of the milk system, which reduces the calcium phosphate activity coefficient and increass the solubility of calcium (Huppertz & Fox, 2006; Gaucheron, 2011b).

2.8. Bovine milk protein gelation

The process by which denatured milk protein molecules aggregate to form an ordered protein network structure is called gelation. Gelation is a very important functional property of milk protein and plays an important role in food manufacturing of cheese and fermented milk products (Varnam, & Sutherland, 1994; Lucey, 2020; Walsh-O'Grady *et al.*, 2001).

Milk protein gelation is considered to be irreversible, which can be induced by manipulation of pH or temperature, or addition of enzymes or metal salts (Lucey, 2020). The gelation of milk protein not only forms a solid elastic gel but also can thicken the product or improve the water absorption by controlling the gelation conditions (Varnam, & Sutherland, 1994; Jaros & Rohm, 2017; Lucey, 2017; Phadungath, 2005; Ramasubramanian *et al.*, 2008).

2.8.1. Gelation by acid

Milk acidification gelation is the basis for yogurt manufacture from the reduction of milk pH. The reduction of pH can influence the stability of casein micelles in two ways (Figure 2.10). The first situation is with the pH decreasing, the CCP in the casein micelle is dissociated which then results in release of calcium ions. CCP only slightly dissociates into the serum at around pH 6.0 but completely dissociates into the serum phase at around pH 5.0. With the release of calcium ions into the serum, this can lead to an increase in the electrostatic interactions between casein micelles (Fox *et al.*, 1998; Lucey, 2017). The second effect relates to the isoelectric point of casein (pH 4.6). At the native milk pH (6.6 - 6.8), the casein micelles have net negative charges. It ensures the casein micelles repel each other and stay separated (Damodaran & Parkin, 2017). However, with the pH decreasing, the positively charged hydrogen ions will associate with the casein micelles and reduce the electrostatic repulsion between the micelles. On further decrease in pH to 5.0, the acidic condition results in less electrostatic repulsion between the casein micelles. Due to the reduction in electrostatic repulsion and attraction of hydrophobic interactions at low pH, coagulation of the micelles starts occuring (Lucey & Singh, 1997; Lucey, 2017; Vasbinder *et al.*, 2003). Moreover, when the pH drops to 4.6, the negative charges on the casein micelles will be neutralised, which results in a zero net charge on the micelle surface and further drop results in the greatest coagulation of the casein micelles, hence the pH of 4.6 is also called the iso-electric point for casein micelle (Fox *et al.*, 1998).

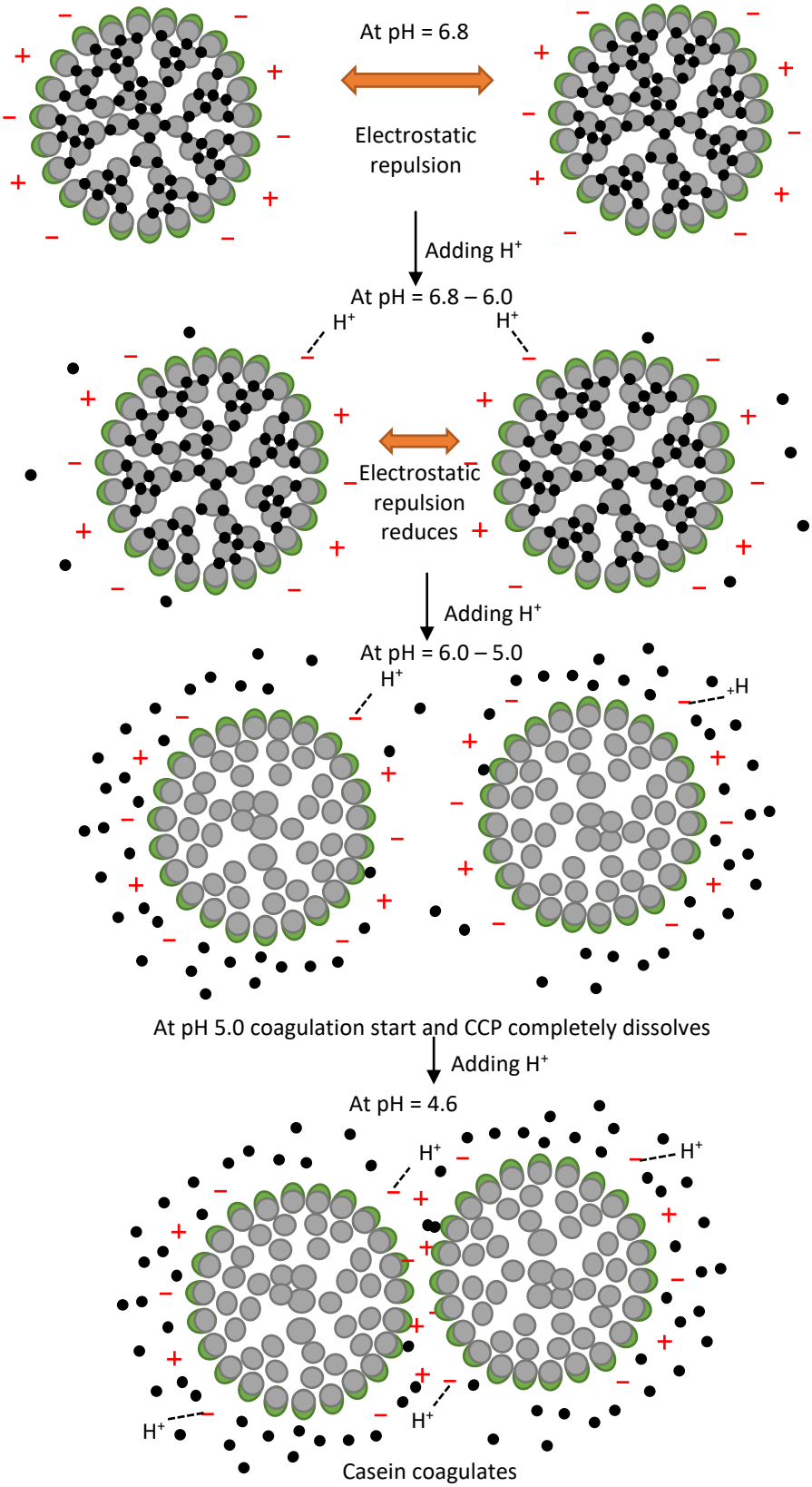


Figure 2.10: The mechanism changes of casein micelles during acidification, (κ - casein rich sub micelle), (κ - casein poor sub micelle), (CCP). Adapted from (Damodaran & Parkin, 2017; Fox *et al.*, 1998; Lucey & Singh, 1997; Lucey, 2017; Vasbinder *et al.*, 2003)

The acid-induced gelation of milk can be influenced by many factors, including milk heat treatment conditions, incubation temperature, cooling conditions, and storage conditions (Lucey, 2020). Preheat treatment, 90°C, 10 min, is commonly used in yogurt manufacture to improve the texture, microstructure and rheological properties (Lucey & Singh, 1997). Interaction occurring between β - lg and κ - casein by disulphide bond reactions during heating (Section 2.5.1) is one of the key reactions to help improve the yogurt texture. Compared to preheated yogurt, the acid induced gels without preheat treatment will only form at lower pH, at which the resultant yogurt has a very weak structure (Li & Dalgleish, 2006; Lucey, 2020). The incubation temperature for acidified milk is another important factor that affects the acid gel properties. In the industry, most of the yogurt manufacturers use 46°C as the incubation temperature. However, Lee and Lucey (2004) found a slightly lower incubation temperature (40°C) extended the gelation time and provided a firmer gel. It should be noted that although acidification gelation can happen at any temperature, when the temperature of gelation is higher than 50°C, the formed gel will be more stringy (Fox *et al.*, 1998).

2.8.2. Gelation with enzymes

The addition of proteolytic enzymes can lead to casein micelle aggregation. One typical example in the dairy industry is cheese making by the addition of rennet (chymosin), which can cleave the C-terminal part (hairy layer) of casein micelles from the κ - casein molecules to form para-κ-casein and a macropeptide by hydrolysation (Lucey, 2020). As discussed in Section 2.4.1, κ - casein is present on the surface of the casein micelle and stabilises the calcium sensitive caseins. Once the κ-casein is hydrolysed, α_{s1} - casein, α_{s2} - casein and β - casein are gradually exposed to the presence of free calcium ions in the serum phase. Then the calcium sensitive caseins will associate with Ca^{2+} and form calcium bridges with other proteins. Consequently, the casein micelle is no longer stable and will aggregate (Fox *et al.*, 1998; Horne, 2006; Jaros & Rohm, 2017; Phadungath, 2005). According to Sandra *et al.* (2007) and Lucey (2020), casein micelles only begin to approach one another when more than 70% κ-casein is hydrolysed.

The enzyme-induced gelation can be affected by temperature, pH, free calcium ion concentration, casein concentration and whey protein concentration (Lucey, 2020). With temperature increase, the cleavage of κ - casein increases and this results in an increase in the casein coagulation rate as moderate temperature facilitates the enzymatic raction rate (Jaros & Rohm, 2017). However, when the temperature increases to a point where the enzyme starts to denature, the reaction rate decreases rapidly. Similarly, each enzyme has its optimum active pH, either higher or lower pH can inhibate its gelation activity (Wong *et al.*, 1988).

2.8.3. Gelation by heat

The heat-induced milk gelation is the result of several changes happening during the heat treatment. As discussed in Section 2.5.1, whey protein denaturation starts when the temperature is higher than 70°C, which can lead to association of the denatured whey protein with the κ - casein that is present in the serum phase and on the surface of the casein micelles (Anema, 2008; Datta & Deeth, 2001; Fox *et al.*, 1998; Oldfield *et al.*, 1998). Consequently, the surface properties of casein micelles can be affected and the ability of κ - casein to stabilise calcium-sensitive casein is reduced (Zittle *et al.*, 1962). The reduction in pH due to the increase in temperature also influences milk gelation. As discussed in Section 2.7.3, the dissociation of κ - casein during heating is minimal when the skim milk pH is between 6.3 and 6.5, thus, most of the κ - casein remains on the surface of casein micelles (Anema, 2009). At these pHs, only 40% of the whey protein remains in the serum phase after heating at 90°C (Anema, 2008). Thus, the decrease of milk pH from heating results in more denatured whey protein associated with the κ - casein and an increased susceptibility of the milk to gel (Huppertz, 2016). Wong *et al.* (1988) also reported when milk at pH less than 6.5 and heated at 100°C for about 20-30 minutes, protein coagulation occurred.

2.8.4. Gelation by salt addition

Addition of mineral salts can lead to milk gelation. The milk gelation by adding calcium salts has been studied. It has been found Ca^{2+} forms crosslinks between negatively charged groups of the caseins which leads to aggregation of proteins and gelation (Wong *et al.*, 1988; Lin *et*

al., 2018; Mellema *et al.*, 1999; Ramasubramanian *et al.*, 2008). It has been reported that the addition of magnesium ions can interact with casein in much the same way as calcium ions and result in milk gelation when the temperature of milk is higher than 70°C (Oh, & Deeth, 2017). As a divalent cations, zinc has the same capability as calcium and magnesium, which can also promote milk protein coagulation (Singh *et al.*, 1989a, 1989b). Singh *et al.* (1989b) reported milk protein coagulation was observed for a skim milk sample with 16 mmol L^{-1} of zinc chloride added and after holding at 30°C for 2 hours. As discussed in Section 2.7.5, the addition of mineral salt into bovine milk can increase the ionic strength and decrease the milk pH (Gaucheron, 2005, 2011b; Udabage *et al.*, 2000). The decrease in pH caused by the addition of salts will destabilise the casein micelle, which has a similar effect as when acid is added. Concomitantly, as the addition of salts binds with the negatively charged parts on caseins, both zeta potential (negative charge) and the electrostatic repulsion between casein micelles are reduced (Lin *et al.*, 2018; Tsioulpas *et al.*, 2007). Furthermore, the formation of salt bridges between casein micelles is another main cause of the salt-added milk gelation. Cross links are formed between casein particles when the added cations bind with the negatively charged groups on caseins, which results in tighter, more compact curds and forms larger curd particles (Ahmad *et al*., 2009; Fox *et al.*, 1998; Horne, 2006; Jaros & Rohm, 2017; Phadungath, 2005).

The effect of adding different types of calcium salt on bovine milk have been studied (Crowley *et al.*, 2014; Lin *et al.*, 2018; Philippe *et al*., 2004). Lin *et al.* (2018) evaluated the effects of addition of different calcium salts on milk gelation properties. The authors suggested the Hofmeister effect of anions may explain the different milk gel properties caused by adding different calcium salts. The Hofmeister series was proposed firstly around the 1800s based on the salt concentration required to result in the egg white protein precipitate (Kunz *et al.*, 2004).

2.9. Zinc deficiency for humans

Zinc is an essential trace element required by the human body. It is critical for physiological processes such as human growth, reproductive health, immunity and the endocrine system. (Kahraman & Ustunol, 2012; Pabón & Lönnerdal, 1993). Zinc is involved in the activity and

composition of various proteins, nucleic acids synthesis and catabolic enzymes (Islam & Ahmed, 2013). Therefore, zinc plays an indispensable role in the growth and development of humans, especially children. The recommended dietary intake for zinc is listed in Table 2.8. For pregnant females, a higher zinc intake is recommended to support foetus growth (Trumbo *et al.*, 2001). According to Caulfield and Black (2004), the estimated global prevalence of zinc deficiency is 31% of children under five years of age. Based on the data published by the International Zinc Nutrition Consultative Group (2019), at least 17% of the world population is at risk of inadequate zinc intake. Severe zinc deficiency in childhood can lead to short stature or even dwarfism (Blakeborough *et al.*, 1983). Zinc also assists the body's immune functions, therefore malnutrition of zinc will reduce resistance to infection (Gibson, 2012; Kahraman & Ustunol, 2012). Zinc deficiency can increase the risk of incidence for diarrhoeal disease, pneumonia and malaria by 1.28, 1.52 and 1.56 times, respectively (Caulfield & Black, 2004). Mild to moderate zinc deficiency is more common than severe zinc deficiency worldwide (Darnton-Hill, 2013). However, mild and moderate zinc deficiency is difficult to be diagnosed, because the symptoms of zinc deficiency such as weakened immunity and stunted growth can also be caused by deficiencies from other nutrients. (De Benoist *et al.*, 2007).

Table 2.8: Recommended dietary intakes for zinc (Trumbo *et al.*, 2001).

Age	Male	Female	Pregnancy	During lactation
0 - 6 months	2 mg	2 mg		
7 - 12 months	3 mg	3 mg		
1 - 3 years	3 mg	3 mg		
4 - 8 years	5 mg	5 mg		
9 - 13 years	8 mg	8 mg		
14 - 18 years	11 mg	9 mg	12 mg	13 mg
19+ years	11 mg	8 mg	11 mg	12 mg

2.10. Zinc concentration in different kinds of food

The zinc content in some food products has been listed in Table 2.9. Table 2.10 presents the zinc content in different animal milks. As can be seen in Table 2.9, the best food to supply zinc is oyster which contains 36.10 mg zinc in every 100g oysters, followed by the red meat in which zinc content ranges from 3.5 to 1.5 mg/100g (Scherz, & Kirchhoff, 2006). For vegetarianism, cereals are a good source of zinc. Although the zinc content in cows milk is

only 0.37 mg per 100g (Scherz & Kirchhoff, 2006), the bioavailability of zinc from animal food sources is higher than from plant food sources (Gibson, 2012; Wong *et al.*, 1988).

Table 2.9: Zinc content in some foods (Scherz & Kirchhoff, 2006).

Food	Zinc content (mg/100g)
Milk	
Cow-milk, whole-milk	0.37
Meat	
Beef, muscle, average	3.45
Pork, muscle, average	2.67
Chicken, meat, average	1.54
Salt-water fish	
Flatfish (Sole, Plaice)	0.45
Herring (Atlantic)	0.99
Mackerel	0.70
Fresh-water-fish	
Eel	1.62
Trout	0.66
Molluscs crustaceans	
Mussel	1.43
Shrimp	1.11
Oyster	36.10
Cereals	
Wheat kernel	2.19
Barley kernel	3.04
Oat kernel	3.05
Vegetables, roots, tubers	
Carrot	0.25
Celeriac	0.33
Beetroot	0.35
Potato	0.29
Vegetables leaves, steams, flowers	
Lettuce	0.19
Spinach	0.45
Cabbage, white	0.18
Cauliflower	0.28
Onions	0.17
Vegetable fruits	
Tomato	0.13
Cucumber	0.13
Fruits	
Apple	0.02
Strawberry	0.16
Grape	0.05
Orange	0.06
Banana	0.17

Considering the zinc content in different animal milks (Table 2.10), camel milk contains the highest zinc content which is 0.51 ± 0.02 mg/100g, followed by bovine milk with 0.37 ± 0.01 mg/100g (Scherz & Kirchhoff, 2006; Soliman, 2005). In bovine milk, the zinc concentration is highly related to the period of lactation the cow is in. Colostrum is the first milk produced after the cow has given birth to the calf. The zinc content in colostrum milk is in the range of 1.2 - 1.8 mg/100g, but in milk from later in the season contains less zinc (~ 0.3 - 0.6 mg/100g) (Jensen, 1995; Soliman, 2005). Vaillancourt and Allen (1990) postulated the high zinc concentration in colostrum was because of the increase in glucocorticoids during parturition, which leds to more zinc transfer into the mammary glands from the blood.

Table 2.10: Zinc content in different animal milk (Soliman, 2005).

Milk	Zinc content (mg/100g)
Camel	0.51 ± 0.02
Buffalo	0.24 ± 0.01
Bovine	0.38 ± 0.01
Goat	0.32 ± 0.03
Human	0.17 ± 0.02

2.10.1. Native zinc distribution in bovine and human milk

According to Parkash and Jenness (1967), about 88% of the total zinc found in the bovine milk is bound to the colloidal casein micelles in the milk and only 12% of the total zinc is present in the serum. One study by Cousins and Smith (1980) reported no zinc is associated with the low molecular weight fractions (<10kDa) in native bovine milk. Blakeborough *et al.* (1983) also confirmed this result by reporting there was no detectable amount of zinc associated with the low molecular fractions in bovine milk and over 95% of the total zinc in bovine milk associated with the casein micelles. Singh *et al.* (1989b) reported ~ 90% of the native zinc in bovine milk is sedimented after ultracentrifugation as it was associated with casein micelles, and 10% of the total zinc present was found in the serum phase. Approximately half of the zinc in the serum phase was not dialysable, as it was associated with high molecular weight fractions and which were probably the non-sedimented proteins. Singh *et al.* (1989b) concluded that only 5% of the total zinc in milk associates with low molecular weight ligands. In conclusion, there is considerable evidence to show that the zinc in the native bovine milk is mostly associated with high molecular weight fractions such as the casein micelles.

Research has also been interested in the zinc content in human milk. Differring from bovine milk, the zinc is almost evenly distributed in human milk. Lönnerdal *et al.* (1982) reported that in human milk, about 14% of the total zinc associates with casein, 28% with serum albumin, 29% with the low molecular weight fractions and the remaining zinc (~ 29%) associates with fat. Singh *et al.* (1988) also studied the distribution of zinc in skim (low fat) human milk. They reported ~ 34% of the total zinc in a mother's milk associates with the low molecular weight fractions, 36% zinc with whey protein and 30% with the casein micelles. Cousins and Smith (1980) and Blakeborough *et al.* (1983) also reported a higher fraction of zinc associates with low molecular fractions in human milk (10%) compared with bovine milk (~ 0%). The typical distribution of zinc in human and bovine milk is shown in Table 2.11. The difference in zinc distribution between bovine milk and human milk is likely due to the protein composition difference in these two types of milk (Blakeborough *et al.*, 1983; Cousins & Smith, 1980; Lönnerdal *et al.*, 1982; Singh *et al.*, 1989a).

Table 2.11: Distribution of zinc in human and bovine skim milks (Singh *et al.*, 1988).

Fraction	Zn concentration (mg L^{-1})	
	Human skim milk	Bovine skim milk
Total	1.55	3.80
Colloidal calcium phosphate	0.14	2.38
Casein	0.32	1.18
Whey proteins	0.56	trace
Low molecular weight	0.53	0.23

2.10.2. Binding of native zinc to casein in bovine milk

As reported most of the zinc in bovine milk associates with the casein micelle, it is therefore, important to understand the native zinc-casein binding in bovine milk. By adding different amounts of ethylenediaminetetraacetate (EDTA) into bovine milk to extract zinc, there was found two states of zinc in the sediment phase after ultrafiltration (Parkash & Jenness, 1967; Singh *et al.*, 1989b). This indicated that there may be two types of bonds between zinc and casein (Parkash & Jenness, 1967; Singh *et al.*, 1989b). Singh *et al.* (1989b) reported 1.18 mg zinc per litre in bovine milk (about 32% of the total zinc content in milk) was directly bound to the casein and about 2.38 mg L^{-1} (~ 63%) of the zinc associates with CCP. When 0.2 mM

EDTA was added, more than 80% of the zinc dissolved into the serum in the CCP-free milk, whereas only 40% of the zinc dissolved in the skim milk. This result indicated that part of zinc may be loosely bound to the casein and part of zinc was tightly bound with CCP. Another study was carried out by Parkash and Jenness (1967), who found that ~ 1.7 mg L^{-1} (49%) of zinc was loosely bound with casein and ~ 1.8 mg L^{-1} (51%) of zinc was tightly bound with CCP. Harzer and Kauer (1982) reported that zinc is only released from casein by acid precipitation, not rennin or calcium precipitation. Therefore, zinc is not likely to be bound to the C-terminal from κ - casein. Many studies have suggested the negatively charged phosphate groups in the casein and the CCP present are most likely to be associated with zinc (Cousins & Smith, 1980; Harzer & Kauer, 1982; Parkash & Jenness, 1967; Singh *et al.*, 1989a, 1989b).

2.10.3. Binding of added zinc in bovine milk

Several *in vitro* studies have reported in bovine milk, the added zinc is mostly bound with casein and only small amounts of zinc are bound to whey protein or other low molecular weight fractions (Cousins & Smith, 1980; Harzer & Kauer, 1982; Parkash & Jenness, 1967; Philippe *et al.*, 2005; Singh *et al.*, 1989a, 1989b). Singh *et al.* (1989b) reported the zinc concentration in casein micelle increases with increasing concentration of added zinc chloride. When more than 16 mmol L^{-1} of zinc chloride was added to bovine milk, coagulation of the casein micelle occurred when the sample was heated to 30°C and held for 2 hours. At this concentration of added zinc, 40% of the calcium in the micellar phase was replaced by zinc in the casein micelles (Singh *et al.*, 1989b). Philippe *et al.* (2005) reported the association of zinc with casein micelle was stronger than with calcium. This could explain why calcium can be partically replaced by zinc in the casein micelle.

Cousins and Smith (1980) reported increasing the concentration of added zinc not only increased the zinc concentration in the sediment, but also increased the zinc concentration in the serum phase. More zinc was found in the serum phase when the addition of zinc increased from 0.378 mg ml^{-1} (5.78 mmol L^{-1}) to 3.780 mg ml^{-1} (57.80 mmol L^{-1}), from 4 to 26% (w/w), respectively. The most likely explanation for the increasing concentration of zinc in the serum was that the high molecular weight fractions which had the ability to bind with zinc

became saturated, hence, the extra zinc ions had to present in the supernatant, if the zinc concentration in the bovine milk was sufficiently high (Cousins & Smith, 1980).

Singh *et al.* (1989a) studied the zinc binding capacities of each bovine milk protein (Table 2.12). The number of binding sites for zinc in each casein was similar to the number of phosphoserine residues in each casein (Table 2.3). Another study by Harzer and Kauer (1982) determined the zinc binding capacity of bovine casein was 8.4 μg zinc per mg casein at pH 7.4. Both Harzer and Kauer (1982) and Singh *et al.* (1989a) have reported the zinc binding capacity of casein was significantly reduced by dephosphorylation, and they suggested the phosphoserine residues are the primary binding sites for zinc in the caseins.

Table 2.12: Number of binding sites and precipitation concentration for free Zn to bovine milk proteins (Singh *et al.*, 1989a).

Protein	Zinc binding capacity (atoms Zn/mol protein)	Precipitate free Zn concentration (mmol L^{-1})
Whole casein	6	2.0
α_{s1} - casein	11	2.0
β - casein	8	2.5
κ - casein	1 - 2	2.5
Bovine serum albumin (BSA)	5	-
α - Lactalbumin	0.4	-
β - Lactoglobulin	0.6	-

2.10.4. Effects of added zinc cation on bovine milk

Philippe *et al*. (2005) studied the physicochemical property changes of whey protein free bovine milk when zinc chloride stock solution (0.25 mmol L^{-1}) was added, the results are summaried in Table 2.13. The whey protein free bovine milk was prepared by dispersing phosphocaseinate powder in a milk ultrafiltrate. The final zinc chloride concentration in the whey protein free bovine milk was adjusted from 2.5 to 8.0 mmol kg^{-1}. The pH of the zinc added whey protein free bovine milk was adjusted to 6.75 after one hour and left for 24 hours at room temperature before analysis.

Table 2.13: Influence of added zinc cation concentration (2.5 to 8 mmol kg^{-1} zinc) on physicochemical properties of whey protein free bovine milk (Philippe *et al.*, 2005).

Physicochemical properties	Changes	Percentage changed
Amount of zinc associated with casein micelle	Increases	171%
Inorganic phosphate concentration in the serum phase	Decreases	46%
Ca^{2+} concentration in casein micelle	Decreases	15%
Casein micelle hydration	Decreases	23%
Zeta potential of casein micelle	Almost the same	-
Particle size of casein micelle	Almost the same	-

Rana *et al*. (2018) studied the physicochemical properties for zinc fortified fresh whole bovine milk. Zinc acetate and zinc sulphate was added into the milk as a powder at 45°C with a uniform mixing for 15 minutes before analysis. The final zinc concentration in the milk was adjusted to 15 - 22.5 ppm (0.23 - 0.34 mmol L^{-1}). Rana *et al*. (2018) found the addition of zinc acetate and zinc sulphate did not significantly (p>0.05) change the milk pH, but there is a significant increase (p<0.05) in milk apparent viscosity with the addition of zinc salts regardless of the zinc salt type. They also reported the weight of the dry insoluble materials increased significantly (p<0.05) with increasing amount of added both zinc salts.

2.10.5. Effect of pH, ionic strength and temperature on zinc binding

As most of the zinc associates with casein micelle, the change of milk pH, ionic strength, thermal treatment and the presence of other ions may have a significant influence on zinc binding (Damodaran & Parkin, 2017; Fox *et al.*, 1998; Harzer & Kauer, 1982; Seiquer *et al.*, 2000; Singh *et al.*, 1989a, 1989b).

2.10.5.1. Effect pH

Several studies have investigated the effect of added zinc concentration in the serum phase with changing pH. With a decrease in pH (from 7.0 to 4.6), the zinc concentration in the serum phase increased. When the pH dropped to 4.6 (isoelectric point for casein), almost all of the zinc present in the micellar phase was released into the serum phase (Harzer & Kauer, 1982; Singh *et al.*, 1989a, 1989b). Zinc binds with the negatively charged phosphoserine residues in the casein. With a decrease in pH , the hydrogen ions can partially replace the zinc ions that bind with the negatively changed phosphoserine residues and lead to micellar zinc released into the serum phase (Harzer & Kauer, 1982).

Singh *et al.* (1989a) reported that the amount of zinc bound with α_{s1}-casein increases significantly with an increase in pH from pH 5.4 to 7.0. Another investigation by Baumy and Brule (1988) found that zinc binding to β - casein decreased with a reduction in pH, which was similarly observed with α_{s1} - casein. When pH decreased , the ionic charge of phosphoserine groups reduced which then lowered the β - casein mineral binding capabilities.

2.10.5.2. Ionic strength

The ionic strength in milk serum as exerted by the milk salts was estimated to be approximately 80 mmol L^{-1} (Fox *et al.*, 1998). When adding extra zinc salt, the ionic strength of the solution will increase. Zinc binding with α_{s1} - casein and β - casein has been found to decrease with increasing ionic strength. The increase in ionic strength can slightly decrease the pH of the milk and reduce the ionic charge of the phosphoserine residues then zinc is released from the casein micelle (Baumy & Brule, 1988; Singh *et al.*, 1989a).

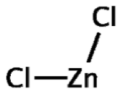
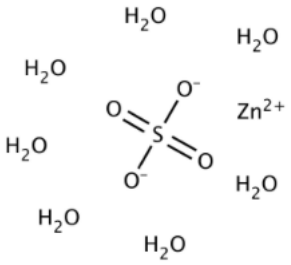
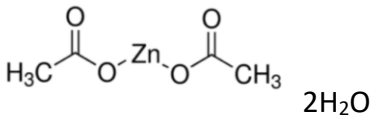
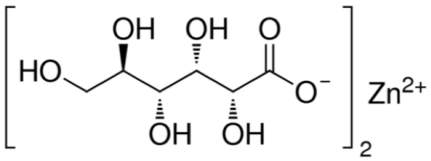
2.10.5.3. Thermal treatment

Seiquer *et al.* (2000) reported the zinc concentration in the serum phase decreases after heat treatment. In addition, the protein desaturation and association with each other by heating may also influence the zinc binding (Varnam, & Sutherland, 1994; Damodaran & Parkin, 2017; Fox *et al.*, 1998). However, there is limited research reported the effect of heat treatment on zinc binding in milk system.

2.11. Mineral zinc sources and their bioavailability

Zinc is an metal atom with a molecular weight of 65.4 g mol^{-1} (James, 2017). Zinc is classed as a group 12 element in the periodic table which has an electronic configuration of $1s^2 2s^2 2p^6 3s^2 3p^6 3d^{10} 4s^2$ (James, 2017). Some studies classified zinc as a transition metal as it is located in the *d*-block in the periodic table (Blackman *et al.*, 2012; James, 2017). However, according to the IUPAC definition, zinc has a complete 3d atomic orbital (10 electrons) which is classified as a non-transition metal (McNaught & Wilkinson, 1997). Zinc has two electrons in its outermost atomic orbital (4s) which it loses to form a zinc ion with a charge of +2 (Zn^{2+}), hence the behaviour of zinc is similar to alkaline-earth metals such as calcium and magnesium (House & House, 2015). It is insoluble in water as a metal (Goodwin, 2000). To increase the zinc ion concentration in the bovine milk, soluble zinc salts are necessary. Table 2.14 lists the solubility of four soluble zinc salts normally used for fortifying food with zinc. Zinc chloride and zinc sulphate are the two common inorganic zinc salts that are used in zinc fortified milk studies (Kahraman & Ustunol, 2012; Singh *et al.*, 1989a, 1989b). Both of these salts have high water solubility. According to Wong *et al.* (1988), the chloride and sulphate are completely soluble and ionised (100%) in bovine milk. However, early studies have reported that organic zinc compounds are better absorbed than inorganic zinc in the human digestive tract (Gibson, 2012; Sapota *et al.*, 2014; Seal & Heaton, 1983). After comparing the bioavailability of zinc gluconate, zinc citrate and zinc sulphate, Sapota *et al.* (2014) reported zinc gluconate is the most efficient compound as a fortifying zinc, while zinc sulphate has the lowest bioavailability. Seal and Heaton (1983) found zinc sulphate is more easily absorbed by a rat's intestinal sacs than zinc chloride, and the uptake of zinc acetate was slightly higher than zinc sulphate. In conclusion, based on the published research, the order of zinc compounds' bioavailability from greatest to lowest is zinc gluconate > zinc acetate > zinc sulphate > zinc chloride. Although some studies reported the bioavailability of organic zinc salt as better than inorganic salt, there are a lack of studies on the effect of adding organic zinc salt into milk.

Table 2.14: The solubility of four zinc compounds (Granum, 2018).

Zinc compounds	Formula	Solubility in water at 25°C (mol L^{-1})	Structural formula
Zinc chloride	$ZnCl_2$	31.69	
Zinc sulphate heptahydrate	$ZnSO_4 \cdot 7H_2O$	3.34	
Zinc acetate dihydrate	$Zn(C_2H_3O_2) \cdot 2H_2O$	1.82	
Zinc gluconate	$Zn\ C_{12}H_{22}O_{14}$	0.22	

2.12. Application of rheology in milk gelation study

Dynamic rheology is very applicable to the study of milk gelation. It can provide simply viscosity measurements, characterise flow behaviour, and provide information about the structure of the material (Iličić *et al.*, 2015; Walsh-O'Grady *et al.*, 2001). Milk gels are viscoelastic bodies and the study of rheological properties of milk gels can help to understand the gel properties and gel formation process (Walsh-O'Grady *et al.*, 2001). There are two parameters that can be used to describe the rheological properties of a viscoelastic materials. Storage modulus (*G'*) is a measure of elasticity, and loss modulus (*G''*) is a measure of viscosity (Iličić *et al.*, 2015). The ratio of the viscous to elastic properties can be described as tan δ (Equation 2-2), which describes the overall structure of the material (Lucey, 2002). When tan δ is smaller than 1 (*G''* < *G'*), the deformation of the test sample is essentially elastic and

recoverable, and the sample behaves more like a solid. When tan δ is larger than 1 (*G''* > *G'*), the test sample behaves more like a liquid (Iličić *et al.*, 2015).

$$tan\ \delta = \frac{G''}{G'}$$

Equation 2-2

The milk gelation rheology properties are often highly dependent on the time scale (Fox *et al.*, 1998). The point of gelation (gelation point) can be determined by measuring *G'*, but there are different experimental definitions for choosing gelation point. According to Walsh-O'Grady *et al.* (2001), the initial formation of milk gel can be estimated as the rapid increase in *G'*. However, Liu *et al.* (2014) and Ramasubramanian *et al.* (2014) suggested when the *G'* larger than 1 Pa the milk transformed from a liquid to a gel. Zhao and Corredig (2020) had another point of view, they decided the gel forms when the tan δ is ≥ 1. As the experimental definition of gelation point is decided arbitrarily, experiment results would vary with the decision making.

2.13. Methods for zinc determination in milk

As discussed previously, zinc is an essential trace element for physiological processes such as human growth, reproductive health, immunity, and the endocrine system. However, excess intake of zinc can be harmful and toxic to human (Islam & Ahmed, 2013). In addition, zinc distribution in milk is another important factor which may influence the zinc absorption by the human body. Thus, it is necessary to determine the zinc distribution and quantify its concentration in the milk.

Milk is an organic matter which has a very complex composition. The zinc in the milk usually associates with different components and forms complex matrices as discussed in Section 2.10.2. Thus, in order to accurately determine the zinc concentration, pre-treatment of the sample is necessary to release zinc from its complexes. Furthermore, sample preparation is necessary for separating the bovine milk proteins in the serum and colloidal phases. In order to be able to study the zinc distribution in bovine milk, the detection methods of metal elements can include atomic absorption dpectrometry (AAS), inductively coupled plasma

mass spectrometry (ICP-MS), atomic emission spectrometry (AES), spectrophotometric and complexometric titration methods.

2.13.1. Sample preparation for zinc determination

2.13.1.1. Dry ashing

Ashing is a method to destroy the sample matrix especially the organic matter. By means of dry ashing the samples are dried first in the crucible, and then put into the high temperature furnace (~500℃) for several hours. The ashed powder is then usually dissolved in dilute acid for further determination (Akinyele & Shokunbi, 2015; Association of Official Analytical Chemists, 2005). Dry ashing results in a sample that dissolves quickly, consumes less acid, and is suitable for handling large quantities of samples.

2.13.1.2. Wet digestion

Wet digestion usually uses strong oxidizing acid to destroy the organic matter in the sample, and then retains the inorganic component for determination. Commonly used acids are nitric acid, perchloric acid, hydrochloric acid and sulfuric acid. This method can reduce the loss of volatile elements, but digestion time is also long, and more reagent is required in this method (Akinyele & Shokunbi, 2015). According to Saracoglu *et al.* (2007), the recovery percentage of zinc from baby food via dry ashing (92%) and wet ashing (93%) is not significantly different.

2.13.1.3. Microwave digestion

Microwave digestion is a direct heating method which acts on the sample organic molecules. Usually, it only requires a small amount of strong acid and a few minutes to complete the digestion. Compared with the dry and wet ashing methods, microwave digestion is faster, and only requires a small amount of reagent (Luo *et al.*, 2010; Saracoglu *et al.*, 2007).

2.13.2. Atomic absorption spectrophotometry (AAS)

Atomic absorption spectrometry (AAS) is currently the most commonly used method for the detection of heavy metal elements in food (Luo *et al.*, 2010). This method was established by

taking advantage of the fact that gaseous atoms can absorb at certain wavelengths of light and cause the electrons in the outer layers of atoms to jump from the ground state to the excited state. The absorbed wavelength varies for different metal atoms. This can be used as the qualitative basis of identifying elements, and the intensity of absorbed radiation can be used as the quantitative basis (Sikirić *et al.*, 2003). According to the different ways of atomization, it can be divided into flame atomic absorption spectroscopy (FAAS) and graphite furnace atomic absorption spectroscopy (GF-AAS). FAAS is simple to operate and is fast, but its sensitivity is relatively low (Luo *et al.*, 2010). GF-AAS is a commonly used for trace analysis technology with high sensitivity, but it is not suitable for multi-element analysis (Aceto *et al.*, 2002).

2.13.3. Atomic emission spectroscopy (AES)

AES method has a fast analysis speed and can quantitatively analyse a number of elements at the same time in a few minutes. It has high accuracy and low detection limit, as low as μg/L with an inductively coupled plasma (ICP) light source. Inductively coupled plasma emission spectrometer (ICP-AES) is widely used to determine metal content in the food sample. However, the instrument itself and operation cost is high (Aceto *et al.*, 2002).

Microwave plasma atomic emission spectrometer (MP-AES) is a new generation of AES. In order to use this method, digestion needs to be carried out first and the solid sample needs to be brought into solution. When heat is applied to the sample, the metal element gains energy and excites into a higher energy level. However, the element in the excited state is less stable and will move back to the lower energy level by emitting radiation in the form of photons. Then the monochromator separates the radiation into different wavelengths and this is recorded by the detector. The metal can be determined based on their wavelength, and the intensity of the wavelength can be used for the metal concentration determination (Guerin *et al.*, 2012). There is no need to use expensive or flammable (required by FAAS) gases for this instrument which allows unattended, overnight operation. Agilent 4100 MP-AES is a cheap, fast and unattended multi-element sequential atomic emission spectroscopic technique using a nitrogen plasma (Nham & Taylor, 2012).

2.13.4. Inductively coupled plasma mass spectroscopy (ICP-MS)

Inductively coupled plasma mass spectrometry (ICP-MS) is a trace metal determination method, offering both sensitivity and accurate results compared with AAS and AES (Luo *et al.*, 2010). However, the price of ICP-MS and the cost of operation limits its wide application.

2.13.5. Colourimetry / Spectrophotometric

Colourimetric /spectrophotometric methods are well-developed methods to determine zinc content in food. It is based on the reaction of zinc with other reagents to form a coloured complex. Then the presence of the colour complex is measured by measuring the absorbance of the sample solution at a specific wavelength (Association of Official Analytical Chemists, 1976; Islam & Ahmed, 2013; Song *et al*, 1976).

Dithizone is a common colourimetric reagent used for determining heavy metals. After the sample is digested, zinc ions can form a purple-red complex with dithizone which absorbs at the wavelength of 555nm (for zinc only) (Song *et al*, 1976). This method does not require a specific instrument, but the detection limit of this method is slightly higher than others and usually the toxic reagent, carbon tetrachloride, is required to extract dithizone (Association of Official Analytical Chemists, 1976).

2.13.6. Complexometric titration

Novick (1997) reported a simple titration method for zinc quantification analysis by ethylene diamine tetraacetic acid (EDTA), which can remove the zinc from the protein without digestion by its chelating ability (Harzer & Kauer, 1982). Complexometric titrations with EDTA have traditionally been performed to determine the calcium or magnesium content of water at a basic pH. In contrast, the determination of metal ions (e.g., Zn^{2+}, Fe^{3+}, Cu^{2+}, Ni^{2+}, Pb^{2+}, Al^{3+}) with EDTA to form complexes at low pH has not been reported extensively. EDTA titration is not only performed at high pH, but also can be used at low pH for different metal ion detections (Novick, 1997). Since EDTA is an acid substance with four weak acid dissociations, the reactions with metal ions are pH dependent (Chaberek Jr *et al.*, 1955). Zinc reacts strongly

with EDTA, thus, it can be titrated in an acidic solution (pH 5.5). However, calcium and magnesium reacts more weakly with EDTA and must be titrated in alkaline solutions (Waters *et al.*, 2001). To ensure consistent results for titrations, the pH of the solutions must be controlled by using buffer solutions.

2.14. Conclusion

Milk contains approximately 4 mg Zn / L milk and the bioavailability of zinc in milk is relatively high. In the native state, most of the zinc is associated with the casein micelles and is present in the micellar phase. The zinc ions released from the added zinc salts are primarily associated with high molecular fractions, but also can be found in the serum phase when the high molecular fractions are saturated with zinc. When a high concentration of zinc salt is added to the milk system, casein micelle coagulation was found to occur. The milk property changes after addition of calcium salts have been mostly described. Information on how the physicochemical property changes of skim milk after zinc salt is added, such as pH, zinc and calcium distribution have not been not well understood yet. Although casein micelle coagulation after zinc salt addition has been reported, the milk rheology property changes; viscosity change or formation of gels have not been studied extensively. Consequently, this research focused on studying the milk physicochemical property changes after zinc salt added and investigating the rheology properties of milk with the addition of zinc salts.

Chapter 3. Materials and Methodology

3.1. Materials

Low heat skim milk powder (32.91% protein, 0.93% fat, 54.50% carbohydrate, 7.90% ash, 3.79% moisture (w/w)) was purchased from Fonterra Co-operative Group, New Zealand and resealed into small pouches (~200g skim milk powder per pouch) then stored at 4 ^{o}C before use. Zinc chloride (reagent grade, ≥ 98%), zinc sulphate heptahydrate (reagent grade, > 99.5%) and zinc acetate dihydrate (reagent grade, ≥ 98%) were purchased from Sigma-Aldrich, New Zealand. Zinc gluconate hydrate (≥97%) was purchased from Fisher Scientific, New Zealand.

3.2. Preparation of the reconstituted skim milk

Low heat skim milk powder (SMP) was reconstituted to 13% (w/w) total solids with distilled water at 20 ± 1^{o}C. Sodium azide (>99.0%, SERVA Electrophoresis GmbH, Heidelberg, Germany) was added at 0.02% w/w as a preservative. The skim milk sample was stirred for 3 hours using a magnetic stirrer. The reconstituted skim milk solution was left for at least 10 hr to ensure complete hydration before use. Skim milk samples were stored at 20 ± 1^{o}C and used within 48 hr of preparation. The 13% (w/w) reconstituted skim milk density was measured by recording the weight of one ml 13% (w/w) reconstituted skim milk at 20 ± 1^{o}C. The average density was determined to be 1038 ± 6 kg m^{-3} (n=4).

3.3. Preparation of the zinc salt solutions

The degree of hydration used for calculation was based on the manufacturer's specification for zinc sulphate heptahydrate and zinc acetate dihydrate. For zinc gluconate hydrate ($ZnC_{12}H_{22}O_{14}$. xH_2O), the degree of hydration (x) was not specified on the product specification. A known amount of zinc gluconate hydrate was dissolved in distilled water, and the zinc concentration was determined by EDTA titration (Section 3.8). The degree of hydration of zinc gluconate hydrate was 3.14. Calculations to determine the actual degree of hydration can be found in Appendix 1. All the three zinc salt stock solutions were prepared to

a final concentration of 200 mmol Zn/L solution. The required mass of each zinc salt to prepare 500 ml of 200 mmol L^{-1} zinc salt stock solution can be found in Appendix 2. The zinc concentration in the zinc stock solutions prepared from different zinc salts was confirmed by EDTA titration (Section 3.8).

3.4. Preheat treatment of reconstituted skim milk

A hot water bath was set up by placing a large beaker (2000 ml) with tap water on top of a hot plate (Magnetic stirrer / hot plate thermostat control, Industrial Equipment & Control Pty. Ltd, Australia) and then heated to 95 ± 2°C. The 13% (w/w) reconstituted skim milk was transferred into a 250 ml conical flask. The conical flask with skim milk was place into the hot water bath (95 ± 2°C) and heated to 90 ± 2°C within 5 minutes accompanied with a continuous stirring by a magnetic stirrer. When the skim milk sample temperature reached 90 ± 2°C, the skim milk sample was transferred into a hot water bath (GD100, Grant Instruments Ltd, Cambridge, England) at 90 ± 1°C and left for 10 minutes. Then the skim milk was cooled to 20 ± 1°C within 5 mins in a 4 ± 1°C water bath. A thermometer (Thermocouple, Fisher Scientific, New Zealand) was placed in the reconstituted skim milk during the preheating process to monitor the sample temperature. The zinc salt stock solutions were added after the preheated skim milk was cooled to 20 ± 1°C.

3.5. Preparation of skim milk with zinc salts added

A required amount of 13% (w/w) reconstituted skim milk was added into 100 ml beakers, followed by addition of the 200mmol L^{-1} zinc salt stock solution to prepare the skim milk samples with final added zinc concentrations between 5 to 40 mmol L^{-1}. The volume of 13% (w/w) skim milk varied to account for the difference of the density of zinc salt stock solution. Distilled water was then added to the sample, and the final skim milk concentration was adjusted to 10% total solid (w/w). The amount of each skim milk sample, zinc stock solution and distilled water added to make 50 ml skim milk with added zinc salt is shown in Tables 3.1 to 3.3. The samples were prepared with magnetic stirring. The calculation example of the volume added for each solution can be found in Appendix 3.

Table 3.1: The volume of skim milk, zinc acetate stock solutions (200 mmol L^{-1}) and distilled water added to make up 50 mL of zinc+skim milk with a final concentration of 10% total solids (w/w).

Concentration of added zinc acetate in the skim milk (10 % w/w) (mmol L^{-1})	Volume of 13% (w/w) skim milk added (ml)	Volume of zinc acetate stock solution added (ml)	Volume of distilled water added (ml)
0	38.115	0	11.885
5	38.150	1.250	10.600
10	38.190	2.500	9.310
15	38.225	3.750	8.025
17.5	38.245	4.375	7.380
20	38.265	5.000	6.735
22.5	38.285	5.625	6.090
25	38.300	6.250	5.450
30	38.340	7.500	4.160
35	38.375	8.750	2.875
40	38.415	10.000	1.585

Table 3.2: The volume of skim milk, zinc sulphate stock solutions (200 mmol L^{-1}) and distilled water added to make up 50 mL of zinc+skim milk with a final concentration of 10% total solids (w/w).

Concentration of added zinc sulphate in the skim milk (10 % w/w) (mmol L^{-1})	Volume of 13% (w/w) skim milk added (ml)	Volume of zinc acetate stock solution added (ml)	Volume of distilled water added (ml)
0	38.115	0	11.885
10	38.170	2.500	9.330
15	38.200	3.750	8.050
20	38.225	5.000	6.775
22.5	38.240	5.625	6.135
25	38.255	6.250	5.495
30	38.280	7.500	4.220
35	38.310	8.750	2.940
40	38.335	10.000	1.665

Table 3.3: The volume of skim milk, zinc gluconate stock solutions (200 mmol L^{-1}) and distilled water added to make up 50 mL of zinc+skim milk with a final concentration of 10% total solids (w/w).

Concentration of added zinc gluconate in the skim milk (10 % w/w) (mmol L^{-1})	Volume of 13% (w/w) skim milk added (ml)	Volume of zinc acetate stock solution added (ml)	Volume of distilled water added (ml)
0	38.115	0	11.885
10	38.225	2.500	9.275
15	38.275	3.750	7.975
20	38.330	5.000	6.670
22.5	38.355	5.625	6.020
25	38.385	6.250	5.365
30	38.440	7.500	4.060
35	38.490	8.750	2.760
40	38.545	10.000	1.455

3.6. pH measurement and pH adjustment for skim milk samples

The zinc+skim milk samples prepared, according to Section 3.5, were stirred for 1 min with a magnetic stirrer and left at 20 ± 1°C for 10 min to equilibrate before measuring the pH. The pH of the zinc+skim milk samples was measured at 20 ± 1°C using a pH meter (PB-20, Sartorius, New Zealand) with a pH electrode (Fisher Scientific, New Zealand) after the zinc salt stock solution was added. The pH probe was calibrated using pH 4.00 and pH 7.00 buffer solutions (Lab serve-Biolab, Australia).

The pH of the skim milk with added zinc was restored to 6.73 ± 0.03 (native 10% skim milk pH) by using 2 M sodium hydroxide (NaOH) where required. The total solid was always maintained at 10% (w/w) for the zinc+skim milk saples regardless of the pH adjustement. Preliminary experiments were carried out to determine the volume of NaOH needed to restore the zinc+skim milk pH with 40 mmol L^{-1} zinc salt added. In the preliminary experiment, the method used to prepare the zinc+skim milk was a modified method to that described in Section 3.5. A block diagram is shown in Figure 3.1 to identify the order of each component added to prepare the pH adjusted skim milk sample with zinc salt added. To prepare 50 ml zinc+skim milk samples, firstly, a required amount of 13% reconstituted skim milk was added to the beaker, followed with 10ml 200 mmol L^{-1} zinc salt stock solution. Sodium hydroxide (2

mol L^{-1}) was carefully added as the next solution to adjusted the sample pH to 6.73 ± 0.03. After pH adjustment, a small amount of distilled water was added to adjust the final skim milk concentration to 10% total solid (w/w) while keep the pH at 6.73 ± 0.03. The detail of the amounts of each component added to make the 10% (w/w) zinc+skim milk with a pH of 6.73 ± 0.03 is shown in Appendix 4. The samples in which the pH was restored to 6.73 ± 0.03 was referred to as "adjusted pH" samples.

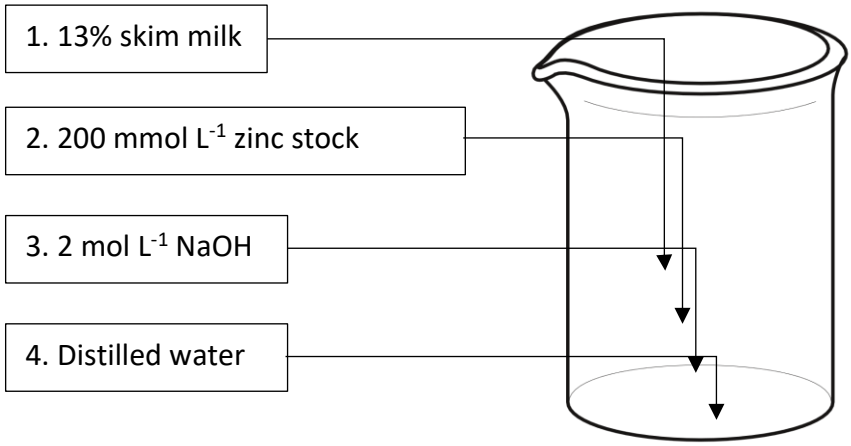


Figure 3.1: A diagram showing the order of each part of the solution added to prepare the pH adjusted skim milk samples with zinc salt added to and final total solid of 10% (w/w).

A second experiment was to adjust the native 10% skim milk pH to 5.34±0.02 (the 10% skim milk pH with 40 mmol L^{-1} zinc acetate added) by carefully adding 1M hydrochloric acid (HCl). The volume of each solution added to make the 10% (w/w) skim milk with different pH is shown in Appendix 4.

3.7. Milk serum and collodial phase separation

About 1.8 g of the 10% (w/w) zinc+skim milk sample was transferred into a 2.0 ml clear micro-centrifuge tube (Bio-Strategy, New Zealand) and centrifuged at 17,000 x *g* for 90 minutes at 20 ± 2°C in a high-speed micro-centrifuge (Heraeus™ Pico™ 17 Microcentrifuge, Fisher Scientific, New Zealand). The clear supernatant (serum phase) after centrifugation was pipetted off and transferred into a conical flask for zinc and calcium quantification analysis. The insoluble materials left in the microtubes were recorded as wet sediment and will be referred to as the colloidal phase. The wet sediment was placed into a drying oven

(Heratherm™ General Protocol Ovens, Fisher Scientific, New Zealand), at 65±2 °C. The weight of the sediment was measured every four hours until the weight became stable (± 0.0005 g). The dry sediement phase was collected for zinc quantification analysis by MPAES (Section 3.10).

3.8. Complexometric (EDTA titration) method for zinc quantification analysis

The zinc concentrations in skim milk and serum phase were determined using an ethylenediamine tetra-acetic acid (EDTA) titration method described by Novick (1997). The $Na_2EDTA{\cdot}2H_2O$ (analytical reagent, Sigma-Aldrich, USA) was dried in the oven for one hour to evaporate all the water and then cooled to the room temperature before use. The EDTA solution (0.01 mol L^{-1}) was prepared by dissolving 0.93 g of dried $Na_2EDTA{\cdot}2H_2O$ in a 250 ml volumetric flask with distilled water. The acetate buffer solution was prepared by weighing 5.40 g of glacial acetic acid (analytical reagent grade, ≥ 99.7%, Fisher Scientific, New Zealand) and 68.04 g of sodium acetate trihydrate (Reagent grade, ≥ 99.0%, Sigma Aldrich, New Zealand) and diluted to 1 L with distilled water. The acetate buffer solution pH was then adjusted to pH 5.5 using 1M NaOH (analytical reagent grade, Fisher Chemical, UK) or glacial acetic acid. Xylenol orange solution at 0.1% (w/v) was prepared by dissolving 0.1 g xylenol orange (≥ 96%, Laboratory reagents, BDH chemicals, England) in 100 ml of distilled water and used as the indicator for determining the endpoint of the titration.

A standard curve was prepared with a series of standard zinc acetate aqueous solutions ranging from 0.13 to 3.92 mg zinc in 1 g solution (Figure 3.2). The limit of detection of this method was found to be 0.03 (mg Zn / g skim milk). The calculation for the EDTA titration method limit of detection can be found in Appendix 5. About 1 - 3 ml of the sample (skim milk or serum milk sample) was transferred into a conical flask, and the weight of the sample was recorded. The sample was then diluted with 20 ml acetate buffer solution. The pH of the solution was determined by a pH meter. A blank solution was prepared by adding 1 ml distilled water instead of a skim milk sample. The titration result from the blank solution was subtracted from the titration of the samples. Five drops of Xylenol Orange solution (0.1% w/v) was added into each sample solution as an indicator for determining the endpoint of the titration method. The sample was then titrated with a 0.01 mol L^{-1} EDTA solution until the

initial pink colour changed to bright yellow. The mass of the zinc in each sample was determined from the standard curve:

For skim milk samples:

$$Concentration\ of\ zinc\ in\ skim\ milk\left(\frac{mg\ Zn}{g\ skim\ milk}\right) = \frac{mg\ zinc\ (from\ standard\ curve)}{Mass\ of\ skim\ milk\ taken}$$

Equation 3.1

For serum samples:

$$Concentration\ of\ zinc\ in\ serum\left(\frac{mg\ Zn}{g\ skim\ milk}\right) = \frac{mg\ zinc\ (from\ standard\ curve)}{Mass\ of\ serum\ taken} \times Mass\ of\ serum\ in\ 1\ g\ skim\ milk$$

Equation 3.2

The mass of serum in 1 g skim milk can be found in Appendix 6.

For sediment samples:

$$Concentration\ of\ zinc\ incolloidal\ phase\left(\frac{mg\ Zn}{g\ skim\ milk}\right) = concentration\ of\ Zn\ in\ skim\ milk - concentration\ of\ Zn\ in\ serum$$

Equation 3.3

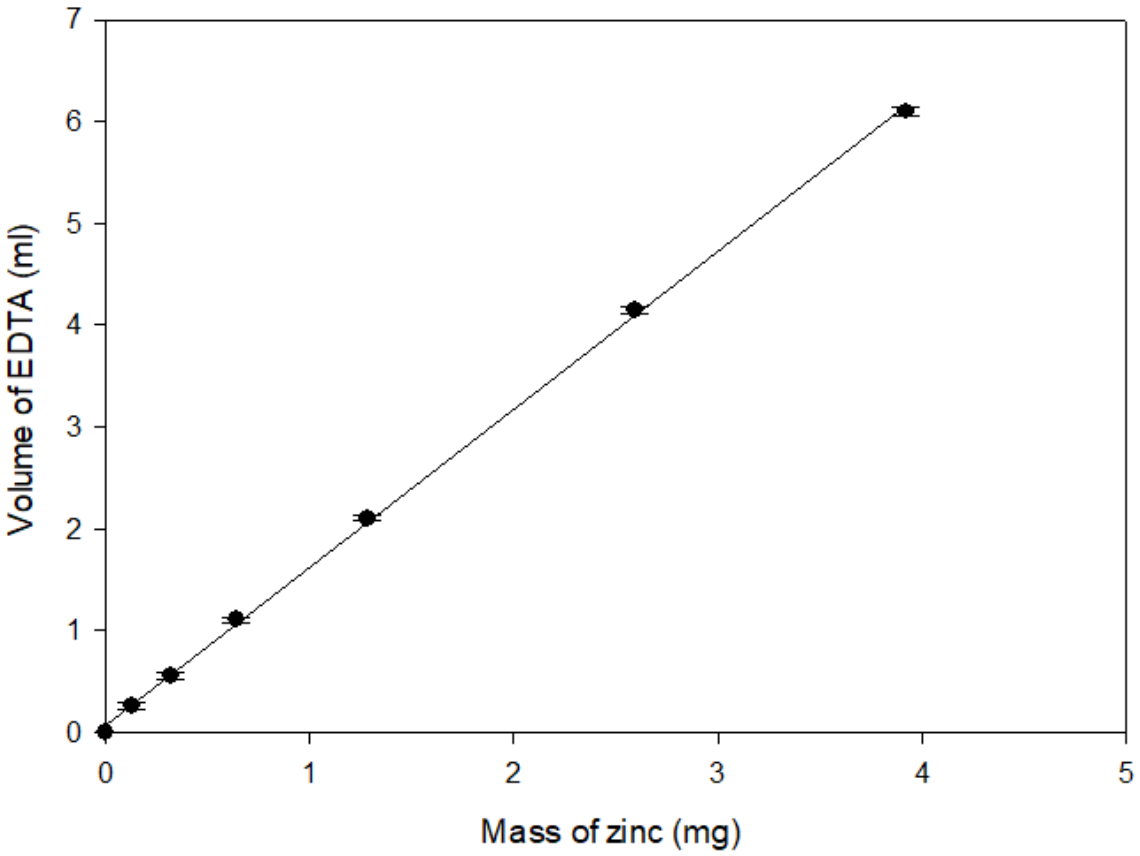


Figure 3.2: An example of an EDTA titration standard curve identifies the relationship between the volume of EDTA used for titration and mass of zinc in the standard zinc acetate aqueous solutions. The points presented are means ± standard deviation (n = 3).

3.9. Complexometric (EDTA titration) method for calcium quantification analysis

A method described by Lin *et al.* (2018) derived from Wendell and Patton (1959) was followed to determine the calcium quantification in the zinc+skim milk and serum phase. The calcium determination by EDTA titration method measures the total calcium content in the skim milk sample from both the serum and colloidal phase. The calcium concentration in the colloidal phase phase was calculated by subtracting the amount of calcium in the serum from the amount of calcium in the skim milk. The standard curve was prepared with a series of calcium chloride (≥ 99.0%, Sigma Aldrich, New Zealand) standard solutions ranging from 0 to 50 mmol L^{-1} (0 to 2.0 mg g^{-1}) (Figure 3.3). Patton-Reeder Indicator (0.5% w/v) was used as an indicator to determine the endpoint of the titration.

To start the analysis, one ml of the sample was transferred into the conical flask and diluted with 50 ml distilled water. Then 3 ml of the 8 mol L^{-1} NaOH were added into each sample to increase the solution pH to 13.40 ± 0.02. The samples were then left for 5 min to allow the magnesium present in the solution to precipitate as magnesium hydroxide (Pearce, 1977). A few drops of the 0.5% (w/v) Patton-Reeder indicator were added and mixed well. The samples were then titrated with 0.01 mol L^{-1} EDTA solution (the same EDTA solution prepared in Section 3.8) until the initial purple colour changed to blue. The mass of the calcium in each sample was determined from the standard curve:

For skim milk samples:

$$Concentration\ of\ calcium\ in\ skim\ milk\left(\frac{mg\ Ca}{g\ skim\ milk}\right) = \frac{mg\ Ca\ (from\ standard\ curve)}{Mass\ of\ skim\ milk\ taken}$$

Equation 3.4

For serum samples:

$$Concentration\ of\ calcium\ in\ serum\left(\frac{mg\ Ca}{1\ g\ skim\ milk}\right) = \frac{mg\ Ca\ (from\ standard\ curve)}{Mass\ of\ serum\ taken} \times Mass\ of\ serum\ in\ 1\ g\ skim\ milk$$

Equation 3.5

For sediment samples:

$$Concentration\ of\ calcium\ in\ colloidal\ phase\left(\frac{mg\ Ca}{1\ g\ skim\ milk}\right) = concentration\ of\ Ca\ in\ skim\ milk - concentration\ of\ Ca\ in\ serum$$

Equation 3.6

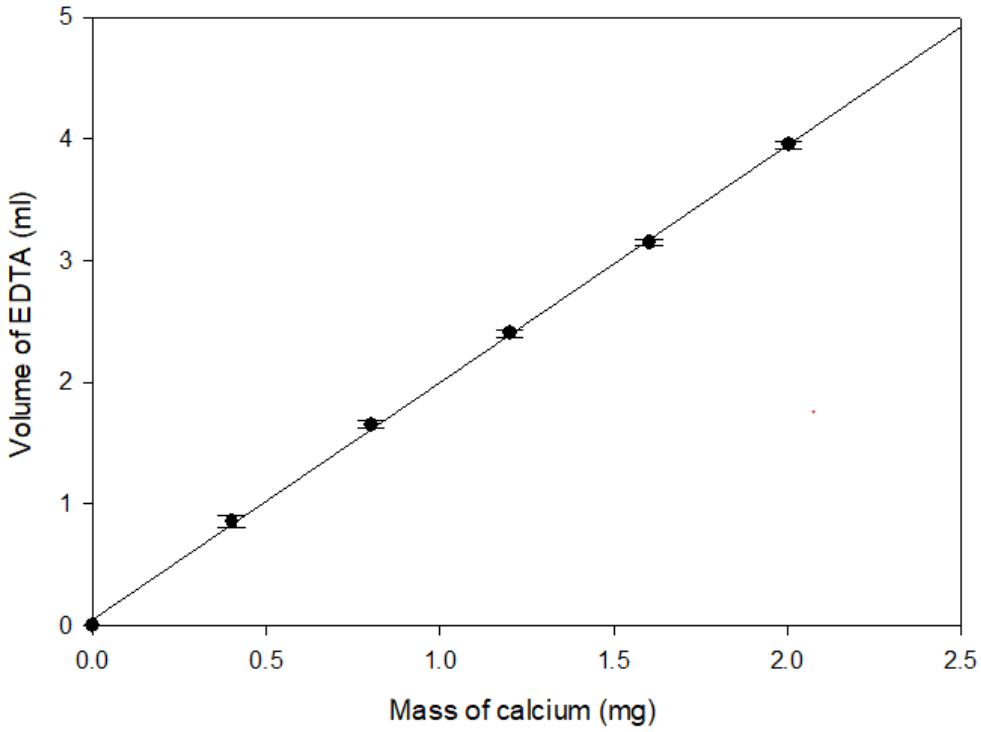


Figure 3.3: An example of an EDTA titration standard curve identifies the relationship between the volume of EDTA used for titration and mass of calcium in the standard calcium chloride aqueous solutions. The points presented are means ± standard deviation (n = 3).

3.10. Microwave Plasma Atomic Emission Spectroscopy (MPAES)

A modified method from Agilent Technologies (2016) was used for determining the zinc concentration in milk samples by MPAES.

3.10.1. Reagents and standard curve preparation

The 5% (w/w) and 6.5 % (w/w) HNO_3 was prepared by diluting the 68% (w/w) nitric acid (PrimarPlus-Trace analysis grade, Fisher Scientific, United Kingdom) with distilled water. The reference zinc standard solutions with low zinc concentration (0.08 to 0.2 mg Zn / L solution) and high zinc concentration (1 to 5 mg Zn / L solution) were prepared by diluting the zinc stock solution containing 1000 ± 2 mg/L of Zn (Zinc Standard for ICP, Sigma Aldrich, New Zealand) with 5% (w/w) nitric acid and 6.5% (w/w) nitric acid, respectively. The standard curves prepared with low and high zinc concentration were shown in Figures 3.4 and 3.5. The calculation of limit of detection of MPAES method can be found in Appendix 7 and was found to be 0.03 (mg Zn / g skim milk).

3.10.2. Sample digestion

About 0.96 g liquid samples (skim milk and serum samples) or 0.20 g solid samples (dry sediment or standard skim milk powder (ERM-BD150, European Commission, Joint Research Centre, Consumers and Reference Materials, Belgium) were transferred into the digestion tube, and the weight was recorded. The digestion tubes were then transferred into the digestion vessels in the digestion rotor (Rotor 16MF100, Anton Paar, New Zealand) and 2 ml Milli Q water was added to the liquid samples and 3 ml Milli Q water was added to the solid samples. Then 4 ml of 68% (w/w) nitric acid was added into each tube. The digestion vessel caps were then closed, and the digestion rotor was then transferred into the microwave reaction system (Multiwave 5000, Anton Paar, New Zealand). To perform the digestion, the samples were heated from ambient temperature up to 190°C in 15 minutes and then held at 190°C for 20 mins, finally cooled down to 65°C within 29 minutes. A temperature and pressure sensor was placed in the digestion vessels to measure the vessel's temperature and pressure. The maximum temperature and pressure inside the vessels were set to 210°C and 35 bar (absolute pressure). After the digestion process finished, the digestion rotor was removed from the microwave reaction system and placed in the fume hood. Nitrogen dioxide gases were carefully released from each digestion tube, and then the vessel caps were removed.

3.10.3. Procedure for zinc concentration determination

The contents of each digestion tube were transferred into a 30 ml beaker, and each of the tube and cap was rinsed twice with MilliQ water (Milli-Q® Reference Water Purification System, Merck, Germany). The digests were then transferred into 25 ml (for the samples without addition of zinc salt) or 50 ml volumetric flasks (for all the samples with added zinc salt) and diluted to volume with MilliQ water. MPAES can only work at a certain acid range. Thus, a titration was carried out to determine the acid concentration in the solution. Carefully, 1 ml digests were transferred into a 250 ml conical flask and diluted to 50 ml with MilliQ water then a few drops of Bromothymol Blue (0.5% w/v) was added as an indicator to determine the endpoint of the titration. The solution was then titrated by 0.1961 M sodium hydroxide (NaOH) until the colour changed from yellow to blue. The nitric acid concentration can be calculated by:

$$Concentration\ of\ nitric\ acid\ \left(\frac{mol}{L}\right) = \frac{C_{NaOH}\ \times V_{NaOH}}{V_{sample}}$$

Equation 3.7

Where:
C_{NaOH}: Concentration of NaOH used in the titration (0.1961 M)
V_{NaOH}: Volume of NaOH used to titrate the sample (L)
V_{sample}: Volume of sample transferred into the conical flask (L)

The acid concentration in the digests diluted to 25 ml was found to be 6.5% w/w (as Nitric acid), and the digests diluted to 50 ml were found to be 5% w/w (as Nitric acid). Both of the acid concentrations were within the detection range for MPAES. MPAES could only accurately detect the sample zinc concentration in the range of 1 - 10 mg Zn/L solution. Therefore, further dilution was necessary for the samples which expected zinc concentration above the maximum detection limit. The digested solutions were diluted with 6.5% or 5% HNO_3 by the dilution factors listed in Table 3.4 to meet the zinc determination range for MPAES. MPAES then accomplished zinc quantification determination at a wavelength of 213.857 nm. The zinc concentration of the sample was then determined from the standard curves:

$$Concentration\ of\ zinc\ \left(\frac{mg\ Zn}{g\ sample}\right) = \frac{\frac{mg\ zinc\ /\ L\ (from\ standard\ curve) \times V_2}{1000\ V_t} \times V_1}{Mass\ of\ sample\ digested}$$

Equation 3.8

Where:
V_1: Digest volume after first dilution (ml)
V_2: Further diluted volume (ml)
V_t: Volume took for further dilution (ml)

The MPAES method was validated by comparing the detected zinc concentration of the standard skim milk powder (ERM®-BD150, European Reference Materials, Belgium) with the product certificate of analysis. The zinc concentration for standard skim milk powder was determined to be 44.5 ± 0.3 mg Zn/kg skim milk powder while the zinc concentration from the ERM® certificate of analysis (Appendix 8) was 44.8 mg Zn/kg skim milk powder (European Commission, Joint Research Centre, Consumers and Reference Materials, Belgium). Thus, the zinc concentration in the standard skim milk powder (ERM®-BD150) measured by MPAES method agreed with the concentration reported on the product specification.

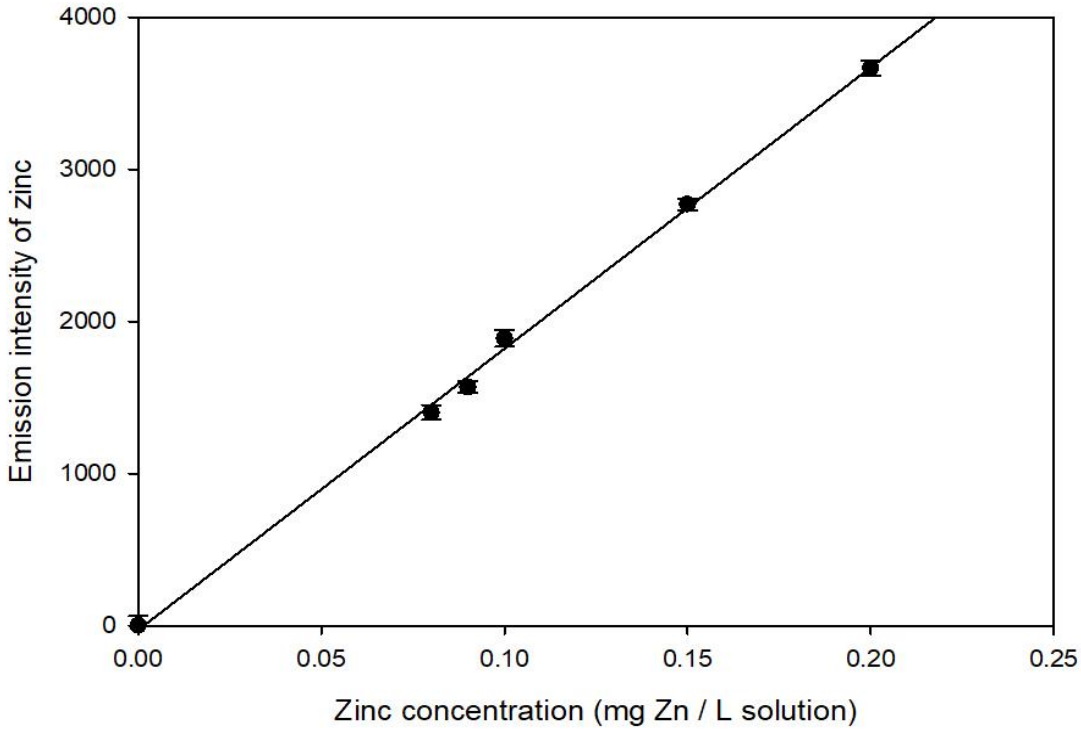


Figure 3.4: A standard curve shows the relationship between the microwave plasma quantification and zinc concentration in the solution for the low zinc concentration range (0.08 - 0.2 mg Zn /L solution). The points presented are means ± standard deviation (n = 10).

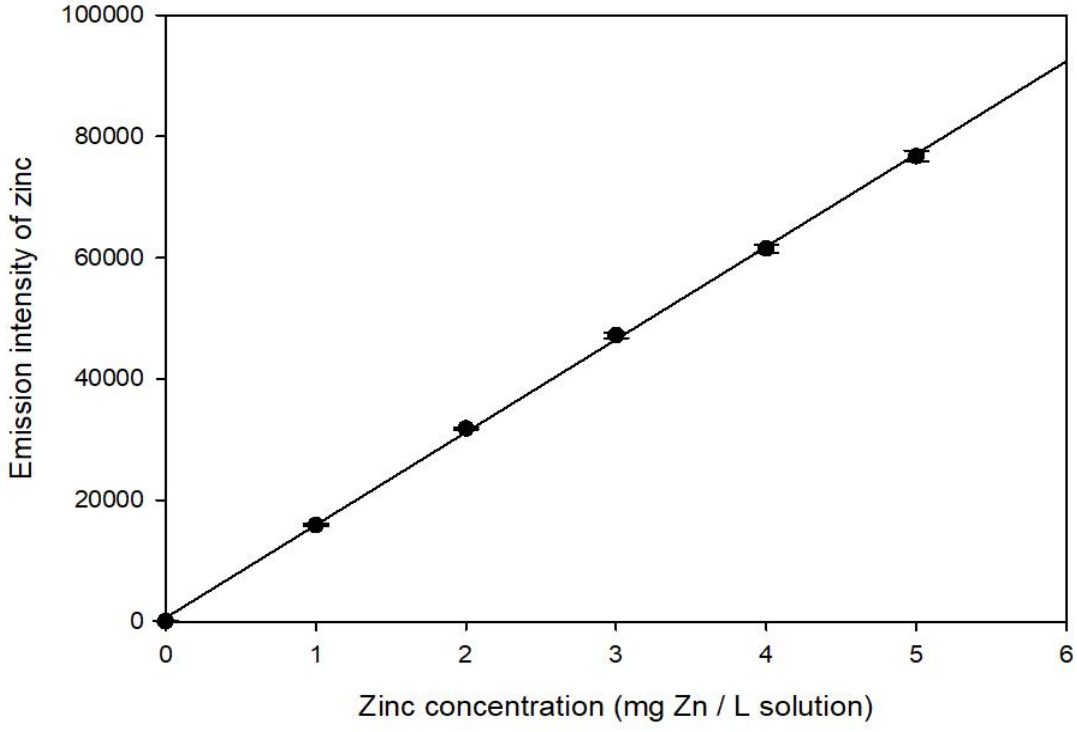


Figure 3.5: A standard curve shows the relationship between the microwave plasma quantification and zinc concentration in the solution for the high zinc concentration range (1 - 5 mg Zn /L solution). The points presented are means ± standard deviation (n = 10).

Table 3.4: The dilution factors for each sample measured by MPAES.

Sample	Digest volume after first dilution (ml), V_1	Volume took for further dilution (ml) V_t	Further diluted volume (ml) V_2	Concentration of HNO_3 used for dilution
Skim milk (0mM zinc acetate)	25	-	-	-
Skim milk (15mM zinc acetate)	50	2	25	5%
Skim milk (25mM zinc acetate)	50	2	25	5%
Skim milk (40mM zinc acetate)	50	2	25	5%
Serum (0mM zinc acetate)	25	-	-	-
Serum (15mM zinc acetate)	50	5	25	5%
Serum (25mM zinc acetate)	50	5	25	5%
Serum (40mM zinc acetate)	50	5	25	5%
Sediment (0mM zinc acetate)	25	3	25	6.5%
Sediment (15mM zinc acetate)	50	1	25	5%
Sediment (25mM zinc acetate)	50	0.8	25	5%
Sediment (40mM zinc acetate)	50	0.4	25	5%
Standard skim milk powder (ERM®-BD150)	25	5	10	6.5%

Sediment = colloidal phase

3.11. Visual assessment of zinc+skim milk samples

Skim milk (10% w/w) with added zinc salts (zinc acetate, zinc gluconate and zinc sulphate) at concentrations ranging from 0 to 40 mmol L^{-1} was prepared in 25 ml test tubes and mixed for 1 minute by vortex mixer (ZX3 Advanced Vortex Mixer, VELP Scientifica, USA). The tubes were then placed in the hot water bath (GD100, Grant Instruments Ltd, Cambridge, England) set at different temperatures (20°C, 30°C, 40°C, 60°C, 70°C and 80 ± 1°C) for 60 minutes. After holding at the different required temperatures, the tubes were cooled to 20 ± 1°C within 5 mins in a 4 ± 1°C water bath. Finally, the zinc+skim milk samples were visually assessed.

3.12. Rheological measurements by rheometer

Rheological measurements of skim milk samples were carried out by using a rheometer (Discovery HR-3, TA Instruments, USA) and TRIOS software (TA Instruments, Version # 4.3.1., USA) was used to review data. The analysis was performed using a stainless steel (SS) single

gap cylinder cup (15 mm radius) and a vane geometry (28 mm diameter, 42 mm length). The skim milk samples after zinc salt added and adjusted to 10% total solids were mixed for 2 minutes with a magnetic stirrer and left to equilibrate for another 3 minutes at room temperature (20 ± 1^{o}C), then the samples were transferred into the rheometer cup, a total 5 minutes delay was required from the addition of the zinc salts to commencement of the rheological measurements of the zinc+skim milk samples. A thin layer of Canola oil (Countdown Home brand, New Zealand) was carefully applied on the surface of the sample, and an evaporation trap was used to minimise sample evaporation during measurement.

3.12.1. Fluid behaviour and viscosity analysis

Shear stress was measured over shear rate 0.1 s^{-1} to 50 s^{-1} at 20°C in 30 min to determine the skim milk fluid flow behaviour. The change in apparent viscosity with time was determined for the skim milk with zinc salt added at a constant shear rate of 25 s^{-1} and were held at 20 °C for 3 hours. Oscillatory measurements were performed for the zinc+skim milk samples with high zinc salt concentration (viscoelastic properties) to determine the change in storage modulus (*G'*).

3.12.2. Gelation properties measurement

To investigate the effect of different conditions on zinc+skim milk gelation, different methods were used for different purposes.

To determine the effect of zinc concentration and different zinc salts on zinc+skim milk gelation, non-preheated skim milk with different zinc salts added (20 - 40 mmol L^{-1}) was used. A time sweep was carried first at 20°C for 3 hours at a constant strain of 0.05% and a frequency of 0.1 Hz. For the samples which formed gel (*G'* > 1 Pa after the 3 hours), a frequency sweep was performed at 20°C by increasing the frequency from 0.01 to 5 Hz at a constant strain of 0.05%.

To determine the effect heat treatment on zinc+skim milk gelation, both preheated and non-preheated skim milk with 5 to 20 mmol L^{-1} zinc acetate added was used. A temperature sweep

from 20°C to the required temperature (40°C, 60°C and 80°C) was carried first, the temperature increasing rate was set to be 5°C/min. After the temperature reached the required temperature (40°C, 60°C and 80°C), a time sweep at 80°C for 60 mins was done. A cooling temperature sweep from required temperature (40°C, 60°C and 80°C) to 20°C was performed after holding at a temperature decreasing rate of 5°C/min. All the three steps were carried at a constant strain of 0.05% and a frequency of 0.1 Hz. Finally, a frequency sweep was performed at 20°C by increasing the frequency from 0.01 to 10 Hz at a constant strain of 0.05%.

3.13. Statistical analysis

All experiments in this study were carried out in at least triplicate. Standard deviation and pooled standard deviation were used to describe the variability between repeated experiments. One-way ANOVA (Tukey Pairwise Comparisons test) and t-test in the Minitab 19 Statistical software (Minitab Inc, Pennsylvania, USA) was used as the statistical analysis for determining the significant difference between the result means.

Chapter 4. Effect of zinc acetate concentration on skim milk

4.1. Introduction

The salts in the bovine milk are in dynamic equilibria between the serum and micellar phase and contribute to improving the nutritional value of bovine milk. The equilibria of salts between serum and micellar phase can be affected by a number of factors including temperature, pH, the concentration of mineral salts and ionic strength (Fox *et al*., 1998; Mekmene *et al*., 2009; Udabage *et al*., 2000). Addition of minerals can further improve the bovine milk nutritional value (Hilliam, 1998); however, the addition of mineral salts can influence the bovine milk salt equilibria and lead to physicochemical changes in milk properties. Many studies have reported the addition of cations like Ca^{2+}, Mg^{2+} and Zn^{2+} significantly influence the mineral-protein interactions and result in the change of bovine milk pH, mineral distribution, viscosity and other properties (Begum, 2019; Lin, 2019; Phillippe *et al*., 2005; Singh *et al*., 1989a, 1989b; Tsioulpas *et al*., 2007). Bovine milk is an excellent source of zinc for humans, where one serve of milk can provide approximately 9% of the recommended daily dose of zinc for adults (Gibson, 2012; Wong *et al.*, 1988). There are several studies that reported that most of the native zinc is present in the sediment (colloidal phase) in bovine milk (Blakeborough *et al.*, 1983; Johnson & Evans, 1978; Parkash & Jenness, 1967; Singh *et al.*, 1988) and any added zinc ions bind with casein micelles until this high molecular weight fraction becomes saturated with zinc (Blakeborough *et al.*, 1983; Harzer & Kauer, 1982; Philippe *et al.*, 2005; Singh *et al.*, 1989a, 1989b; Sugiarto, 2004). However, the effect of the addition of zinc to bovine milk and its effect on the rheological properties of a skim milk system has not been studied in detail. This study was focused on the physicochemical and rheological properties of non-preheated skim milk with added zinc acetate.

4.2. Experimental Design

A summary of the experimental plan to investigate the effect of added zinc acetate concentration, pH and heat treatment on zinc+skim milk rheological properties is shown in Figure 4.1. Three sets of experiments were carried out. Firstly, the effect of zinc acetate concentration on skim milk pH, zinc and calcium distribution and rheological properties was assessed, with no preheat or pH adjustment after addition of zinc acetate. The samples were prepared by using non-preheated skim milk with the addition of 200 mmol L^{-1} zinc acetate to form skim milk with different final added zinc acetate concentrations. Secondly, the effect of pH on zinc+skim milk rheological properties was assessed for the samples without preheat treatment and adjusted to have the same added zinc acetate concentration as the first set of experiment. The pH of zinc+skim milk was adjusted back to pH 6.73 ± 0.03 which was the native pH for 10% (w/w) skim milk determined in this study. Then the 10% (w/w) skim milk pH was adjusted to 5.34 ± 0.02 with no added zinc salt; this was the pH the zinc+skim milk with 40 mmol L^{-1} zinc acetate added to it decreased to. This experiment aimed to determine the effect of pH on skim milk rheological properties without addition of zinc acetate. Thirdly, the effect of heat treatment was investigated. Heat treatment was applied to the skim milk in different experiments in this chapter as outlined in Figure 4.1. To investigate the effect of preheating and holding temperature on zinc+skim milk visually, the preheated and non-preheated zinc+skim milk was held at different temperatures (20, 30, 40, 60, 70 and 80°C ± 1°C) for 60 minutes before observations were carried out. The detail of the methodology used in this chapter can be found in Chapter 3.

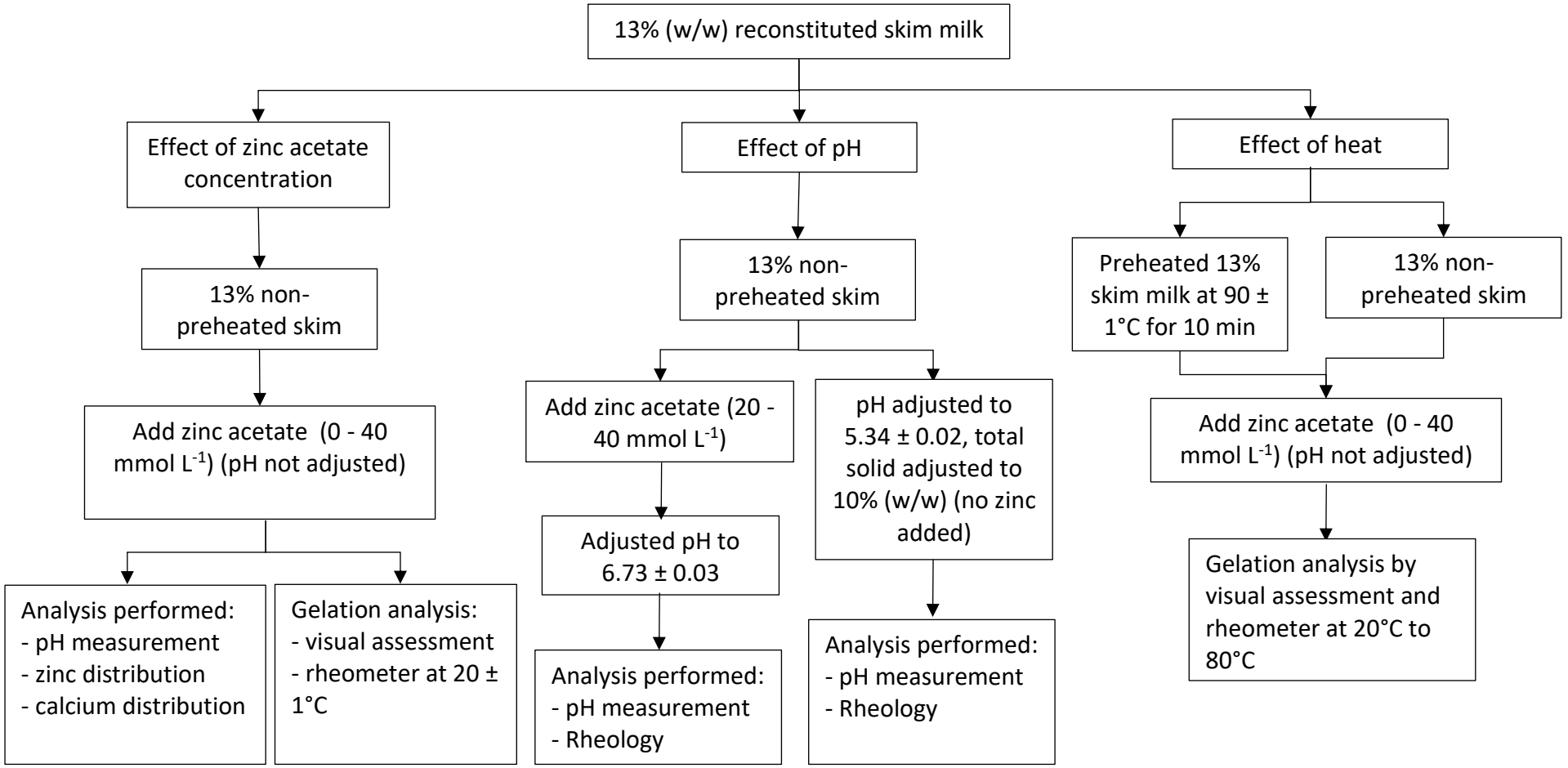


Figure 4.1: Summary of the experimental plan to investigate the effect of different zinc salts and pH.

4.2.1. Zinc+skim milk sample preparation

Both preheated and non-preheated reconstituted skim milk was used in the study for this chapter, the non-preheated and preheated skim milk was prepared according to Section 3.2 and 3.4. Zinc salt stock solutions were prepared according to Section 3.3 with the zinc concentration of 200 mmol L^{-1}. Different amounts of zinc salt stock solutions were added to the 13% (w/w) reconstituted skim milk to prepare 10% (w/w) skim milk (both preheated and non-preheated) with different added zinc concentrations (0 to 40 mmol L^{-1}). The detail of the zinc+skim milk preparation can be found in Section 3.5.

4.2.2. Analysis of zinc+skim milk samples

This chapter reports on the effect of adding different zinc salts on the physicochemical and rheological properties of skim milk. The method of analyses used in this chapter can be found in Section 3.6 to 3.13. The effect of the concentration of added zinc acetate on skim milk textural properties were investigated at 20 ± 1°C both visually and in a rheometer. The rheometer was used to further study the zinc+skim milk texture properties with heat treatment. A heating temperature sweep from 20°C to 80°C was carried first, followed with a time sweep at 80°C then treated with another cooling temperature sweep from 80°C to 20°C. A frequency sweep was carried ou as the last test for the samples which formed a gel ($G' > 1$ Pa). Details of the visual assessment and rheology analyses was described in Section 3.11 and 3.12.

4.3. Effect of zinc acetate concentration

4.3.1 Visual assessment of zinc+skim milk samples

This study was focused on the non-preheated skim milk gelation after the addition of zinc acetate at room temperature (20 ± 1°C). Depending on the added zinc acetate concentration, the non-preheated zinc+skim milk solution either stayed liquid or thickened after holding, with no further heat treatment (Figure 4.2). After holding, the skim milk samples with the addition of zinc acetate at 20 ± 1°C for 60 minutes, the test tubes were inverted (Figure 4.2b)

to check the sample states. From the visual observation, when 20 mmol L^{-1} zinc acetate (equivalent to 1.10 mg zinc per g of skim milk) was added, thickening of skim milk was visible (Figure 4.2b). At higher concentrations of added zinc acetate a visible amount of milk adhered to the top of the inverted test tubes. Therefore, the skim milk was thickening with increasing concentrations of zinc acetate. Visually serum separation was observed in skim milk with added zinc acetate concentrations ≥ 30 mmol L^{-1} which may indicate a gel structure formed in this sample (Figure 4.2a). However, the gels were very weak as no gel adhered to the top of the inverted test tubes (Figure 4.2b). Serum separation refers to the appearance of whey liquid on the surface of milk gel (Vasbinder *et al.*, 2003; Yuliarti *et al.*, 2019). Serum separation often occurs if milk protein structural rearrangement occurs.

For acid gels, high fermentation temperature (43 - 44°C) or rapid acidification of milk (high culture concentration) are two important reasons for whey separation. The decrease in electrostatic repulsion between casein micelles and the resulting interaction between casein molecules can lead to serum separation, one situation when this can occur is when the milk pH drops to 4.6 or below (Lucey *et al.*, 1999). From previous studies by Begum (2019) and Lin *et al.* (2018), magnesium and calcium added to skim milk did not form thickened milk or form a gel without heat treatment. Whereas the addition of zinc, a divalent ion, has resulted in skim milk thickening and gel formation without heat treatment.

a) b)

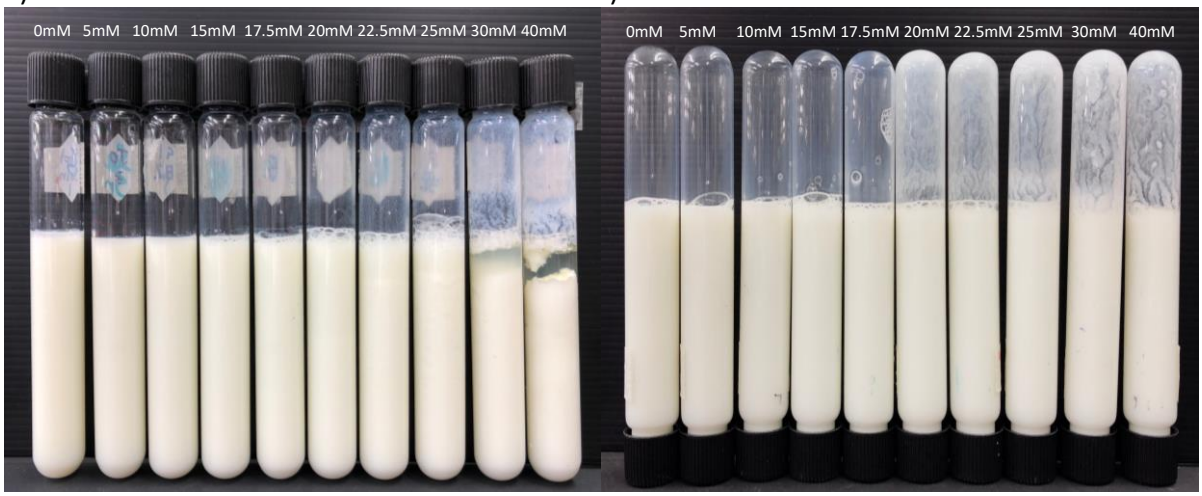


Figure 4.2: Visual observations of skim milk with added zinc acetate at different concentrations (0 - 40 mmol L^{-1}). (a) test tubes after 60 minutes, (b) test tubes after 60 minutes inverted. This experiment was repeated three times, and the same results were observed each time.

4.3.2. Skim milk pH after zinc acetate addition

The pH of non-preheated skim milk decreased significantly (p<0.05) as the concentration of added zinc acetate was increased from 0 to 40 mmol L^{-1} (Table 4.1). The change in milk pH with added zinc salts (zinc sulphate and zinc acetate) has been reported by Rana *et al.* (2018), they reported the milk pH decreased but not significantly (p>0.05) from 6.79 to 6.73 and from 6.68 to 6.63 for the milk with 0 and 22.5 ppm Zn (0 - 0.34 mmol Zn / L sample) zinc acetate and zinc sulphate added, respectively. The reported insignificant change of milk pH by Rana *et al*. (2018) may be due to a low concentration of added zinc salts. The decrease in milk pH with increasing soluble calcium and magnesium salts has been reported previously by Begum (2019), Lin *et al. (*2018) and Ramasubramanian *et al. (*2014). As was found with the addition of calcium and magnesium salt to skim milk, the addition of zinc acetate may also affect the equilibrium of the ionic species in milk. The addition of zinc ions can lead to a decrease in serum phosphate ions (PO_4^{3-}) and citrate ions ($Citrate^{3-}$) (Philippe *et al.*, 2005). The zinc ions released from added zinc acetate may disrupt the ionic equilibrium in the milk system and result in pH decreasing. The added zinc ions may associate with the phosphate ions (HPO_4^{3-}) and citrate ions ($Citrate^{3-}$) in the serum and formed new salts, zinc-citrate and zinc-phosphate (Equation 4-1 and 4-2). The newly formed zinc salts are very slightly soluble in water (Goodwin, 2000; Wegmüller *et al.,* 2014) which decreases the concentration of citrate ions and phosphate ions in the serum. A decrease in citrate and phosphate ions in the milk serum phase after zinc chloride was added was reported by Philippe *et al.*, (2005). Taking phosphate ions as an example, the reduction of phosphate ions can lead to the conversion of $H_2PO_4^-$ to HPO_4^{2-} to restore the equilibrium (Equation 4-3), the concomitant release of H^+ ions could lead to a decrease in the zinc+skim milk pH (Table 4.1) (Begum, 2019; Lin *et al.*, 2018; Philippe *et al.*, 2005).

Table 4.1: The effect of zinc acetate concentration on skim milk pH. Different superscript letters (a, b, c, d, e, f, g, h, I, j) indicate significant difference across the different concentrations of added zinc acetate (95% confidence level). Results presented are means ± standard deviation (n = 3 to 6).

Concentration of added salt concentration (mmol L^{-1})	Zinc acetate[1]	Calcium chloride[2] (Lin *et al.*, 2018)	Magnesium chloride[1] (Begum, 2019)
0	6.73±0.01[a]	6.60	6.70
5	6.31±0.01[b]	6.43	6.62
10	5.93±0.01[c]	6.30	6.50
15	5.69±0.01[d]	6.22	6.41
17.5	5.61±0.02[e]		
20	5.53±0.01[f]	6.18	6.35
22.5	5.47±0.01[g]		
25	5.43±0.01[h]		
30	5.39±0.01[i]	6.02	6.28
35	5.36±0.01[j]		
40	5.34±0.01[j]	5.95	6.22

[1]: Non-preheated skim milk with a final total solids of 10% (w/w).
[2]: Preheated skim milk with a final total solids of 9.6% (w/w).

$$Zn^{2+} + HPO_4^{2-} \rightleftharpoons ZnHPO_4 \quad \text{Equation (4-1)}$$

$$3Zn^{2+} + 2Citrate^{3-} \rightleftharpoons Zn_3(Citrate)_2 \quad \text{Equation (4-2)}$$

$$H_2PO_4^- \rightleftharpoons HPO_4^{2-} + H^+ \quad \text{Equation (4-3)}$$

A comparison of the effect of calcium chloride (Lin *et al.*, 2018), magnesium chloride (Begum, 2019) and zinc acetate addition on milk pH is also shown in Table 4.1. The skim milk pH decreased to a greater extent when zinc acetate was added compared to the addition of calcium chloride and magnesium chloride (Table 4.1). The skim milk pH after the addition of a divalent cation from lowest to highest at the same added cation salt concentration was found to be Zn^{2+}<Ca^{2+}<Mg^{2+}.

This effect can be explained by comparing the pKa values of the three metal ions where zinc ion has the smallest pKa value; as the pKa of zinc, magnesium and calcium are 8.96, 11.4 and

12.6, respectively (James, 2017). The smaller the pKa value, the more hydrogen ions are released from the metal ion-water complexes (Blackman *et al*., 2012). By looking into the properties of the three cations, Zn^{2+}, Mg^{2+} and Ca^{2+}, the metal ions can act as a Lewis acid and interact with water (a Lewis base), this results in a hydrated metal ion surrounded by water molecules. Zinc ions in water typically have associated with them, six water molecules which form a hydration shell (Brown, 2018). Based on the Brønstede-Lowry theory, the hydration shell around all three metal ions can lose hydrogen ions to then result in a hydrated metal with exposed hydroxide sites (Krężel & Maret, 2016). The magnitude of this effect depends on the charge and radius of the metal ion (House & House, 2015). All three cations (Zn^{2+}, Mg^{2+}, Ca^{2+}) have the same charge, but zinc has the smallest radius. The Van der Waals radii of zinc, magnesium and calcium are 137 pm, 160 pm and 197 pm, respectively. Therefore, zinc has the largest charge-to-radius ratio (Housecroft & Sharpe, 2008; James, 2017). This results in a stronger bond between zinc and the oxygen of the water molecules, therefore, weakening the oxygen to hydrogen bonds resulting in the ease of hydrogen ion release compared to calcium and magnesium (Krężel & Maret, 2016). This results in a greater pH drop when zinc salts are added to an aqueous solution compared to magnesium and calcium (Table 4.1).

4.3.3. Zinc and calcium distribution in skim milk

4.3.3.1. Zinc distribution

The zinc concentration in the skim milk and serum were determined by EDTA titration and MPAES, while zinc concentration in the collodial phase was determined by difference or by MPAES determination. The MPAES method was also used to validate the EDTA results. Table 4.2 shows the zinc distribution in skim milk when various concentrations of zinc acetate were added. By comparing the zinc concentration results determined by EDTA and MPAES methods, there was no significant difference (p>0.05) for the skim milk and serum samples regardless of the added zinc acetate concentration. However, one significant difference (p<0.05) was observed for the sediment sample with 25 mmol L^{-1} zinc acetate added (Table 4.2). The colloidal phase zinc concentration results from the EDTA method was calculated by the difference between skim milk and serum zinc concentration. Thus, the uncertainty of colloidal phase zinc concentration from the EDTA method was influenced by both skim milk and serum

zinc concentration. In conclusion, these findings indicated the zinc concentration results determined by EDTA titration method agreed with the MPAES results. Thus, the EDTA titration method was a good method for zinc concentration determination in milk samples.

From the MPAES method (Table 4.2), there was 0.0040 ± 0.0006 mg g^{-1} zinc in the native 10% (w/w) reconstituted skim milk which agreed with the zinc concentration in bovine milk reported by Jensen (1995) of 3 - 6 mg L^{-1} zinc in the mature milk. In the native 10% (w/w) reconstituted skim milk, most of the zinc 0.0038 ± 0.006 mg/g was found in the colloidal phase which equates to 95% of the total zinc present. Cousins and Smith (1980) reported that no zinc is associated with the non-protein compounds of low molecular fractions (<10kDa) in native bovine milk, and several articles reported most of the zinc (88% to 95%) associates with the casein micelle (Blakeborough *et al.*, 1983; Parkash & Jenness, 1967; Singh *et al.*, 1989a, 1989b).

Table 4.2: Zinc concentration in skim milk, serum and colloidal phase (calculated) at different concentrations of added zinc acetate determined by EDTA titration and MPAES methods (n=3 to 10). Different superscript letters (a, b, c, d, e, f, g, h, I, j) indicate significant difference across the different concentrations of added zinc acetate. Different superscript Greek letters (χ, γ) indicate significant difference across EDTA and MPAES determination method (95% confidence level).

Concentration of added zinc acetate (mmol Zn/L skim milk)	Mass of zinc added (mg/g skim milk)	Determination by EDTA titration method[1]				Determination by MPAES method[2]		
		Concentration of zinc (mg zinc/1 g skim milk)			% of zinc in serum	Concentration of zinc (mg zinc/1 g skim milk)		
		Skim milk	Serum	Colloidal phase[3]		Skim milk	Serum	Colloidal phase[4]
0	0	ND	ND	ND		0.0040 ± 0.0006^{a}	0.0009 ± 0.0006^{a}	0.0038 ± 0.0006^{a}
5	0.32	0.33^{a}	0.05^{a}	0.28^{a}	15%			
10	0.63	0.65^{b}	0.14^{b}	0.51^{b}	21%			
15	0.95	$0.96^{c,\chi}$	$0.26^{c,\chi}$	$0.70^{c,\chi}$	27%	$0.95\pm0.01^{b,\chi}$	$0.26\pm0.01^{b,\chi}$	$0.70\pm0.01^{b,\chi}$
17.5	1.10	1.08^{d}	0.31^{d}	0.78^{d}	28%			
20	1.26	1.28^{e}	0.38^{e}	0.90^{e}	30%			
22.5	1.42	1.39^{f}	0.42^{f}	0.97^{f}	30%			
25	1.58	$1.56^{g,\chi}$	$0.50^{g,\chi}$	$1.06^{g,\chi}$	32%	$1.57\pm0.01^{c,\chi}$	$0.53\pm0.01^{c,\chi}$	$1.10\pm0.01^{c,\gamma}$
30	1.89	1.88^{h}	0.68^{h}	1.19^{h}	36%			
35	2.21	2.19^{i}	0.87^{i}	1.32^{i}	40%			
40	2.52	$2.52^{j,\chi}$	$1.07^{j,\chi}$	$1.44^{j,\chi}$	43%	$2.49\pm0.01^{d,\chi}$	$1.05\pm0.01^{d,\chi}$	$1.46\pm0.01^{d,\chi}$

[1]: Results presented were mean (n=3), pooled standard deviation: ± 0.02, the limit of detection was 0.03 mg/1 g skim milk.

[2]: Results presented were mean ± standard deviation, the limit of detection was 0.0006 mg/1 g skim milk (n=10).

[3]: Colloidal phase zinc mass by EDTA titration method was calculated the difference in zinc mass in skim milk and in serum.

[4]: Colloidal phase zinc mass by MPAES method was detected by assay after ashing.

ND: not determined as below the minimum level of detection for this assay.

The addition of zinc acetate (0 to 40 mmol L^{-1}) resulted in a significant (p<0.05) increase in the colloidal phase zinc concentrations from very low levels 0.0038 to 1.46 (mg Zn)/(g skim milk) for the non-preheated skim milk samples (Table 4.2). It has been reported previously that the colloidal phase zinc concentration increased with increasing added zinc salt concentration (mainly zinc chloride and zinc sulphate) (Cousins & Smith, 1980; Harzer & Kauer, 1982; Parkash & Jenness, 1967; Philippe *et al.*, 2005; Singh *et al.*, 1989a, 1989b). Studies have suggested that there are two binding sites for zinc in the casein micelle, one is the negatively charged phosphoserine residues and another is the CCP (Harzer & Kauer, 1982; Parkash & Jenness, 1967; Singh *et al.*, 1989a, 1989b). Singh *et al.* (1989a) reported that the zinc and calcium binding capacity to casein micelles was largely reduced with the dephosphorylation of casein, and concluded that the phosphoserine residues are the primary binding sites for zinc, as they are for calcium. Singh *et al.* (1989b) reported that zinc and calcium could also bind to other negatively charged groups like histidine and carboxylic groups as the zinc binding capacity of dephosphorylated casein is not zero. When zinc acetate was added, the zinc ions may have associated with negatively charged groups or bind to CCP in the casein micelles which form part of the colloidal phase after centrifugation. Thus, the zinc concentration in the colloidal phase increased after zinc acetate was added (Table 4.2).

As discussed in Section 4.3.2, the zinc ions released from added zinc acetate may associate with serum phosphate and citrate ions which have low solubility. According to Gaucheron (2005), the calcium ions released from added calcium salt can associate with citrate and phosphate ions to form calcium-citrate and calcium-phosphate in the serum phase, which leads to the movement of the newly formed calcium salt into the casein micelles. It was postulated that as the addition of calcium chloride and zinc chloride both can lead to phosphate concentration decrease in the serum phase (Philippe *et al.*, 2005), a similar equilibrium is assumed to occur with zinc. The newly formed zinc-citrate or zinc-phosphate in the serum phase may shift to the colloidal phase due to the low solubility and then contributed to the colloidal phase zinc concentration.

The addition of zinc acetate (0 to 40 mmol L^{-1}) not only resulted in colloidal phase zinc concentration increasing, but also a significant increasie (p<0.05) in serum zinc concentration (Table 4.2). It was noted that when 5 mmol L^{-1} zinc acetate was added, the majority of the

zinc ended up in the colloidal phase. When further zinc acetate was added to skim milk, with each further addition of 5 mmol L^{-1} zinc acetate the amount of zinc ending up in the colloidal phase was never as much as when the first 5mmol L^{-1} was added. When 40 mmol L^{-1} zinc acetate was added, the colloidal phase zinc concentration compared to the sample with 35 mmol L^{-1} zinc acetate added only resulted in 0.12 mg increase in zinc in the colloidal phase. As more zinc acetate was added it reached a point when the sediment (colloidal phase) was saturated and any additional zinc remained in the serum. Based on the results from the EDTA titration method, with increasing concentration of zinc acetate from 5 mmol L^{-1} to 40 mmol L^{-1}, the percentage of zinc in the serum phase increased from 15% to 43% (Table 4.2). Cousins and Smith (1980) reported the increase in serum zinc percentage from 4% to 26% with added zinc sulphate concentration increasing from 5.78 mmol L^{-1} and 57.80 mmol L^{-1}. The increase in serum zinc concentration was likely due to the corresponding saturation of the high molecular weight fractions with zinc when the concentration in the bovine milk was sufficiently high (Cousins & Smith, 1980).

4.3.3.2. Calcium distribution

From the EDTA titration method, the calcium concentration in the 10% (w/w) reconstituted non-preheated skim milk was 1.20 $\pm$ 0.03 (mg Ca)/(g skim milk) and showed no significant change in concentration (p>0.05) with increasing addition of zinc acetate concentration (results can be found in Appendix 9). However, as the concentration of zinc acetate added to the skim milk was increased (0 - 40 mmol L^{-1}) there was a significant decrease (p<0.05) in the calcium concentration in the colloidal phase, and a significant increase (p<0.05) in serum calcium concentration (Figure 4.3). Since the total calcium concentration in skim milk did not change significantly with the added zinc acetate concentration, the serum calcium concentration increasing indicated the calcium presented in the colloidal phase was released into the serum phase with the addition of zinc acetate. When 40 mmol L^{-1} zinc acetate was added, 57% of the added zinc presented in the colloidal phase but 33% of the calcium in the micellar phase was released into the serum phase, the calcium concentrations in each phase can be seen in Figure 4.3 and in Appendix 9.

Lucey (2017) has reported that CCP can dissociate from the casein micelle into the serum phase when skim milk pH is decreased. Experimentally, the skim milk pH was decreased with an increasing amount of zinc acetate added (Table 4.1). Thus, the pH decreasing caused by the addition of zinc acetate may lead to the dissociation of CCP from the casein micelle and result in the release of calcium ions from the colloidal phase into the serum phase. According to Philippe *et al.* (2005), the zeta potential of casein micelles stayed constant when 8 mmol L^{-1} zinc chloride was added, however, there was a significant decrease in zeta potential when calcium chloride was added. They suggested any surface modification of the casein micelles, when 8 mmol L^{-1} zinc chloride was added, was too low to be detected. From the results reported here, this may be caused of the calcium originally associated with the casein micelle was partially replaced by the added zinc ions. Singh *et al.* (1989a, 1989b) have studied the calcium distribution for zinc+skim milk and reported there is a decreasing trend of colloidal phase calcium concentration with increasing added zinc salt concentration. When 16 mmol L^{-1} zinc chloride was added into bovine milk, 40% of the calcium in the micellar phase was replaced by zinc in the casein micelles. Singh *et al*. (1989a) have studied the effect of added zinc chloride on calcium binding to α_{s1} - casein at pH 6.6. Singh *et al* (1989a) suggested that the addition of zinc ions partially replaced the calcium ions on the α_{s1} - casein by competing for the binding sites to phosphoseryl residues and also partially displaced the calcium from CCP which associated with casein micelles. Although the added zinc can partially replace the calcium that is natively associated with casein micelles (Figure 4.3), the increasing amount of zinc in the colloidal phase was proportionally greater than the decrease in calcium concentration in the colloidal phase. Consequently, the added zinc not only associated with the casein micelles by replacing calcium, but also may associate with other negatively charged groups and CCPs on the casein micelles.

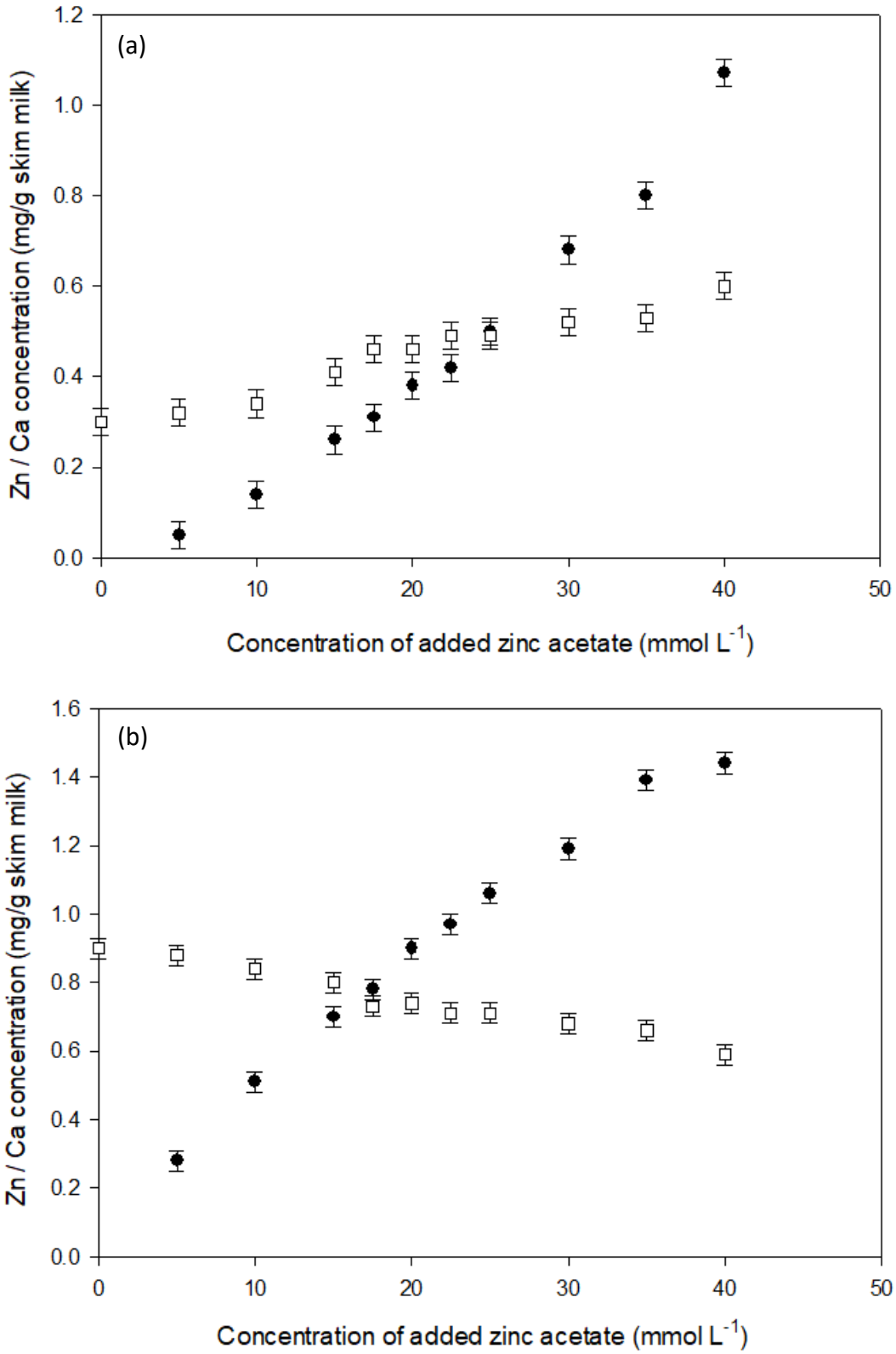


Figure 4.3: Zinc (●) and calcium (□) concentration in the (a) skim milk serum phase; (b) skim milk colloidal phase at different concentration of added zinc acetate determined by EDTA titration. The zinc and calcium concentration in colloidal phase was calculated by the difference between skim milk and serum. Results presented are means ± standard deviation (n = 3 - 4).

Singh *et al.* (1989a) reported that the displacement of bound calcium by zinc does not happen in reverse, which indicates that zinc binds much strongly to casein than calcium. Baumy and Brule (1988) and Philippe *et al.* (2005) studied the association of several divalent cations with casein micelles, they have reported zinc has a stronger association with casein micelles than calcium at native milk pH (6.70). This could be due to the electronegativity difference between zinc and calcium (Gordy, 1946). The electronegativity for zinc is about 1.65, but for calcium it is only 1.00 (Blackman *et al.*, 2012; James, 2017). The larger the electronegativity of the atom, the more it can attract electrons (Gordy, 1946). Therefore, zinc may have a higher affinity to casein micelles than calcium which results in the replacement of the native calcium associated with casein micelles when zinc ion added.

Many studies have reported milk is supersaturated with respect to calcium phosphate (Fox *et al.*, 1998; Wong *et al.*, 1988). However, Philippe *et al.* (2005) reported a reduction of serum phosphate concentration when zinc chloride was added. Such observations may also occur when other zinc salts are added as the newly formed zinc phosphate may precipitate when zinc acetate was added which would then lead to a decreased serum phosphate concentration. Lin (2019) has reported when serum phosphate concentration decreases, more calcium ions may be accommodated in the serum phase before the ion activity product exceeds the solubility product. Thus, the serum calcium concentration was able to increase when zinc acetate was added (Figure 4.3).

4.3.4. Rheological properties of zinc+skim milk samples

From the visual observations in Section 4.3.1, visible thickening and serum separation occurred in non-preheated 10% (w/w) skim milk when added zinc acetate concentration was ≥ 20 mmol L^{-1}. To evaluate the viscosity, gelation point and gel strength of the zinc+skim milk, the samples were prepared in the rheometer. Different amounts of 200 mmol L^{-1} stock solution of zinc acetate was added into the non-preheated reconstituted skim milk to achieve different added zinc concentrations and the resulting skim milk was 10% (w/w) total solids. The sample preparation in the rheometer can be found in Section 3.12.

4.3.4.1. Fluid behaviour and viscosity analysis

It was hypothesised that the addition of zinc acetate will change the skim milk viscosity. To test this hypothesis, zinc+skim milk samples were placed in the sample cup in the rheometer and the shear stress was monitored with increasing shear rate to generate a flow curve from 0.01 to 50 s^{-1}. Skim milk at 10% (w/w) skim milk with concentrations of added zinc acetate between 0 to 22.5 mmol L^{-1} were evaluated and the results are shown in Figure 4.4. If the shear stress of a fluid is linearly related to the shear rate, then the fluid is Newtonian (Prentice, 1992). The flow behaviours of the skim milk samples with added zinc acetate were found to be Newtonian fluids when the concentration was between 0 to 20 mmol L^{-1} over the shear rate range of 0.1 to 50 s^{-1}. The R^2 for the regression lines were higher than 0.99 (Table 4.3 for R^2 values). There was a transition from a Newtonian fluid to a non-Newtonian fluid at 22.5 mmol L^{-1} added zinc acetate (Figure 4.4). When the concentration of added zinc acetate increased to 22.5 mmol L^{-1}, a non-linear relationship between shear stress and shear rate was observed (Figure 4.4) and the R^2 was less than 0.99 (Table 4.3). The flow curve for the shear stress versus shear rate of the sample with 22.5 mmol L^{-1} zinc acetate added was concave and the viscosity decreased with increasing shear rate indicating shear-thinning behaviour (Mitchell, 2013). A similar transition from Newtonian to non-Newtonian behaviour was reported by Bienvenue *et al.* (2003) in concentrated skim milk; the skim milk behaved like a Newtonian fluid when the total solids was below 45% (w/w) but non-Newtonian shear-thinning behaviour was found for the samples with 45% (w/w) total solids. The increase in milk concentration resulted in a reduction in the interparticle spacing and enhanced micelle-micelle interactions which caused an increase in sample viscosity (Prentice, 1992).

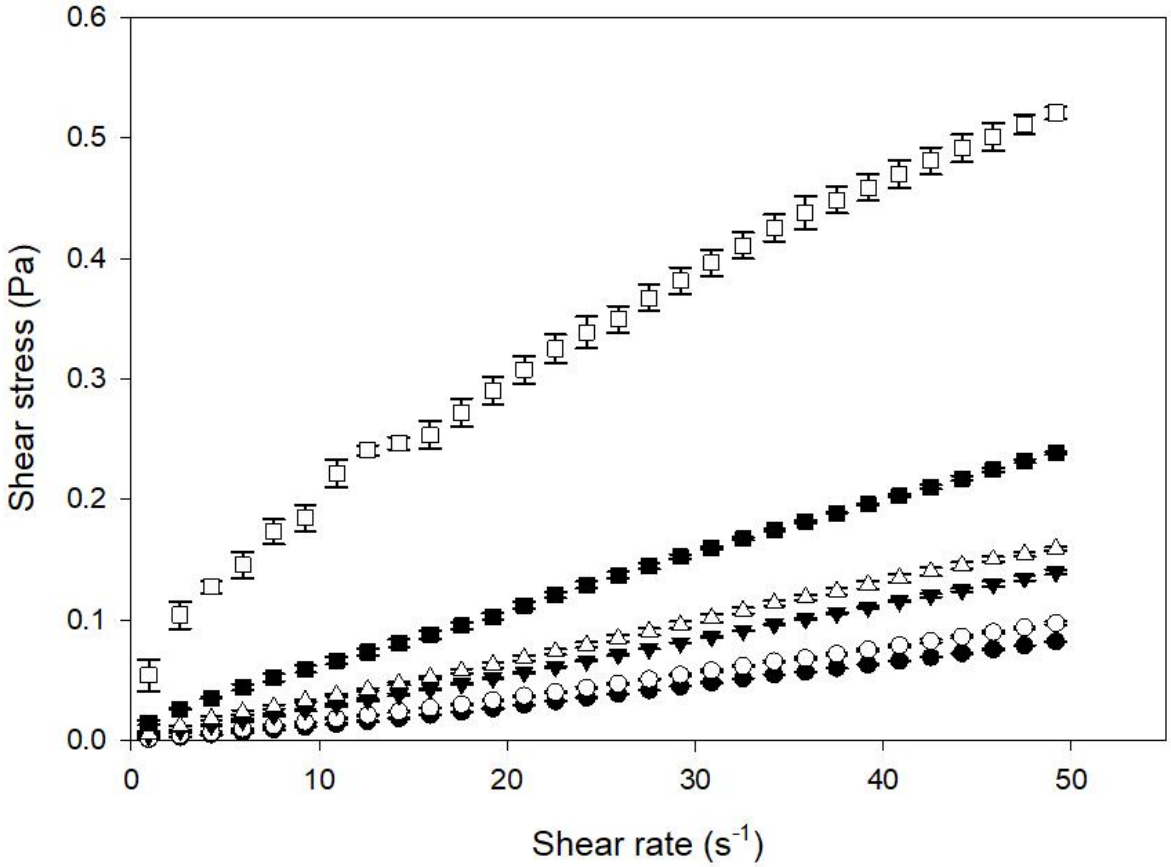


Figure 4.4: The shear stress versus shear rate at 20 ± 1°C for 10% (w/w) total solid non-preheated skim milk with different concentrations of added zinc acetate: 0 mmol L^{-1} (●); 10 mmol L^{-1} (○); 15 mmol L^{-1} (▼); 17.5 mmol L^{-1} (△); 20 mmol L^{-1} (■) and 22.5 mmol L^{-1} (□). Results presented are mean ± standard deviation (n=3).

Table 4.3: The absolute viscosity obtained from flow curves for zinc+skim milk samples with added zinc acetate ≤ 20 mmol L^{-1} and R^2 values the flow curves in Figure 4.4. Different superscript letters (a, b, c, d, e, f) indicate significant difference across the different concentrations of added zinc acetate.

	Added zinc concentration (mmol L^{-1})	R^2	Absolute viscosity (mPa·s)
NP skim milk	0	0.9963	1.72^{a}
NP skim milk + zinc acetate	10	0.9980	2.01^{b}
	15	0.9991	2.84^{c}
	17.5	0.9995	3.21^{d}
	20	0.9980	4.53^{e}

NP: non-preheated.

Skim milk exhibited Newtonian behaviour with the addition of zinc acetate up to 20 mmol L^{-1}, the absolute viscosity increased as more zinc salt was added (Figure 4.4 and Table 4.3). The viscosity of the zinc+skim milk increased more significantly at higher added zinc acetate concentrations. When more than 10 mmol L^{-1} zinc acetate was added, a small change of the added zinc acetate concentration led to a dramatic change in sample viscosity.

Visual observations (Section 4.3.1) did not show a change in milk texture until 20 mmol L^{-1} zinc acetate was added (Figure 4.2). The significant increase (p<0.05) in absolute viscosity for the samples with 15 and 17.5 mmol L^{-1} zinc acetate added (Table 4.3) may be too small to be visually observed. Rana *et al.* (2018) has reported an increase in milk viscosity with increasing zinc acetate concentration from 0 to 22.5 ppm Zn (0 - 0.34 mmol Zn / L sample), the increase in milk viscosity with addition of zinc salt was reported to be mainly due to the specific properties of the salt ion with casein micelles not the decrease in pH (Rana *et al.*, 2018). Singh *et al.* (1989b) reported that when more than 16 mmol L^{-1} of zinc chloride was added to bovine milk, casein micelles were reported to have coagulated after being heated to 30°C and held for 2 hours. It is therefor postulated that the findings in this study, with the significant increase in zinc+skim milk viscosity may be due to the aggregation of the casein micelle when zinc acetate was added.

4.3.4.2. Gelation properties by rheological measurements

To investigate the effect of added zinc acetate on skim milk texture, the zinc+skim milk samples prepared in the rheometer were firstly analysed with a time sweep at 20°C for 3 hours, followed by a frequency sweep for the samples which formed gels, those samples whose final *G'*> 1 Pa after the time sweep (Liu *et al.*, 2014; Ramasubramanian *et al.,* 2014). The method used in the rheometer can be found in Section 3.12.2. The change of *G'* with time for the skim milk with concentrations of zinc acetate ≥ 20 mmol L^{-1} added is shown in Figure 4.5 (data not shown for samples with < 20 mmol L^{-1}). The gelation point was defined as the point when the *G'* reaches 1 Pa (Ramasubramanian *et al.*, 2014). From the preliminary experiment, no increase in *G'* was observed after 3 hours holding at room temperature (20°C) for samples with less than 20 mmol L^{-1} added zinc acetate. A small but significant increase (p<0.05) in *G'* was found for the sample with 20 mmol L^{-1} added zinc acetate during holding,

but the final *G'* was <1 Pa. Hence no gelation occurred. Gelation was only observed in the samples with at least 22.5 mmol L^{-1} added zinc acetate. The samples with added zinc acetate concentrations of 22.5, 25, 30 and 40 mmol L^{-1} reached a *G'* of 1 Pa after 70, 16, 2 and 2 min holding, respectively. For the skim milk with zinc acetate concentration of 30 mmol L^{-1} and 40 mmol L^{-1}, the *G'* increased higher than 1 Pa in less than 5 min which indicated the gel network formation was very rapid at high zinc acetate concentrations. The zinc-added skim milk gelation was shown to be a time-dependent process. The final *G'* of the gels after 3 hours holding increased with zinc acetate concentration. The highest final *G'* was 6.86 Pa for the sample with 40 mmol L^{-1} zinc acetate added (Figure 4.5).

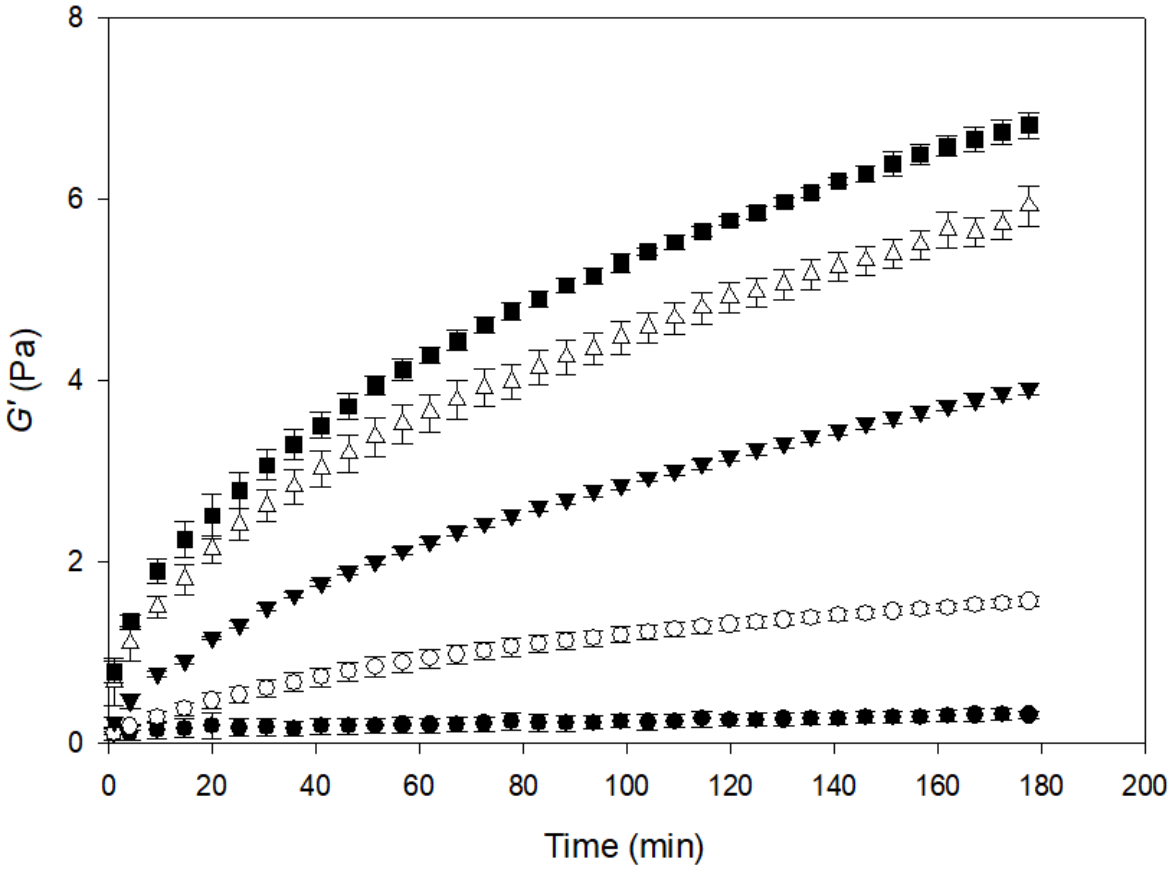


Figure 4.5: The change of storage modulus *G'* with time for 10% non-preheated skim milk with different concentrations of added zinc acetate: 20 mmol L^{-1} (●); 22.5 mmol L^{-1} (○); 25 mmol L^{-1} (▼); 30 mmol L^{-1} (△) and 40 mmol L^{-1} (■) during holding at 20 ± 1°C for 3 hours. Results presented are means ± standard deviation (n = 3).

These results show that skim milk gels can be formed with the addition of zinc acetate with no heating. It was postulated that only casein was likely to be involved in the gelation of milk

for the non-preheated sampleas, as the structure of whey proteins remained unchanged. Preheat can cause whey protein denaturation and lead to the formation of whey-casein complexes which contribute together to form the gel structure (Damodaran & Parkin, 2017; Fox *et al.*, 1998). Many studies have reported that preheating of the milk can increase the gel strength (G') of the acid-induced gel (Lucey *et al*., 1999; Lucey, 2017; Vasbinder *et al*., 2004) and mineral salt-induced milk gel properties like calcium and magnesium salts (Begum, 2019; Lin, 2019; Ramasubramanian *et al.*, 2014; Vasbinder *et al.*, 2004).

According to Lin *et al.* (2018) and Totosaus *et al.* (2002), the negative charge of the casein micelle will reduce when cations associate with them and then reduce the electrostatic repulsion between casein micelles. The decrease in skim milk pH after zinc acetate addition (Table 4.1) may have contributed to gelation occurring as the electrostatic repulsion between casein micelles decreases with a reduction in pH (Figure 2.9). Consequently, the distances between casein micelles were reduced and this encouraged the interactions between casein molecules and then resulted in a gel network.

Philippe *et al.* (2005) has reported the addition of zinc salt can decrease the hydration of casein micelles. The hydration interaction is a repulsive interaction between casein micelles when the casein micelles come close to each other, the larger the hydration of the casein micelle, the greater is the hydration repulsion between two casein micelles (Bryant & McClements, 1998). With the decrease in casein micelle hydration when zinc ions were added, the hydration repulsion between casein micelles may reduce, and casein micelle aggregation becomes easier and more possible.

Lin *et al*. (2018) suggested that the calcium ions (Ca^{2+}) can associate with two neighbouring casein micelles and form a salt-bridge with the negatively charged groups. As zinc and calcium are competing for the same binding site on the casein micelle, a similar mechanism is postulated as the zinc-casein complexes may be formed by electrostatic interaction between oppositely charged ions and resulted in salt bridges between casein micelles leading to the formation of a zinc-casein network. In relation to the results found in this study, when 17.5 mmol L^{-1} zinc acetate was added, the zinc-casein network may have been sufficient to cause an increase in viscosity, and when zinc acetate concentration increased to 22.5 mmol L^{-1} the

zinc salt bridges were enough to form a gel structure. However, there was lack of information reported for the real picture of skim milk gel with the addition of zinc salts. Thus, further research is required to gain a better understanding of the zinc-added skim milk gel structure.

To investigate the viscoelastic properties of zinc-added skim milk gels, a frequency sweep was carried out between 1 - 5 HZ for the samples with a final *G'* higher than 1 Pa after 3 hours holding at 20°C (Figure 4.6). According to Lapasin and Pricl (1995), a gel is formed by weak interactions if the difference between *G'* and *G"* is less than one log, while a larger difference would be expected for stronger gels. The difference between *G'* and *G"* was smaller than one log for all gels, thus, all skim milk gels with added zinc acetate may be held together by weak interactions. As the concentration of zinc acetate increased the gel strength increased (Figure 4.6).

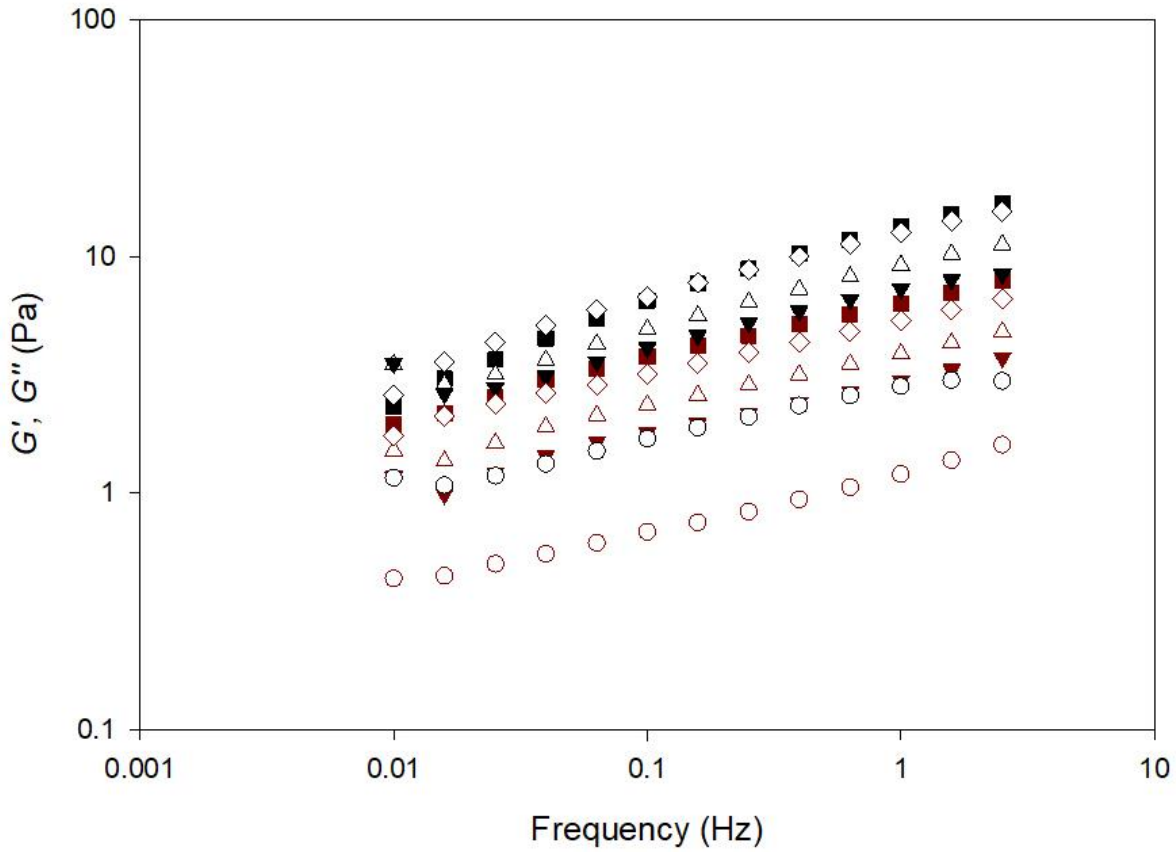


Figure 4.6: A representative plot showing the *G'* (black symbols) and *G"* (red symbols) of the zinc-added non-peheated skim milk gels (after a 3 hours time sweep at 20 ± 1°C) as a function of frequency with various concentrations of added zinc acetate: the concentration of added zinc acetate was 22.5 mmol L^{-1} (○,○); 25 mmol L^{-1} (▼,▼); 30 mmol L^{-1} (△,△) and 40 mmol L^{-1} (■,■).

4.4. Effect of pH adjustment on rheological properties

To determine if the decrease in pH due to zinc acetate addition was the primary reason for the thickening or gelation in the skim milk, when 20 - 40 mmol L^{-1} of zinc acetate was added, the pH of the skim milk after zinc acetate addition was restored back to pH 6.73 $\pm$ 0.03 (native skim milk pH). The final *G'* for the pH adjusted samples with different zinc acetate concentrations added is shown in Figure 4.7. It was noted that at the same added zinc acetate concentration, a lower final *G'* was observed for the sample adjusted back to pH 6.73 $\pm$ 0.03. Gelation was observed for the unadjusted pH zinc+skim milk samples at the added zinc acetate concentration ≥ 22.5 mmol L^{-1}. Gelation was only observed at a higher concentrations of added zinc acetate (≥ 30 mmol L^{-1}) for the pH adjusted zinc+skim milk samples with a finl *G'* of 1.13 $\pm$ 0.05 Pa (Figure 4.7). This result may be explained by the fact that the decrease in skim milk pH contributed to the gel structure formation of skim milk with zinc acetate added. A similar decrease in final *G'* was reported by Lin *et al.* (2018) when comparing skim milk at pH 6.60 with skim milk with added calcium salt at pH 5.94. As discussed in Section 2.8.1, decreasing the skim milk pH increases the hydrogen ion concentration which can associate with the casein micelle and reduce the electrostatic repulsion of casein micelles and encouraging the coagulation of the casein micelles (Lucey & Singh, 1997; Lucey, 2017; Vasbinder *et al.*, 2003).

A separate experiment was carried out to determine the effect of pH on skim milk gelation. The pH of skim milk was adjusted to 5.34 ± 0.02 which was the pH of skim milk after 40 mmol L^{-1} zinc acetate was added. There was no gelation (*G'*> 1) observed for the pH 5.34 sample without zinc acetate added after holding at 20°C for 3 hours. For the unheated skim milk, the casein coagulation only happens when the pH is less than 4.8 (Vasbinder *et al.*, 2003). Thus, pH 5.34 may not be sufficient to reduce the electrostatic repulsion between casein micelles and cause casein micelle coagulation. It can then be suggested that the addition of zinc acetate was the primary cause of gelation and the pH decreased by the addition of zinc acetate also contributed to the strength of the gel.

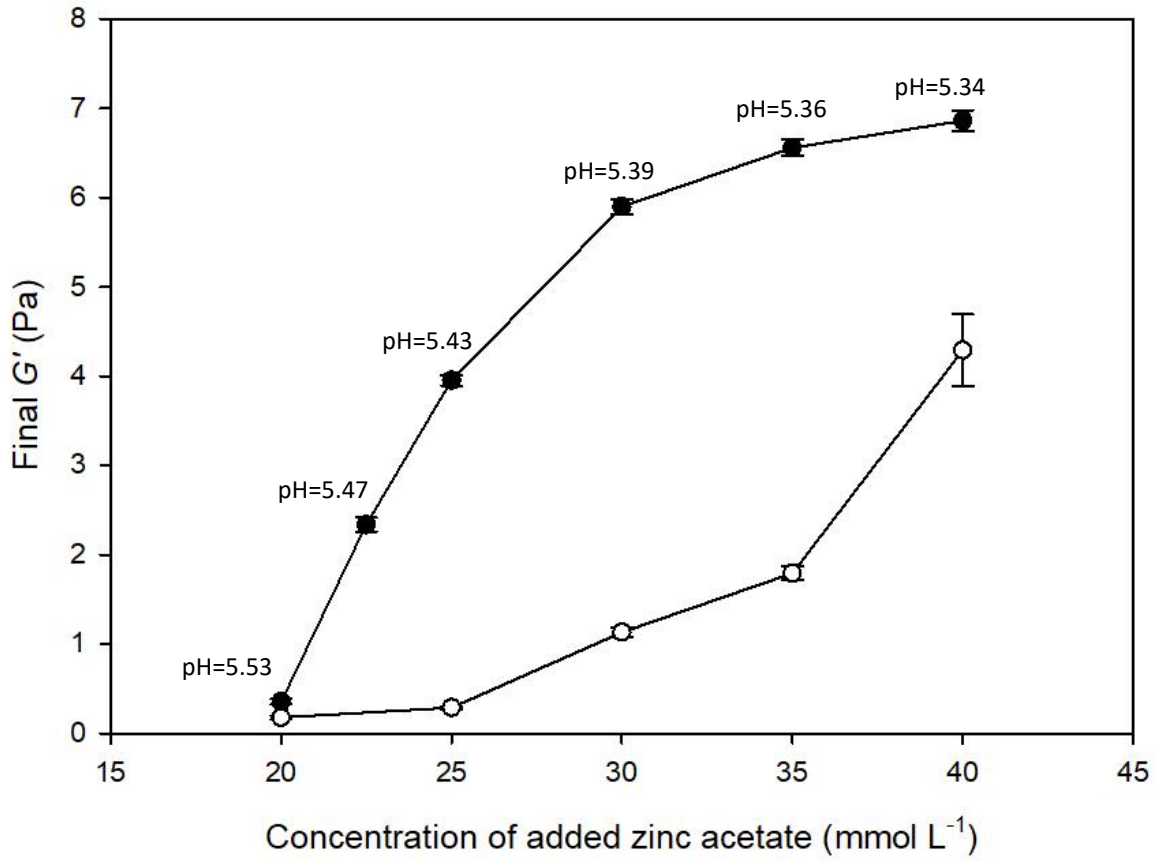


Figure 4.7: Final *G'* after holding at 20 ± 1°C for 3 hours of 10% (w/w) non-preheated skim milk with various addition concentrations (20 to 40 mmol L^{-1}) of zinc acetate; close symbols (●) represent unadjusted pH samples and open symbols (○) represent adjusted pH samples (pH=6.73 ± 0.03). Results presented were mean ± standard deviation (n=3).

4.5. Effect of heat treatment on zinc+skim milk

4.5.1. Visual assessment of zinc+skim milk

Visual observation results of non-preheated and preheated zinc+skim milk with different added zinc acetate concentrations (0 to 40 mmol L^{-1}) held at different temperatures (20, 30, 40, 60, 70 and 80 ± 1°C) for 60 minutes are presented in Table 4.4. The skim milk samples without zinc acetate added did not thicken or form gels at the heating temperature range from 20 to 80 ± 1°C regardless of preheating (Table 4.4). For the preheated zinc+skim milk, thickening or gelation started at lower added zinc acetate concentrations with the same holding temperature compared with non-preheated skim milk samples. For example, when

the holding temperature was 60 ± 1°C, the addition of 15 mmol L^{-1} zinc acetate was required for the non-preheated zinc+skim milk sample to form a gel, but gelation was observed for the preheated zinc+skim milk with only 10 mmol L^{-1} zinc acetate added (Figure 4.8c, 4.8b). At the same added zinc acetate concentration and the same holding temperature, the gels obtained from the non-preheated skim milk samples were weaker than gels observed in the preheated zinc+skim milk samples. For example, for the zinc+skim milk with 17 mmol L^{-1} zinc acetate added and held at 30 ± 1°C, the gel of the preheated sample remained on the top part of the inverted test tubes, but the gel of non-preheated zinc+skim milk broke when the tube was inverted (Table 4.4, Figure 4.8a, 4.8b).

For the zinc+skim milk held at a higher temperatures, gelation occurred at lower added zinc acetate concentrations (Table 4.4). At a holding temperature of 30 ± 1°C, at least 20 mmol L^{-1} added zinc acetate was required for both preheated and non-preheated skim milk samples to lead to any observed gelation (Table 4,4, Figure 4.8a, 4.8b). However, when the holding temperature increased to 80 ± 1°C, the required concentration of zinc acetate to form a gel was reduced to 10 mmol L^{-1} for both preheated and non-preheated samples (Table 4.4, Figure 4.8e and 4.8f). Gel formation was obtained with addition of 10 mmol L^{-1} zinc acetate and a holding temperature of 60 ± 1°C (Table 4.4). To produce gels for zinc+skim milk, either high concentrations of zinc acetate at lower holding temperatures or low concentrations of zinc acetate with higher holding temperatures was required. Preheat may encourage the zinc+skim milk gelation, but without the addition of zinc acetate preheating itself cannot lead to milk gelation.

Table 4.4: Visual observations of gelation in preheated and non-preheated skim milk samples after holding for 60 min at different holding temperatures and different concentrations of zinc acetate (n=3). The heating rate was about 12°C/min. Milk samples were cooled to 20 ± 1°C before observation.

Hold temp	Skim milk type	Zinc acetate concentration (mmol L^{-1})								
		0	5	10	15	17	20	25	30	40
20°C	NP	Liquid	Liquid	Liquid	Liquid	Liquid	Thickened	Thickened	Serum separated	Serum separated
	P	Liquid	Liquid	Liquid	Thickened	Thickened	Thickened	Thickened	Serum separated	Serum separated
30°C	NP	Liquid	Liquid	Liquid	Liquid	Thickened	Gel	Gel	Serum separated	Serum separated
	P	Liquid	Liquid	Liquid	Thickened	Soft gel	Gel	Gel	Serum separated	Serum separated
40°C	NP	Liquid	Liquid	Liquid	Thickened	Gel	Gel	Gel	Serum separated	Serum separated
	P	Liquid	Liquid	Liquid	Gel	Gel	Gel	Gel	Serum separated	Serum separated
60°C	NP	Liquid	Liquid	Liquid	Gel	Gel	Gel	Gel	Serum separated	Serum separated
	P	Liquid	Liquid	Gel	Gel	Gel	Gel	Gel	Serum separated	Serum separated
70°C	NP	Liquid	Liquid	Thickened	Gel	Gel	Gel	Gel	Serum separated	Serum separated
	P	Liquid	Liquid	Gel	Gel	Gel	Gel	Serum separated	Serum separated	Serum separated
80°C	NP	Liquid	Liquid	Gel	Gel	Gel	Gel	Gel	Serum separated	Serum separated
	P	Liquid	Thickened	Gel	Gel	Gel	Gel	Serum separated	Serum separated	Serum separated

NP = Non-preheated, P = Preheated.
Liquid: No milk can adheres to the top of the inverted test tube visually.
Thickened: Milk flows slow enough to coat the walls of test tube when inverted.
Soft gel: The gel was broken when inverted, part of the gel maintained on the top of the inverted test tube, part on the bottom.
Gel: All the gel remained at the top of the inverted test tube.
Serum separation: a visible transparent or semi-transparent top layer of liquid was observed after holding and before inverting the tubes.

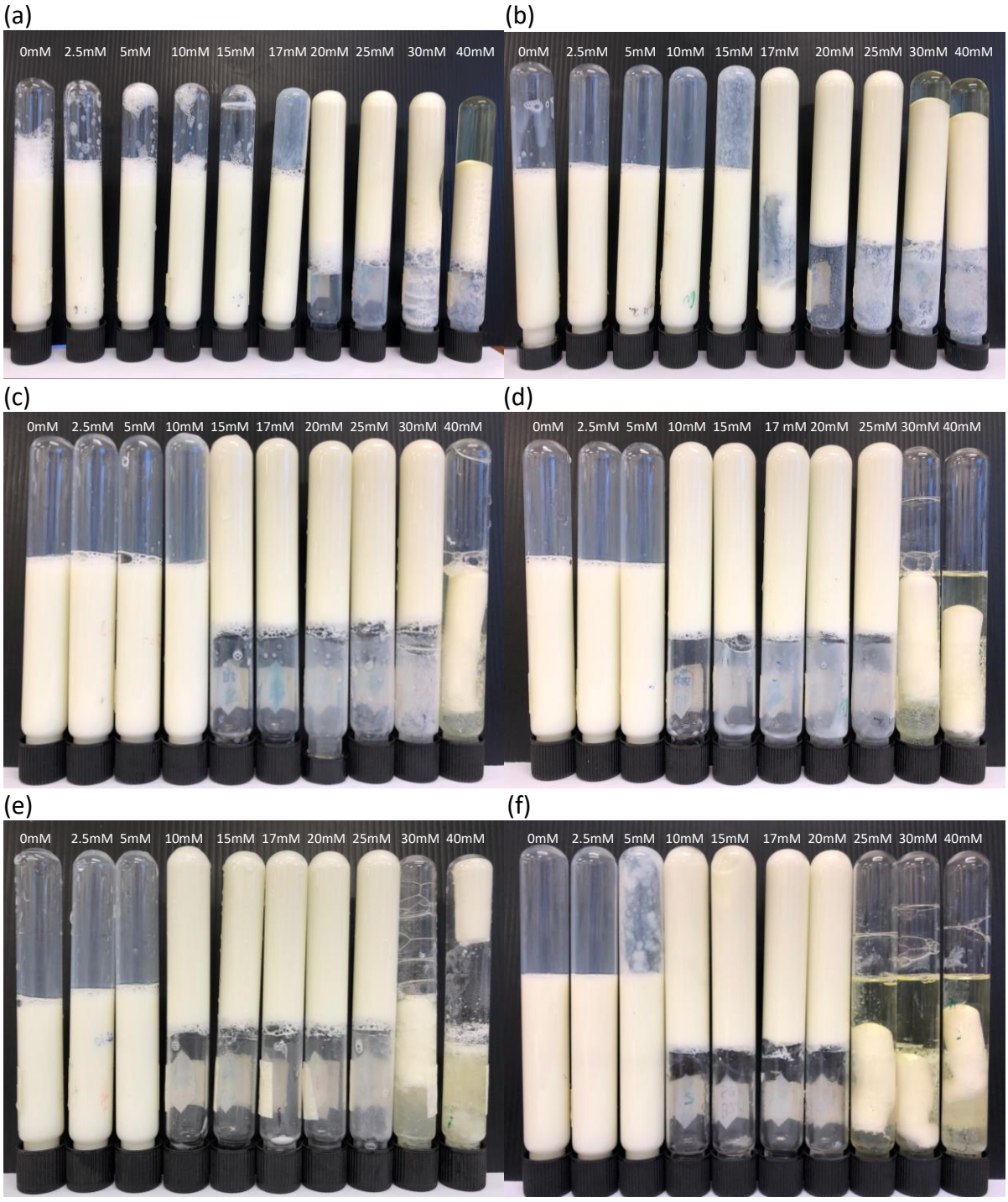


Figure 4.8: Zinc+skim milk samples with different added zinc acetate concentration (0 to 40 mmol L^{-1}) obtained after holding at (a and b) 30 ± 1°C, (c and d) 60 ± 1°C and (e and f) 80 ± 1°C. (a), (c) and (e) for non-preheated zinc+skim milk, and (b), (d) and (f) for preheated zinc+skim milk samples. This experiment was repeated three times and the same results were observed each time.

4.5.2. Rheological properties of zinc+skim milk with heat treatment

4.5.2.1. Effect of preheating on zinc+skim milk gelation

To determine the effect of preheating on zinc+skim milk gelation, the 13% reconstituted skim milk was preheated at 90^{o}C for 10 mins prior to the addition of zinc acetate, the preheating method can be found in Section 3.4. Unlike the rheological analysis method used in Section 4.3 and 4.4, the zinc+skim milk samples were analysed firstly by carrying out a heating temperature sweep from 20^{o}C to 80^{o}C, then a time sweep for 60 mins at a constant holding temperature, followed with a cooling temperature sweep from 80^{o}C to 20^{o}C, and finally, a frequency sweep was applied for the samples which formed gels (*G'*>1 Pa). The rheology methods can be found in Section 3.12.2.

The change in *G'* for the preheated and non-preheated zinc+skim milk during the heating temperature sweep from 20^{o}C to 80^{o}C with different concentrations of zinc acetate added is shown in Figure 4.9. For the zinc+skim milk with heat treatment applied, gelation occurred at a lower added zinc acetate concentration (Figure 4.9) compare with the zinc+skim milk with no heat treatment (Figure 4.5). There was no change in the *G'* for both preheated and non-preheated skim milk with 5 mmol L^{-1} zinc acetate added during the heating phase. Hence, the change of *G'* for these two samples were not plotted in Figure 4.9. Gelation during heating up was only observed for the non-preheated samples with ≥ 15 mmol L^{-1} zinc acetate added, but gelation was observed at a lower added zinc acetate concentration (10 mmol L^{-1}) for preheated skim milk at the same heating rate, 5^{o}C/min (Figure 4.9). The results also revealed that for the zinc+skim milk sample with added zinc acetate concentration between 10 to 15 mmol L^{-1}, the *G'* of the preheated zinc+skim milk gel was significantly higher (p<0.05) than the *G'* of the non-preheated skim milk gels observed at the same temperature during heating. For the preheated skim milk with 15 mmol L^{-1} zinc acetate added, the *G'* during heating increased more rapidly than the non-preheated one with same added zinc acetate concentration. For the non-preheated sample, the *G'* plateaued out and did not increase further from 75^{o}C onwards. It was noted that the *G'* for the zinc+skim milk sample with 20 mmol L^{-1} zinc acetate added increased initially and then decreased at 65^{o}C for both non-preheated and preheated skim milk samples (Figure 4.9). The decrease in *G'* of the samples with 20 mmol L^{-1} zinc acetate

added could be caused by the syneresis in the samples at the higher temperatures. Thus, the results from the rheometer for these zinc acetate concentrations after 60°C may not be reliable. Therefore, the change of *G'* during holding and cooling phase for the sample with added zinc acetate concentration of 20 mmol L^{-1} was not analysed further on the rheometer.

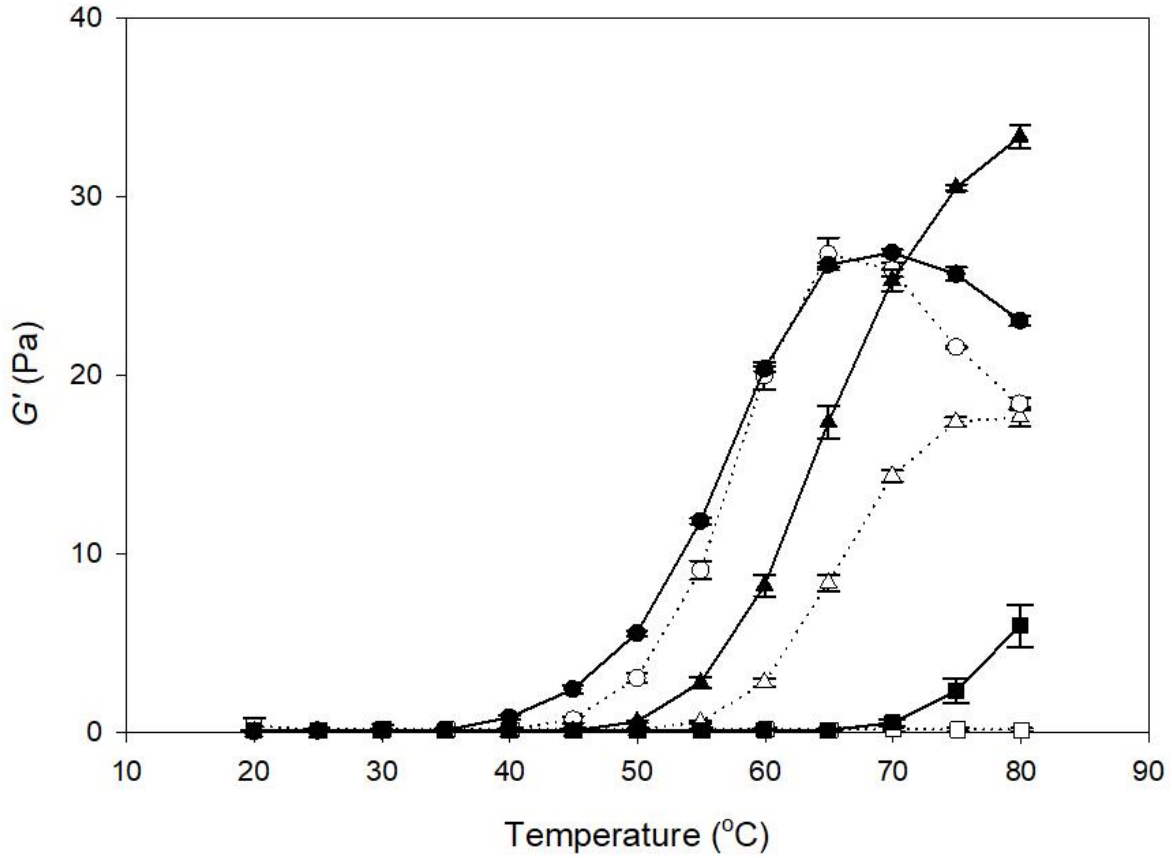


Figure 4.9: The change of storage modulus *G'* with temperature increasing from 20°C to 80°C for 10% non-preheated (open symbols, ·····) and preheated (close symbols, ——) skim milk with different concentrations of added zinc acetate: 10 mmol L^{-1} (□, ■); 15 mmol L^{-1} (△, ▲) and 20 mmol L^{-1} (○, ●). Results presented are means ± standard deviation (n = 3).

During the holding period, no gel formed in the non-preheated skim milk with 5 mmol L^{-1} added zinc acetate. However, for the preheated sample with the same concentration of added zinc acetate, gelation occurred after 43 min holding. For the samples with added zinc acetate concentration between 10 to 15 mmol L^{-1}, the *G'* continued to increase during the holding period (60 mins) regardless if preheated or not. The increase in *G'* was significantly higher for the preheated skim milk compared to the non-preheated samples with the same concentration of zinc acetate added (Figure 4.10). Development of the gel network in

preheated skim milk samples with 15 mmol L^{-1} added zinc acetate occurred mainly during heating and began to plateau after 15 min holding at 80°C (Figure 4.9 and Figure 4.10). The non-preheated skim milk with 15 mmol L^{-1} zinc acetate reached a *G'* of 17.37 ± 0.27 Pa after heating to 75°C, when the sample was heated and held at 80°C , the sample remained at a *G'* of ~ 17 Pa for 10 mins into the holding period then began to increase in *G'* after this (Figure 4.9 and Figure 4.10).

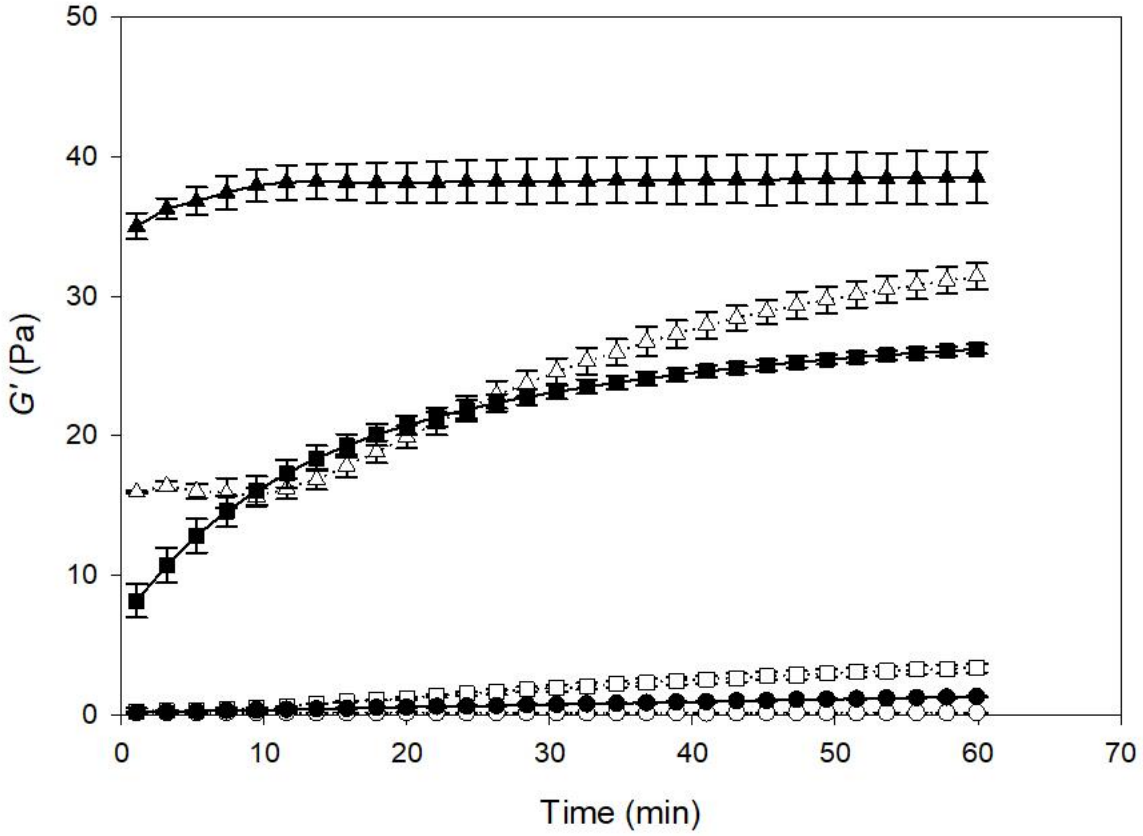


Figure 4.10: The change of storage modulus *G'* with time for 10% non-preheated (open symbols, ⋯⋯) and preheated (close symbols, ——) skim milk with different concentrations of added zinc acetate: 5 mmol L^{-1} (○, ●); 10 mmol L^{-1} (□, ■) and 15 mmol L^{-1} (△, ▲) during holding at 80°C for 60 minutes. Results presented are means ± standard deviation (n = 3).

The plateau period of *G'* observed for the non-preheated skim milk with 15 mmol L^{-1} zinc acetate added, during the first 15 minutes of holding at 80°C may indicate that the gel network reached the maximum strength without the contribution of the denatured whey proteins, and the further increase in *G'* during holding may be due to the cross-link formation between denatured whey protein and casein micelles after extended holding at 80°C, this

would lead to increased the gel strength (Figure 4.10). As discussed earlier, preheat (>70^{o}C) can lead to whey protein denaturation and development of the cross-links between denatured whey protein with casein micelles which results in a higher gel strength (higher *G'*) (Anema, 2008; Datta & Deeth, 2001; Oldfield *et al*., 1998; Wong *et al*., 1988). For the non-preheated zinc+skim milk, the whey protein did not denature before the sample was heated, the unchanged whey proteins were present in an environment which had a low pH 5.69 (Table 4.1), with a serum rich in zinc ions, 0.26 (mg Zn)/(g skim milk), and 0.70 (mg Zn)/(g skim milk) in the colloidal phase (Table 4.2). Lyster (1970) has reported heat-induced whey protein denaturation can occur at pH below 6.2. Law and Leacer (2000) reported whey protein denaturation can also happen during heat treatment at 80^{o}C for skim milk with the addition of calcium chloride before heat treatment. The authors suggested that both pH decreasing and addition of Ca^{2+} can effectively reduce the net negative charge of the whey proteins and then promotes the heat induced whey protein aggregation. For the non-preheated skim milk with <15 mmol L^{-1} zinc acetate added, there was no plateau trend observed during the heating or holding phases. The possible reason was that there was no significant increase (p>0.05) in *G'* during heating (20^{o}C to 80^{o}C) for concentrations of zinc acetate < 10mmol L^{-1}. A significant increase in *G'* was only observed for the sample with 10 mmol L^{-1} zinc acetate added and holding at 80^{o}C after 10 minutes. Since the holding temperature was higher than 70^{o}C, the denatured whey protein may participate in the gel network formation and contribute to the *G'*.

It is postulated that the addition of zinc acetate and the accompanying decrease in pH prior the heat treatment can decreases the net negative charge of the native whey proteins and then promotes whey protein denaturation at high temperatures. Although, the gel network may form without denatured whey protein, the maximum *G'* of the gel formed from association between zinc and casein micelles was 17.37 ± 0.27 Pa after heating to 75^{o}C (Figure 4.9) which was lower than the maximum gel strength when denatured whey protein may have participated, 31.45 ± 0.95 Pa (Figure 4.10). Further research is required to get a better understanding of the whey protein denaturation in the non-preheated zinc+skim milk during heat treatment at high temperature.

The change in *G'* of zinc+skim milk samples during cooling is shown in Figure 4.11. The *G'* increased significantly (p<0.05) for all the samples that formed a gel after holding regardless of the preheat treatment (Figure 4.11). The increase in *G'* during the cooling process has been reported previously for acid-induced, rennet-induced, calcium added and magnesium added skim milk gelation (Lucey, 2017; Van Vliet *et al*., 1989; Lin *et al*., 2018, 2020; Begum, 2019). Van Vliet *et al*. (1989) has reported the increase in storage modulus (*G'*) for rennet-gels during cooling can be caused by the decrease in hydrophobic interactions which result in an increase in the size of casein micelles. Thus, the intermolecular bonds increase, leading to an increase in *G'*. This was confirmed by Lucey (2017), who reported the swelling of casein particles caused by weak hydrophobic interactions may explain the increase in storage modulus (*G'*) during the cooling phase. A similar process may be happening in the zinc+skim milk gels during cooling and resulted in a significant increase in *G'*.

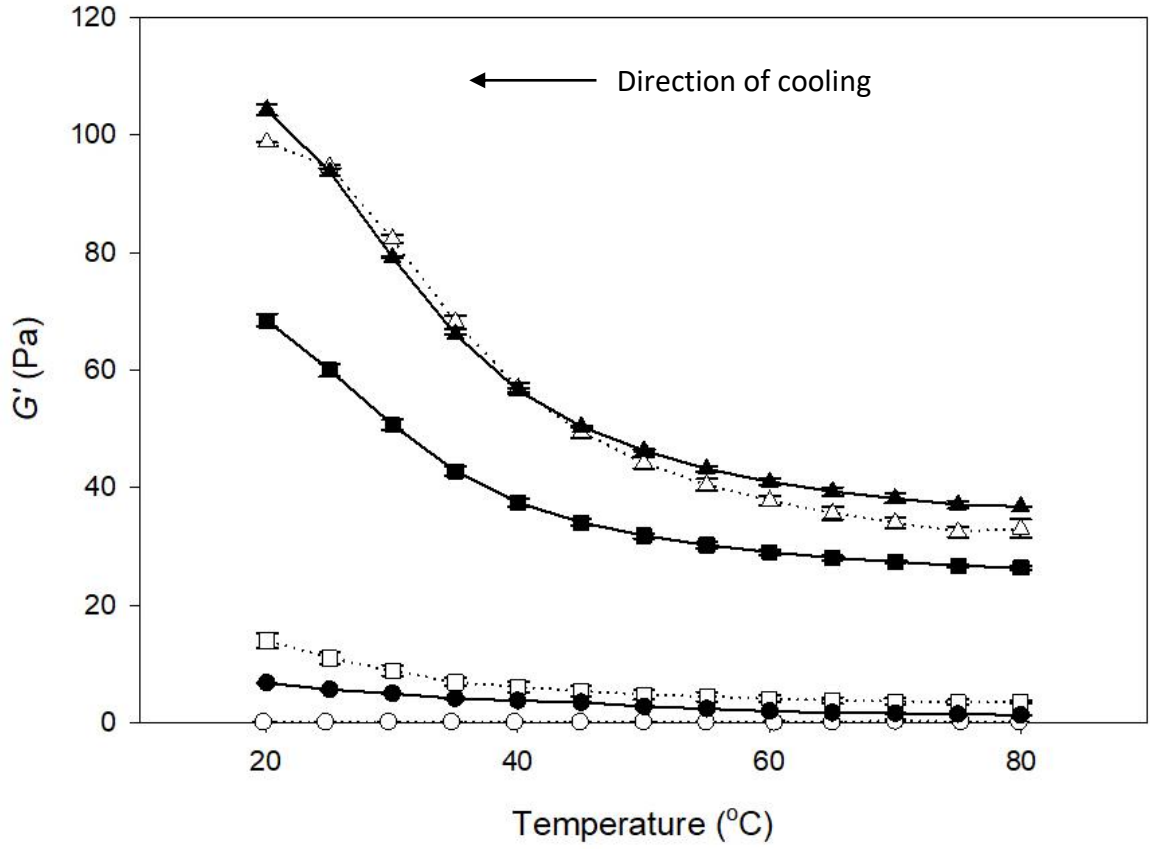


Figure 4.11: The change of storage modulus *G'* with temperature decreased from 80°C to 20°C for 10% non-preheated (open symbols, ⋯⋯) and preheated (close symbols, ——) skim milk with different concentrations of added zinc acetate: 5 mmol L^{-1} (○, ●); 10 mmol L^{-1} (□, ■) and 15 mmol L^{-1} (△, ▲). Results presented are means ± standard deviation (n = 3).

The effect of preheat treatment on the final *G'* after cooling to 20°C is shown in Figure 4.12. For the zinc+skim milk samples, the final *G'* increased with the concentration of zinc acetate added between 5 to 15 mmol L^{-1} regardless of the preheat treatment. However, the final *G'* for the preheated samples was higher than the non-preheated samples. Begum (2019), and Ramasubramanian *et al*. (2014) have reported a higher gel strength was observed for the preheated skim milk samples with both magnesium chloride or calcium chloride added at the same concentration. As whey protein will denature at approximately 70°C (Wong *et al*., 1988), during the preheat treatment (90°C for 10 min in this study), the whey proteins are likely to have denatured and associate with the micellar κ - casein by hydrophobic interactions and intermolecular disulphide bonds (Anema, 2008; Datta & Deeth, 2001; Oldfield *et al.*, 1998). Lucey *et al*. (1999) reported the association of denatured whey protein with casein micelles could help the formation of cross-links between protein particles and increase firmness and strength of acid-gels.

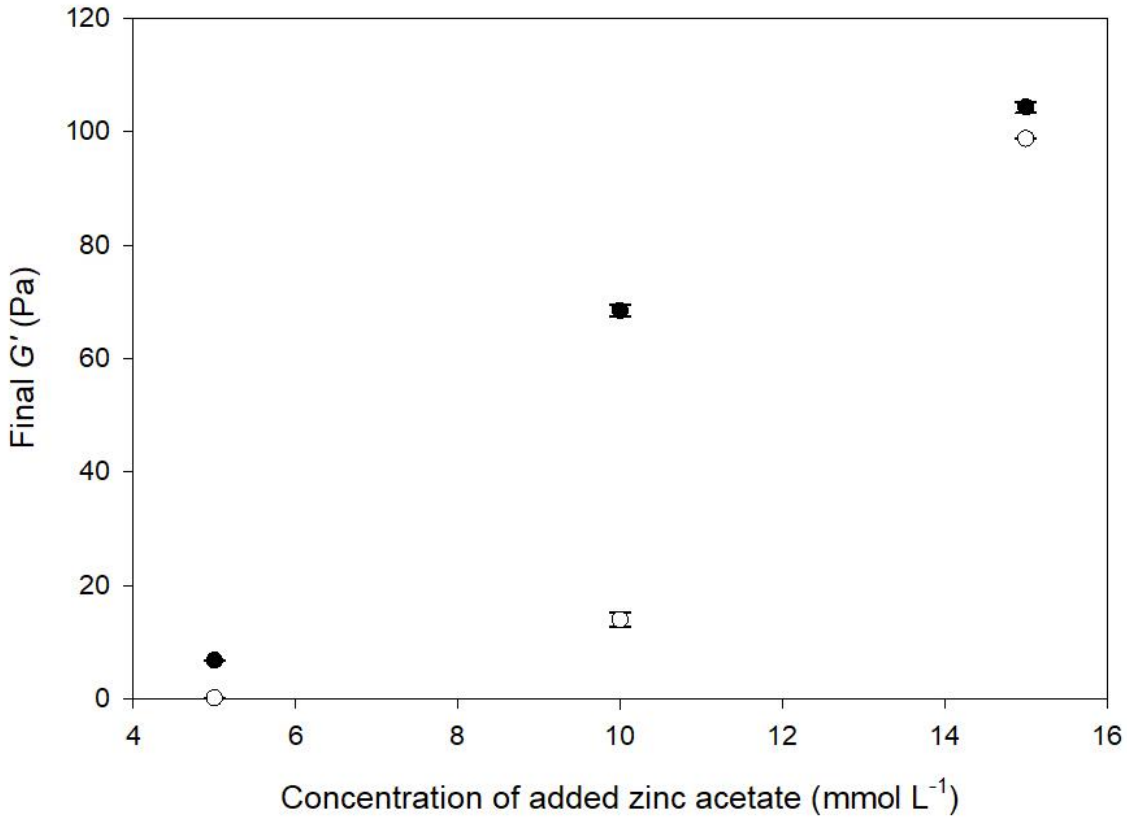


Figure 4.12: Final *G'* after the samples were heated to 80°C and held for 60 minutes then cooled to 20°C for 10% (w/w) non-preheated (open symbols, ○) and preheated (close

symbols, ●) skim milk with various concentrations (5 to 15 mmol L^{-1}) of zinc acetate. Results presented were mean ± standard deviation (n=3).

As observed in Figure 4.9 and 4.10, for the skim milk with 15 mmol L^{-1} added zinc acetate the *G'* of the non-preheated skim milk showed a plateau when *G'* did not increase for 10 minutes after reaching 75°C. After this period the *G'* began to increase for the remainder of the holding period and after cooling to a final *G'* of 98.82 ± 0.02 Pa (Figure 4.10 and Figure 4.11). This final *G'* for the non-preheated skim milk (104.35 ± 0.94 Pa) was close to the final *G'* of the preheated sample with the same concentration of zinc acetate (Figure 4.12).

The frequency sweep results are presented in Figure 4.13 for the zinc+skim milk samples which formed a gel after being cooled to 20°C.

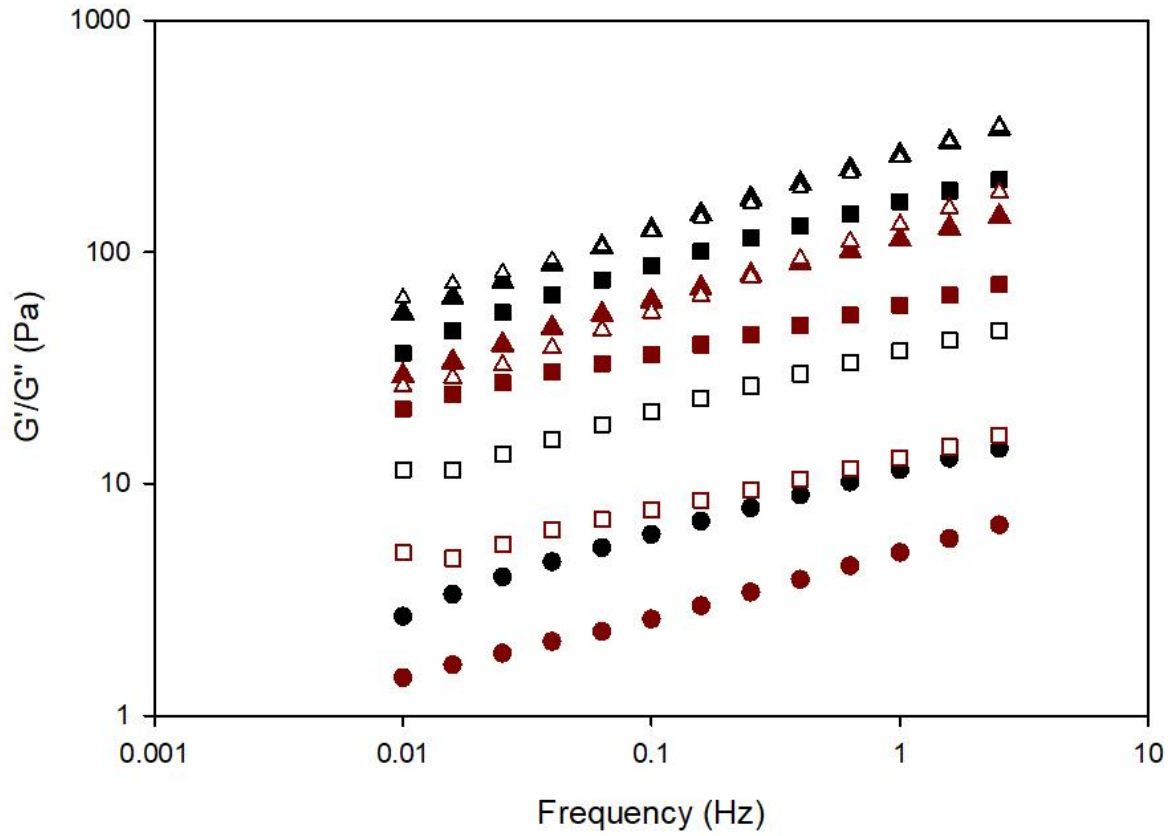


Figure 4.13: A representative plot showing the *G'* (black symbols) and *G''* (red symbols) of the (a) non-preheated (open symbols) skim milk gels and (b) preheated (close symbols) zinc+skim milk gels (after heated to 80 ± 1°C and held for 60 minutes then cooled to 20 ± 1°C) as a function of frequency with various concentrations of added zinc acetate: the concentration of added zinc acetate was 5 mmol L^{-1} (●, ●); 10 mmol L^{-1} (□, □, ■, ■) and 15 mmol L^{-1} (△, △, ▲, ▲).

4.5.2.2. Effect of holding temperature zinc+skim milk gelation

To investigate the effect of holding temperature on zinc+skim milk gelation, the preheated and non-preheated skim milks with 15 mmol L^{-1} zinc acetate added were prepared in the rheometer by firstly heating to the different temperatures, 40°C, 60°C and 80°C (heating temperature sweep), then held at the required temperature for 60 minutes (time sweep) and finally cooled to 20°C (cooling temperature sweep). Detail of the gel preparation in rheometer can be found in Section 3.12.2.

The change of *G'* for non-preheated and preheated skim milk with 15 mmol L^{-1} zinc acetate held at different temperatures during heating, holding and cooling phase is shown in Figure 4.14, Figure 4.15 and Figure 4.16. The preheated and non-preheated zinc+skim milk heated to 40°C did not show significant increase (p>0.05) in *G'* during heating phase (Figure 4.14). However, the *G'* was increased during holding phase (Figure 4.15) and gels were formed after 14 min and 35 min holding for preheated and non-preheated zinc+skim milk. The samples reached final *G'* values of 13.08 ± 0.27 Pa (preheated) and 2.23 ± 0.46 Pa (non-preheated) after cooled to 20°C (Figure 4.16).

For samples heated to 60°C, the *G'* significantly increased (p<0.05) after heating to 50°C for both preheated and non-preheated zinc+skim milk, and gelation was observed at 55°C and 60°C respectively (Figure 4.14). During the holding phase, the *G'* for both the preheated and non-preheated sample increased rapidly in the first 20 min and then plateaued and reached a maximum *G'* of 39.08 ± 0.41 Pa and 20.15 ± 0.17 Pa, respectively (Figure 4.15). There was no further increase in the non-preheated sample's *G'* after the plateau period, which may indicate the whey protein did not denature further and contribute to the gel strength at 60°C for the non-preheated zinc+skim milk.

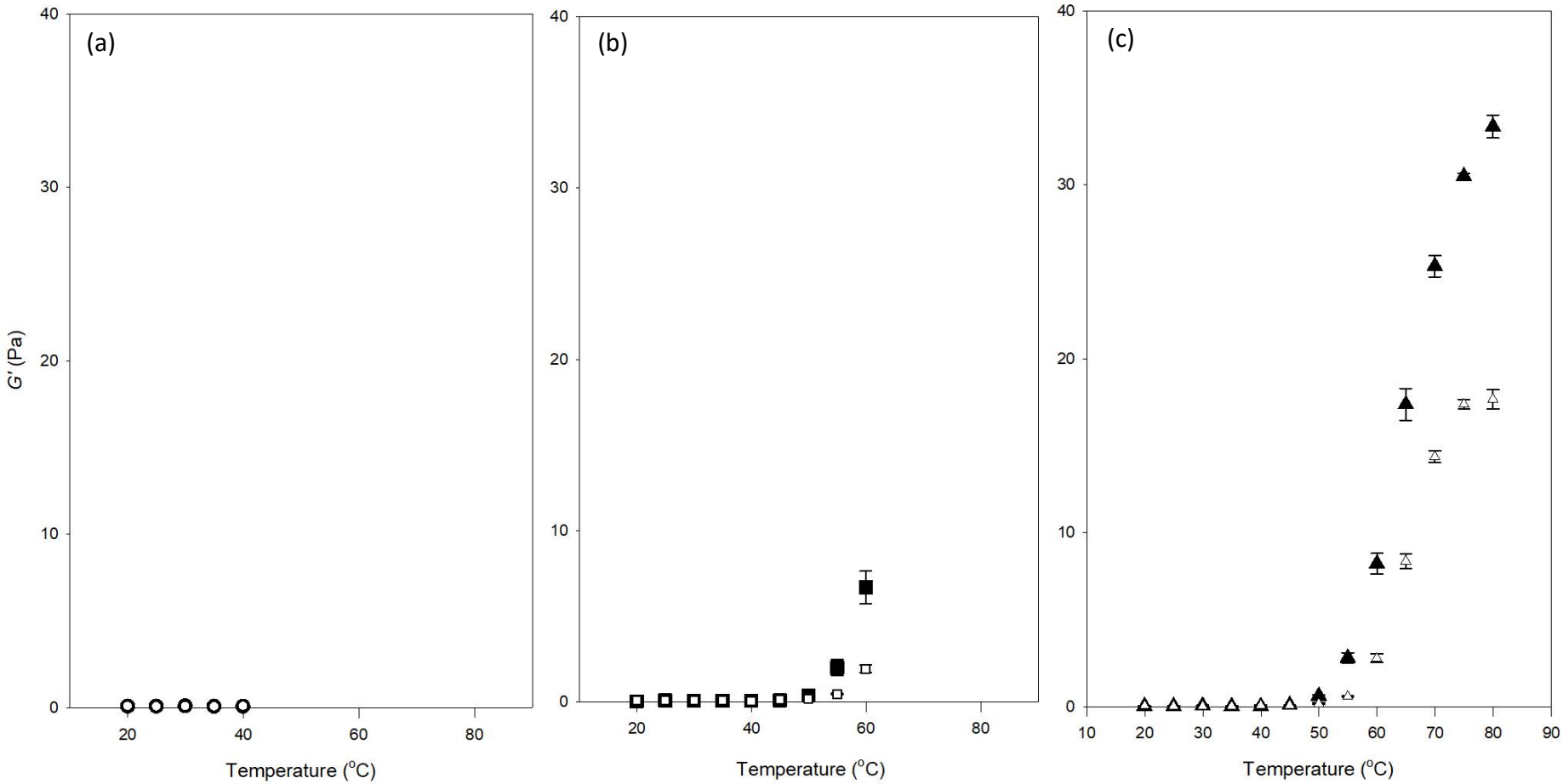


Figure 4.14: The change of storage modulus *G'* with temperature for 10% non-preheated (open symbols) and preheated (close symbols) skim milk with 15 mmol L^{-1} zinc acetate added and heated to different temperature: (a) 40°C (○, ●); (b) 60°C (□, ■) and (c) 80°C (△, ▲). Results presented are means ± standard deviation (n = 3).

The gelation temperature was observed to be 60°C for non-preheated samples and 55 °C for preheated samples (Figure 4.14). The gel strength was developed mainly during the heating phase for the samples heated to 80°C which reached the *G'* values of 33.36 ± 0.66 Pa (preheated) and 17.67 ± 0.55 Pa (non-preheated) after heated to 80°C (Figure 4.14). For the preheated sample, no further increase in *G'* was observed after 15 minutes holding at 80°C (Figure 4.15), with the maximum *G'* ~ 39 Pa. However, for the non-preheated sample, the *G'* plateaued after the first 10 minutes holding but then the *G'* further increased until the end of holding phase (Figure 4.15). The plateau period and subsequent increase in *G'* during holding at 80°C for the non-preheated sample was discussed in Section 4.5.2.1., which may have been due to whey protein denaturation at high temperature (>70°C).

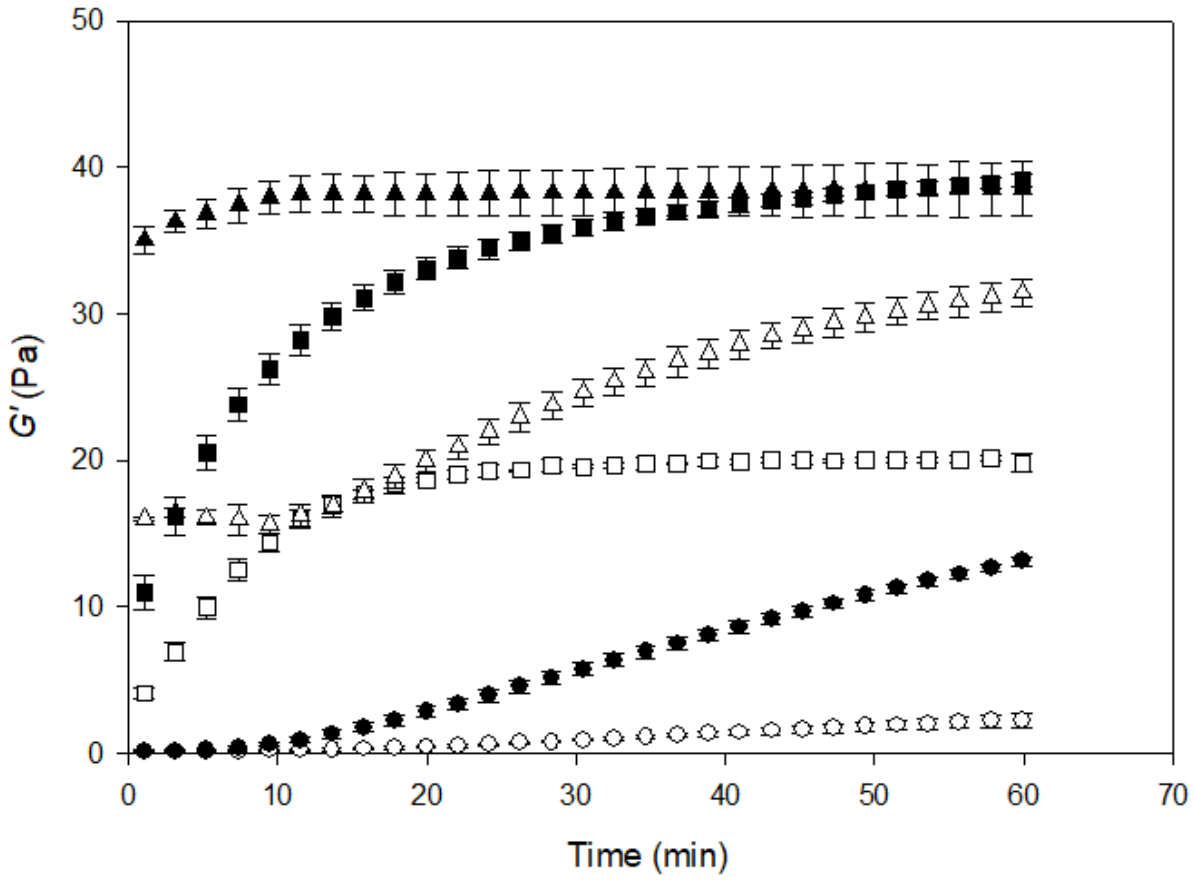


Figure 4.15: The change of storage modulus *G'* with time for 10% non-preheated (open symbols) and preheated (close symbols) skim milk with 15 mmol L^{-1} zinc acetate added and held at a different temperature: 40°C (○, ●); 60°C (□, ■) and 80°C (△, ▲). Results presented are means ± standard deviation (n = 3).

To conclude, the *G'* during heating of both preheated and non-preheated skim milks increased exponentially after 60°C and at a higher temperature the gelation rate was faster. Holding the preheated sample at 60°C, eventually resultiend in a similar *G'* (~ 39 Pa) as at 80°C but it took longer than holding at 80°C. A similar phenomenon was also reported by Begum (2019) and Lin *et al*. (2020) for the preheated skim milk with the addition of magnesium chloride and calcium chloride. The authors suggested the gelation rate of the mineral added preheated skim milk with temperature followed an Arrhenius relationship.

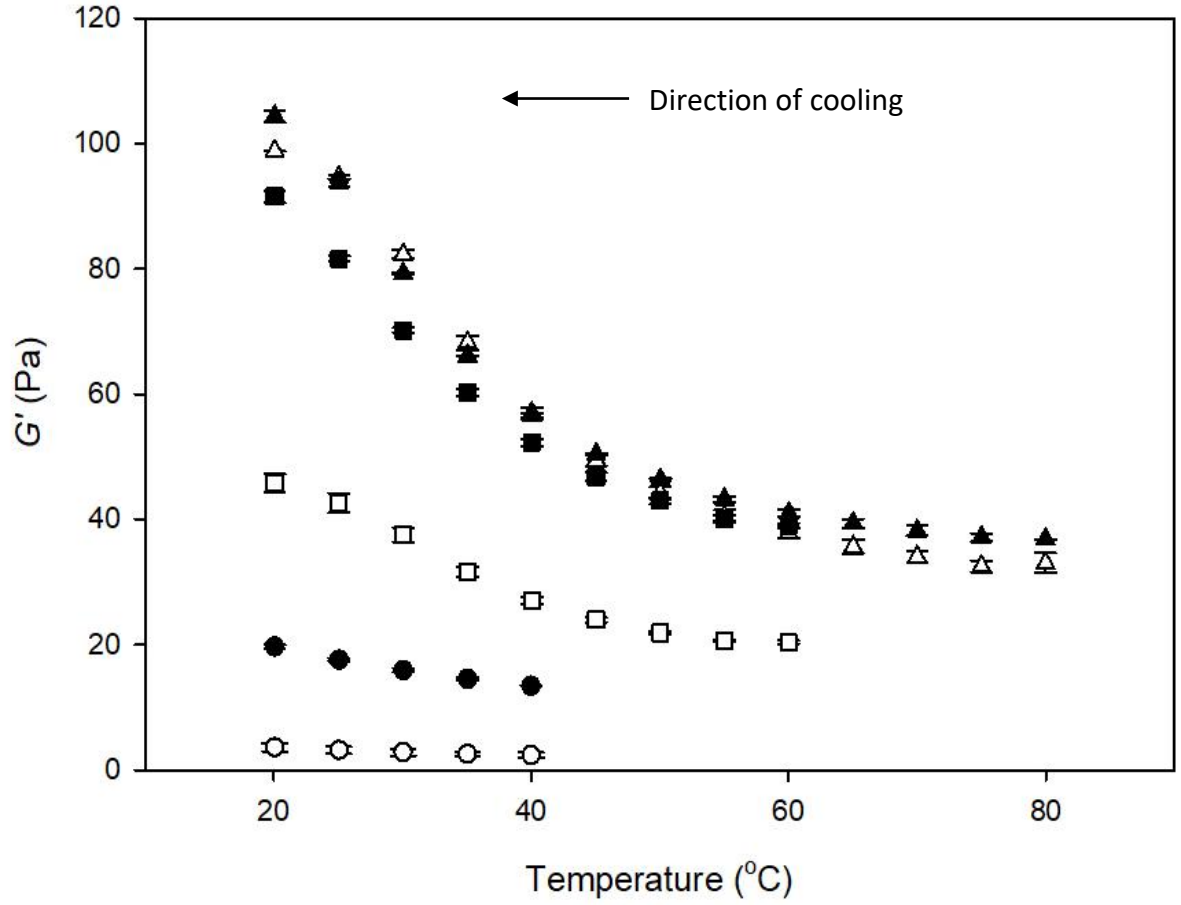


Figure 4.16: The change of storage modulus *G'* with temperature decreased from 80°C (△, ▲); 60°C (□, ■) and 40°C (○, ●) to 20°C for 10% non-preheated (open symbols) and preheated (close symbols) skim milk with 15 mmol L^{-1} zinc acetate added. Results presented are means ± standard deviation (n = 3).

4.6. Conclusions

Overall, the results presented in this chapter showed that the addition of zinc acetate can lead to a change in the ionic equilibrium in the serum and significantly lower the non-preheated skim milk pH. In the native skim milk, most of the zinc is presented in the colloidal phase. When zinc acetate was added to the skim milk, the zinc and calcium distribution changed. The zinc concentration in both serum and colloidal phase increased with the further addition of zinc acetate, but the calcium in the colloidal phase was partially replaced by the zinc added and resulted in a decrease in the colloidal phase calcium concentration. At 20^{o}C, there was a significant increase (p<0.05) in skim milk viscosity when the concentration of added zinc acetate increased to 17.5 mmol L^{-1}, and the skim milk behaviour transformed from a Newtonian fluid to a non-Newtonian fluid when more than 20 mmol L^{-1} zinc acetate was added. High concentrations, ≥ 22.5 mmol L^{-1} of added zinc acetate lead to skim milk gelation at 20 ^{o}C, and the final *G'* of skim milk increased with zinc acetate concentration. The increase in skim milk viscosity and final *G'* was likely explained by gel network structure formed by the added zinc ions and casein micelles. The gel network formation and development can significantly be influenced by heat treatment. When heat treatment was applied (80^{o}C), the zinc+skim milk gelation can occur at a lower added zinc acetate concentration (5 mmol L^{-1} for the preheated sample and 10 mmol L^{-1} for the non-preheated sample). Preheat treatment prior to the addition of zinc acetate can lead to whey protein denaturation and result in stronger gel strength. Holding temperature is another condition that can influence the zinc+skim milk gelation, higher holding temperature can lead to faster gelation rate. However, further research is required to determine the gel structure of non-preheated skim milk with the addition of zinc acetate.

Chapter 5. Effect of different soluble zinc salts on skim milk

5.1. Introduction

Zinc chloride and zinc sulphate are the two common zinc salts that have been used in zinc added milk studies due to their high solubility (Kahraman & Ustunol, 2012; Singh *et al.*, 1989a, 1989b). Several studies have found the organic zinc salts have higher bioavailability than the inorganic zinc salts (Seal & Heaton, 1983; Gibson, 2012). Hydrolysis of zinc chloride occurs at low concentrations where the salt can be hyrolysed to zinc oxychloride (Philippe *et al*., 2005; Peacock & Peacock, 1918). A few drops of hydrochloric acid can inhibit the hydrolysis but will also lower the solution pH (Peacock and Peacock, 1918). To avoid the significant pH effect on the zinc-induced skim milk, zinc chloride was not chosen for this study. Zinc sulphate was chosen as the inorganic zinc salt, zinc acetate and zinc gluconate were chosen as the organic zinc salts to study the effect of zinc salt type on skim milk solution properties. The order of zinc bioavailability from highest to lowest is zinc gluconate > zinc acetate > zinc sulphate (Seal & Heaton, 1983; Sapota *et al.*, 2014). Studies have reported the addition of different calcium salts can influence milk protein stability and lead to protein precipitation differently (Crowley *et al.*, 2014; Lin *et al.*, 2018). The Hofmeister series of ions was first proposed many years ago based on the salt and salt concentration required to cause egg white protein precipitation in aqueous solutions (Kunz *et al.*, 2004). Based on the Hofmeister effect, different anions released from different zinc salts may influence the milk protein stability differently (Gao, 2012). There is a lack of information in the literature about the effects of different zinc salts on milk physicochemical properties. Thus, this chapter aimed to focus on the effect of different zinc salts on the pH, zinc distribution and rheological properties of skim milk.

5.2. Experimental design

A summary of the experimental plan to investigate the effect of different zinc salts and pH on the zinc+skim milk physicochemical and rheological properties is shown in Figure 5.1. Two sets of experiments were carried out and only non-preheated skim milk was used for the studies in this chapter. Firstly, the effect of different zinc salts on skim milk pH, zinc distribution and rheological properties was assessed, with no pH adjustment. The samples were prepared by using non-preheated skim milk with the addition of 200 mmol L^{-1} zinc acetate, zinc sulphate or zinc gluconate to form zinc+skim milk with different final added zinc salt concentrations (0 - 40 mmol L^{-1}). Secondly, the effect of pH on skim milk rheological properties with and without addition of zinc salt was assessed. For the zinc+skim milk samples, the pH was adjusted to 6.73 ± 0.03 after addition of zinc salts to study the effect of pH for zinc+skim milk samples with different zinc salts added. To determine the effect of pH reduction on rheological properties, the pH of 10% (w/w) skim milk, with no added zinc salt, was adjusted to 5.03 ± 0.02, which is the lowest zinc+skim milk pH when 40 mmol L^{-1} zinc sulphate added to skim milk. The detail of the methodology used in this chapter can be found in Chapter 3.

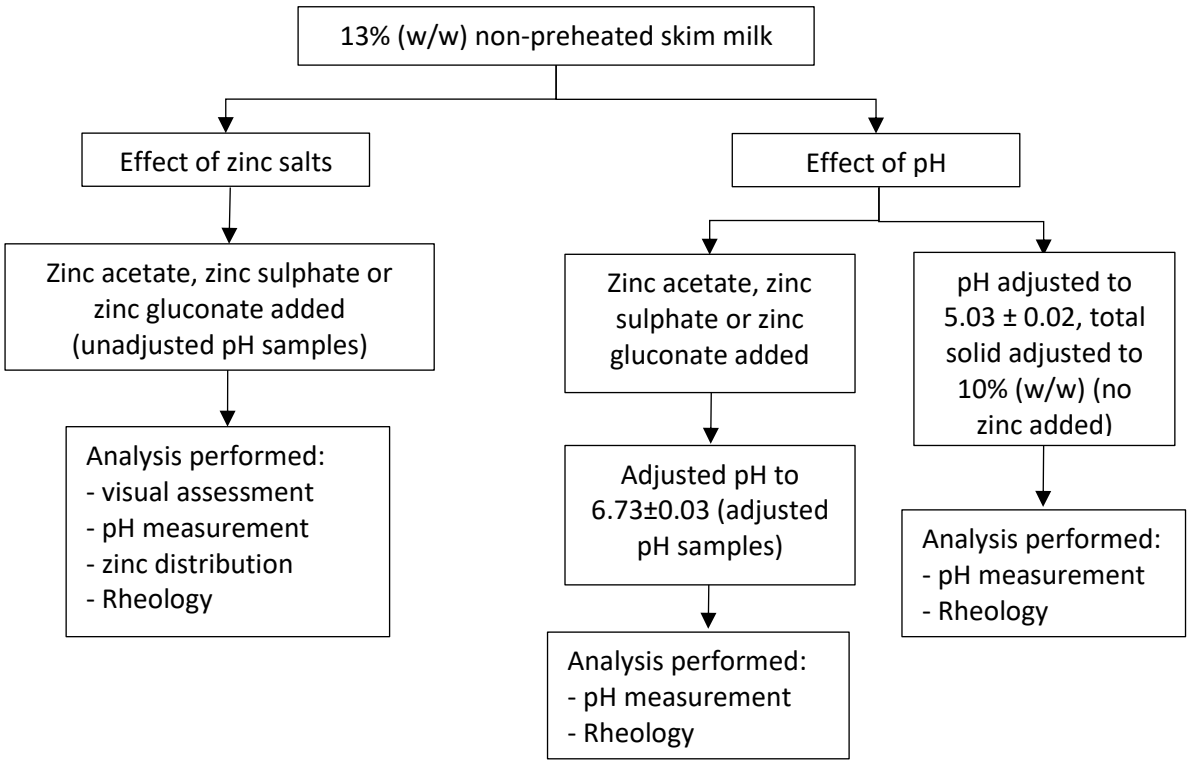


Figure 5.1: Summary of the experimental plan to investigate the effect of different zinc salts and pH.

5.2.1. Zinc+skim milk sample preparation

The 13% (w/w) non-preheated reconstituted skim milk samples were prepared according to Section 3.2. Zinc salt stock solutions (zinc acetate, zinc gluconate and zinc sulphate) were prepared according to Section 3.3 with the same zinc concentration of 200 mmol L^{-1}. Different amounts of zinc salt stock solutions were added to the 13% (w/w) non-preheated reconstituted skim milk to prepare 10% (w/w) non-preheated skim milk with different added zinc concentrations (0 to 40 mmol L^{-1}). The detail of the zinc+skim milk preparation can be found in Section 3.5.

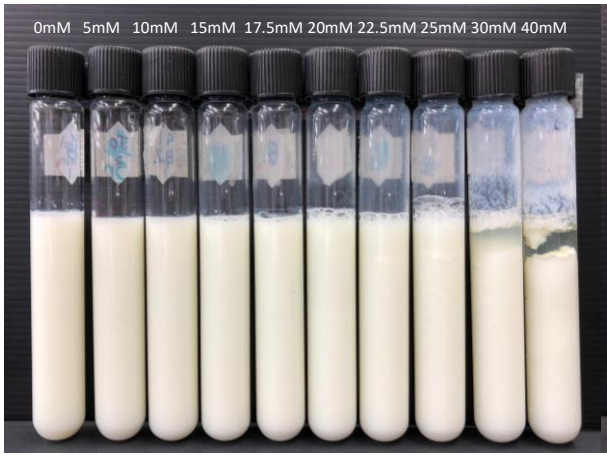
5.2.2. Analysis of zinc+skim milk samples

This chapter reports the effect of adding different zinc salts on the physicochemical and rheological properties of skim milk. The analyses methodology used in this chapter can be found in Section 3.6 to 3.9, visual assessment and rheology analysis were as described in Section 3.11 and 3.12. No heat treatment was applied to all the zinc+skim milk samples during preparation, analysis or characterisation in this chapter.

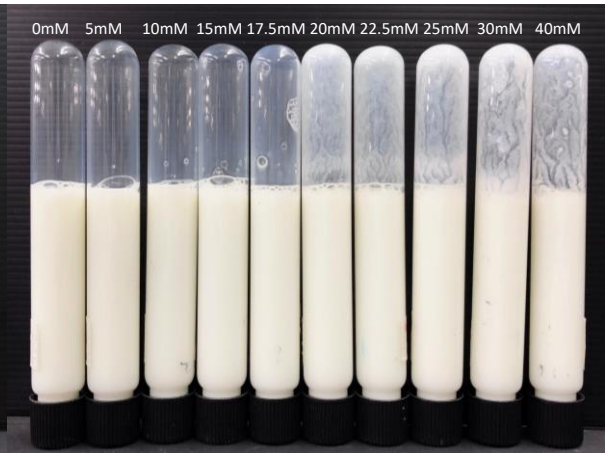
5.3. Visual assessment of zinc+skim milk samples

To investigate the effect of different soluble zinc salts on skim milk, the samples were observed after adding different concentrations of zinc acetate, zinc sulphate and zinc gluconate. The photographs are presented in Figure 5.2, and the summaries of observation are presented in Table 5.1. Skim milk appeared more viscous after 20 mmol L^{-1} zinc acetate was added. For skim milk with added zinc sulphate and zinc gluconate, similar thickening was observed at the higher addition level of 25 mmol L^{-1}. Serum separation was observed with the addition of 30 mmol L^{-1} zinc salts, regardless of the type.

(a) zinc acetate



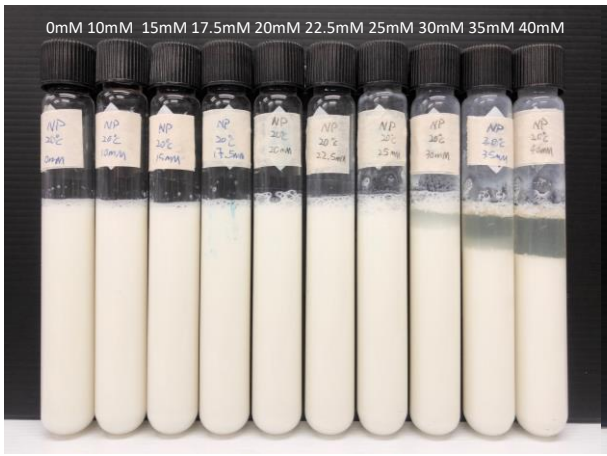
(b) zinc acetate – inverted



(c) zinc sulphate

(d) zinc sulphate - inverted

(e) zinc gluconate



(f) zinc gluconate - inverted

Figure 5.2: Skim milk samples were held at 20 ± 1°C for 60 min with (a) and (b) zinc acetate; (c) and (d) zinc sulphate; (e) and (f) zinc gluconate: concentration of zinc slat added from 0 to 40 mmol L^{-1}.

Table 5.1: Observations of non-preheated skim milk with different added zinc salt concentrations after holding for 60 min at 20 ± 1°C (n=3).

Added zinc salt concentration (mmol L^{-1})	Zinc salt added		
	Zinc acetate	Zinc sulphate	Zinc gluconate
0	Liquid	Liquid	Liquid
5	Liquid	-	-
10	Liquid	Liquid	Liquid
15	Liquid	Liquid	Liquid
17.5	Liquid	Liquid	Liquid
20	Thickened	Liquid	Liquid
22.5	Thickened	Liquid	Liquid
25	Thickened	Thickened	Thickened
30	Serum separated	Serum separated	Serum separated
35	-	Serum separated	Serum separated
40	Serum separated	Serum separated	Serum separated

Thickened: Milk flows slow enough to coat the walls of test tubes when inverted.
Serum separation: a visible transparent or semi-transparent top layer of liquid was observed after holding and before inverting the tubes.
-: Not tested

5.4. pH of zinc+skim milk samples

The change in pH after different zinc salts were added to skim milk are shown in Figure 5.3, the number of skim milk pH can be found in Appendix 10. As discussed in Section 4.3.2, the addition of zinc acetate may disrupt the ionic equilibrium of the skim milk, and the pH of zinc+skim milk decreased with increasing zinc acetate concentration (Table 4.1). It was found the addition of zinc sulphate resulted in the greatest pH drop in non-preheated skim milk, followed by zinc gluconate and zinc acetate. Since the change in skim milk pH was different when different types of zinc salt were added, the pH of the zinc+skim milk may depend not only on the zinc concentration, but also due to the difference in the hydrolysis of the anion from the zinc salt (Krężel & Maret, 2016).

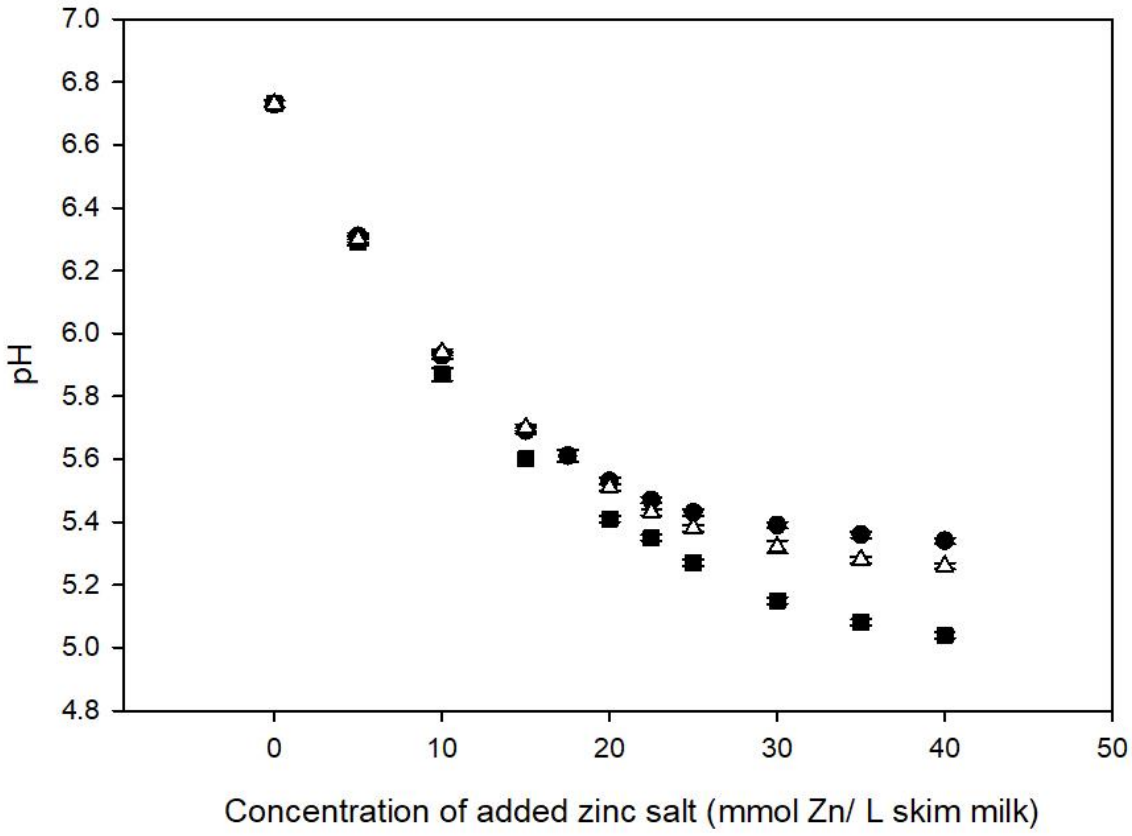


Figure 5.3: The pH of 10% (w/w) skim milk after addition of various concentrations of zinc acetate (●); zinc sulphate (■); zinc gluconate (△). Results presented are means ± standard deviation (n=3).

For strong acid anions (such as sulphate ions), the anions are extremely weak bases and will not cause water hydrolysis which means they will not affect the solution pH (Housecroft & Sharpe, 2008; Krężel & Maret, 2016). Thus, the zinc sulphate solution pH is only influenced by the zinc cations, which leads to the most significant decrease in skim milk pH at the same added zinc salt concentration. In contrast, weak acid anions like acetate and gluconate ions are stronger bases which can cause water hydrolysis and influence the solution pH. Using zinc acetate as an example, both zinc cations and acetate anions can cause hydrolysis of water (Equation 5.1 and 5.2) (Brown, 2018).

$$Zn^{2+} + 6H_2O \rightleftarrows [Zn(OH)(H_2O)_5]^+ + H_3O^+$$

Equation 5.1

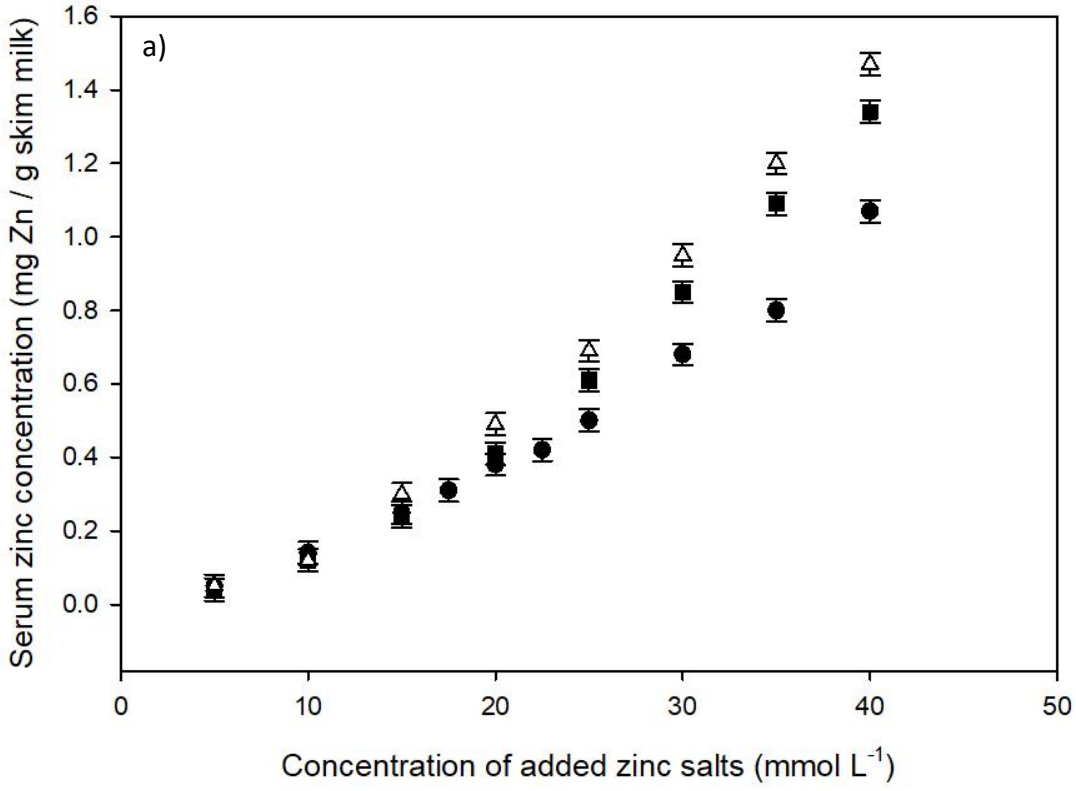
$$CH_3COO^- + H_2O \rightleftarrows CH_3COOH + OH^-$$

Equation 5.2

The hydrolysis of water caused by the addition of zinc ions may lower the solution pH, but the acetate anions may react like conjugate bases and lead to water hydrolysis which increases the solution pH. The pK_a (acidity coefficient) for zinc hydrolysis is approximately 8.96 (James, 2017), which is close to the pK_b (basicity factor) for an acetate anion pair, 9.2 (Krężel & Maret, 2016). Thus, the effect of water hydrolysis caused by zinc ions and acetate ions on solution pH is almost counteracted and leading to a zinc acetate aqueous solution pH close to the natural pH of water (Krężel & Maret, 2016). The basicity of gluconate ions is lower than the acetate ions and results in less water hydrolysis (Ramachandran *et al.*, 2006). Thus, the pH of the skim milk with the addition of zinc gluconate at the same molar concentration will be lower than that of zinc acetate solution.

5.5. Zinc distribution in zinc+skim milk

It has been reported that when zinc is added to milk, the distribution of zinc in each fraction can vary (Blakeborough *et al.*, 1983; Cousins and Smith, 1980; Lin, 2019; Parkash & Jenness, 1967; Singh *et al.*, 1989a, 1989b). To investigate the zinc distribution when different zinc salts were added, the serum and sediment (colloidal phase) zinc concentration was determined by EDTA titration method. As shown in Section 4.3.3.1, the EDTA titration method was verified by MPAES method and can be used to measure the zinc concentration in a liquid sample with a limit of detection of 0.03 mg zinc / g sample. Figure 5.4 shows the zinc concentration in the serum and colloidal phase with different types of zinc salts added. The zinc concentration in the colloidal phase was calculated by subtracting the serum zinc concentration from the skim milk zinc concentration (Equation 3.3). With the added zinc concentration increasing, the zinc concentration in skim milk, serum and colloidal phase increased regardless of the zinc salt type (Figure 5.4).



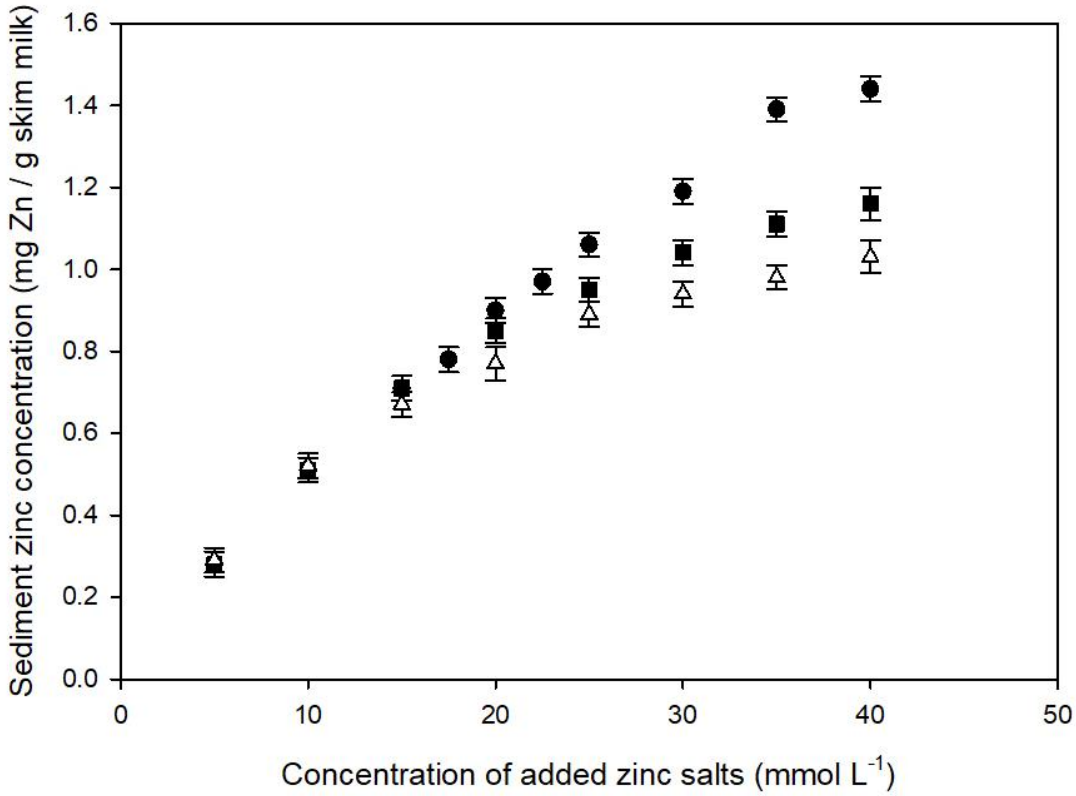


Figure 5.4: Serum (a) and sediment (colloidal phase) (b) zinc concentration in skim milk with various addition concentration of zinc acetate (●); zinc sulphate (■); zinc gluconate (△) determined by EDTA titration method. Results presented were means ± sd (n=3).

The skim milk, serum and colloidal phase zinc concentrations with different concentrations of added zinc salts can be found in Appendix 11. At the same added zinc salt concentration, no significant difference (p>0.05) was observed for the measured zinc concentration in skim milk across all the different added zinc salt types (Appendix 11). For the skim milk with low added zinc salt concentrations (≤ 10 mmol L^{-1}), there was no significant difference (p>0.05) observed in the serum and colloidal phase zinc concentrations for all zinc salts. When the added zinc salt concentration increased to greater than 15 mmol L^{-1}, a significant difference (p<0.05) in the serum zinc concentration was found between zinc acetate and zinc gluconate added samples, with higher values observed with zinc gluconate. When the added zinc salt concentration was greater than 25 mmol L^{-1}, there was a significant difference (p>0.05) in serum zinc concentration between all three different zinc salts. The concentration of zinc in the serum in order of highest to lowest at the same addition level (≥ 25 mmol L^{-1}) was zinc gluconate > zinc sulphate > zinc acetate (Figure 5.4a). The zinc concentration in the colloidal phase followed the reverse order to the serum (Figure 5.4b). Since the colloidal phase will sediment during microcentrifugation, the zinc concentration in the serum was considered to be the soluble zinc concentration.

The difference in serum and colloidal phase zinc concentration caused by the addition of different zinc salts at the same zinc concentration may be explained by the stability constants (K) of the three zinc salts. According to Smith & Martell (1976), the smaller the stability constants of a metal complex implies a lower affinity of the anion for Zn^{2+}, therefore, more free zinc ions (Zn^{2+}) are released into the solution. Larger amounts of Zn^{2+} released from the added zinc salt may result in more zinc ions available to associate with the casein micelles and therefore, are sedimented during microcentrifugation, which results in the increased zinc concentration in the colloidal phase. The stability constants for the three zinc salts are listed in Table 5.2. The zinc and anions released from added zinc salts do not fully exist as free ions but can also form zinc-anion complexes (Eby, 1997; Krężel & Maret, 2016; Vavrusova *et al*., 2014). According to James (2017), no zinc acetate ($Zn(OAc)_2$) is present in an aqueous solution, hence only $Zn(OAc)^+$ is formed and its stability constant is low at 8.51 (Krężel & Maret, 2016). The formation of zinc sulphate requires one step, but the stability constant for zinc sulphate is 218.78 (Krężel & Maret, 2016). There are two steps required for the zinc gluconate

association (Table 5.2). The first step's stability constant can be determined from Equation 5.3:

$$K_1 = \frac{[ZnL^+]}{[Zn^{2+}][L^-]}$$

Equation 5.3

The second step's stability constant can be determined from Equation 5.4:

$$K_2 = \frac{[ZnL^2]}{[ZnL^+][L^-]}$$

Equation 5.4

The cumulative or overall stability constant for zinc gluconate can be calculated from equation 5.5:

$$K_{zinc\ gluconate} = \frac{[ZnL^2]}{[Zn^{2+}][L^-]^2}$$

Equation 5.5

Where ZnL^2 is the zinc gluconate, L^- is the gluconate group and ZnL^+ indicates the intermediate complex of the gluconate anionic group with zinc.

Eby (1997) has reported the first step stability constant for zinc gluconate is 41.69. However, there was lack of information about the second step stability constant and the cumulative stability constant. Compared to zinc sulphate and zinc gluconate, the smallest stability constant for zinc acetate indicates that most of the zinc acetate dissociates into free zinc ions and free acetate ions, and a small amount of zinc-acetate ions ($Zn(OAc)^+$) in the skim milk solution. The positively charged $Zn(OAc)^+$ may also associate with casein micelles and increase the colloidal phase zinc concentration. Lin *et al.* (2018) reported that when calcium gluconate was added into skim milk, not only the dissociated calcium ions associated with casein micelles, but also the calcium-gluconate ions (CaL^+). Since zinc and calcium are competing for the same binding sites on casein micelles (Section 4.3.3), the positively charged ZnL^+ may also associate with casein micelles and increase the colloidal phase zinc concentration.

Table 5.2: Zinc salts stepwise formation and stability constants (Eby, 1997; Krężel & Maret, 2016).

Zinc salts	Structural formula	Stability constant K
Zinc acetate dihydrate ($Zn(C_2H_3O_2) \cdot 2H_2O$)	$[Zn]^{2+} + OAc^{-} \rightleftharpoons [Zn(OAc)]^{+}$	K = 8.51 [a]
Zinc sulphate heptahydrate ($ZnSO_4 \cdot 7H_2O$)	$[Zn]^{2+} + SO_4^{2-} \rightleftharpoons [ZnSO_4]$	K = 218.78 [b]
Zinc gluconate hydrate ($ZnC_{12}H_{22}O_{14}$)	$[Zn]^{2+} + L^{-} \rightleftharpoons [ZnL]^{+}$ $[ZnL]^{+} + L^{-} \rightleftharpoons [ZnL_2]$	K_1=41.69 [c]

OAc: acetate group
L: gluconate group.
[a] 25°C, ionic strength = 0.1 mol L^{-1}
[b] 25°C, ionic strength = unknown
[c] First stability constant K_1, not the cumulative stability constant, pH = 7.4

5.6. Rheological properties of zinc+skim milk samples

5.6.1. Fluid flow behaviour and viscosity analysis

From visual assessment, skim milk with zinc sulphate and zinc gluconate added thickened at a higher added zinc salt concentration (25 mmol L^{-1}) compared with the addition of zinc acetate (20 mmol L^{-1}) (Table 5.1 and Figure 5.2). To investigate the skim milk flow behaviour with low zinc salt concentrations (0 to 25 mmol L^{-1}), flow curve analysis (shear stress versus shear rate) at room temperature (20 ± 1°C) (Section 3.12.1),the results are shown in Figure 5.5. For the samples with zinc acetate (22.5 mmol L^{-1} and 25 mmol L^{-1}) and zinc sulphate (25 mmol L^{-1}) added, analysis with a flow curve was not carried out as a gel was formed. The absolute viscosity of the zinc+skim milk increased with added zinc salt concentration increasing from 0 to 20mmol L^{-1} regardless of the added zinc salt type (Figure 5.5). It was noted that at the same added zinc salt concentration, the order of zinc+skim milk absolute viscosity from high to low was zinc acetate > zinc sulphate > zinc gluconate (Figure 5.5).

As presented in Chapter 4, there was a transition from Newtonian behaviour to non-Newtonian behaviour when the addition of zinc acetate concentration reached 20 mmol L^{-1}. A similar phenomenon was observed for the zinc+skim milk samples with the addition of zinc sulphate and zinc gluconate. However, the transition from Newtonian fluid to non-Newtonian fluid happened at a higher added zinc concentration, 22.5 mmol L^{-1} for zinc sulphate and 25 mmol L^{-1} for zinc gluconate (Figure 5.5 and Appendix 12). To conclude, the concentration of zinc salt added to skim milk resulting in a change from Newtonian to non-Newtonian behaviour from low to high was zinc acetate (20 mmol L^{-1}) < zinc sulphate (22.5 mmol L^{-1}) and zinc gluconate (25 mmol L^{-1}).

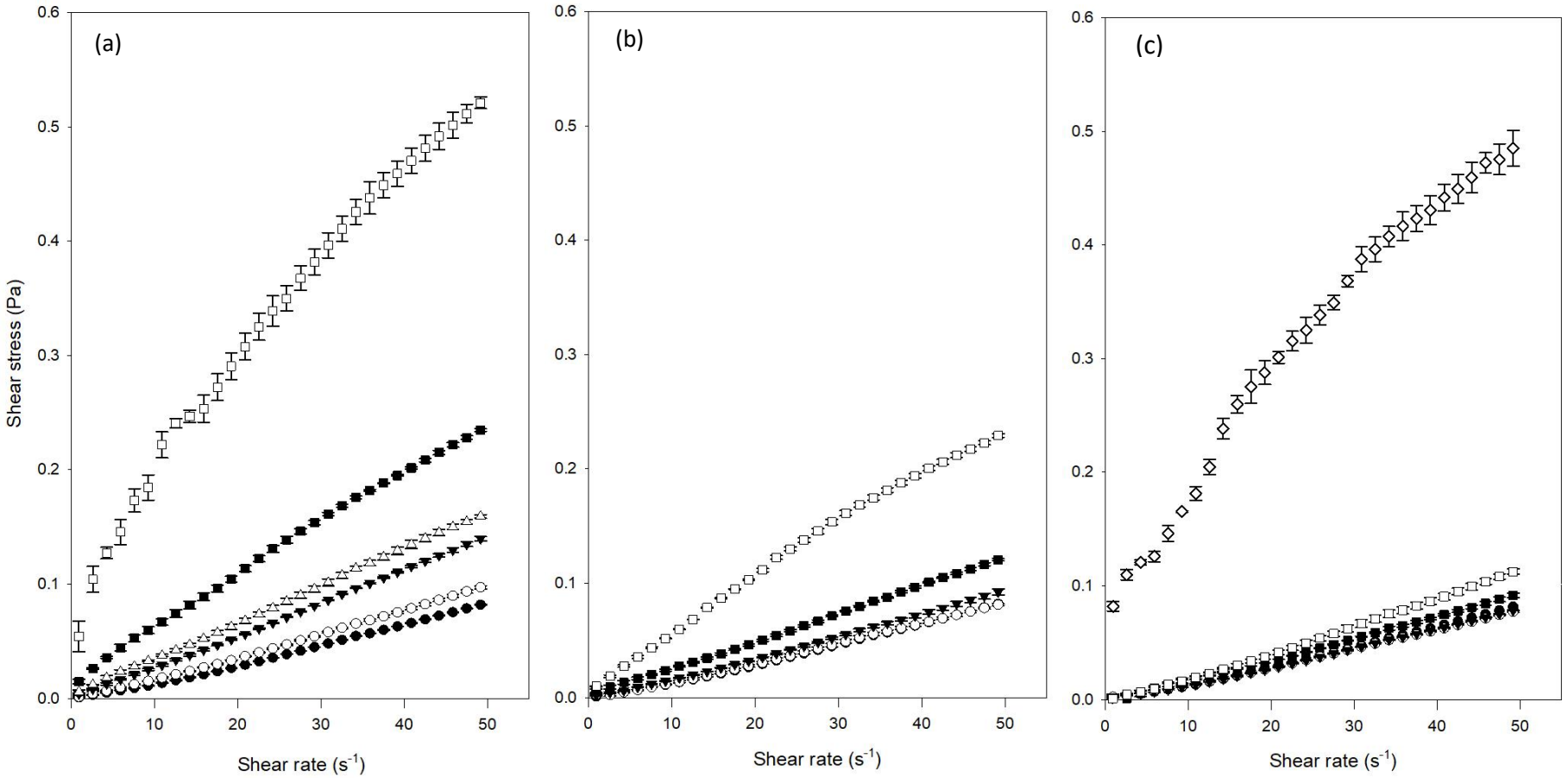


Figure 5.5: The shear stress versus shear rate at 20 ± 1°C for 10% (w/w) total solid skim milk with various addition concentration of (a) zinc acetate; (b)zinc sulphate and (c) zinc gluconate: 0 mmol L^{-1} (●); 10 mmol L^{-1} (○); 15 mmol L^{-1} (▼); 17.5 mmol L^{-1} (△); 20 mmol L^{-1} (■); 22.5 mmol L^{-1} (□) and 25 mmol L^{-1} (◇). Results presented are means ± standard deviation (n=3).

To investigate the effect of the type of zinc salt on zinc+skim milk viscosity, the change of zinc+skim milk's viscosity with time was analysed at constant shear rate of 25 s^{-1} and held at 20°C for 3 hours. The final absolute or apparent viscosity after 3 hours holding is presented in Figure 5.6. It was noted that a higher final absolute or apparent viscosity for zinc+skim milk was observed when higher concentrations of zinc salt were added, regardless of the zinc salt type. However, the degree of increase in the final absolute or apparent viscosity was different with different zinc salts added. At the same molar concentration of added zinc salt, the order of skim milk final absolute or apparent viscosity from high to low was zinc acetate > zinc sulphate > zinc gluconate. An increase in milk absolute viscosity with added zinc salts was reported previously by Rana *et al.*, (2018). However, Rana *et al.* (2018) found zinc sulphate fortified (0.34 mmol Zn / L sample) whole milk showed higher absolute viscosity changes compared to zinc acetate, which is opposite to what was observed in this study. The possible explanation for the inverted order may be the relatively low added zinc salt concentrations used by Rana *et al*. (2018) (0.34 mmol Zn / L sample), the absolute viscosity at that low added zinc salt concentrations was not measured in this study. In addition, the state of fat globules and the heat treatment difference may also influence the absolute viscosity of samples, since Rana *et al*.(2018) used preheated (at 63°C for 30 minutes) whole milk and the zinc+milk was mixed at 45°C for 15 minutes before incubating at 20°C.

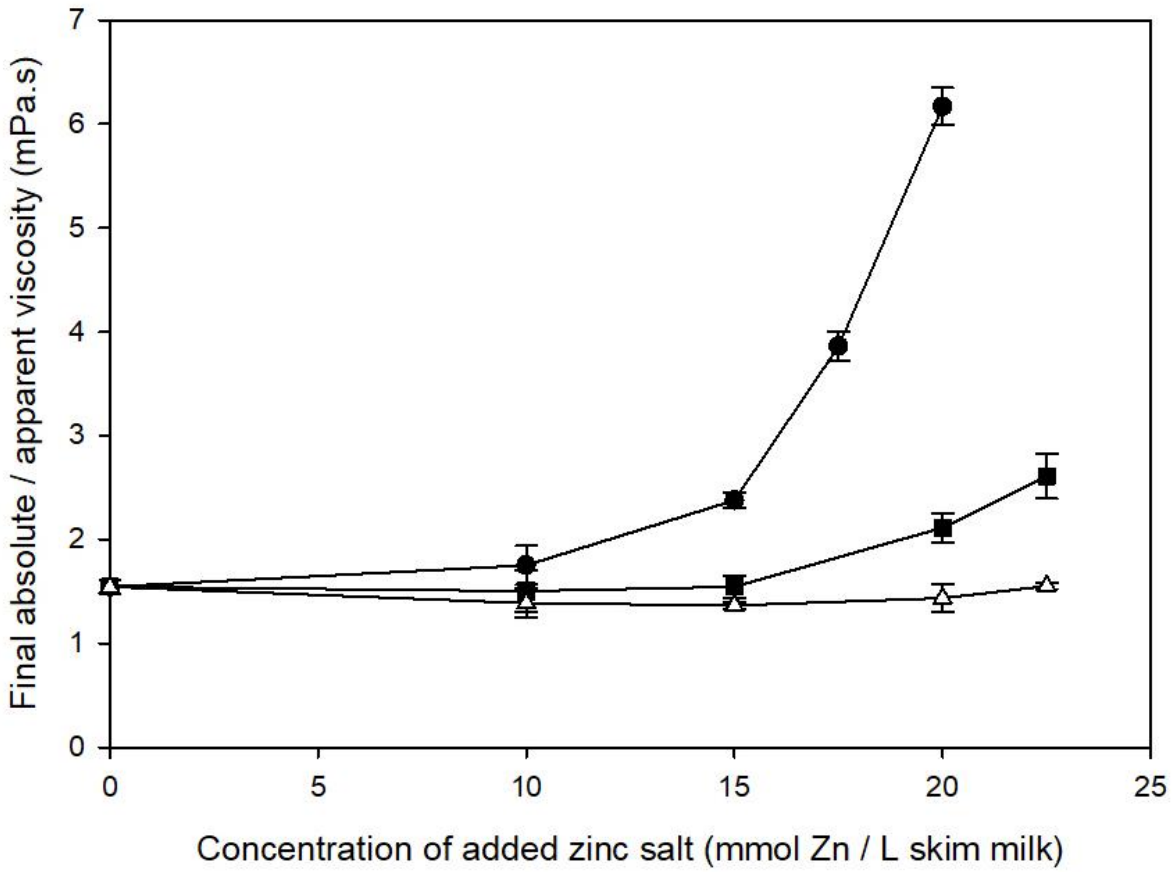


Figure 5.6: Final absolute or apparent viscosity determined after held at 20 ± 1°C for 3 hours for 10% (w/w) skim milk with various concentrations of zinc acetate (●); zinc sulphate (■); zinc gluconate (△). A constant shear rate of 25 s^{-1} was used for this analysis. Results presented were mean ± standard deviation (n=3).

5.6.2. Gelation properties by rheological measurement

A time sweep at 20 ± 1°C for 3 hours was carried out for zinc+skim milk samples with different zinc salt concentrations (20 mmol L^{-1} to 40mmol L^{-1}) and a frequency sweep was applied for the samples which formed a gel after holding (final *G'*>1 Pa). The *G'* at the end of the time sweep was considered as final *G'*. The *G'* of zinc+skim milk samples increased with increasing concentration of added zinc salts (Figure 5.7), the number of final G' can be found in Appendix 13. For skim milk with added zinc acetate, gelation occurred when the added zinc salt concentration was 22.5 mmol L^{-1}, while for zinc sulphate or zinc gluconate at 30 mmol L^{-1}. At the same concentration of added zinc salt, the order of the final *G'* from highest to lowest was zinc acetate > zinc sulphate > zinc gluconate.

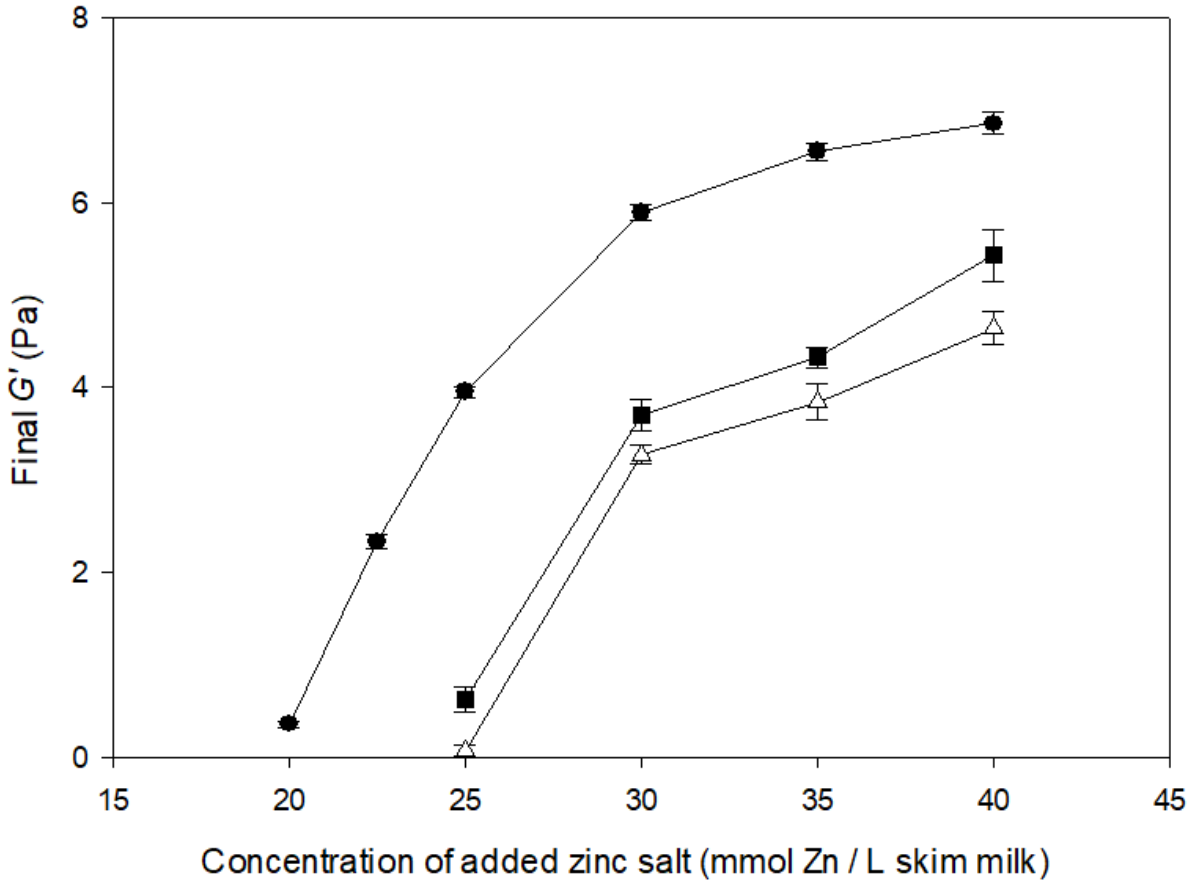


Figure 5.7: Final *G'* of 10% (w/w) skim milk with various addition concentration (20 to 40 mmol L^{-1}) of zinc acetate (●); zinc sulphate (■); zinc gluconate (△). Results presented were mean ± standard deviation (n=3).

As discussed in Chapter 4, the amount of zinc associated with casein micelles contributed mostly to the zinc-added skim milk gel strength. It was noted that the final *G'* increased with increase in colloidal phase zinc concentration regardless of the types of zinc salt. At the same added molar concentration of zinc salt, the sample with higher colloidal phase zinc concentration (Figure 5.4b) resulted in the higher final *G'* (Figure 5.7).

The metal salt anions may also influence protein stability (Yang, 2009). The dissolution of salts in water will change a series of physical and chemical properties, such as hydrogen bond network structure between water molecules, viscosity coefficient of water and surface tension (Yang, 2009). These changes can affect the dissolution of proteins and other biological macromolecules in water (Gao, 2012). As early as 1888, Hofmeister found that different salts have different effects on the blood serum and egg white proteins precipitation and found the required concentration of salts to cause protein precipitation was different (Kunz *et al.*, 2004).

Based on these findings, different salt ions can be arranged in a sequence, which is known as the Hofmeister Series (Collins *et al.*, 2007; Kunz *et al.*, 2004). The order of anions in terms of those that had the greatest affect on protein solubility was found to be: SO_4^{2-} > HPO_4^{2-} > CH_3COO^- > F^- > Cl^- > Br^- > NO_3^- > I^- > ClO_4^- > SCN^- (Yang, 2009). Studies of this sequence suggest that the ions on the left side (like SO_4) tend to have a stronger interaction with water molecules and snatch the water molecules that originally associated with the protein surface (Hyde *et al.*, 2017). The hydrophobic effect of the protein will then increase and result in a reduction of the protein surface area exposed to the solvent (Yang, 2009). The decrease in available surface area may lead to fewer zinc binding sites exposed and a reduced number of zinc salt bridges formed between casein micelles. Unlike the SO_4^{2-}, Arakawa and Timasheff (1982) have reported the association of acetate ions (OAc^-) with the bovine serum albumin protein-water interface cannot be neglected. The association of OAc^- with the protein-water interface can destabilise the protein by reducing the hydrophobic effect and lead to protein stretching (Yang, 2009). Thus, with zinc acetate, more zinc binding sites may become exposed in the skim milk resulting in more zinc ions bound to the protein and the formation of more salt bridges which increases the gel strength. Therefore, at the same added zinc concentration, the skim milk with zinc sulphate added resulted in a lower final *G'* compared to the skim milk with acetate added at the same addition of zinc concentration (Figure 5.7). There was lack of information in the literature on the gluconate ions and zinc-gluconate ions (ZnL^+) association with milk proteins and how the ions influenced the milk gelation properties. However, Lin *et al.* (2018) reported that the particle size of the casein micelle after addition of organic calcium salts like calcium lactobionate and calcium gluconate was significantly larger than the inorganic calcium salts like calcium chloride, but the final *G'* for the gels was in the reverse order to the observed particle size. The possible reason is the interaction between calcium-organic ligand ions (CaL^+) and proteins may hinder protein aggregation and gelation due to steric hindrance (Lin *et al.*, 2018). Similar mechanisms may be applied to this study to explain the lowest final *G'* for skim milk with the addition of zinc gluconate at the same added zinc concentration compared with skim milk + zinc acetate and skim milk + zinc sulphate (Figure 5.7). The ZnL^+ associated with the zinc binding sites on casein micelles may prevent the formation of zinc salt bridges between casein micelles as it only has one positive charge and resulted in the lowest final *G'* at the same added zinc molar concentration.

The frequency sweep results are presented in Figure 5.8 for the zinc+skim milk samples which formed gel after 3 hours holding (final G' > 1 Pa). The difference between G' and G'' was less than one log which indicated the zinc+skim milk gels were held together by weak interactions formed between zinc ions and milk proteins.

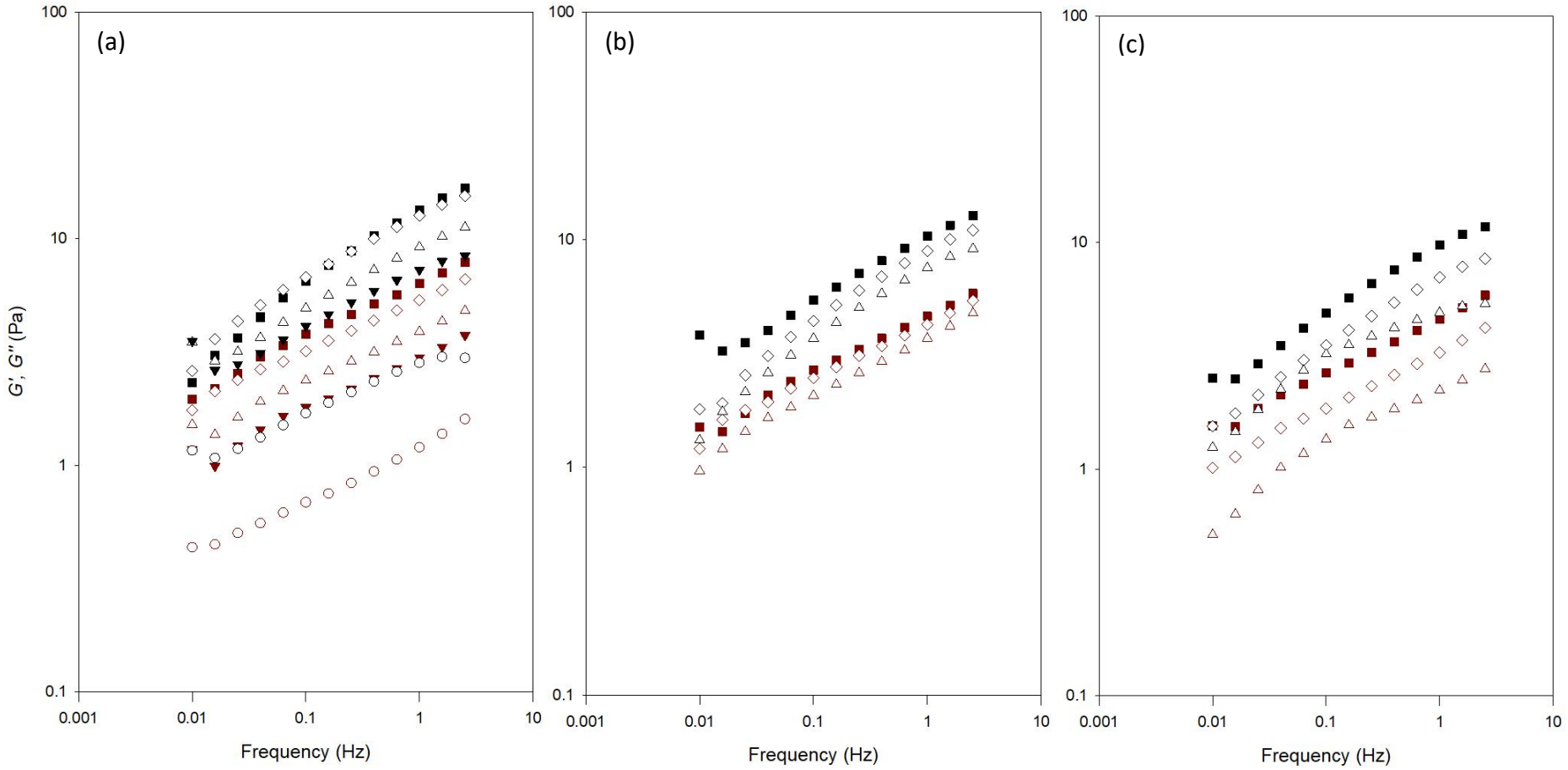


Figure 5.8: A representative plot showing the *G'* (black symbols) and *G''* (red symbols) of the zinc-added non-peheated skim milk gels (after a 3 hours time sweep at 20 ± 1°C) a function of frequency with various concentrations of added (a) zinc acetate; (b) zinc sulphate and (c) zinc gluconate: 22.5 mmol L^{-1} (○,○); 25 mmol L^{-1} (▼,▼); 30 mmol L^{-1} (△,△); 35 mmol L^{-1} (◇,◇) and 40 mmol L^{-1} (■,■).

5.6.3. Effect of pH

As discussed in Chapter 4, the skim milk pH decreasing can have an influence on gel strength. The drop in pH was more significant (p<0.05) when zinc sulphate and zinc gluconate was added compared to when zinc acetate was added (Figure 5.2), to offset the pH drop caused by the addition of zinc salt, the pH of skim milk samples was adjusted back to pH 6.73 ± 0.03 after adding 40 mmol L^{-1} zinc salt (Section 3.6). The final *G'* of the zinc-added sample with or without pH adjustment is shown in Table 5.3. The final *G'* of the sample with pH adjustment was lower than that of the sample without pH adjustment regardless of the zinc salt type. The adjusted pH skim milk sample with zinc acetate added had the highest final *G'*, followed by zinc sulphate and zinc gluconate. This is in the same order for the zinc-added skim milk without pH adjustment. A second experiment was carried out, adding 1M HCl to adjust the skim milk pH to 5.03 ± 0.02 (the lowest zinc+skim milk pH when 40 mmol L^{-1} zinc salt (zinc sulphate) added) without the addition of zinc salts. The final *G'* for this sample was found to be 0.11 ± 0.01 Pa, which was less than 1 Pa. Thus, no gelation was observed for the skim milk adjusted pH to 5.03 ± 0.02 by 1M HCl which indicated decreasing skim milk pH to 5.03 ± 0.02 without the addition of zinc salt cannot lead to skim milk gelation. In conclusion, the gel formation was not primarily dependent on the drop in skim milk pH.

Table 5.3: The final *G'* of skim milk samples with or without pH adjustment. Various zinc salts were added at 40 mmol L^{-1} to all the samples, 2M NaOH was used to adjust the sample pH to 6.73 ± 0.03. Results presented were means ± standard deviation (n = 3).

Zinc salt	Un-adjusted pH samples		Adjusted pH samples	
	Final *G'*	pH	Final *G'*	pH
Zinc acetate	6.86±0.12	5.34±0.01	4.29±0.40	6.73±0.03
Zinc sulphate	5.43±0.28	5.04±0.01	1.60±0.15	6.73±0.03
Zinc gluconate	4.64±0.18	5.26±0.01	1.04±0.07	6.73±0.03

5.7. Conclusions

This study showed that at the same added zinc concentration, the order of resultant skim milk pH from high to low after different zinc salts were added was zinc acetate > zinc gluconate > zinc sulphate. At the same added zinc concentration, the skim milk with addition of zinc

acetate had the highest colloidal phase zinc concentration and highest final *G'*. In contrast, the skim milk with zinc gluconate added had the lowest colloidal phase zinc concentration and lowest final *G'* at the same added zinc salt concentration. The colloidal phase zinc concentration difference was a clear indicator of likely final *G'* and the likelihood of a strong gel. The effect of the different added zinc salts on the zinc+skim milk gelation may also be influenced by the difference in the association between anions (SO_4^{2-}, OAc^-) and water in the protein-water interface. The interactions between the intermediate zinc complex (ZnL^+) with casein micelles may also influence the zinc+skim milk gelation. To understand the results obtained with zinc gluconate, further research is required to determine the stability constant of zinc gluconate, study the gluconate and ZnL^+ interaction with milk proteins to gain a better understanding of the organic zinc salt effect on skim milk gelation properties.

Chapter 6. Overall discussion

The overall aim of this study was to investigate the physicochemical and rheological properties of zinc+skim milk. The skim milk physicochemical properties such as pH, zinc and calcium distribution were determined after zinc salt was added. The EDTA titration method for zinc concentration determination was validated by comparing the results with the MPAES method (Chapter 4). The skim milk ionic species equilibrium change and zinc ion interaction with skim milk proteins after zinc acetate was added was investigated in Chapter 4. The effects of heat treatment (preheat and holding temperature treatment) were also presented in Chapter 4 to identify how processing conditions can affect the zinc+skim milk gelation. The results of preheat treatment (Chapter 4) indicated that the denatured whey protein may associate with casein micelles and form cross-link which influence the rheological properties of zinc+skim milk (Chapter 4). In Chapter 5, the effect of different types of zinc salts on skim milk properties was determined, and found not only did the added zinc ions influence the zinc+skim milk properties, but also the anions released from the added zinc salts had an influence on the physicochemical and rheological properties of the zinc+skim milk. Overall, it was found the changes of chemical properties may influence the rheological properties of the zinc+skim milk.

6.1. Equilibrium changes for the skim milk with zinc acetate added

The physicochemical changes of skim milk when zinc acetate was added was investigated in Chapter 4. When zinc acetate was added, the skim milk pH decreased significantly and both serum and colloidal phase zinc concentrations increased significantly. The total calcium concentration in the zinc+skim milk was maintained as constant but there was a significant decrease in colloidal phase calcium concentration indicating a shift into the serum. The proposed mechanism explanation of the mineral equilibria changes after addition of zinc acetate is shown in Figure 6.1. Zinc ions were released from the added zinc acetate and associated with phosphate and citrate in the serum phase to form zinc phosphate and zinc citrate which are highly insoluble (Figure 6.1, ① and ⑦) (Goodwin, 2000; Wegmüller *et al.,* 2014). This resulted in decreasing the serum phosphate and citrate concentrations, and also

increased the colloidal phase zinc concentration (Philippe *et al.*, 2005). Taking the phosphate as an example, to restore the equilibrium change of serum phosphate concentration decreasing, more $H_2PO_4^-$ was converted to HPO_4^{2-} and H^+ which lowed the zinc+skim milk pH (Figure 6.1, ⑤). The pH decreasing caused by addition of zinc acetate may lead to dissociation of CCP from the micellar phase (Figure 6.1, ⑥) (Lucey, 2017). The added zinc ions also can associate with the CCP and negatively charged groups on the casein micelle (Figure 6.1, ①), which increased the colloidal phase zinc concentration (Harzer & Kauer, 1982; Parkash & Jenness, 1967; Singh *et al.*, 1989a, 1989b). The added zinc ions also partially replaced the calcium in the micellar phase (Figure 6.1, ② and ③) since the association between zinc and casein micelle is greater than with calcium (Philippe *et al.*, 2005; Singh *et al.*, 1989a, 1989b). The released calcium from the micellar phase may further associate with the serum phosphate and citrate which further decreased the sample pH (Figure 6.1, ④).

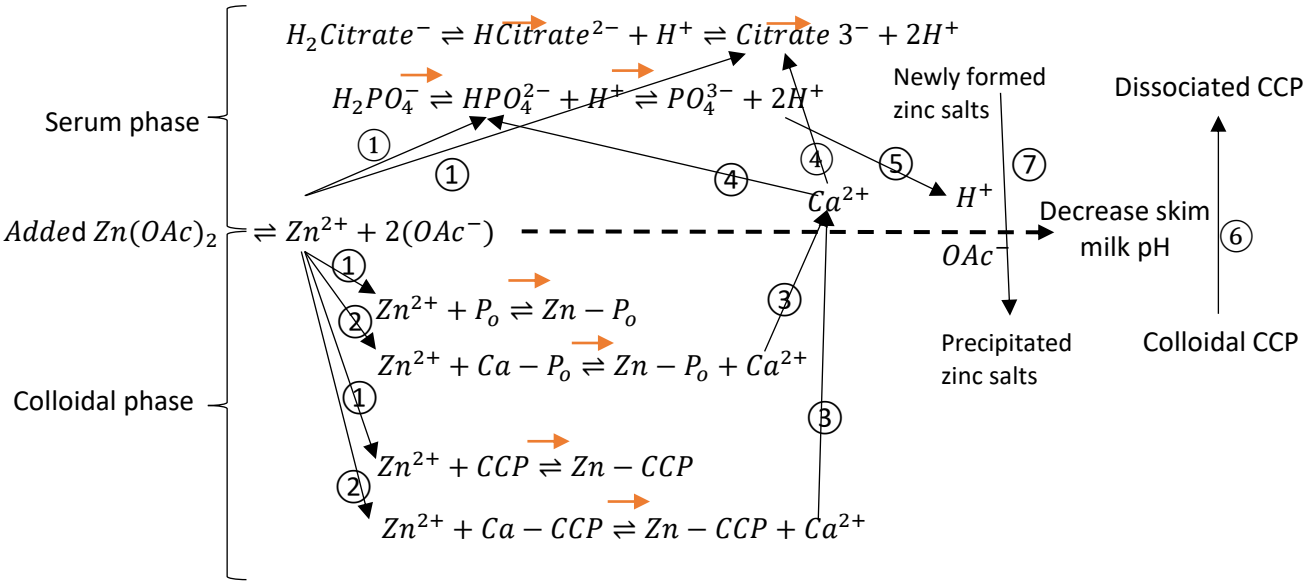


Figure 6.1: Proposed mechanism explaining the changes in the mineral equilibria when adding zinc acetate. →: Equilibrium toward direction. ①: Displacement of added zinc ions with phosphoserine residual (P_o), CCP and serum phosphate groups. ②: Replacement of calcium by added zinc. ③: Increasing of calcium ions in serum phase by zinc replacement. ④: Formation of calcium phosphate salts. ⑤: Releasing of hydrogen ions and decrease the milk. ⑥: Dissociation of CCP due to the pH decreasing. ⑦: Newly formed zinc salts precipitated into the colloidal phase.

6.2. Ion-protein interactions in zinc+skim milk

6.2.1. The ion-protein interaction in zinc+skim milk without heat treatment

The addition of zinc salt can affect the skim milk protein stability by associating with casein micelles via electrostatic interaction or by increasing the ionic strength which results in casein micelle dehydration (Harzer & Kauer, 1982; Philippe *et al*., 2005; Singh *et al*, 1988; Singh *et al*., 1989a). As discussed in Section 6.1., the addition of zinc acetate can lead to the mineral equilibrium change in skim milk. From the rheology results, the non-preheated skim milk with zinc acetate concentration higher than 15 mmol L^{-1} showed a significant increase in the apparent viscosity and gelation occurred after 3 hours holding at room temperature for the sample with 22.5 mmol L^{-1} zinc acetate added. The gel strength increased with increasing concentrations of added zinc acetate 22.5 to 40 mmol L^{-1}. The possible mechanisms of the changes in the rheological properties with different zinc acetate concentrations will be separated into three stages.

The first stage is the mineral equilibrium change caused by added zinc acetate, discussed in Section 6.1. The second stage is zinc-casein micelle association and formation of the salt bridges. In Figure 4.4 and 4.6, the skim milk viscosity increased with increasing added zinc concentration. With the increase in added zinc ions, the ions may associate with the negatively charged groups and CCP on the casein micelles via electrostatic interactions (Singh *et al.*, 1989a, 1989b). When zinc ions with positive charges associated with casein micelles, the net negative charges of casein micelles will be reduced and result in a reduction of electrostatic repulsion casein micelles (Bryant & McClements, 1998). As mentioned earlier, the decrease in skim milk pH may also contribute to the electrostatic repulsion between casein micelles (Lucey & Singh, 1997). In addition, the addition of zinc ions also leads to a reduction of hydration repulsion between casein micelles, which further encouraged casein micelle coagulation (Bryant & McClements, 1998; Philippe *et al*., 2005). The decrease in electrostatic repulsion resulted in the reduction of the distance between casein micelles and therefore increased the interaction between casein micelle particles which lead to progressively increasing skim milk viscosity.

The third stage is the formation of salt bridges between zinc ions and casein micelles and the formation of the three-dimensioned gel structure. For the skim milk with 22.5 mmol L^{-1} zinc acetate added, the yield stress increased to 0.10 Pa which implied three-dimensioned structures were being formed (Prentice, 1992). The added zinc ions are polyvalent ions which can simultaneously bind to the negatively charged groups in two casein micelles and formed salt bridges between these two casein micelles (Bryant & McClements, 1998). The number of salt bridges formed may increase with the increased concentration of added zinc ions. Results showed the gel strength increased (Figure 4.6) with the increase in added zinc concentration. Overall, with the increasing amount of added zinc ions to non-preheated skim milk leaded to decreasing pH and increase in zinc ion interaction with casein micelles leading to increase in viscosity and with further increase in zinc concentration the formation of self-supporting gels.

6.2.2. The ion-protein interaction in heat treated zinc+skim milk

Zinc+skim milk gelation was also prepared in the rheometer with heat treatment. It was found the zinc+skim milk gelation can occur at lower added zinc acetate concentrations of 5 mmol L^{-1} for the preheated skim milk and 10 mmol L^{-1} for the non-preheated skim milk, when the sample was heated and held at 80°C. The preheat effect on zinc+skim milk gelation was studied in Chapter 4, and found that at the same concentration of zinc acetate added, the preheated zinc+skim milk had a higher final gel strength. This may be as a result of the participation of denatured whey protein in the gel network. The preheat treatment induced whey protein denaturation and association with the κ - casein by hydrophobic interactions and disulphide linkages (Anema, 2008; Oldfield *et al.*, 1998). The association of denatured whey protein would reduce the net negative charge of casein micelles, lower the isoelectric point of the casein micelles and also reduce the ability of κ - casein to stabilise calcium-sensitive casein (Oldfield *et al.*, 1998; Zittle *et al.*, 1962). The denatured whey protein can form linkages to whey proteins that are already bound to casein micelles and increase the gel strength (Vasbinder *et al*., 2003). Begum (2019) and Ramasubramanian *et al*. (2014) have reported that denatured whey protein caused by preheat treatment were likely to coagulate in the presence of magnesium and calcium ions. It was found a higher holding temperature resulted in faster gel network development for the preheated zinc+skim milk (Chapter 5). The

higher temperatures may lead to rapid movement of the molecules hence increasing the gelation rate of the zinc+skim milks (Baldwin, 1986).

6.3. Comparison of the addition of zinc, calcium and magnesium to added skim milk gelation

The addition of zinc acetate to skim milk from this study was compared to results published on the addition of calcium chloride and magnesium chloride to skim milk (Begum, 2019, Lin 2019). The skim milk with different additions of divalent cation salts were prepared and analysed in a similar way in the rheometer. All of the cation+skim milks were heated from 20°C to 80°C with a heating rate of 5°C/min, then held at 80°C for 60 mins (for zinc+skim milk and calcium+skim milk) or 45 mins (for the magnesium+skim milk), followed by a cooling phase from 80°C to 20°C with a cooling rate of 5°C/min. Begum (2019) reported there was no significant difference in final *G'* observed for the sample which was held at 80°C for 45 min or 60 min. Thus, the results of final *G'* for the skim milk with different divalent cations added were considered to have the same treatment and the results can then be compared.

Figure 6.2 shows the final *G'* of preheated zinc+, calcium+ and magnesium+skim milks from results presented in this thesis, and from Lin (2019) and Begum (2019). It was noted that higher final *G'* was observed for the calcium+skim milk and magnesium+skim milk gel with the added salt concentration up to 20 mmol L^{-1}. However, for the zinc+skim milk with 20 mmol L^{-1} zinc acetate showed syneresis during the heating phase (Chapter 4), therefore was not plotted in Figure 6.2.

Considering the added salt concentrations between 5 to 15 mmol L^{-1} only, it was noted that the final gel strength for the zinc+ and magisium+skim milk was higher than the calcium+skim milk regardless of the added cation salt concentration. However, the order of gel strength for the zinc+skim milk and magnesium+skim milk was different with the added cation salt concentration. When 5 mmol L^{-1} salt added, there was no significant different for the zinc+skim milk gel strength and magnesium+skim milk gel strength. The gel with 10 mmol L^{-1} magnisium chloride added was significantly softer compared to the zinc acetate gel. The order of gel strength changed when 15 mmol L^{-1} salt was added as the magnesium+skim milk gel was firmer than the zinc+skim milk gel. Philippe *et al*. (2004) has reported the order of

association of cations with casein micelles (in the whey protein free milk) from strongest to weakest is Zn^{2+}>Ca^{2+}>Mg^{2+} at native milk pH (6.7). This is not in the same order as increasing gel strength observed in this study. However, Phillippe *et al*. (2004) used whey protein free skim milk, but preheated skim milk was used in these three studies. The denatured whey protein may play an important role in the formation of cation+skim milk and influence the final gel strength. More research is required to determine the order of association of cation with the casein micelle in preheated skim milk.

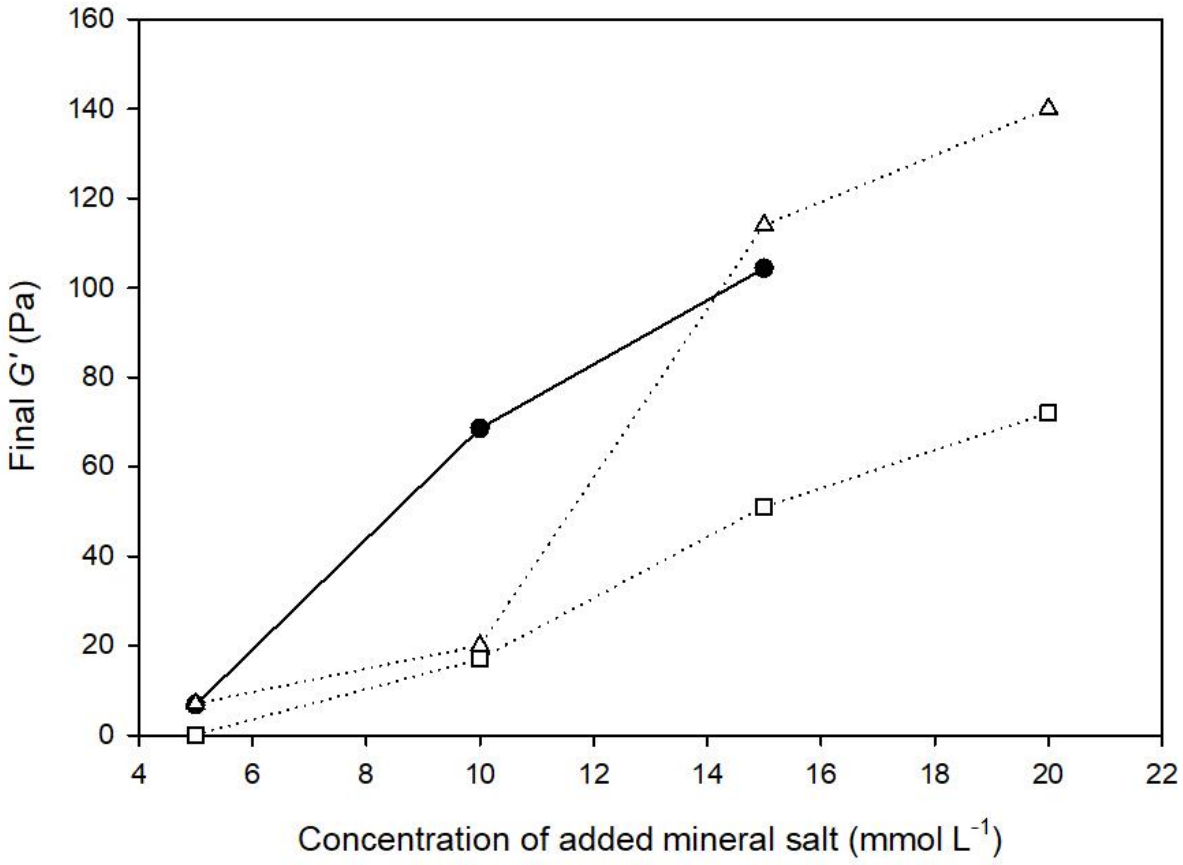


Figure 6.2: A representative plot shows the final *G'* after heating to 80°C, holding for 45 – 60 min and cooling to 20°C for 10% (w/w) preheated skim milk with different concentrations of zinc acetate (●), calcium chloride (□) (Lin, 2019) and magnesium chloride (△) (Begum, 2019). Close symbols indicated the data collected from the current study, open symbols indicated the published results. Results presented are mean values ± standard deviation (n = 3)

Chapter 4 showed that the non-preheated skim milk with addition of zinc acetate was able to form a gel network without any heat treatment (Figure 4.1 and 4.6), while added calcium and

magnesium salts only helped to induce gelation in pre-heated skim milks (Begum, 2019; Lin, 2019). For non-preheated skim milk, no gelation was observed for the samples with calcium chloride or magnesium chloride with up to 40 mmol L^{-1} added salt, until the holding temperature was increased to 65°C (Begum, 2019; Lin, 2019). The formation of a gel in the zinc+skim milk without heat treatment may be caused by the stronger association of zinc with casein micelles. Zinc has the largest electronegativity (1.65) which means it may have the strongest affinity with the casein micelle compared with calcium (1.00) and magnesium (1.31) (Blackman *et al.*, 2012; James, 2017). In addition, zinc has the smallest atom radius of the three elements (Housecroft & Sharpe, 2008; James, 2017), which may result in a shorter salt bridges between casein micelles and result in a tighter network between casein micelles. Also , the skim milk pH with zinc acetate added was the lowest pH compared to the skim milk with calcium chloride and magnesium chloride added at the same concentration (Table 4.1). The decrease in pH can contribute to the strength of the gel (Lin *et al.*, 2018; Lucey, 2017). Consequently, the large reduction in skim milk pH reduced the electrostatic repulsion between casein micelles and the added zinc ions associated strongly with casein micelles and may form shorter salt bridges which resulted in the skim milk gel structure network without heat treatment.

6.4. The effect of zinc salt type on non-preheated skim milk properties

In Chapter 5, it was found that the addition of different types of zinc salt (zinc acetate, zinc sulphate and zinc gluconate) can change the skim milk properties differently. The skim milk pH was decreased when additional zinc salts were added to the skim milk, however, the degree of pH decreasing was different for different zinc salts added (Figure 5.1). At the same molar concentration of added zinc salt, the skim milk pH from low to high was found to be zinc sulphate > zinc gluconate > zinc acetate. This could possibly be caused by the difference in the hydrolysis reactions of the anions in solution. Zinc ions released from added zinc salt can cause water hydrolysis and lower the solution pH by acting as a Lewis acid (Brown, 2018; Krężel & Maret, 2016). Some of the anions released from the added zinc salt like acetate and gluconate ions have strong basicity which can also result in water hydrolysis and increase the solution pH (Krężel & Maret, 2016; Ramachandran *et al*., 2006). However, sulphate ions have really weak basicity which does not lead to the hydrolysis of water, therefore resulting in a

reduction in pH due to the zinc cation (Housecroft & Sharpe, 2008; Krężel & Maret, 2016). Thus, the skim milk with zinc sulphate added showed the lowest pH (Figure 5.1). From Chapter 4, the reduction in skim milk pH provide an environment conducive to the formation of salt bridges between added zinc ions and casein micelles, but the decrease in pH as seen with zinc sulphate was not the primary cause for zinc-added skim milk gelation. It was found at the same molar concentration of zinc salt, the firmer gel defined by final *G'* after holding for the skim milk with different types of zinc salts was not in the same as the solution with the lowest pH. As zinc acetate produced firmer gels than zinc sulphate. Therefore, the final gel strength difference with different types of zinc salt added was not mainly caused by the anion hydrolysis, that is, the release of H^+.

In Chapter 5, zinc salts with different cumulative stability resulted in different colloidal phase zinc concentrations. At the same concentration of added zinc salts, zinc acetate which has a high stability constant and the highest zinc concentration in the colloidal phase while zinc gluconate which had a relatively low stability constant and the lowest zinc concentration in the colloidal phase. Rheology analysis showed the skim milk with a higher colloidal phase zinc concentration led to higher final *G'* after heating and holding. At the same molar concentration of added zinc salt, the order of final *G'* from highest to lowest of skim milk was zinc acetate > zinc sulphate > zinc gluconate. A lower cumulative stability constant which zinc acetate has, suggested that there were more free zinc ions released from the added zinc salt (Smith & Martell, 1976). Thus, more zinc ions were able to associate with the casein micelles and form salt bridges (Philippe *et al*., 2005). The formation of salt bridges between casein micelles were likely to be the primary cause for the skim milk gelation with addition of zinc salts. The different anions dissociated from zinc salts have different effects on the protein stability (Gao, 2012). According to the Hofmeister series, SO_4^- has a higher affinity for water molecules on the protein hydration shells and increases the protein hydrophobic effect which can increase the protein stability (Hyde *et al*., 2017). Increased protein stability will not lead to strong protein to protein interactions during gel formation, this is shown in the results as zinc sulphate addition resulted in the weaker gels than zinc acetate. For zinc acetate, OAc^- can associate with the protein-water interface and reduce the hydrophobic interaction of the protein (Yang, 2009). Thus, the protein stretched and more zinc binding sites were exposed for binding. More zinc bridges may form between casein micelles and resulted in a higher final

G' of the zinc-added skim milk. The results show stronger gels were formed with zinc acetate at the same concentrations as the other added zinc salt. The association of gluconate and the intermediate cation complex, ZnL^+, may also associate with the casein micelles and influence the zinc-casein micelle binding. The hypothesis was the ZnL^+ can associated with the zinc binding sites on casein micelles which may reduce the number of zinc salt bridges formed between two milk proteins, and hinder protein aggregation and gelation due to steric hindrance. This could be the reason why zinc gluconate formed the weaker of all gels compared to zinc acetate and zinc sulphate. However, there was limited information available on the zinc gluconate dissociation in solution and interaction with proteins, further investigation was required to gain a better understanding of the organic zinc salt association with milk proteins.

Chapter 7. Conclusions and recommendations

7.1. Conclusions

This study showed the addition of zinc salts (0 - 40 mmol L^{-1}) to skim milk can influence the physicochemical properties of skim milk, such as decreased skim milk pH, increased serum and colloidal phase zinc concentration and decreased the colloidal phase calcium concentration. These is changes may result in the reduction of protein stability and lead to casein micelle coagulation and gelation at room temperature.

This study has found the addition of zinc salts can lead to zinc+skim milk gelation at room temperature. It was postulated that the added zinc salts mainly associated with casein micelle and reduced the electrostastic repulsion between casein micelles and resulted in the formation of salt bridges which may lead to gel network formation. The addition of different zinc salts can alter the zinc+skim milk rheological properties differently, which may be explained by the Hofmeister effect of different anions.

The pH decrease caused by the addition of zinc salts was not the dominating factor in the formation of zinc+skim milk gels as gelation also was observed for the sample which had pH adjusted to the native skim milk pH. The colloidal phase zinc concentration may be the primary factor that influenced the zinc+skim milk gel strength as higher *G'* was observed for the sample which had higher colloidal phase zinc concentration.

It was found that preheating can increase the zinc+skim milk gel strength, which may be caused by the participation of denatured whey protein. The heat treatment during gelation also influences the zinc+skim milk gelation as higher temperature during gelation, the higher rate of movement of particles which resulted in faster gel network formation.

7.2. Recommendations

It was postulated that the added zinc associated with casein micelles and formed salt bridges which may lead to gel network formation and resulted in zinc+skim milk viscosity increase and even gelation. However, further research is recommended to comfirm that where are the binding sites on casein micelles for added zinc and does the added zinc can partially replace the micellar calcium.

One of the most important effects of adding zinc salts on skim milk is the change in the salt equilibrium. Only the H^+, Zn^{2+} and Ca^{2+} distribution between serum and colloidal phase had been studied in this work. Investigation of phosphate concentration is recommended to better understand the movement of zinc phosphate after zinc salts added. In addition, a study of ZnL^+ distribution and stability constant in zinc+skim milk after zinc gluconate added is also recommended. This may help better understand the effect of ZnL^+ on zinc equilibrium and interactions with casein micelle.

The contribution of pH to the zinc+skim milk gelation has been studied in this work, but only one pH was studied (pH 6.73), and the effect of pH on zinc and calcium distribution was not assessed. Thus, it would be interesting to extend the adjusted pH range such as 7.0 to 5.4 and then determine the effect of pH on gelation and zinc distribution in the zinc+skim milk.

It was found that heat treatment can influence the zinc+skim milk gelation, and the whey protein denaturation during heat treatment plays an important role in the gelation. Thus, it would be useful to investigate the distribution of whey protein in zinc+skim milk and studying the effect of whey protein concentration on zinc+skim milk gelation. In addition, a study of the effect of zinc on whey protein gelation may also be helpful to build a full picture of the role of whey protein in zinc+skim milk gelation.

This work compared the gel strength for zinc+skim milk, magnesium+skim milk and calcium+skim milk, but the actual picture of gel structure has not been investigated and compared by any studies. It is recommended to determine the gel structure by using confocal laser scanning microscopy or scanning electron microscope. It is also recommended that the

cryogenic electron microscopy and X-ray scatter may also be useful to understand the internal structural change of CCP nanoclusters of casein micelles in the future study.

References

Aceto, M., Abollino, O., Bruzzoniti, M. C., Mentasti, E., Sarzanini, C., & Malandrino, M. (2002). Determination of metals in wine with atomic spectroscopy (flame-AAS, GF-AAS and ICP-AES); a review. *Food Additives & Contaminants*, *19*(2), 126-133.

Agilent Technologies, (2016). *Microwave Plasma Atomic Emission Spectroscopy (MP-AES) Application eHandbook* (pp. 42-45). Agilent Technologies. https://www.agilent.com/cs/library/applications/5991-7282EN_MP-AES-eBook.pdf

Ahmad, S., Piot, M., Rousseau, F., Grongnet, J. F., & Gaucheron, F. (2009). Physico-chemical changes in casein micelles of buffalo and cow milks as a function of alkalinisation. *Dairy Science & Technology*, *89*(3), 387-403.

Akinyele, I., & Shokunbi, O. (2015). Comparative analysis of dry ashing and wet digestion methods for the determination of trace and heavy metals in food samples. *Food Chemistry*, *173*, 682-684.

Anema, S. G. (2008). On heating milk, the dissociation of [kappa]-casein from the casein micelles can precede interactions with the denatured whey proteins. *The Journal of Dairy Research*, *75*(4), 415-421.

Anema, S. G. (2009). Effect of milk solids concentration on the pH, soluble calcium and soluble phosphate levels of milk during heating. *Dairy Science and Technology*, *89*(5), 501-510.

Aoki, T., Kawahara, A., Kako, Y., & Imamura, T. (1987). Role of individual milk salt constituents in cross-linking by colloidal calcium phosphate in artificial casein micelles. *Agricultural and Biological Chemistry*, *51*(3), 817-821.

Arakawa, T., & Timasheff, S. N. (1982). Preferential interactions of proteins with salts in concentrated solutions. *Biochemistry*, *21*(25), 6545-6552.

Association of Official Analytical Chemists. (1976). Official Method 944.09: Zinc in Food. *Association of Official Analytical Chemists* (15th ed.) (pp. 272-273).

Association of Official Analytical Chemists. (2005). Official Method 999.11: Determination of Lead, Cadmium, Copper, Iron, and Zinc in Foods. *Association of Official Analytical Chemists* (18th ed.).

Baldwin, R. L. (1986). Temperature dependence of the hydrophobic interaction in protein folding. *Biochemistry, 83*, 8609-8072.

Baumy, J., & Brule, G. (1988). Effect of pH and ionic strength on the binding of bivalent cations to β-casein. *Le Lait*, *68*(4), 409-417.

Begum, N. (2019). *Magnesium enrichment of skim milk: a thesis presented in partial fulfilment of the requirements for the degree of Master of Food Technology at Massey University.* Massey University.

Berton, A., Rouvellac, S., Robert, B., Rousseau, F., Lopez, C., & Crenon, I. (2012). Effect of the size and interface composition of milk fat globules on their in vitro digestion by the human pancreatic lipase: Native versus homogenized milk fat globules. *Food Hydrocolloids*, *29*(1), 123-134.

Bienvenue, A., Jiménez-Flores, R., & Singh, H. (2003). Rheological properties of concentrated skim milk: importance of soluble minerals in the changes in viscosity during storage. *Journal of Dairy Science*, *86*(12), 3813-3821.

Blackman, A., Bottle, S. E., Schmid, S., Mocerino, M., & Wille, U. (2012). *Chemistry* (4th ed.) (pp. 749-821). Wiley.

Blakeborough, P., Salter, D. N., & Gurr, M. (1983). Zinc binding in cow's milk and human milk. *Biochemical Journal*, *209*(2), 505-512.

Brown, T. L. (2018). *Chemistry: the central science* (14th ed.) (pp. 708-749). Pearson Education.

Bryant, C. M., & McClements, D. J. (1998). Molecular basis of protein functionality with special consideration of cold-set gels derived from heat-denatured whey. *Trends in Food Science & Technology*, *9*(4), 143-151.

Caulfield, L. E., & Black, R. E. (2004). Zinc deficiency. *Comparative quantification of health risks: global and regional burden of disease attributable to selected major risk factors*, *1*, 257-280.

Chaberek Jr, S., Bersworth, F., & Martell, A. E. (1955). Chelating agents as metal buffers in biological systems. I. Principles and use of ethylenediaminetetraacetic acid. *Archives of biochemistry and biophysics*, *55*(2), 321-337.

Collins, K. D., Neilson, G. W., & Enderby, J. E. (2007). Ions in water: characterizing the forces that control chemical processes and biological structure. *Biophysical chemistry*, *128*(2-3), 95-104.

Council, N. R. (1988). Factors affecting the composition of milk from dairy cows. In *Designing foods: animal product options in the marketplace*. National Academies Press (US).

Cousins, R. J., & Smith, K. T. (1980). Zinc-binding properties of bovine and human milk in vitro: influence of changes in zinc content. *The American Journal of Clinical Nutrition*, *33*(5), 1083-1087.

Crowley, S. V., Kelly, A. L., & O'Mahony, J. A. (2014). Fortification of reconstituted skim milk powder with different calcium salts: Impact of physicochemical changes on stability to processing. *International Journal of Dairy Technology*, *67*(4), 474-482.

Dalgleish, D. G. (2011). On the structural models of bovine casein micelles—review and possible improvements. *Soft matter*, *7*(6), 2265-2272.

Damodaran, S., & Parkin, K. (2017). *Fennema's food chemistry* (5th ed.) (pp. 907-954). Taylor & Francis Group

Darnton-Hill. (2013). Zinc supplementation and growth in children. World Health Organization. https://www.who.int/elena/titles/bbc/zinc_stunting/en/

Datta, N., & Deeth, H. (2001). Age gelation of UHT milk—a review. *Food and Bioproducts Processing*, *79*(4), 197-210.

De Benoist, B., Darnton-Hill, I., Davidsson, L., Fontaine, O., & Hotz, C. (2007). Conclusions of the joint WHO/UNICEF/IAEA/IZiNCG interagency meeting on zinc status indicators. *Food and Nutrition Bulletin*, *28*, S480-S484.

De Kruif, C., & Holt, C. (2003). Casein micelle structure, functions and interactions. In *Advanced dairy chemistry—1 proteins* (pp. 233-276). Springer.

Eby, G. A. (1997). Zinc ion availability--the determinant of efficacy in zinc lozenge treatment of common colds. *The Journal of Antimicrobial Chemotherapy*, *40*(4), 483-493.

Fox, P. F., McSweeney, P. L., & Paul, L. (1998). *Dairy chemistry and biochemistry* (pp. 1-20, 146-264, 347-379). Blackie Academic & Professional.

Gao, Y. Q. (2012). Simple theory for salt effects on the solubility of amide. *The Journal of Physical Chemistry B*, *116*(33), 9934-9943.

Gaucheron, F. (2005). The minerals of milk. *Reproduction Nutrition Development*, *45*(4), 473-483.

Gaucheron, F. (2011a). Milk and dairy products: a unique micronutrient combination. *Journal of the American College of Nutrition*, *30*(5), 400S-409S.

Gaucheron, F. (2011b). Milk salts: Distribution and analysis. In Fuquay, J. W., Fox, P. F., McSweeney, P. L. H., (Eds.), *Encyclopedia of Dairy Sciences* (pp. 908-916): Springer Link.

Gibson, R. S. (2012). A historical review of progress in the assessment of dietary zinc intake as an indicator of population zinc status. *Advances in Nutrition*, *3*(6), 772-782.

Goodwin, F. E. (2000). Zinc compounds. In J. Kroschwits (ed.), *Kirk-Othmer Encyclopedia of Chemical Technology* (pp. 840-853). Wiley.

Gordy, W. (1946). A relation between bond force constants, bond orders, bond lengths, and the electronegativities of the bonded atoms. *The Journal of Chemical Physics*, *14*(5), 305-320.

Granum, B. (2018). Opinion of the Scientific Committee on Consumer safety (SCCS)–Final opinion on water-soluble zinc salts used in oral hygiene products. *Regulatory Toxicology and Pharmacology*, *99*, 249-250.

Grufferty, M. B., & Fox, P. F. (1985). Effect of added NaCl on some physicochemical properties of milk. *Irish Journal of Food Science and Technology, 9,* 1-9.

Guerin, A., Steeg, U., & Abdelnour, Y. (2012). Determination of exchangeable cations in soil extracts using Agilent 4100 Microwave Plasma-Atomique Emission Spectrometer. *Application note*, 1-8.

Harzer, G., & Kauer, H. (1982). Binding of zinc to casein. *The American Journal of Clinical Nutrition*, *35*(5), 981-987.

Hilliam, M. (1998). The market for functional foods. *International dairy journal*, *8*(5-6), 349-353.

Horne, D. S. (1998). Casein interactions: casting light on the black boxes, the structure in dairy products. *International dairy journal*, *8*(3), 171-177.

Horne, D. S. (2006). Casein micelle structure: models and muddles. *Current opinion in colloid & interface science*, *11*(2-3), 148-153.

House, J. E., & House, K. A. (2015). *Descriptive inorganic chemistry*. Academic Press.

Housecroft, C. E., & Sharpe, A. G. (2008). Structures and Energetics of Metallic and Ionic Solids. *Inorganic Chemistry*, 164-171.

Huppertz, T. (2016). Heat Stability of Milk. In P. L. H. McSweeney & J. A. O'Mahony (Eds.), *Advanced Dairy Chemistry: Volume 1B: Proteins: Applied Aspects* (pp. 179-196). Springer.

Huppertz, T., & Fox, P. F. (2006). Effect of NaCl on some physico-chemical properties of concentrated bovine milk. *International Dairy Journal*, *16*(10), 1142-1148.

Hyde, A. M., Zultanski, S. L., Waldman, J. H., Zhong, Y.-L., Shevlin, M., & Peng, F. (2017). General principles and strategies for salting-out informed by the Hofmeister series. *Organic Process Research & Development*, *21*(9), 1355-1370.

Iličić, M., Milanović, S., & Carić, M. (2015). Rheology and texture of fermented milk products. *Rheology: Principal, application and environmental impacts. New York: Nova Science*, 27-64.

International Zinc Nutrition Consultative Group (2019). *Country level data on zinc deficiency*. International Zinc Nutrition Consultative Group. https://www.izincg.org/riskzincdeficiency

Islam, M. T., & Ahmed, M. J. (2013). A Simple Spectrophotometric Method for the Trace Determination of Zinc in Some Real, Environmental, Biological, Pharmaceutical, Milk and Soil Samples Using 5, 7-Dibromo-8-hydroxyquinoline. *Pakistan Journal of Analytical & Environmental Chemistry*, *14*(1), 1-15.

James, G. S. (2017). *Lange's handbook of chemistry* (7th ed.) (pp. 150-154, 161-162, 166-167, 377). McGraw-Hill Education.

Jaros, D., & Rohm, H. (2017). Rennets: applied aspects. In McSweeney, P.L.H., Fox, P. F., Cotter, P. D., & Evertt, D. W., (Eds.), *Cheese* (pp. 53-67). Elsevier.

Jensen, R. G. (1995). *Handbook of milk composition* (pp. 464-468, 577-592). Academic press.

Johnson, P. E., & Evans, G. W. (1978). Relative zinc availability in human breast milk, infant formulas, and cow's milk. *The American Journal of Clinical Nutrition*, *31*(3), 416-421.

Kahraman, O., & Ustunol, Z. (2012). Effect of zinc fortification on Cheddar cheese quality. *Journal of dairy science*, *95*(6), 2840-2847.

Krężel, A., & Maret, W. (2016). The biological inorganic chemistry of zinc ions. *Archives of biochemistry and biophysics*, *611*, 3-19.

Kunz, W., Henle, J., & Ninham, B. W. (2004). 'Zur Lehre von der Wirkung der Salze'(about the science of the effect of salts): Franz Hofmeister's historical papers. *Current Opinion in Colloid & Interface Science*, *9*(1-2), 19-37.

Lapasin, R., Pricl, S., 1995. *Rheology of Industrial Polysaccharides: Theory and Applications.* Blackie Academic and Professional, Glasglow, UK.

Law, A. J., & Leaver, J. (2000). Effect of pH on the thermal denaturation of whey proteins in milk. *Journal of Agricultural and Food Chemistry*, *48*(3), 672-679.

Lee, W., & Lucey, J. (2004). Structure and physical properties of yogurt gels: Effect of inoculation rate and incubation temperature. *Journal of dairy science*, *87*(10), 3153-3164.

Li, J., & Dalgleish, D. G. (2006). Controlled proteolysis and the properties of milk gels. *Journal of agricultural and food chemistry*, *54*(13), 4687-4695.

Lin, L. (2019). *A study on the mechanisms of calcium-induced gelation in skim milk: a thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Food Technology at Massey University*. Massey Unniversity.

Lin, L., Wong, M., Deeth, H., & Oh, H. (2018). Calcium-induced skim milk gels using different calcium salts. *Food Chemistry*, *245*, 97-103.

Lin, L., Wong, M., Deeth, H. C., & Oh, H. E. (2020). Calcium-induced skim milk gels: Impact of holding temperature and ionic strength. *International Dairy Journal*, *104*, 104657.

Liu, X., Zhang, H., Wang, F., Luo, J., Guo, H., & Ren, F. (2014). Rheological and structural properties of differently acidified and renneted milk gels. *Journal of Dairy Science*, *97*(6), 3292-3299.

Lönnerdal, B., Hoffman, B., & Hurley, L. (1982). Zinc and copper binding proteins in human milk. *The American Journal of Clinical Nutrition*, *36*(6), 1170-1176.

Lucey, J. (2002). Formation and physical properties of milk protein gels. *Journal of Dairy Science*, *85*(2), 281-294.

Lucey, J. (2017). Formation, structural properties, and rheology of acid-coagulated milk gels. In McSweeney, P.L.H., Fox, P. F., Cotter, P. D., & Evertt, D. W., (Eds.), *Cheese* (pp. 179-197). Elsevier.

Lucey, J. (2020). Milk protein gels. In Singh, H., Boland, M., & Tompson, A., (Eds.), *Milk proteins* (pp. 599-632). Elsevier.

Lucey, J., & Singh, H. (1997). Formation and physical properties of acid milk gels: a review. *Food Research International*, *30*(7), 529-542.

Lucey, J., Munro, P., & Singh, H. (1999). Effects of heat treatment and whey protein addition on the rheological properties and structure of acid skim milk gels. *International Dairy Journal*, *9*(3-6), 275-279.

Luo, Y., Zhang, B., Chen, M., Wang, J., Zhang, X., Gao, W.-y., Huang, J.-f., & Fu, W.-l. (2010). Rapid and simultaneous determination of essential minerals and trace elements in human milk by improved flame atomic absorption spectroscopy (FAAS) with microwave digestion. *Journal of Agricultural and Food Chemistry*, *58*(17), 9396-9400.

Lyster, R. L. J. (1970). The denaturation of α-lactalbumin and β-lactoglobulin in heated milk. *Journal of Dairy Research*, *37*(2), 233-243.

Lyster, R. (1972). Section C. Chemistry of milk proteins. *Journal of Dairy Research*, *39*(2), 279-318.

McNaught, A. D., & Wilkinson, A. (1997). Transition element. In *Compendium of chemical terminology* (Vol. 1669). Blackwell Science Oxford.

McSweeney, P. L., & Fox, P. F. (2013). *Advanced dairy chemistry: volume 1A: proteins: basic aspects* (4th ed.) (pp. 1-261). Springer.

Mekmene, O., Le Graët, Y., & Gaucheron, F. (2009). A model for predicting salt equilibria in milk and mineral-enriched milks. *Food Chemistry*, *116*(1), 233-239.

Mellema, M., Leermakers, F., & De Kruif, C. (1999). Molecular mechanism of the renneting process of casein micelles in skim milk, examined by viscosity and light-scattering experiments and simulated by model SCF calculations. *Langmuir*, *15*(19), 6304-6313.

Mitchell, G. (2013). *Rheology : Theory, Properties, and Practical Applications*. Nova Science Publishers, Inc.

Nham, T., & Taylor, C. (2012). Cost-Effective Analysis of Major, Minor, and Trace Elements in Foodstuffs Using the 4100 MP-AES. *Spectroscopy-Eugene*, *25*, 1-8.

Novick, S. (1997). Complexometric titration of zinc: An analytical chemistry laboratory experiment. *Journal of Chemical Education*, *74*(12), 1463.

Oh, H. E., & Deeth, H. C. (2017). Magnesium in milk. *International Dairy Journal*, *71*, 89-97.

Oldfield, D. J., Singh, H., Taylor, M. W., & Pearce, K. N. (1998). Kinetics of denaturation and aggregation of whey proteins in skim milk heated in an ultra-high temperature (UHT) pilot plant. *International Dairy Journal*, *8*(4), 311-318.

Ortega-Requena, S., & Rebouillat, S. (2015). Retracted Article: Bigger data open innovation: potential applications of value-added products from milk and sustainable valorization of by-products from the dairy industry. *Green Chemistry*, *17*(12), 5100-5113.

Pabón, M. L., & Lönnerdal, B. (1993). Effect of citrate on zinc bioavailability from milk, milk fractions and infant formulas. *Nutrition Research*, *13*(1), 103-111.

Parkash, S., & Jenness, R. (1967). Status of zinc in cow's milk. *Journal of Dairy Science*, *50*(2), 127-134.

Peacock, J. C., & Peacock, B. L. D. (1918). Some observations the dissolving of zinc chloride and several suggested solvents. *Journal of the American Pharmaceutical Association*, *7*(8), 689-697.

Phadungath, C. (2005). Casein micelle structure: a concise review. *Songklanakarin Journal of Science and Technology*, *27*(1), 201-212.

Philippe, M., Gaucheron, F., & Le Graet, Y. (2004). Physicochemical characteristics of calcium supplmented skin milk: comparison of three soluble calcium salts. *Milchwissenchaft-Milk Science International,* 59(9-10), 498-502.

Philippe, M., Le Graët, Y., & Gaucheron, F. (2005). The effects of different cations on the physicochemical characteristics of casein micelles. *Food Chemistry*, *90*(4), 673-683.

Pouliot, Y., Boulet, M., & Paquin, P. (1989a). Observations on the heat-induced salt balance changes in milk I. Effect of heating time between 4 and 90 C. *Journal of Dairy Research*, *56*(2), 185-192.

Pouliot, Y., Boulet, M., & Paquin, P. (1989b). Observations on the heat-induced salt balance changes in milk II. Reversibility on cooling. *Journal of Dairy Research, 56*(2), 193-199.

Prentice, J. H. (1992). *Dairy rheology: a concise guide* (pp. 1-29, 49-56, 123-137). VCH Publishers.

Ramachandran, S., Fontanille, P., Pandey, A., & Larroche, C. (2006). Gluconic acid: Properties, applications and microbial production. *Food Technology & Biotechnology*, *44*(2), 185-195.

Ramasubramanian, L., D'Arcy, B. R., Deeth, H. C., & Oh, H. E. (2014). The rheological properties of calcium-induced milk gels. *Journal of Food Engineering*, *130*, 45-51.

Ramasubramanian, L., Restuccia, C., & Deeth, H. (2008). Effect of calcium on the physical properties of stirred probiotic yogurt. *Journal of Dairy Science*, *91*(11), 4164-4175.

Rana, B., Kaushik, R., Kaushal, K., Arora, S., Kaushal, A., Gupta, S., Upadhyay, N., Rani, P., & Kaushik, P. (2018). Physicochemical and electrochemical properties of zinc fortified milk. *Food Bioscience*, *21*, 117-124.

Sandra, S., Alexander, M., & Dalgleish, D. G. (2007). The rennet coagulation mechanism of skim milk as observed by transmission diffusing wave spectroscopy. *Journal of Colloid and Interface Science*, *308*(2), 364-373.

Sapota, A., Daragó, A., Skrzypińska-Gawrysiak, M., Nasiadek, M., Klimczak, M., & Kilanowicz, A. (2014). The bioavailability of different zinc compounds used as human dietary supplements in rat prostate: a comparative study. *Biometals*, *27*(3), 495-505.

Saracoglu, S., Saygi, K. O., Uluozlu, O. D., Tuzen, M., & Soylak, M. (2007). Determination of trace element contents of baby foods from Turkey. *Food Chemistry*, *105*(1), 280-285.

Scherz, H., & Kirchhoff, E. (2006). Trace elements in foods: zinc contents of raw foods—a comparison of data originating from different geographical regions of the world. *Journal of Food Composition and Analysis*, *19*(5), 420-433.

Seal, C., & Heaton, F. (1983). Chemical factors affecting the intestinal absorption of zinc in vitro and in vivo. *British Journal of Nutrition*, *50*(2), 317-324.

Seiquer, I., Valverde, A., Delgado-Andrade, C., & Navarro, M. P. (2000). Influence of heat treatment of casein in presence of reducing sugars on Zn solubility and Zn uptake by Caco-2 cells after *in vitro* digestion. *Journal of Physiology and Biochemistry*, *56*(3), 237-246.

Sikirić, M., Brajenović, N., Pavlović, I., Havranek, J. L., & Plavljanić, N. (2003). Determination of metals in cow's milk by flame atomic absorption spectrophotometry. *Czech J. Anim. Sci*, *48*(11), 481-486.

Singh, H., & Fox, P. F. (1985). Heat stability of milk: pH-dependent dissociation of micellar κ-casein on heating milk at ultra-high temperatures. *Journal of Dairy Research*, *52*(4), 529-538.

Singh, H., Flynn, A., & Fox, P. F. (1988). Binding of zinc to colloidal calcium phosphate in human and cow's milks. In Hurley, L. S., & Keen, C. L., *Trace Elements in Man and Animals 6* (pp. 565-566). Springer.

Singh, H., Flynn, A., & Fox, P. F. (1989a). Binding of zinc to bovine and human milk proteins. *Journal of Dairy Research*, *56*(2), 235-248.

Singh, H., Flynn, A., & Fox, P. F. (1989b). Zinc binding in bovine milk. *Journal of Dairy Research*, *56*(2), 249-263.

Smith, R. M., & Martell, A. E. (1976). *Critical stability constants: inorganic complexes* (Vol. 4). Springer.

Soliman, G. Z. (2005). Comparison of chemical and mineral content of milk from human, cow, buffalo, camel and goat in Egypt. *The Egyptian Journal of Hospital Medicine*, *21*(1), 116-130.

Song, M. K., Adham, N. F., & Rinderknecht, H. (1976). A simple, highly sensitive colorimetric method for the determination of zinc in serum. *American Journal of Clinical Pathology*, *65*(2), 229-233.

Sugiarto, M. W. (2004). *Studies on the binding of iron and zinc to milk protein products: a thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Food Technology, Riddet Centre and Institute of Food, Nutrition and Human Health.* Massey University.

Totosaus, A., Montejano, J. G., Salazar, J. A., & Guerrero, I. (2002). A review of physical and chemical protein-gel induction. *International Journal of Food Science & Technology*, *37*(6), 589-601.

Trumbo, P., Yates, A. A., Schlicker, S., & Poos, M. (2001). Dietary reference intakes: vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium, and zinc. *Journal of the Academy of Nutrition and Dietetics*, *101*(3), 294-301.

Tsioulpas, A., Lewis, M. J., & Grandison, A. S. (2007). Effect of minerals on casein micelle stability of cows' milk. *Journal of Dairy Research*, *74*(2), 167-173.

Udabage, P., Mckinnon, I., & Augustin, M. (2000). Mineral and casein equilibria in milk: effects of added salts and calcium chelating agents. *Journal of Dairy Research*, *67*(3), 361-370.

Vaillancourt, S., & Allen, J. (1990). Elevation of zinc concentration in milk and colostrum by glucocorticoids. *Journal of Dairy Science*, *73* (Supplement 1).

Van Dijk, H. M. (1991). The properties of casein micelles. IV: The effect of the addition of NaCl, $MgCl_2$ or NaOH on the partition of Ca, Mg and PO_4 in cows' milk. *Nederlands Melk en Zuiveltijdschrift*, *45*(4), 241-251.

Van Vilet, T., Roefs, S. P. F. M., Zoon, P., & Walstra, P. (1989). Rheological properties of casein gels. *Journal of Dairy Research*, *56*(3), 529-534.

Varnam, A. H., & Sutherland, J. P. (1994). *Milk and milk products : technology, chemistry and microbiology* (pp. 42-102, 159-182, 275-386). Springer US.

Vasbinder, A. J., van de Velde, F., & de Kruif, C. G. (2004). Gelation of casein-whey protein mixtures. *Journal of Dairy Science*, *87*(5), 1167-1176.

Vasbinder, A., Rollema, H., Bot, A., & De Kruif, C. (2003). Gelation mechanism of milk as influenced by temperature and pH; studied by the use of transglutaminase cross-linked casein micelles. *Journal of Dairy Science*, *86*(5), 1556-1563.

Vavrusova, M., Liang, R., & Skibsted, L. H. (2014). Thermodynamics of dissolution of calcium hydroxycarboxylates in water. *Journal of Agricultural and Food Chemistry*, *62*(24), 5675-5681.

Walsh-O'Grady, C. D., O'Kennedy, B. T., Fitzgerald, R. J., & Lane, C. N. (2001). A rheological study of acid-set" simulated yogurt milk" gels prepared from heat-or pressure-treated milk proteins. *Le Lait*, *81*(5), 637-650.

Waters, R. S., Bryden, N. A., Patterson, K. Y., Veillon, C., & Anderson, R. A. (2001). EDTA chelation effects on urinary losses of cadmium, calcium, chromium, cobalt, copper, lead, magnesium, and zinc. *Biological Trace Element Research*, *83*(3), 207-221.

Wegmüller, R., Tay, F., Zeder, C., Brnić, M., & Hurrell, R. F. (2014). Zinc absorption by young adults from supplemental zinc citrate is comparable with that from zinc gluconate and higher than from zinc oxide. *The Journal of Nutrition, 144*(2), 132-136.

Wendell, R., & Patton, J. C. (1959). *U.S. Patent No. 2,910,349*. Washington, DC: U.S. Patent and Trademark Office.

Wong, N. P., Jenness, R., Marth, E. H., & Keeney, M. (1988). *Fundamentals of dairy chemistry* (3rd ed.) (pp. 1-169, 409-460, 583-654). Springer Science & Business Media.

Yang, Z. (2009). Hofmeister effects: an explanation for the impact of ionic liquids on biocatalysis. *Journal of Biotechnology*, *144*(1), 12-22.

Yuliarti, O., Mei, K. H., Ting, Z. K. X., & Yi, K. Y. (2019). Influence of combination carboxymethylcellulose and pectin on the stability of acidified milk drinks. *Food Hydrocolloids*, *89*, 216-223.

Zhao, Z. T., & Corredig, M. (2020). Effects of pH-modification on the rennet coagulation of concentrated casein micelles suspensions. *Food Chemistry*, *316*, 126199.

Zittle, C., Thompson, M., Custer, J., & Cerbulis, J. (1962). κ-Casein—β-Lactoglobulin Interaction in solution when heated. *Journal of Dairy Science*, *45*(7), 807-810.

Appendices

Appendix 1 – Determination of the degree of hydration for zinc gluconate hydrate ($ZnC_{12}H_{22}O_{14}\cdot xH_2O$)

Zinc gluconate hydrate ($ZnC_{12}H_{22}O_{14}\cdot xH_2O$) (4.556 g) was weighed into a 50 ml volumetric flask and fill to the mark with distilled water. The zinc concentration was determined by the EDTA method (Section 3.8). The zinc concentration for this zinc gluconate solution was calculated to be 177.95 ± 0.03 mmol L^{-1}. Since one mole of zinc gluconate when dissolved can release one mole of zinc, Equation 3.9 (Eby, 1997), the zinc gluconate concentration in the solution should be the same as the zinc concentration, which was 177.95 ± 0.03 mmol L^{-1}.

$$Zn(C_6H_{11}O_7)_2 \leftrightarrow [Zn]^{2+} + 2[C_6H_{11}O_7]^-$$

Equitation 3.9

$$\begin{aligned} &Total\ moles\ of\ zinc\ in\ the\ 50ml\ zinc\ gluconate\ solution \\ &= zinc\ gluconate\ concentration\ \times\ volume\ of\ the\ solution \\ &= 177.9\ \times 10^{-3}\ \times\ 50\ \times 10^{-3} = 8.90\ \times 10^{-3}\ mol \end{aligned}$$

$$\begin{aligned} &Experimental\ molecular\ mass\ of\ zinc\ gluconate\ hydrate \\ &= \frac{mass\ of\ zinc\ gluconate\ added}{moles\ of\ zinc\ gluconate\ in\ the\ solution} = \frac{4.556}{8.90\ \times\ 10^{-3}} = 512.17\ g/mol \end{aligned}$$

$$\begin{aligned} &Degree\ of\ hydration \\ &= (\text{experimental molecular mass of zinc gluconate hydrate} \\ &- \text{molecular mass of zinc gluconate (anhydrate)}/molecular\ mass\ of\ water \\ &= (512.17 - 455.69)/18 = 3.14 \end{aligned}$$

Appendix 2 – Mass of each zinc salt required to prepare 500 ml 200 mmol L^{-1} zinc stock solutions and the measured density.

Similar to zinc gluconate, one mole of zinc acetate and zinc sulphate can release one mole of zinc when dissolved, Equation 3.10 and Equation 3.11 (Krężel, & Maret, 2016).

$$Zn(C_2H_3O_2)_2 \leftrightarrow Zn^{2+} + 2[C_2H_3O_2]^-$$

Equation 3.11

$$ZnSO_4 \leftrightarrow [Zn]^{2+} + [SO_4]^{2-}$$

Equation 3.12

Table A1: Weight of each zinc salt to prepare 200 mmol L^{-1} (500ml) zinc stock solutions and the measured density for each zinc stock solution.

Zinc salt	Weight of zinc salt (g)	Density (kg m^{-3})
Zinc sulphate heptahydrate	28.76	1027 ± 1
Zinc acetate dihydrate	21.95	1037 ± 3
Zinc gluconate	51.22	1055 ± 1

Appendix 3 – Example of calculation of the volume added for each solution to make up 50ml of zinc+skim milk with a final concentration of 10% total solids (w/w)

e.g. to prepare 20 mmol L^{-1} solution

$$Volume\ of\ zinc\ stock\ solution\ needed = \frac{final\ zinc\ salt\ concentration\ \times\ amount\ of\ sample\ required\ to\ make}{zinc\ stock\ solution\ concentration}$$

$$= \frac{20 \times 10^{-3} \times 50 \times 10^{-3}}{200\ \times 10^{-3}} = 5\ \times 10^{-3}\ L = 5\ ml$$

Volume of 13% (w/w) skim milk needed ($V_{13\%}$)

$$10\%\ \left(\frac{w}{w}\right) = \frac{Total\ solid\ mass\ in\ 13\%\ skim\ milk}{Mass\ of\ sample}$$

$$= \frac{Total\ solid\ mass\ in\ 13\%\ skim\ milk}{Mass\ of\ water + Mass\ of\ zinc\ stock\ solution + Mass\ of\ 13\%\ skim\ milk}$$

$$= \frac{V_{13\%}\rho_{13\%} \times 13\%}{[(V_s - V_{Zn} - V_{13\%})\rho_w] + V_{Zn}\rho_{Zn} + V_{13\%}\rho_{13\%}}$$

$$V_{13\%} = \frac{10\%\ \times V_s\rho_w - 10\%V_{Zn}\rho_w + 10\%V_{Zn}\rho_{Zn}}{\rho_{13\%} \times 13\% + 10\%\rho_w - 10\%\rho_{13\%}}$$

Volume of distilled water needed

$$V_w = V_s - V_{13\%} - V_{Zn}$$

Where

$V_{13\%}$: Volume of 13% (w/w) skim milk needed

$\rho_{13\%}$: Density of 13% (w/w) skim milk 20 ± 1 °C

V_s: Volume of sample needs to be made

V_{Zn}: Volume of 200 mmol L^{-1} zinc stock solution needed

ρ_{Zn}: Density of 200 mmol L^{-1} zinc stock solution 20 ± 1 °C

V_w: Volume of distilled water needed

ρ_w: Density of water at 20 ± 1°C

Appendix 4 – Amount of each component added to prepare the pH-adjusted skim milk samples with zinc salts added

Table A2: Amount of each component required to make 50ml adjusted pH skim milk (pH 6.73 ± 0.03) with zinc salts added and final total solid of 10% (w/w).

	13% (w/w) reconstituted skim milk (ml)	0.2 mol L^{-1} zinc stock solution (ml)	2 mol L^{-1} NaOH (ml)	Distilled water (ml)
Zinc acetate	38.414	10	0.725	0.861
Zinc gluconate	38.546	10	0.730	0.725
Zinc sulphate	38.336	10	0.744	0.920

Table A3: The volume of each solution added to form 50ml 10% (w/w) skim milk with pH adjusted to 5.34 ± 0.02 and 5.03 ± 0.02.

Final pH	13% (w/w) reconstituted skim milk (ml)	First part of distilled water (ml)	1M HCl (ml)	Second part of distilled water (ml)
5.34 ± 0.02	38.115	10	1.125	0.760
5.03 ± 0.02	38.115	10	1.650	0.235

Appendix 5 – Calculation for EDTA titration method limit of detection (LOD) (Forootan *et al.*, 2017)

For zinc quantification analysis:

The standard curve equation was

$$Volume\ of\ EDTA = 0.0789 + 1.54 * Mass\ of\ zinc$$

$$Mass\ of\ zinc = \frac{Volume\ of\ EDTA - 0.0789}{1.54}$$

The LOD was determined as the mass of zinc can be titrated by the lowest volume of the titre (0.01M EDTA) was 0.05 ml (1 drop).

$$LOD = The\ zinc\ mass\ can\ be\ titrated\ by\ 1\ drop\ (0.05ml)\ 0.01M\ EDTA$$
$$= \frac{0.05-0.0789}{1.54} - \frac{0-0.0789}{1.54} = 0.03\ mg\ Zn/1g\ skim\ milk$$

For calcium quantification analysis:

The standard curve equation was

$$Volume\ of\ EDTA = 0.0488 + 1.95 * Mass\ of\ calcium$$

$$Mass\ of\ calcium = \frac{Volume\ of\ EDTA - 0.0488}{1.95}$$

R^2 was 0.9996, the standard error was 0.0402

The LOD was determined as the mass of calcium can be titrated by the lowest volume of the titre (0.01M EDTA) was 0.05 ml (1 drop).

$$LOD = The\ calcium\ mass\ can\ be\ titrated\ by\ 1\ drop\ (0.05ml)\ 0.01M\ EDTA$$
$$= \frac{0.05 - 0.0448}{1.95} - \frac{0 - 0.0448}{1.95} = 0.03\ mg\ Ca/1g\ skim\ milk$$

Appendix 6 – Mass of serum and dry sediment in 1 g skim milk with zinc salt added

There were small but significant (p<0.05) changes of sediment mass when different concentrations of zinc acetate, zinc sulphate and zinc gluconate were added.

Table A4: The mass of serum and dry sediment in 1 g skim milk with various concentrations of added zinc acetate. Results presented are means ± standard deviation (n = 3-4).

Concentration of added zinc acetate (mmol L^{-1})	Mass of serum (g)	Mass of sediment (g)
0	0.9678±0.0001	0.0322±0.0001
5	0.9678±0.0003	0.0322±0.0003
10	0.9672±0.0003	0.0328±0.0003
15	0.9663±0.0001	0.0337±0.0001
17.5	0.9661±0.0003	0.0339±0.0003
20	0.9658±0.0001	0.0342±0.0001
22.5	0.9655±0.0001	0.0345±0.0001
25	0.9648±0.0004	0.0352±0.0004
30	0.9642±0.0002	0.0358±0.0002
35	0.9641±0.0003	0.0359±0.0003
40	0.9637±0.0001	0.0363±0.0001

Table A5: The mass of serum and dry sediment in 1 g skim milk with various concentrations of added zinc sulphate. Results presented are means ± standard deviation (n = 3-4).

Concentration of added zinc sulphate (mmol L^{-1})	Mass of serum (g)	Mass of sediment (g)
0	0.9678±0.0001	0.0322±0.0001
5	0.9678±0.0002	0.0322±0.0002
10	0.9671±0.0003	0.0329±0.0003
15	0.9664±0.0002	0.0336±0.0002
20	0.9660±0.0001	0.0340±0.0001
25	0.9652±0.0001	0.0348±0.0001
30	0.9649±0.0004	0.0351±0.0004
35	0.9644±0.0002	0.0356±0.0002
40	0.9628±0.0001	0.0367±0.0001

Table A6: The mass of serum and dry sediment in 1 g skim milk with various concentrations of added zinc gluconate. Results presented are means ± standard deviation (n = 3-4).

Concentration of added zinc gluconate (mmol L^{-1})	Mass of serum (g)	Mass of sediment (g)
0	0.9678±0.0001	0.0322±0.0001
5	0.9678±0.0001	0.0322±0.0001
10	0.9675±0.0008	0.0325±0.0008
15	0.9667±0.0001	0.0333±0.0001
20	0.9656±0.0003	0.0344±0.0003
25	0.9654±0.0003	0.0346±0.0003
30	0.9652±0.0002	0.0348±0.0002
35	0.9648±0.0008	0.0352±0.0008
40	0.9645±0.0006	0.0355±0.0006

Appendix 7 – Calculation for MPAES method limit of detection (LOD) (Armbruster, & Pry, 2008)

Standard curve:

$Emission\ intensity\ of\ zinc\ =\ 18627.98 * zinc\ concentration - 51.85$

$Zinc\ concentration = \frac{Emission\ intensity\ of\ zinc - 51.85}{18627.98}$

$LOD = mean\ of\ blank + k(standard\ diviation\ of\ the\ blank)$, k = 10 at 95% confidential (Armbruster, & Pry, 2008)

$LOD = 138.44 + 10 \times 26.66 = 405.04$ (emission intensity of zinc at 213.857 nm wavelength)

$LOD = \frac{405.04 - 51.85}{18629.98} = 0.02\ mg\ Zn/\ L\ solution$

Convert the LOD from mg Zn / L solution to mg Zn / g sample by Equation 3.7,

LOD = 0.0006 mg Zn / g sample

Appendix 8 – Certificate of analysis for the standard skim milk powder (ERM-BD150)

JOINT RESEARCH CENTRE
Directorate F – Health, Consumers and Reference Materials

CERTIFICATE OF ANALYSIS
ERM®- BD150

SKIMMED MILK POWDER		
	Mass Fraction	
	Certified value [1,2) [g/kg]	Uncertainty [2,3) [g/kg]
Ca	13.9	0.8
Cl	9.7	2.0
K	17.0	0.7
Mg	1.26	0.10
Na	4.18	0.19
P	11.0	0.6
	Certified value [1,2) [mg/kg]	Uncertainty [2,3) [mg/kg]
Cd	0.0114	0.0029
Cu	1.08	0.06
Fe	4.6	0.5
Hg	0.060	0.007
I	1.73	0.14
Mn	0.289	0.018
Pb	0.019	0.004
Se	0.188	0.014
Zn	44.8	2.0

1) Unweighted mean value of the means of accepted sets of data, each set being obtained in a different laboratory and/or with a different method of determination. The certified value and its uncertainty are traceable to the International System of Units (SI).

2) Certified mass fractions are corrected for the water content of the material (and expressed as dry mass), determined as described in the section "Instructions for use and intended use".

3) The uncertainty is expanded with a coverage factor k = 2 corresponding to a level of confidence of about 95 % estimated in accordance with ISO/IEC Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM:1995), ISO, 2008.

This certificate is valid for one year after purchase.
Sales date:
The minimum amount of sample to be used is 500 mg for Fe and 200 mg for all other elements.

Accepted as an ERM®, Geel, August 2013
Latest revision: January 2017

Signed:

Dr Doris Florian
European Commission, Joint Research Centre
Directorate F – Health, Consumers and Reference Materials
Retieseweg 111
B-2440 Geel, Belgium

NOTE

European Reference Material ERM®-BD150 was produced and certified under the responsibility of the European Commission's Joint Research Centre according to the principles laid down in the technical guidelines of the European Reference Materials® co-operation agreement between BAM-IRMM-LGC. Information on these guidelines is available on the internet (http://www.erm-crm.org).

DESCRIPTION OF THE MATERIAL

The sample consists of about 20 g of skimmed milk powder in a brown glass bottle with a plastic neck insert and screw cap.

ANALYTICAL METHODS USED FOR CERTIFICATION

Cold-Vapour Atomic Absorption Spectrometry
Electro-thermal Atomic Absorption Spectrometry
Flame Atomic Absorption Spectrometry
Hydride-Generation Atomic Absorption Spectrometry
High-pressure Liquid Chromatography Inductively Coupled Plasma Quadrupole Mass Spectrometry
Inductively Coupled Plasma Optical Emission Spectrometry
Inductively Coupled Plasma Quadrupole Mass Spectrometry
Ion chromatography
Isotope-Dilution Inductively Coupled Plasma Mass Spectrometry
Neutron Activation Analysis (radiochemical and k_0)
Pyrolysis Atomic Absorption Spectrometry (Mercury)
Inductively Coupled Plasma Sector-Field Mass Spectrometry
Titrimetry

PARTICIPANTS

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE
(Accredited to ISO Guide 34 for production of certified reference materials; BELAC No. 268-RM)
(Measurements performed under ISO/IEC 17025 accreditation; BELAC No. 268-TEST)

Australian Nuclear Science and Technology Organisation, Kirawee (AU)

ALS Scandinavia AB, Luleå (SE)
(Measurements performed under ISO/IEC 17025 accreditation; SWEDAC 1087)

Ceinal, S.A. (Silliker), Área Análisis Físico-Químicos, Barcelona (ES)
(Measurements performed under ISO/IEC 17025 accreditation; ENAC 257/LE413)

The Food and Environment Research Agency, York (UK)
(Measurements performed under ISO/IEC 17025 accreditation; UKAS 1642)

Helmholtz Zentrum München - Deutsches Forschungszentrum für Gesundheit und Umwelt GmbH, München (DE)

Institut "Jozef Stefan", Ljubljana, (SI)
(Measurements performed under ISO/IEC 17025 accreditation; Slovenska Akreditacija LP-090)

Laboratoire national de métrologie et d'Essais, Paris (FR)
(Measurements performed under ISO/IEC 17025 accreditation; COFRAC 22)

LGC Ltd., Teddington (UK)
(Measurements performed under ISO/IEC 17025 accreditation; UKAS 0003)

muva kempten, Kempten (DE)
(Measurements performed under ISO/IEC 17025 accreditation; DAkkS D-PL-14429-01)

SCK-CEN, Mol (BE)
(Measurements performed under ISO/IEC 17025 accreditation; BELAC 015-TEST)

Umweltbundesamt GmbH, Wien (AT)
(Measurements performed under ISO/IEC 17025 accreditation; Wirtschaftsministerium 92714/499-IV/9/01)

SAFETY INFORMATION

The usual laboratory safety precautions apply.

INSTRUCTIONS FOR USE AND INTENDED USE

This material is intended for quality control and assessment of method performance. As any reference material, it can also be used for control charts or validation studies.

Certified mass fractions are corrected for the water content of the material (dry mass): To determine dry mass, accurately weigh an aliquot of at least 1 g on an analytical balance and dry the sample in an oven at atmospheric pressure, at 102 °C ± 1 °C, until constant mass is attained. Weighing of the samples for dry mass determination and weighing for the analysis shall be done at the same time to avoid differences due to possible take up of moisture by the material.

Dispose in accordance with good laboratory practice.

STORAGE

The materials shall be stored at -20 °C ± 5 °C in the dark. Care shall be taken to avoid change of the moisture content once the units are open.

However, the European Commission cannot be held responsible for changes that happen during storage of the material at the customer's premises, especially of opened samples.

LEGAL NOTICE

Neither the European Commission, its contractors nor any person acting on their behalf:

(a) make any warranty or representation, express or implied, that the use of any information, material, apparatus, method or process disclosed in this document does not infringe any privately owned intellectual property rights; or

(b) assume any liability with respect to, or for damages resulting from, the use of any information, material, apparatus, method or process disclosed in this document save for loss or damage arising solely and directly from the negligence of the Joint Research Centre Directorate F – Health, Consumers and Reference Materials, in Geel.

NOTE

A detailed technical report is available on https://crm.jrc.ec.europa.eu. A paper copy can be obtained from the Joint Research Centre Directorate F – Health, Consumers and Reference Materials on request.

European Commission – Joint Research Centre
Directorate F – Health, Consumers and Reference Materials
Retieseweg 111, B - 2440 Geel (Belgium)
Telephone: +32-(0)14-571.705 - Fax: +32-(0)14-590.406

Appendix 9 – calcium distribution in skim milk with the addition of zinc acetate

Table A7: Concentration of calcium in skim milk, serum and collodial phase (calculated) at different concentrations of added zinc acetate determined by EDTA titration. Results presented are means ± standard deviation (n = 3-4). Different superscript letters (a, b, c, d, e, f, g, h, I, j) indicate significant difference across the different concentration of added zinc salt.

Concentration of added zinc acetate (mmol L^{-1})	Mass of zinc added (mg)	Determination by EDTA titration method			
		Concentration of calcium (mg/1 g skim milk)			% of zinc in serum
		Skim milk	Serum	colloidal phase*	
0	0.00	1.20[a]	0.30[a]	0.90[a]	25%
5	0.32	1.20[a]	0.32[a]	0.88[a]	27%
10	0.63	1.18[a]	0.34[b]	0.84[b]	29%
15	0.95	1.20[a]	0.41[c]	0.80[c]	34%
17.5	1.10	1.19[a]	0.46[d]	0.73[d]	39%
20	1.26	1.20[a]	0.46[e]	0.74[e]	39%
22.5	1.42	1.20[a]	0.49[f]	0.71[f]	41%
25	1.58	1.19[a]	0.49[g]	0.71[g]	41%
30	1.89	1.19[a]	0.52[h]	0.68[h]	43%
35	2.21	1.20[a]	0.53[i]	0.66[i]	45%
40	2.52	1.19[a]	0.60[j]	0.59[j]	51%

Pooled standard deviation was ± 0.02, the limit of detection was ± 0.03 mg Zn / 1g skim milk.

Appendix 10 – Skim milk pH with addition of zinc salts

Table A8: The effect of adding zinc acetate, zinc gluconate and zinc sulphate on skim milk pH at . The results presented were mean± standard deviation, (n=3). Different superscript letters (a, b, c, d, e, f, g, h, I, j, k) indicate significant difference across the different concentration of added zinc salt. Different superscript Greek letters (χ, γ, ϕ) indicate significant difference across the different added zinc salt type at each concentration of added zinc salt (95% confidence level).

Concentration of added zinc salt (mmol L^{-1})	Zinc salt added		
	Zinc acetate	Zinc sulphate	Zinc gluconate
0	$6.73\pm0.01^{a,\chi}$	$6.73\pm0.01^{a,\chi}$	$6.73\pm0.01^{a,\chi}$
5	$6.31\pm0.01^{b,\chi}$	$6.29\pm0.01^{b,\chi}$	$6.30\pm0.01^{b,\chi}$
10	$5.93\pm0.01^{c,\chi}$	$5.87\pm0.02^{c,\gamma}$	$5.94\pm0.01^{c,\chi}$
15	$5.69\pm0.01^{d,\chi}$	$5.60\pm0.01^{d,\gamma}$	$5.70\pm0.01^{d,\chi}$
17.5	$5.61\pm0.02^{e,\chi}$	-	-
20	$5.53\pm0.01^{f,\chi}$	$5.41\pm0.01^{f,\gamma}$	$5.52\pm0.01^{f,\phi}$
22.5	$5.47\pm0.01^{g,\chi}$	$5.35\pm0.01^{g,\gamma}$	$5.43\pm0.01^{g,\phi}$
25	$5.44\pm0.01^{h,\chi}$	$5.27\pm0.01^{h,\gamma}$	$5.38\pm0.01^{h,\phi}$
30	$5.39\pm0.01^{i,\chi}$	$5.15\pm0.01^{i,\gamma}$	$5.32\pm0.02^{i,\phi}$
35	$5.36\pm0.01^{j,\chi}$	$5.08\pm0.01^{j,\gamma}$	$5.28\pm0.01^{j,\phi}$
40	$5.34\pm0.01^{k,\chi}$	$5.04\pm0.01^{k,\gamma}$	$5.26\pm0.01^{k,\phi}$

-: Not tested.

Appendix 11 – Concentration of zinc in skim milk, serum and colloidal phase

Table A9: Concentration of zinc in skim milk, serum and colloidal phase (calculated) at different concentrations of added zinc salt determined by EDTA titration method. Results presented are means ± standard deviation (n = 3). For each type of sample (skim milk, serum or colloidal phase), different superscript letters (a, b, c, d, e, f, g, h, I, j) indicate significant difference across the added zinc concentrations for each type of zinc salt. For each type of sample, (skim milk, serum or colloidal phase), different superscript Greek letters (χ, γ, ϕ) indicate significant difference across the different types of zinc salt at each added zinc concentration (95% confidence level).

Zinc salt	Concentration of added zinc salt (mmol zinc/L skim milk)	Mass of zinc added (mg/g skim milk)	Determination by EDTA titration method			
			Concentration of zinc (mg/1 g skim milk)			% of zinc in serum
			Skim milk	Serum	Colloidal phase*	
Zinc acetate	5	0.32	$0.33^{a,\chi}$	$0.05^{a,\chi}$	$0.28^{a,\chi}$	15%
	10	0.63	$0.65^{b,\chi}$	$0.14^{b,\chi}$	$0.51^{b,\chi}$	21%
	15	0.95	$0.96^{c,\chi}$	$0.25^{c,\chi}$	$0.71^{c,\chi}$	26%
	17.5	1.10	$1.08^{d,\chi}$	0.31^{d}	0.78^{d}	28%
	20	1.26	$1.28^{e,\chi}$	$0.38^{e,\chi}$	$0.90^{e,\chi}$	30%
	22.5	1.42	$1.39^{f,\chi}$	0.42^{f}	0.97^{f}	30%
	25	1.58	$1.56^{g,\chi}$	$0.50^{g,\chi}$	$1.06^{g,\chi}$	32%
	30	1.89	$1.88^{h,\chi}$	$0.68^{h,\chi}$	$1.19^{h,\chi}$	36%
	35	2.21	$2.19^{i,\chi}$	$0.80^{i,\chi}$	$1.39^{i,\chi}$	37%
	40	2.52	$2.52^{j,\chi}$	$1.07^{j,\chi}$	$1.44^{j,\chi}$	43%
Zinc sulphate	5	0.32	$0.32^{a,\chi}$	$0.04^{a,\chi}$	$0.28^{a,\chi}$	12%
	10	0.63	$0.63^{b,\chi}$	$0.12^{b,\chi}$	$0.51^{b,\chi}$	19%
	15	0.95	$0.95^{c,\chi}$	$0.24^{c,\chi}$	$0.71^{c,\chi}$	26%
	20	1.26	$1.26^{d,\chi}$	$0.41^{d,\chi}$	$0.85^{d,\chi}$	33%
	25	1.58	$1.56^{e,\chi}$	$0.61^{e,\gamma}$	$0.95^{e,\gamma}$	39%
	30	1.89	$1.89^{f,\chi}$	$0.85^{f,\gamma}$	$1.03^{f,\gamma}$	45%
	35	2.21	$2.20^{g,\chi}$	$1.08^{g,\gamma}$	$1.11^{g,\gamma}$	48%
	40	2.52	$2.51^{h,\chi}$	$1.34^{h,\gamma}$	$1.16^{h,\gamma}$	54%
Zinc gluconate	5	0.32	$0.34^{a,\chi}$	$0.05^{a,\chi}$	$0.29^{a,\chi}$	15%
	10	0.63	$0.64^{b,\chi}$	$0.12^{b,\chi}$	$0.52^{b,\chi}$	19%
	15	0.95	$0.97^{c,\chi}$	$0.30^{c,\phi}$	$0.67^{c,\phi}$	31%
	20	1.26	$1.26^{d,\chi}$	$0.49^{d,\phi}$	$0.77^{d,\phi}$	39%
	25	1.58	$1.57^{e,\chi}$	$0.69^{e,\phi}$	$0.89^{e,\phi}$	44%
	30	1.89	$1.89^{f,\chi}$	$0.95^{f,\phi}$	$0.94^{f,\phi}$	51%
	35	2.21	$2.19^{g,\chi}$	$1.20^{g,\phi}$	$0.98^{g,\phi}$	54%
	40	2.52	$2.50^{h,\chi}$	$1.47^{h,\phi}$	$1.03^{h,\phi}$	59%

Pooled standard deviation was ± 0.02, the limit of detection was ± 0.03 mg Zn / 1g skim milk.

Appendix 12 – R^2 and slope of the shear stress versus shear rate

Table A10: The R^2 and slope of the shear stress versus shear rate curves plotted in Figure 5.5. (appendix)

	Added zinc concentration (mmol L^{-1})	R^2	Slope (mPa s)
NP skim milk	0	0.9963	1.72
NP skim milk + zinc acetate	10	0.9980	2.01
	15	0.9991	2.84
	17.5	0.9995	3.21
	20	0.9980	4.53
	22.5	0.9821	-
NP skim milk + zinc sulphate	10	0.9974	1.71
	15	0.9979	1.90
	20	0.9991	2.42
	22.5	0.9894	-
NP skim milk + zinc gluconate	10	0.9972	1.70
	15	0.9958	1.73
	20	0.9987	1.90
	22.5	0.9976	2.35
	25	0.9762	-

NP non-preheated.

-: Apparent viscosity did not presented in this table, since the apparent viscosity is different at different shear rate.

Appendix 13 – Final *G'* of skim milk with addition of zinc salts

Table A11: Final *G'* of skim milk with different added zinc salt concentration. The results presenter were mean ± sd, (n=3). Different letters (a, b, c, d, e, f) indicate significant difference across the added zinc concentrations for each type of zinc salt. Different superscript Greek letters (χ, γ, ϕ) indicate significant difference across the different types of zinc salt at each calcium concentration (95% confidence level).

	Added zinc concentration (mmol L^{-1})	Final *G'* (Pa)
zinc acetate	20	$0.35\pm0.03^{a,\chi}$
	22.5	$2.34\pm0.08^{b,\chi}$
	25	$3.95\pm0.06^{c,\chi}$
	30	$5.86\pm0.14^{d,\chi}$
	35	$6.56\pm0.09^{e,\chi}$
	40	$6.86\pm0.12^{f,\chi}$
zinc sulphate	25	$0.59\pm0.15^{a,\gamma}$
	30	$3.70\pm0.16^{b,\gamma}$
	35	$4.33\pm0.29^{c,\gamma}$
	40	$5.43\pm0.28^{d,\gamma}$
zinc gluconate	25	$0.04\pm0.04^{a,\phi}$
	30	$3.15\pm0.27^{b,\phi}$
	35	$3.57\pm0.10^{c,\phi}$
	40	$4.64\pm0.18^{d,\phi}$