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Exploring the Physicochemical Basis of Cheese Texture, Rheology and Functionality, with Emphasis on Cation-Casein Interactions

Thesis presented by

Darren Richard Cooke, B.Sc. ***(NUI)***

for the degree of

Doctor of Philosophy

in

Food Science and Technology

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DECLARATION BY THE CANDIDATE

Exploring the Physicochemical Basis of Cheese Texture, Rheology and Functionality, with Emphasis on Casein-Cation Interactions

Darren Cooke

I hereby declare that work described in this thesis is my own and has not been submitted for another degree, either in University College Cork, or elsewhere.

D. Cooke

Darren Cooke

20 August 2014

Summary

The rheological properties of Cheddar cheese and rennet-induced milk gels were investigated, with emphasis on the effects of mineral equilibrium alterations. The physicochemical properties of cheese and milk gels are greatly influenced by molecular interactions between the casein proteins involving calcium. Cheese meltability is a very important functional property that is largely influenced by calcium. The insoluble casein-bound calcium phosphate in milk/cheese (CCP) is in dynamic equilibrium with the soluble calcium in the aqueous phase of dairy products. Novel experiments were developed in order to study the relationship between changes in CCP concentration and the rheological behaviour of milk, milk gels and cheese to generate new information and theories on mineral-casein interactions.

Magnesium and strontium are in the same group as calcium in the Periodic Table (alkaline earth metals), and form divalent cations in aqueous solution. Experiments were designed to investigate if supplementation of Cheddar cheese and rennet-induced milk gels with Mg^{2+} or Sr^{2+} had similar effects on their rheological properties as previously reported in literature for Ca^{2+} supplementation. Sr^{2+} displayed behaviour similar to Ca^{2+} as observed by its ability to increase the rigidity of cheese and rennet milk gels and also decrease cheese meltability. Mg^{+2} had no influence on cheese rheological properties and was greatly inferior to Ca^{2+} and Sr^{2+} in its ability to increase rennet milk gel elasticity.

Cheddar cheese was supplemented with the calcium-chelating salts trisodium citrate, disodium hydrogen phosphate or disodium EDTA at salting, in an attempt to reduce the CCP content of cheese and thereby modify its rheological and functional properties. TSC and EDTA were successful in decreasing cheese CCP, whereas DSP caused an initial increase in CCP content. All these salts caused an increase in cheese meltability as determined by the Schreiber test, but TSC addition displayed unusual behaviour at high temperatures during small deformation rheology tests.

Cheddar cheese was supplemented with chlorides of iron, copper and zinc at salting to investigate the effects of concentrations of these elements in excess of those found innately or commonly in fortification studies, on the physicochemical properties of cheese with emphasis on mineral equilibria changes and resultant alteration of rheological properties. Zinc addition was the only added metal that significantly

influenced cheese rheological properties, leading to an increase in cheese rigidity and decreased cheese melt at elevated temperatures. Copper caused a major inhibitory effect on lactic acid bacteria populations during ripening. No link was established between mineral equilibria and redox potential.

Reducing the fat content of Cheddar cheese can result in abnormal texture and flavour profiles. Gum tragacanth (GT) was used as a fat-replacer in the manufacture of reduced-fat Cheddar cheese, in an attempt to improve the rheological, functional and sensory properties of reduced-fat Cheddar. GT improved certain textural properties, colour and melt, however, results from a consumer test found that GT is unsuitable for use as a fat-replacer in 'table' cheese. The GT strategy may be more suitable for cheese used as a functional ingredient in prepared foods where flavour is not as important.

Overall, the experimental work reported in this thesis generated new knowledge and theories about how casein-mineral interactions influence rheological properties of casein systems.

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The life of a PhD student is truly a rollercoaster of emotions, some days I felt like I deserved a Nobel Prize while other days left me questioning my integrity and resolve. Many of the people acknowledged here had a greater influence on helping me maintain an affirmative outlook on my work and studies than they realize. As I embark on the next chapter of my life in Switzerland, I will take with me the memories of all the great experiences that have shaped my life over the past four years in UCC.

Thank you all sincerely,

Darren Cooke

Chapter 1: Literature Review

From micelle to melt: the influence of calcium on physicochemical properties of cheese

Abstract

The following review explores how the behaviour of calcium in cheesemilk, during manufacture and during ripening, impacts on the rheological and functional properties of cheese. The functional properties of cheese can be explained in terms of their rheological behaviour, which in turn can be linked to molecular interactions within the cheese matrix. Calcium is known to play a key role in structural integrity of casein micelles as colloidal calcium phosphate nanoclusters crosslink casein molecules and reduce electrostatic repulsion allowing formation of the casein micelles. A similar structural role of the insoluble casein-bound calcium phosphate (CCP) is thought to exist in the *para*-casein matrix of cheese, with the concentration of CCP having a large influence on cheese firmness and meltability. A dynamic equilibrium between the CCP and soluble forms of calcium exists in milk and cheese. Partial solubilization of residual CCP occurs during the ripening of cheese. The realization over the last two decades that the CCP concentration in cheese is more important than total calcium in the context of determining the rheological properties of cheese has led to numerous studies where the 'calcium equilibrium' of cheese has been altered in an attempt to modulate textural and functional properties. Most of these studies have focused on Cheddar and Mozzarella. This review focuses on the overall picture of calcium behaviour from the casein micelles in milk, all the way to the functional performance of cheese, with emphasis on the form and structure of CCP, changes in calcium equilibrium during ripening, modification of calcium equilibrium and the influence of calcium equilibrium on the casein interactions that govern rheological and functional properties.

1.1. Introduction

The calcium content of cheese has a major influence on a number of its physicochemical properties. Calcium influences the rheological and functional properties of cheese due to calcium-dependent interactions between casein proteins (Lucey et al., 2003). Functional properties of cheese such as melting and stretch are of critical importance when the cheese is used as a food ingredient, i.e., as pizza toppings, lasagna layers, slices for hamburgers, etc. (Lucey, 2008). Most textural, rheological and functional properties are dependent on molecular interactions involving calcium in the *para*-casein matrix, the origin of which can be traced back to the behaviour of calcium in the cheesemilk, during manufacture and throughout ripening. It should be noted that the only cheeses discussed in this review are those made from bovine milk.

The natural calcium content of bovine milk depends on numerous factors such as breed, stage of lactation, geography, mastitis and diet (Holt, 1985). Bovine milk typically contains 26-32 mmol Ca/kg (Gaucheron, 2005). About two-thirds of the total calcium in milk exists in insoluble complexes associated with casein micelles known as colloidal calcium phosphate (CCP). The calcium in milk exists in a dynamic equilibrium between the insoluble form (CCP) and the soluble forms (free calcium ions and soluble undissociated calcium complexes with phosphate and citrate) in the aqueous phase (Holt, 1985). The majority of insoluble calcium exists in CCP nanoclusters which are of critical importance to the structure of the casein micelle as they can crosslink numerous casein molecules and reduce electrostatic repulsion, allowing formation of the casein micelle (Horne, 1998). The exact form of calcium phosphate in CCP nanoclusters remains a controversial topic with many

different models suggested and revised (McGann et al., 1983; Holt et al., 1989, 1998; Little and Holt, 2004). The precipitation of calcium phosphate to form CCP nanoclusters is largely influenced by pH and temperature, which is significant in the formation of mineral precipitates on heat exchanger surfaces used for thermal processing of milk (De Jong, 2008; Lucey and Horne, 2009).

Rennet coagulation is the primary manufacturing step involved in the production of most cheese varieties, and calcium has a major influence on this process. Calcium has no influence on the enzymatic phase of rennet coagulation if pH is kept constant, but the aggregation phase is highly dependent on ionic calcium (Ca^{2+}) concentration (Van Hooydonk et al., 1986). Cleavage of the glycomacropeptide from κ-casein reduces net negative charge and steric repulsion of micelles, allowing micelles to come into close contact with each other and aggregate in the presence of Ca^{2+} (Fox et al., 2000). Sufficient Ca^{2+} activity is required for proper rennet coagulation (Udabage et al., 2001). At constant Ca^{2+} activity, a lower CCP content results in longer rennet coagulation time (RCT) (Zoon et al., 1988). At constant pH, a lower CCP also results in increased RCT (Choi et al., 2007). It is well known that addition of calcium to milk can reduce RCT and improve gel properties (Zoon et al., 1988; Udabage et al., 2001).

Throughout the cheese manufacturing process, decreases in pH cause partial solubilization of CCP and the residual CCP concentration in the finished cheese has a major influence on the rheological properties of the cheese (Lucey and Fox, 1993; Lucey et al., 2005; O'Mahony et al., 2005). In Cheddar cheese, the residual CCP remaining in the cheese after manufacture partially solubilizes during the first month

of ripening (Hassan et al., 2004; Lucey et al., 2005; O'Mahony et al., 2005), during which time a pseudoequilibrium between soluble and insoluble calcium phosphate is reached (Hassan et al., 2004). This equilibrium is commonly termed the 'calcium equilibrium' of cheese, and alteration of manufacturing steps, i.e., pH alterations, acid development, addition of calcium salts and calcium-binding salts can alter the calcium equilibrium of cheese (Lee et al., 2005; Choi et al., 2008; Brickley et al., 2009). Addition of calcium at sufficient levels can alter the microstructure of cheese, resulting in an increased density of the *para*-casein matrix (Ong et al., 2013). The decrease in CCP content of cheese during early ripening is principally responsible for the initial softening of cheese and an increase in its meltability (Lucey et al., 2005; O'Mahony et al., 2006). A number of studies have reported a decrease in firmness and increased meltability in cheeses with reduced CCP concentrations due to alterations in manufacturing steps (Joshi et al., 2002; Mizuno and Lucey, 2005; Choi et al., 2008). Softening, meltability and stretch are among the most important functional properties of heated cheese. Understanding the relationship between the rheological behaviour of cheese and its calcium content at the molecular level is of great interest when studying functionality improvements in cheese.

1.2. Calcium equilibrium in bovine milk

1.2.1. Forms of calcium in milk

Bovine milk contains 26-32 mM Ca, with ~69% associated with casein micelles in an insoluble form (CCP) or directly-bound Ca^{2+}, and ~31% present as soluble forms in the aqueous phase (Lucey and Horne, 2009). The majority of soluble calcium exists in undissociated complexes formed mainly with citrate (as Cit^{3-}) and also to a lesser extent with inorganic phosphate (as a mixture of $H_2PO_4^-$ and HPO_4^{2-})

(Gaucheron, 2005) (see Table 1.1). Only about ~2 mM of the soluble calcium exists as free ionic calcium ions (Ca^{2+}) (Van Hooydonk et al., 1986; Tsioulpas et al., 2007). About 90% of the total citrate and half of the total inorganic phosphate in milk is soluble (Holt, 2004). The behaviour of calcium and phosphate in milk dictate the so-called calcium pseudoequilibrium in milk, i.e., the distribution of Ca between the soluble phase and insoluble casein-bound (colloidal) phase (Lucey and Horne, 2009). Of the major forms of inorganic phosphate in the aqueous phase of milk ($H_2PO_4^-$ and HPO_4^{2-}), the $H_2PO_4^-$ form has a low affinity for calcium, whereas the HPO_4^{2-} form has a relatively high affinity for this metal (Mekmene et al., 2009), however, the low concentration of the $CaHPO_4$ complex in the aqueous phase of milk (~0.6 mM) is due to its low solubility (Mekmene et al., 2009). It is noteworthy that a very small proportion of the insoluble calcium in milk is bound to whey proteins (Holt, 1985), principally to α-lactalbumin which binds ~0.5 mmol/kg (Lucey and Horne, 2009); however, this can be considered negligible. The calcium phosphate pseudoequilibrium is the most important aspect of the milk salts system in terms of casein micelle structure (see later), however, the other salts and their ions in the system also have a major effect. Sodium, potassium and chloride are present at high concentrations in milk (Table 1.2), with ~95% of each being present in the soluble phase. Potassium has the highest concentration of all the ions in milk (31-43 mmol/kg) and so has a great influence on milk ionic strength.

In addition to inorganic phosphate, milk also contains various forms of organic phosphate which can be found in phosphoseryl residues of caseins (discussed later), phospholipids, nucleotides, nucleic acids and ATP. The phospholipids are an important component of the milk fat globule membrane that helps prevent globules from coalescing (Ward et al., 2006). Richardson et al. (1980) found that bovine milk

contain ~0.23 μM ATP, with virtually all of it located within the casein micelles, possibly associated with CCP.

1.2.2. Colloidal calcium phosphate

The salts associated with casein micelles in milk are collectively referred to as colloidal calcium phosphate (Holt, 1985). This term encompasses the crosslinking calcium phosphate in nanoclusters (also known as micellar or casein-bound calcium phosphate) and also calcium directly bound to casein molecules (Ca caseinate).

Table 1.1. Calculated concentrations of phosphate and citrate anions as free ions and complexed with calcium (mM) in the aqueous phase of bovine milk (from Holt et al., 1981).

Anion	Free ion	Complexed with Ca^{2+}
H_2Cit^-	+	+
$HCit^{2-}$	0.04	0.01
Cit^{3-}	0.26	6.96
$H_2PO_4^-$	7.50	0.07
HPO_4^{2-}	2.65	0.59
PO_4^{3-}	+	0.01

+ indicates concentrations < 0.005 mM

Table 1.2. Mineral composition of bovine milk (Gaucheron, 2005)

Mineral	Concentration (mg/kg)	Concentration (mmol/kg)
Calcium	1043 – 1283	26 – 32
Magnesium	97 – 146	4 – 6
Inorganic phosphate	1805 – 2185	19 – 23
Total phosphate	930 – 992	30 – 32
Citrate	1323 – 2079	7 – 11
Sodium	391 – 644	17 – 28
Potassium	1212 – 1681	31 – 43
Chloride	772 – 1207	22 – 34

It is generally accepted that CCP nanoclusters are particles of amorphous hydrated calcium phosphate linked to casein phosphoseryl clusters, measuring ~2.5 nm in diameter and are distributed throughout the casein micelle (McGann et al., 1983; Holt, 2004). The spacing between nanoclusters has been estimated to be 18 nm (De Kruif and Holt, 2003). There may be up to ~800 of these nanoclusters in a casein micelle with a radius of 100 nm (Holt, 2004). Choi et al. (2011) estimated that 1,100 nanoclusters could fit in a casein micelle of radius 108 nm. The weight fraction of CCP nanoclusters in an average casein micelle has been estimated to be 0.07 (De Kruif and Holt, 2003). Along with calcium phosphate, McGann et al. (1983) reported that CCP contains citrate, magnesium (Mg) and zinc (Zn) at molar ratios to Ca averaging 0.05, 0.03 and 0.003, respectively.

These CCP nanoclusters can be viewed as having two primary roles in casein micelles, namely neonatal nutrition and micellar structural integrity. CCP nanoclusters allow milk to contain concentrations of calcium and phosphate well in excess of saturation levels. The more important role of CCP in the context of this review is that CCP nanoclusters are proposed to be one of the main crosslinking pathways in the formation of casein micelles. In the dual-binding model of the casein micelle proposed by Horne (1998) (Figure 1.1), polymerization of casein molecules proceeds via two possible pathways: (1) hydrophobic interactions between non-polar residues on adjacent casein molecules, with more than two molecules possibly interacting at these junctions and (2) CCP nanocluster crosslinks between hydrophilic regions of certain caseins. The CCP crosslinks act as bridges between two or more casein molecules that contain phosphoseryl cluster sequences. The interaction of the positively charged CCP nanoclusters with negatively charged

phosphoseryl clusters reduces electrostatic repulsion between casein molecules allowing attractive hydrophobic interactions to dominate.

α_{S1}-, α_{S2}- and β-Casein are multi-phosphorylated proteins and all contain at least one phosphoseryl cluster, whereas κ-casein lacks a phosphoseryl cluster (Figure 1.2). Phosphoseryl clusters are essential for the nucleation and stabilization of the calcium phosphate salts that comprise the core of CCP nanoclusters. Aoki et al. (1992) proposed that at least three phosphoseryl residues are required for a casein molecule to be crosslinked by CCP nanoclusters. These sequences on casein molecules have the specific motif $Ser(P)_3$-Glu_2 (Holt and Sawyer, 1988). So according to this model, only sequences that have three consecutive phosphoseryl residues are involved in the stabilization of CCP nanoclusters, i.e., each of α_{S1}- and β-casein have one of these sequences and α_{s2}-casein has two. Holt (2004) defined phosphate centres (PC) as at least two phosphorylated residues in a short sequence, which would give α_{S1}-, α_{S2}- and β-casein two, three and one of these phosphate centres, respectively. Such discrepancies in defining phosphoseryl clusters/centres can lead to different models of nanoclusters. These negatively charged sequences interact with and stabilize the positively charged CCP nanocluster core. Essentially, casein phosphoseryl clusters convert an intrinsically unstable milk system into a thermodynamically stable system (Holt, 2004).

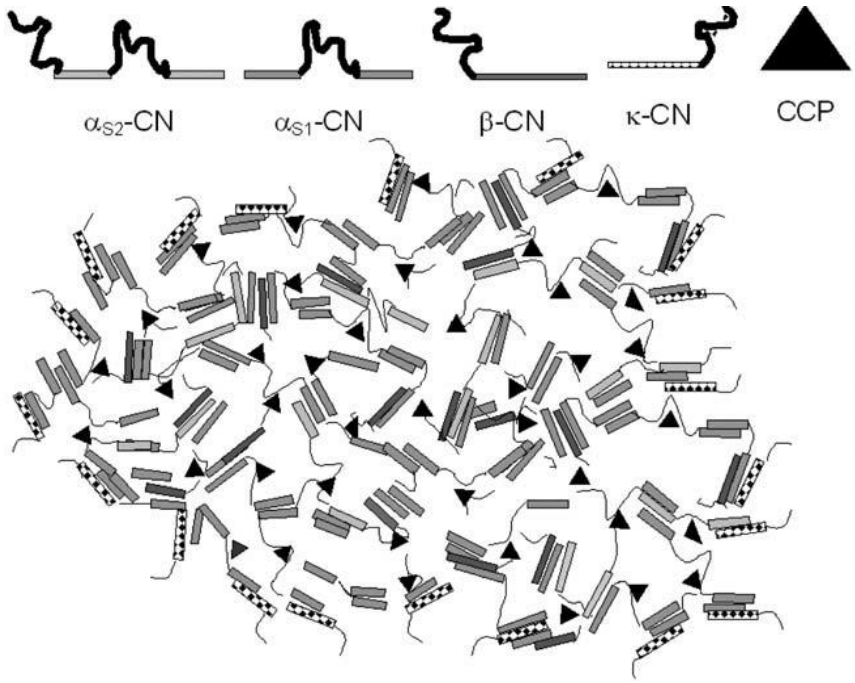


Figure 1.1. Schematic representation of the 'dual-binding model' of the casein micelle (Horne, 1998) revised by Lucey and Horne (2009). CN is casein and CCP is colloidal calcium phosphate nanoclusters.

α_{S1}-casein (8P)

---- - $SerP_{46}$-Glu-$SerP_{48}$- ----- - $SerP_{64}$-Ile-$SerP_{66}$-$SerP_{67}$-$SerP_{68}$- --------- -$SerP_{75}$- ------- --- $SerP_{115}$- -------

α_{S2}-casein (11 or 12P)

--- -$SerP_{8}$-$SerP_{9}$-$SerP_{10}$- --- -$SerP_{16}$- --------- -$SerP_{56}$-$SerP_{57}$-$SerP_{58}$-Glu-Glu-$SerP_{61}$- - ---------- -$SerP_{129}$-Thr-$SerP_{131}$- ----- -$SerP_{143}$- -----

β-casein (5P)

-------- -$SerP_{15}$-Leu-$SerP_{17}$-$SerP_{18}$-$SerP_{19}$- ------- -$SerP_{35}$- ----------

κ-casein (1P)

---------- -$SerP_{149}$- ----------

Figure 1.2. Phosphoseryl residue positions on bovine casein molecules (Horne, 2006).

CCP nanoclusters are thought to exist in a metastable state where growth into a macroscopic phase (leading to tissue calcification) is prevented by the rheomorphic structure of caseins along with their phosphoseryl clusters (Holt, 2004). The exact structure of CCP is still controversial. Two different views of nanoclusters have been envisaged. One view is where the organic phosphates (P_o) from phosphoseryl residues do not contribute to the Ca/P ratio of the calcium phosphate complex, which results in a Ca:P ratio >1.5 (McGann et al., 1983). The other view is that the P_o are integrated into the structure of CCP and not just loosely bound. In the latter model, the Ca:P ratio is lower (<1.5) and therefore the predicted stoichiometric form of the CCP in the nanocluster changes. The Ca:P ratio of a calcium phosphate salt has a major influence on its solution properties (Table 1.3). The aqueous phase of milk is highly supersaturated with respect to hydroxyapatite, to a lesser degree with tricalcium phosphate and octocalcium phosphate, marginally supersaturated with brushite and monotite, and unsaturated with calcium monophosphates (Holt, 1985). Hence, at the natural pH of milk, it would be expected that the most likely form of calcium phosphate in CCP nanoclusters is one of the more basic forms with the least solubility such as tricalcium phosphate (Ca:P = 1.5). However, the interaction of organic phosphate groups from caseins with the mineral complicates this assumption. CCP nanoclusters may persist as amorphous dicalcium phosphate in which a proportion of the HPO_4^{2-} ions are substituted by the RPO_4^{2-} moieties of phosphoseryl clusters on caseins, possibly at surface sites (Holt et al., 1996). Lucey and Horne (2009) also suggested that the form of CCP in milk is more likely to exist as a more basic form such as tricalcium phosphate, rather than an acidic form like brushite. Holt et al. (1998) derived a core-shell model of CCP nanoclusters that comprised a spherical core of ~355 $CaHPO_4.2H_2O$ units with a radius of ~2.3 nm and density of

2.31 g/ml, surrounded by ~49 peptide chains forming a tightly packed shell with an outer radius of ~4.04 nm. Horne et al. (2007) suggested that only four casein molecules are required to stabilize each CCP nanocluster. Cross et al. (2005) proposed a CCP nanocluster model that consists of two calcium phosphate phases: a calcium-poor phase with a Ca:P ratio of 1.5, such as $Ca_3(PO_4)_2$ forming the core, and a calcium-rich phase with a Ca:P ratio of 2.0, such as $Ca_2(PO_4)(OH)$ that interacts with the casein phosphoseryl groups.

Artificial CCP nanoclusters can be prepared *in vitro* by adding phosphoseryl cluster-containing hydrolysates of caseins (casein phosphopeptides) to an undersaturated solution of calcium phosphate salt at ~pH 5.5 and subsequently raising the pH above 6.0 (Holt et al., 1996, 1998). Attempts have been made to estimate the molecular weight of CCP nanoclusters, but differences in the number of phosphoseryl clusters that truly interact with the calcium phosphate core have caused discrepancies in calculations between studies (Ono et al., 1994; Holt et al., 1998; Horne et al., 2007; Choi et al., 2011). Most recently, Choi et al. (2011) used enzymatic digestion of casein micelles followed by size exclusion chromatography to estimate the molecular weight of CCP to be ~7,450 g/mol. This value fell within the range theoretically derived for CCP under the assumption that the form of CCP was brushite with either a tetrahedral- or a bi-pyramidal-shaped crystal structure.

Table 1.3. Chemical formulae and solubility indices of some pure calcium phosphates and other potential solid phases in milk (Holt, 2004)

Compound	Abbreviation	Formula	Ca/P	$-\log_{10}$ (IAP)	SI
Dicalcium phosphate	DCP	$CaHPO_4$	1.0	6.90	3
Dicalcium phosphate dihydrate	DCPD	$CaHPO_4.2H_2O$	1.0	6.59	6
Micellar calcium phosphate hydrate	MCP	$Ca(HPO_4)_{0.7}(PO_4)_{0.2}.xH_2O$	1.1	6.80	1
Octacalcium phosphate	OCP	$Ca_8H_2(PO_4)_6.5H_2O$	1.33	96.6	60
β-Tricalcium phosphate	β-TCP	β-$Ca_3(PO_4)_2$	1.5	28.9	200
Hydroxyapatite	HA	$Ca_5OH(PO_4)_3$	1.67	58.4	8×10^8
Amorphous calcium phosphate	ACP	$Ca_3(HPO_4)_{0.2}(PO_4)_{1.87}.xH_2O$	1.45	24.8	0.2
Tricalcium citrate dihydrate	TCC	$Ca_3(Cit)_2.2H_2O$	–	17.6	0.4
Dimagnesium phosphate	–	$MgHPO_4$	–	5.82	0.3

SI = saturation index
IAP = ion activity product

A proportion of colloidal calcium in milk is also directly bound to casein molecules. α_{S1}-Casein has a higher Ca^{2+}-binding capacity than β-casein which is thought to be a consequence of the higher phosphoseryl content of the former protein (Dickson and Perkins, 1971). However, most of the phosphoseryl clusters that exist in casein micelles are involved in stabilizing CCP nanoclusters. Apart from phosphoseryl residues, Ca^{2+} is also thought to bind to carboxyl groups of glutamic and aspartic acid residues, phenolic groups of tyrosyl residues, sulfhydryl groups of cystedyl residues and imidazole groups of histidyl residues (Dickson and Perkins, 1971; Gaucheron et al., 1997). Direct binding of cations becomes important when cations such as Fe or Cu are added, as these cations are thought to associate with caseins through coordination bonds and displace Ca^{2+} ions in the process (Hegenauer et al., 1979; Gaucheron et al., 1996).

1.2.3. Modification of calcium equilibrium in bovine milk

Changes in milk environmental conditions and solution properties, i.e., alteration of pH, temperature, ionic strength and addition of various mineral salts can have a major effect of the distribution and form of calcium in milk (Figure 1.3 and Table 1.4). Upon addition of calcium to milk, e.g., in the form of $CaCl_2$, an increase in both casein-bound calcium and inorganic phosphate is observed (Van Hooydonk et al., 1986; Udabage et al., 2000, 2001; Philippe et al., 2003). This co-precipitation of calcium and inorganic phosphate to the colloidal phase is indicative of the formation of CCP nanoclusters. It is estimated that ~10% of the total amount of phosphoseryl clusters in the casein micelle are unreacted (Holt, 2004). Philippe et al. (2003) suggested that new CCP nanoclusters formed at unreacted phosphoseryl clusters after Ca^{2+} addition to milk may differ from the natural form of CCP. Addition of calcium

to milk also increases the level of soluble calcium. Addition of inorganic orthophosphate salts (e.g., Na_2HPO_4 and NaH_2PO_4) to milk can decrease Ca^{2+} activity and increase CCP content of casein micelles (Udabage et al., 2000, 2001). Addition of strong calcium sequestering agents such as trisodium citrate and EDTA can decrease both CCP content of casein micelles and Ca^{2+} activity in milk (Udabage et al., 2000, 2001; Choi et al., 2007). The added Ca-binding anion competes with the phosphoseryl residues and CCP in the casein micelle for Ca^{2+} ions (de Kort et al., 2011). These Ca-binding agents sequester Ca from the aqueous phase and a proportion of insoluble Ca from the CCP, forming soluble complexes of Ca citrate, Ca EDTA, etc. It is proposed that the ability of a Ca-binding anion to solubilize CCP from the micelle depends on the saturation state of the calcium-anion complex formed in the aqueous phase (Holt, 1985). Addition of sodium chloride to milk causes an increase in ionic strength and can result in displacement of Ca^{2+} directly bound to casein by Na^+, causing an increase in soluble calcium but CCP nanoclusters are not thought to be effected (Van Hooydonk et al., 1986). The definition of CCP becomes complicated in milks supplemented with cations other than Ca^{2+} that also precipitate with inorganic phosphate or citrate such as Zn^{2+}, Fe^{2+} or Fe^{3+}. In particular, when Zn^{2+} is added to milk, a large proportion is thought to associate with CCP nanoclusters (Singh et al., 1989).

Decreasing the pH of milk causes solubilization of CCP, with all of the CCP being completely soluble at ~pH 5.0 (Lucey and Horne, 2009). The buffering capacity of milk is reliant on this solubilzation of CCP which results in the formation of phosphate ions that combine with H^+ causing buffering (Lucey et al., 1993b). During acidification, milk exhibits maximum buffering capacity at ~pH 5 (Lucey et al.,

1993b; Hassan et al., 2004). Increasing milk pH results in the formation of additional CCP (Lucey and Horne, 2009) as the amount of calcium and phosphate bound by phosphoseryl sequences increases with pH (Cross et al., 2005). The solubility of calcium phosphates decreases at high temperatures which results in the formation of heat-induced CCP, which re-solubilizes when milk is allowed to cool (Lucey and Horne, 2009). Alteration of the calcium equilibrium of milk by any of the above-mentioned mechanisms can have a major influence on the various processing steps involved in cheese manufacture, cheese composition and physicochemical properties of the finished cheese.

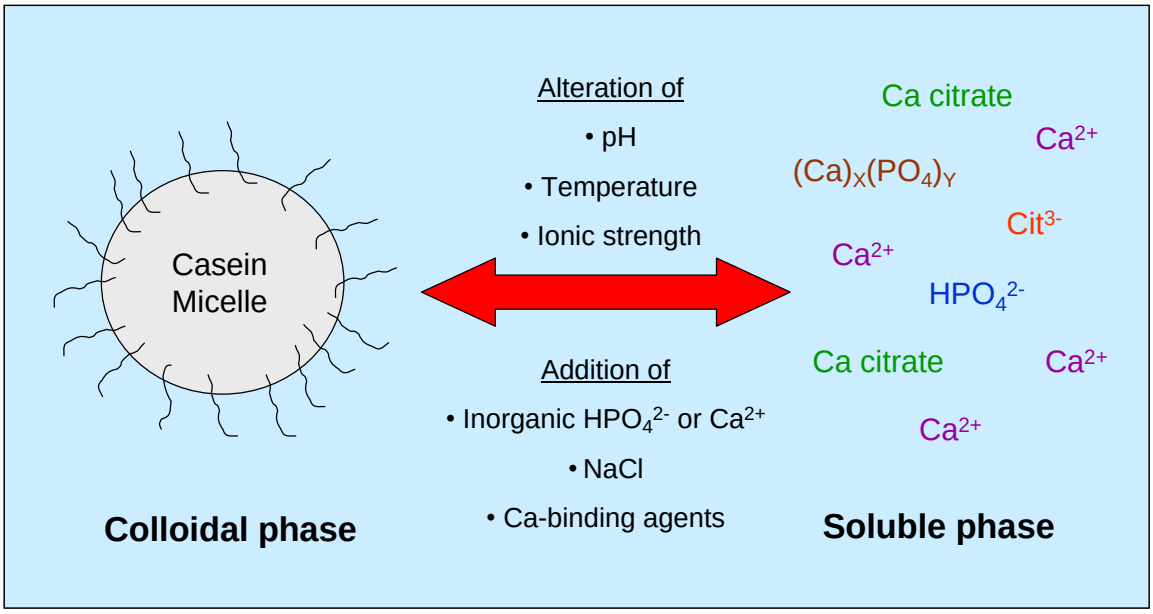


Figure 1.3. Effect of milk environmental conditions and solution properties on calcium phosphate equilibrium. Soluble phase refers to milk serum mineral components. Colloidal phase refers to casein-bound calcium salt complexes or counterions.

High-hydrostatic pressure (HHP) treatment of milk (100-600 MPa) has a major effect on calcium equilibrium. HHP causes casein micelles to disintegrate into small particles or aggregate, the extent of which depends on pressure level, temperature, pH and ionic strength (Lopez-Fandino, 2006.). HHP disrupts electrostatic

interactions, which affects the interaction of casein with CCP, resulting in extensive solubilization of CCP with increasing pressure >100 MPa (Huppertz et al., 2006). Along with CCP solubilization in raw milk, HHP can also solubilize heat-induced CCP from heat-treated milks (Lopez-Fandino, 2006: Huppertz et al., 2002). This solubilization is partially reversed upon release of high pressure, as casein micelles reform due to hydrophobic attractions and reformation of CCP, with the extent of reassociation depending on the pressure applied and temperature of the system (Orlien et al., 2006; Considine et al., 2007).

Table 1.4. Effect of alterations in environmental and solution properties of milk on CCP and soluble Ca content.

Alteration	CCP content	Soluble Ca content
pH increase	↑	↓
pH decrease	↓	↑
Temperature increase	↑	↓
Temperature decrease	↓	↑
NaCl addition	--	↑
Ca addition	↑	↑
Orthophosphate addition	↑	↓
Ca-binding agent addition	↓	↑
Application of HHP	↓	↑

Changes in calcium equilibrium can have a major impact on the physicochemical properties of the casein micelle. A number of studies have found that increasing CCP causes an increase in micellar casein content, a decrease in micellar hydration and a decrease in zeta-potential (Udabage et al., 2000, Philippe et al., 2003, 2005). However, those studies also reported that the average diameter of casein micelles remains constant upon CCP increase. The reduction in zeta-potential may likely arise from conformational changes in the micelle surface layer as a consequence of calcium binding to other parts of the surface and/or to the micelle core (Philippe et

al., 2003). Decreased hydration may indicate an expulsion of water from cavities located in the hydrophobic core of casein micelles, caused by solvent exposure change to casein side chain residues (Philippe et al., 2003). The incorporation of Ca and P_i and proteins into casein micelles and release of water from the casein micelles likely causes an increase in micellar density (Philippe et al., 2003, 2005). Increasing the level of CCP is known to decrease voluminosity of casein micelles, whereas, the opposite effect has been reported for decreases in CCP (Van Hooydonk et al., 1986; De Kort et al., 2011). Large reductions in CCP cause swelling of micelles and increased hydration, and eventually disintegration if enough CCP is lost (Udabage et al., 2000).

1.3. Calcium equilibrium in cheese

1.3.1. Changes in the calcium equilibrium of cheese during ripening

The process of cheese ripening involves numerous microbiological, biochemical and physicochemical changes, many of which are interrelated. Collectively, these changes are responsible for the conversion of the rubbery, bland young cheese into a mature cheese with characteristic flavour, texture and aroma (Fox and McSweeney, 1998; Lucey et al., 2003). One of the major factors governing changes in the structure and texture of cheese is its calcium content. Calcium content varies between cheese varieties due to their unique manufacturing procedures; in particular, the pH at whey drainage has a major influence on the final calcium content of cheese (Lucey and Fox, 1993). Typical calcium contents of Camembert, Cheddar and Emmental are 350, 720 and 970 mg/100 g cheese, respectively (O'Brien and O'Connor, 2004), which are linked to the differences in texture between these varieties. However, the insoluble Ca content of cheese is much more important than total calcium in regard

to cheese structure and inherent textural properties (Lucey and Fox, 1993). As mentioned in Section 1.2.1, a dynamic equilibrium between insoluble calcium bound to the casein micelles (CCP) and soluble calcium in the aqueous phase exists in milk. A similar situation is thought to occur in cheese, where calcium solubilizes from the residual CCP in the *para*-casein matrix during ripening to become part of the aqueous phase of cheese in order to attain a so-called 'pseudoequilibrium' of calcium phosphate between the soluble and insoluble phases of cheese (Hassan et al., 2004). This is commonly termed the 'Ca-equilibrium' of cheese. In Cheddar cheese, the proportion of insoluble calcium decreases during ripening from an initial level of ~72% to ~58% during the first three months of ripening (Hassan et al., 2004; Lucey et al., 2005) with very little change in insoluble calcium observed beyond this time, even when ripened for up to 9 months (Lucey et al., 2005). Most studies have reported that the majority of the changes in calcium equilibrium actually occur within the first month of ripening (Hassan et al., 2004; Lucey et al., 2005; O'Mahony et al., 2005).

1.3.2. Methods of calcium equilibrium determination in cheese

Two efficient methods of determining the insoluble Ca content of cheese have been developed and used successfully during the past two decades. One approach is the cheese juice method (Morris et al., 1988; Hassan et al., 2004; Lee et al., 2005), in which the serum phase is extracted from cheese by applying hydraulic pressure to grated cheese. The extracted serum or 'juice' is assumed to be compositionally equal to the aqueous phase of cheese (Morris et al., 1988) and thus, contains the soluble calcium at the same concentration as the aqueous phase of the cheese. The insoluble calcium content of the cheese can be estimated by comparing the total calcium

content of the cheese to that of the juice. The second method is the acid-base titration method (Lucey et al. 1993a; Hassan et al., 2004; Lucey et al., 2005), which relates the buffering capacity of cheese to its residual CCP content. In this method, the buffering capacity of the cheese and the milk it was made from are determined. The buffering peaks observed in milk between ~pH 5.8 to 4.1 and in cheese from ~pH 5.1 to 4.0 are an index of their CCP content (Lucey et al., 1993a,b; Hassan et al., 2004) (Figure 1.4). Both the buffering capacities and Ca contents of the milk and cheese are used to calculate the insoluble Ca content. Hassan et al. (2004) reported no statistical difference between these two methods for accuracy in their determination of % insoluble Ca in cheese (Figure 1.5).

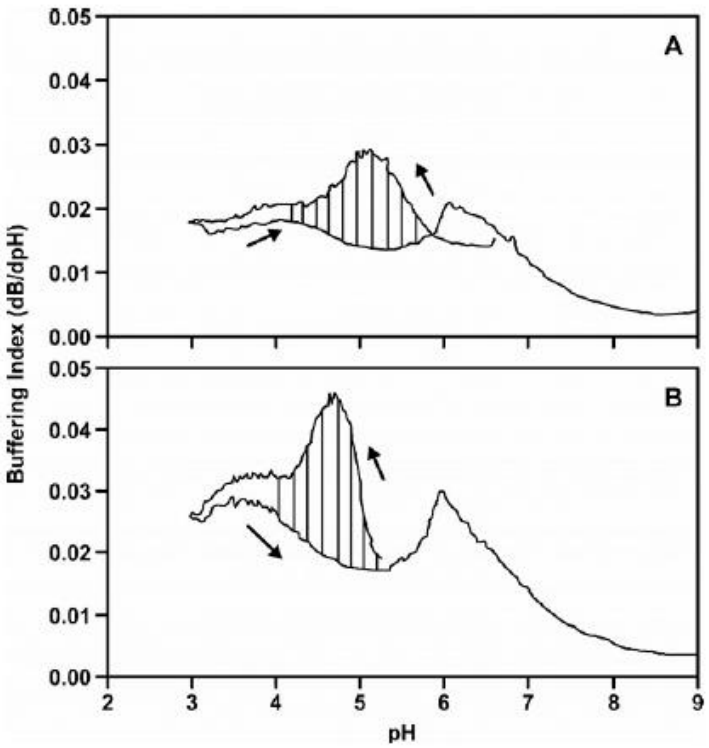


Figure 1.4. Buffering curves of milk (A) and Cheddar cheese (B) titrated from initial pH to pH 3.0 with 0.5 N HCl and then back-titrated to pH 9.0 with 0.5 N NaOH. Hatched area represents the buffering due to colloidal calcium phosphate. Arrows indicate the direction of the titration (Hassan et al., 2004).

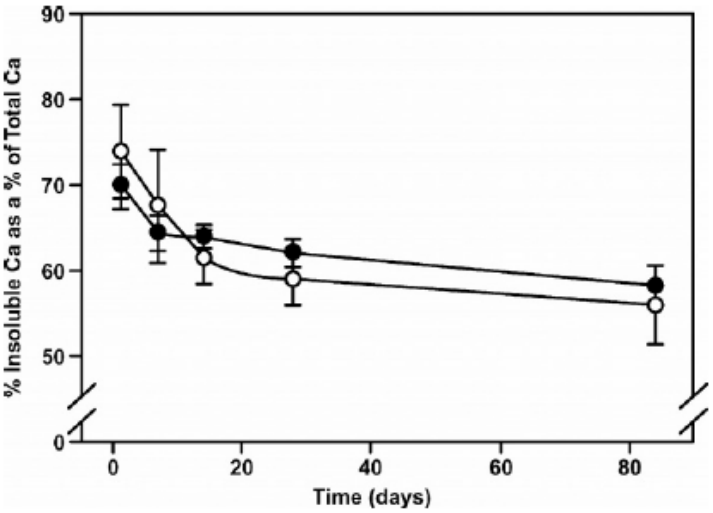


Figure 1.5. Changes in the % insoluble Ca content (expressed as a % of total cheese Ca) as a function of ripening time in Cheddar cheese determined by acid-base titration (○) and cheese juice (●) methods (Hassan et al., 2004).

1.3.3. Manipulation of calcium equilibrium in cheese

A typical manufacturing protocol for Cheddar cheese is shown in Figure 1.6. A decreased total calcium content in cheese is accompanied by a decreased insoluble calcium content (Choi et al., 2008). By altering acid development during manufacture of Cheddar cheese, Lee et al. (2005) produced cheeses with very low pH values (<4.9) that contained lower total calcium and insoluble calcium than cheeses with higher pH values. This study showed that the concomitant decrease in insoluble calcium with decrease in pH that is observed in milk can also be found in cheese. However, in the same study, Lee et al. (2005) reported that the insoluble calcium content did not decrease below ~41% of total calcium during ripening in a cheese with a pH value of ~4.7 (Figure 1.7). This observation highlights the difference in mineral-casein interactions between cheese and milk; i.e., CCP is completely solubilized < pH 5.0 in milk (Lucey and Horne, 2009). Lee et al. (2010) also observed a decrease in insoluble calcium content in Colby cheese with a low pH

induced by alteration of manufacturing pH. Choi et al. (2008) produced directly acidified cheeses with reduced insoluble Ca levels by lowering the pH of the cheesemilk and by addition of EDTA to cheesemilk. Pastorino et al. (2003a) decreased the pH of Cheddar cheese after manufacture by injecting 20% (w/w) glucono-δ-lactone into cheese during early ripening, causing a decrease in insoluble calcium content.

Mizuno and Lucey (2005) supplemented non-fat *pasta filata* cheese with trisodium citrate (TSC) via addition at the dry-salting step of manufacture. Addition of TSC led to a decrease in insoluble calcium level. In a similar study, Brickley et al. (2009) added TSC to Cheddar cheese at the salting stage and observed a decrease in insoluble calcium during the first month of ripening. In both studies, the authors attributed the decrease in insoluble calcium to the calcium-sequestering ability of TSC causing solubilization of a proportion of CCP in the cheeses. Pastorino et al. (2003b) was unsuccessful in altering the insoluble calcium level of Cheddar cheese by injecting the cheese with 40% TSC solution during early ripening. Immersing slices of 4 month-old Cheddar cheese in synthetic Cheddar cheese aqueous phase (SCCAP) solutions of varying calcium concentration, O'Mahony et al. (2006) observed an increase in the CCP concentration of cheese when the calcium concentration of the SCCAP solution exceeded that of the cheese serum. The opposite effect was observed with SCCAP solutions containing lower calcium concentrations than the cheese serum, where solubilization of cheese CCP occurred. These observations occurred due to calcium equilibration between the cheese and solutions in which it was immersed.

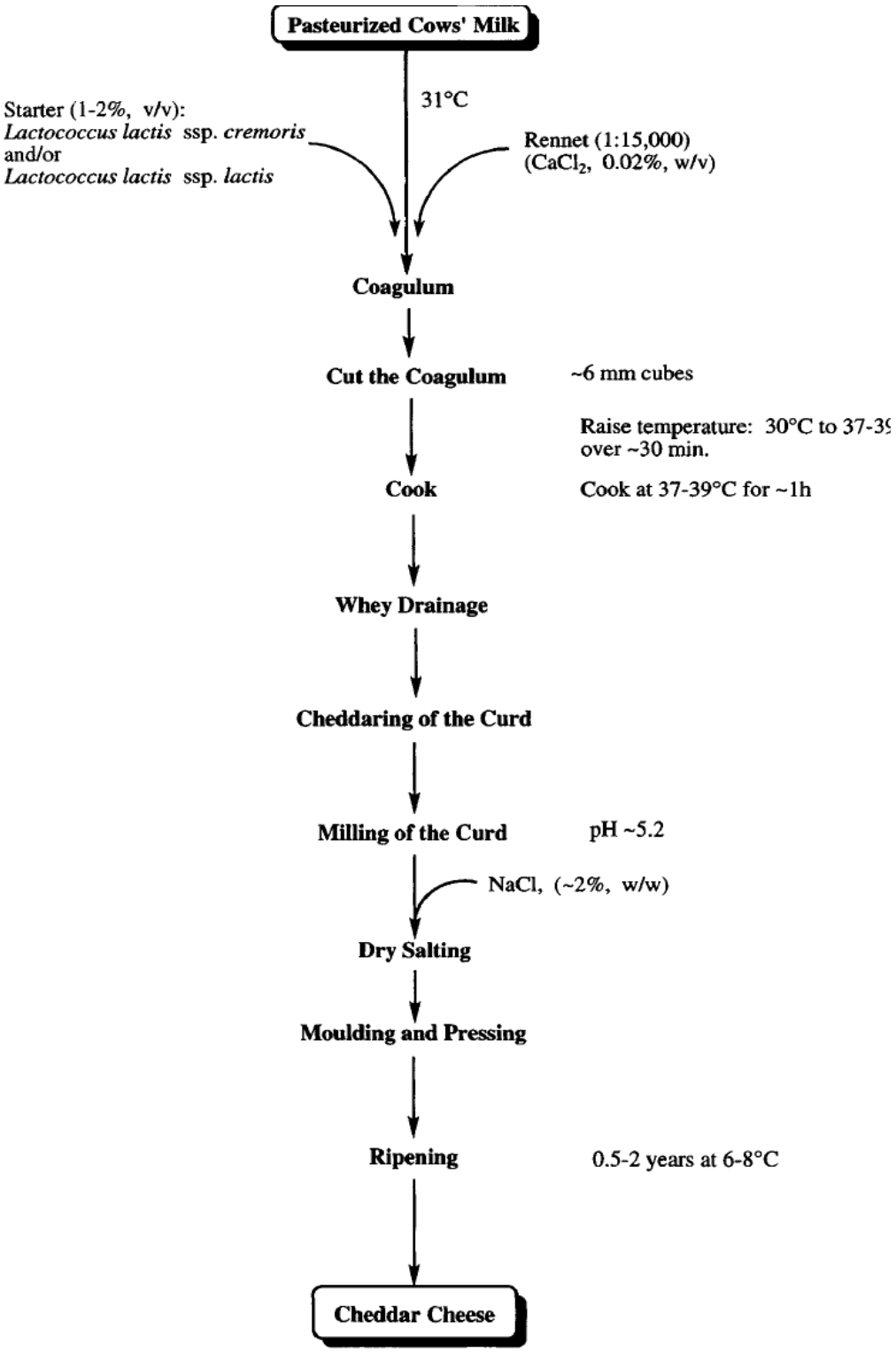


Figure 1.6. Typical manufacturing protocol for Cheddar cheese (Fox et al., 2000)

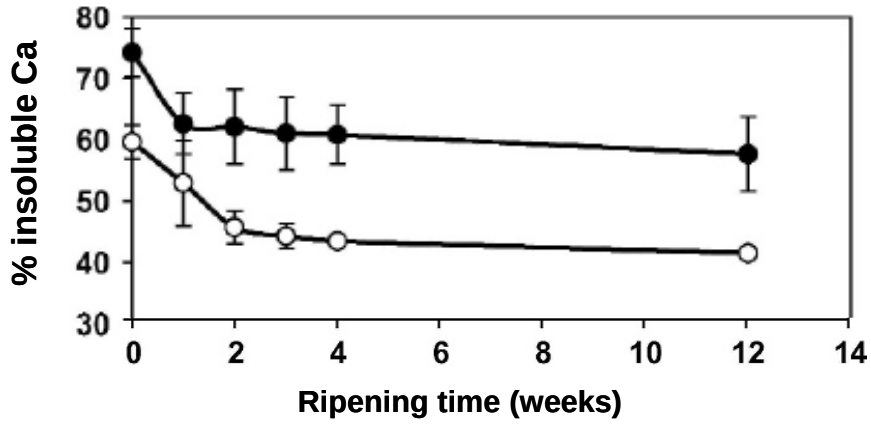


Figure 1.7. Changes in the % insoluble Ca content (% of total Ca in cheese) in Cheddar cheeses with pH decrease from 5.06 to 4.91 (●) and 4.91 to 4.77 (○) during ripening (Lee et al., 2005).

1.3.4. Mechanisms of calcium equilibrium changes during cheese ripening

Comparing the environment surrounding CCP in milk to the CCP in cheese, milk contains ~2.8% casein and ~87% water and has a pH of ~6.6, whereas, Cheddar cheese for example initially contains ~25% casein and 35 to 39% water and a pH of 5.1 to 5.3 (Fox and McSweeney, 1998; Lawrence et al., 2004). The low pH and reduced water content of cheese may have a major effect on the saturation state and form of calcium phosphate. The lower pH in cheese and changes in pH during ripening may affect the form of P_i that is present, i.e. conversion of HPO_4^{2-} to $H_2PO_4^-$ and *vice versa* depending on ripening age. As each of these P_i anionic species have different affinities for calcium (Mekmene et al., 2009), this conversion may alter the composition of CCP nanoclusters. During the first few weeks of Cheddar cheese ripening, starter lactic acid bacteria convert residual lactose to lactic acid (Fox et al., 2000) which can result in a slight decrease in cheese pH depending on salt-in-moisture level that controls microbial growth. The buffering effect of CCP in cheese likely prevents any excessive decrease in pH as acidification causes solubilization of CCP which liberates PO_4^- ions which combine with H^+ causing buffering (Lucey et al., 1993a; Hassan et al., 2004). It is likely that solubilization of CCP by this

mechanism has a contribution to the decrease in insoluble calcium levels during early ripening. Cheeses with very low pH values (<4.9) may likely have an acidic calcium phosphate form in CCP nanoclusters as basic calcium phosphate complexes form over a pH range from ~5.0 to 9.0 (Cross et al., 2005).

The composition of cheese juice from a one-month-old Cheddar cheese was studied in detail by Morris et al. (1988). They reported that approximately 57% of calcium, 89% of P_i and 55% of the citrate was insoluble at this stage of ripening. These levels of insoluble P_i and citrate are much higher than their levels in milk, i.e., ~46 and ~11% for P_i and citrate, respectively (Gaucheron, 2005). These high values were suggested by Morris et al. (1988) to be the consequence of the formation of calcium phosphate or calcium citrate crystals in the aqueous phase of the cheese due to supersaturation of these salts. The crystalline forms were suggested to be tricalcium citrate and brushite, as a more acidic calcium phosphate form would be expected to form at the pH of cheese juice. Morris et al. (1988) proposed that areas with high local pH near or within bacterial colonies may act as nucleation sites for precipitation of calcium phosphate and crystal formation as the degree of supersaturation of calcium phosphate salts would increase in these areas. Lee et al. (2005) observed calcium lactate crystals in Cheddar cheese ripened for ~3 months and proposed that crystallization of calcium salts would effectively decrease soluble calcium level and possibly encourage further loss of calcium from the CCP to balance calcium equilibrium.

Another consideration is that proteolysis in cheese may play some role in changes in calcium equilibrium during ripening. α_{S1}- and β-Casein are the principal structural

components of the *para*-casein matrix of cheese and both of these proteins are hydrolyzed to varying degrees during ripening by certain enzymes. As both of these casein molecules can interact with CCP nanoclusters via their phosphoseryl cluster sequences; the location of these sequences and the sites of enzymatic cleavage on these proteins is of interest as they may have a role to play in calcium equilibrium at some point during ripening. The phosphoseryl cluster sequence with three consecutive phosphoseryl residues in α_{S1}-casein occurs between residues 64 to 68 and in β-casein between residues 15 to 19 (Horne, 2006). α_{S1}- and β-Casein are hydrolysed mainly by chymosin and plasmin, respectively. Hydrolysis products of α_{S1}-and β-casein formed during Cheddar cheese ripening include α_{S1}-CN(f24-98), α_{S1}-CN(f24-101), α_{S1}-CN(f24-109), β-CN(f1-28) and β-CN(f1-105/107) (Sousa et al., 2001), all of which contain a phosphoseryl cluster. Loss of stabilizing phosphoseryl clusters may destabilize CCP nanoclusters. O'Mahony et al. (2005) provided evidence that the solubilization of CCP during the first few weeks of ripening is not influenced by proteolysis of α_{S1}-casein. In this study, inhibition of residual chymosin activity by pepstatin greatly reduced the cleavage of the Phe_{23}-Phe_{24} bond in α_{S1}-casein, allowing ~91% of α_{S1}-casein to remain intact after 180 days of ripening. Based on the findings of this study, it is likely that the rapid decrease in insoluble calcium in cheese within the first few weeks of ripening is not directly related to proteolysis. Gagnaire et al. (2001) reported that 17 peptides out of 91 identified in mature Emmental cheese were phosphopeptides derived from α_{S2}- and β-casein. After extensive hydrolysis of caseins associated with CCP nanoclusters in long ripened cheese, solubilization of phosphopeptides may induce a lack of CCP stabilization and so calcium phosphate may grow into macrocrystals. As cheese ripens, the estimated spacing between CCP nanoclusters increases from ~17 nm to

~40 nm by 6 weeks of ripening (Tunick et al., 1997; Lucey et al., 2003). Lucey et al. (2003) suggested that a type of Ostwald ripening of nanoclusters may occur during ripening. Destabilization of nanoclusters by proteolysis may facilitate such a mechanism.

As calcium can bind directly with caseins, especially at phosphoseryl residues (Dickson and Perkins, 1971), it is likely that hydrolyzed casein fragments that contain a phosphoseryl cluster (casein phosphopeptides) contain bound calcium and possibly have an effect on calcium equilibrium. At the same time, casein phosphopeptides that were already associated with CCP nanoclusters may remain associated, as casein hydrolysates containing phosphoseryl clusters have the ability to stabilize CCP (Holt et al., 1996, 1998; Cross et al., 2005). In the core-shell nanocluster model proposed by Holt et al. (1998), it is proposed that the nanocluster core is surrounded by a peptide 'shell' containing ~49 casein molecules. Presumably, proteolytic breakdown of these associated caseins may interfere with the structure of nanoclusters and possibly destabilise the meta-stable amorphous calcium phosphate core and induce crystal formation. In an attempt to isolate CCP nanoclusters, Choi et al. (2011) digested casein micelles in milk with trypsin and papain, resulting in CCP nanoclusters stabilized by phosphopeptide fragments of caseins. The authors reported that a small proportion of nanoclusters did not survive the digestion/dialysis processes. This finding supports the hypothesis that hydrolysis of caseins may effect calcium equilibrium in cheese. Proteolysis also reduces the 'free' water content of maturing cheese as cleavage of peptide bonds liberates a NH_2^+ group and a COO^- group that both compete for water (Creamer and Olson, 1982; Lucey et al., 2003).

This reduction in free water may concentrate the soluble calcium phosphate in the aqueous phase of cheese.

Formation of calcium lactate crystals in Cheddar cheese is also related to calcium equilibrium. The presence of calcium lactate crystals in Cheddar cheese is a quality defect which occurs as white crystals on the cheese surface. Racemization of L-lactate to D-lactate by non-starter lactic acid bacteria occurs in Cheddar cheese (McSweeney and Fox, 2004). Ca-D-lactate has a lower solubility than Ca-L-lactate and so a higher incidence of calcium lactate precipitation occurs with higher conversion to D-lactate (Chou et al., 2003). As the ratio of D/L lactate increases, cheese becomes increasing susceptible to calcium lactate precipitation (Johnson et al., 1990; Chou et al., 2003). Higher soluble calcium levels and higher levels of residual lactose make cheese more susceptible to the formation of calcium lactate crystals (McSweeney and Fox, 2004; Agarwal et al., 2006).

An interesting case of calcium equilibrium in a cheese system other than Cheddar is the change of calcium distribution during ripening of surface-ripened cheeses such as Brie and Camembert (Figure 1.8). In these cheeses, moulds grow on the cheese surface that metabolize lactate and produce ammonia which causes a pH gradient in the cheese, with a high pH at the surface and a low pH in the centre of the cheese (Karahadian and Lindsay, 1987; Abraham et al., 2007). The high pH at the surface causes calcium phosphate to precipitate, causing soluble calcium phosphate to migrate from the centre to balance the calcium equilibrium at the surface during ripening (Le Graet et al., 1983). The precipitated forms of calcium phosphate at the surface are suggested to be dicalcium or tricalcium phosphate as the Ca:P_i ratio at the

surface is ~1.87:1.00 (Le Graet et al., 1983). This process essentially solubilizes CCP at the centre of the cheese causing reduced attraction between caseins leading to decreased structural integrity of the cheese matrix. Considerable amounts of calcium should also migrate to the surface as calcium lactate, with the lactate being metabolized by the surface flora (Karahadian and Lindsay, 1987). Boutrou et al. (1999) reported a decrease in Ca and P_i concentrations and Ca:P_i ratio of juice extracted from Camembert during ripening, and attributed this to the precipitation of calcium phosphate that occurs near the cheese surface.

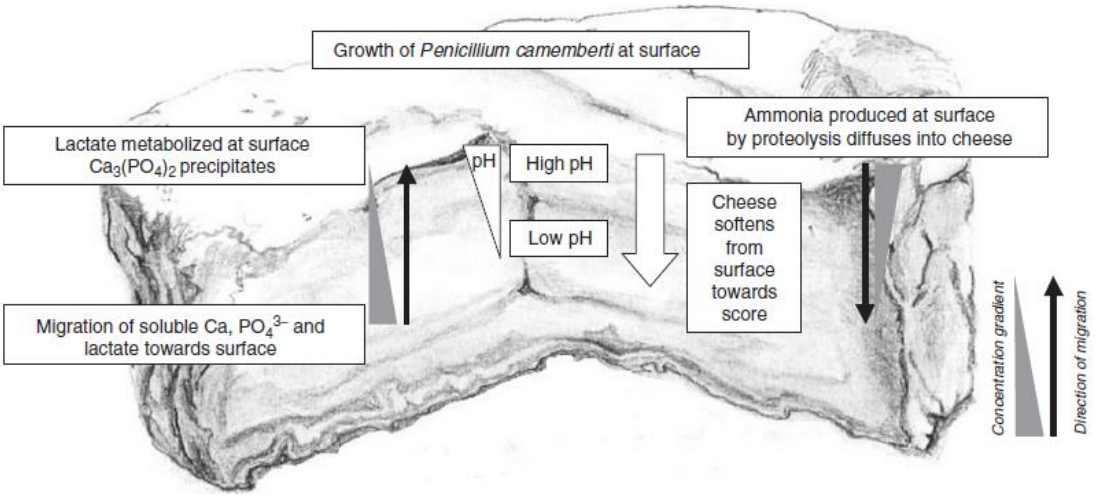


Figure 1.8. Schematic representation of the changes that occur in calcium, phosphate, lactate, ammonia and pH gradients in Camembert-type cheese during ripening (McSweeney and Fox, 2004).

1.4. The influence of calcium on cheese rheology and functionality

Cheese has grown in commercial importance to the food industry over the past few decades as it is being used increasingly as an ingredient in a wide variety of prepared foods (Figure 1.9), for example, pizza toppings and cheese slices on hamburgers (Lucey, 2008). For cheese to be used as a food ingredient, it must have suitable functional properties such as softening, meltability, stretchability, browning, etc. In

particular, Cheddar and Mozzarella have received much attention during the past two decades in the context of modulation of their functional properties relating to calcium, e.g., improvements in meltability. The functional properties of cheese are governed by their rheological properties, and so an intimate understanding of cheese rheology is essential when selecting or designing a cheese for use as a food ingredient.

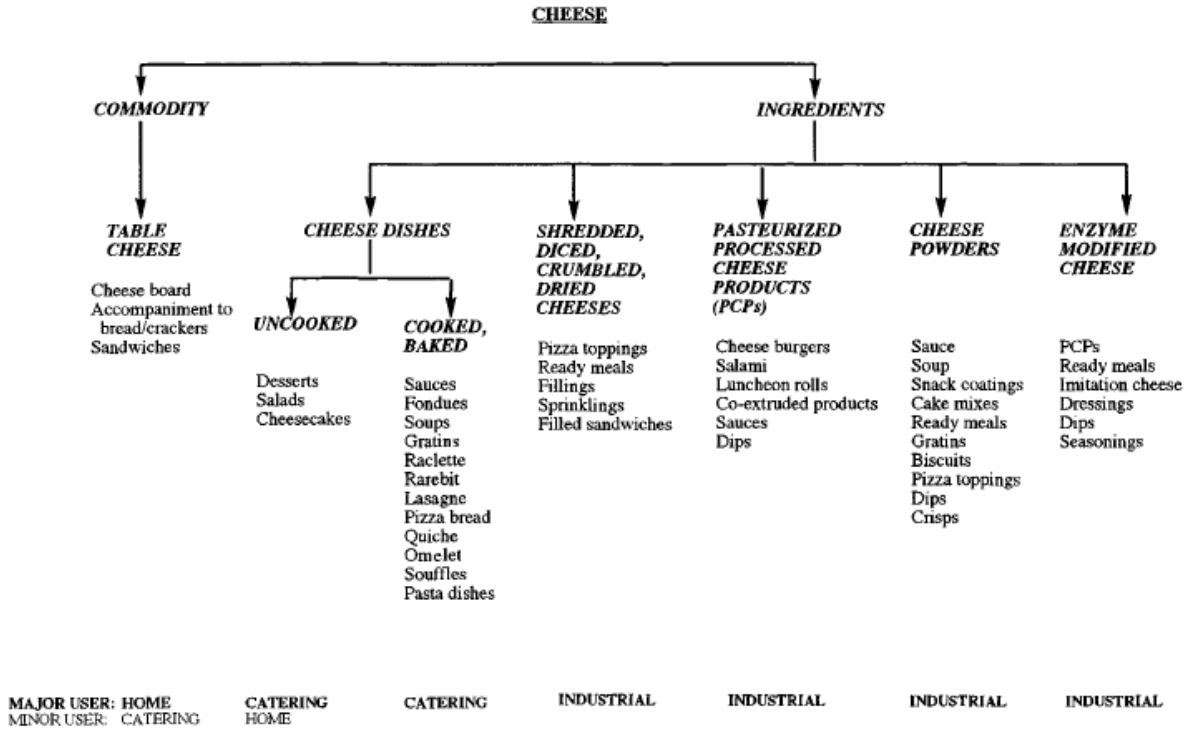


Figure 1.9. Uses of cheese as a food ingredient (Fox et al., 2000).

1.4.1. The influence of Ca equilibrium on cheese microstructure

The microstructure of cheese has a major influence on its textural and functional properties. Physicochemical changes occur in the cheese matrix during ripening which lead to increased hydration of the matrix. Hydration increases due to casein proteolysis, pH changes and solubilization of CCP (Fox et al., 2000). Increased hydration leads to physical expansion or swelling of the matrix which pushes fat globules closer together causing coalescence of the fat globules forming fat pools,

causing an increase in free fat upon melt (Fox et al., 2000). Two of the most popular methods to visualize cheese microstructure are (1) Light microscopy e.g. confocal scanning laser microscopy (CSLM) and (2) Electron microscopy e.g. scanning electron microscopy (SEM) (El-Bakry and Sheehan, 2014). Casein mineralization has a major influence on microstructure as formation of CCP increases matrix density. Supplementing cheesemilk with Ca at 300 and 600 mg/L, Ong et al. (2013) observed cheeses with a greater number of micron-sized pores in the structure and attributed this to reduced micellar fusion. Cheese hardness also increased with >100 mg/L addition levels. This is due to an increased number of CCP crosslinks in the matrix. The influence of increased Ca level on cheese microstructure can be seen in Figure 1.10, where higher Ca contents result in contraction of the *para*-casein matrix. In the study of Pastorino et al. (2003c), injection of 40% $CaCl_2$ solution into cheese post-manufacture resulted in contraction of the matrix and release of water into pores of the matrix. This was attributed to increased casein-casein bonding leading to decreased matrix hydration. Reducing the Ca content of cheesemilk decreases matrix density by allowing more hydration and swelling of matrix, creating more voids in the microstructure (Joshi et al., 2003). From these studies it can be concluded that the CCP content of cheese has a major influence on microstructure.

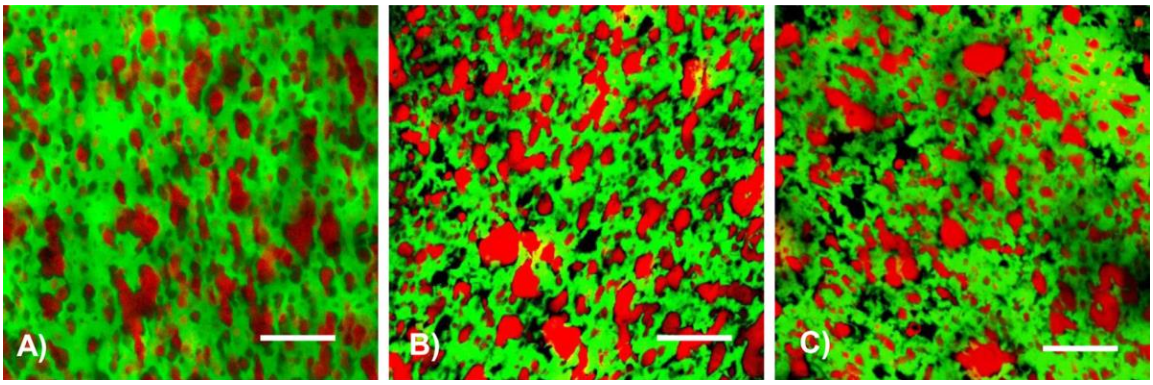


Figure 1.10. The microstructure of Cheddar cheese prepared using cheese-milk with the addition of (A) 0, (B) 300 or (C) 600 mg $CaCl_2$ per litre of milk. The Nile Red stained fat appears red and the Fast Green FCF stained protein appears green in these images. The scale bars are 10 mm in length (Ong et al., 2013)

1.4.2. Determination of the rheological properties of cheese

Rheology is the study of the flow and deformation of matter when subjected to stress or strain. The rheological behaviour of cheese can be categorized as viscoelastic. A viscoelastic material exhibits aspects of both solid (elastic) and liquid (viscous) rheological behaviours under a wide range of conditions. In the relationship between stress and strain, there are three regions of importance for cheese (Figure 1.11). The first region is the linear viscoelastic region, where the strain and stress are directly proportional and Hooke's Law is obeyed. The second region is non-linear, displaying a stress increase that is less proportional to strain increasing the slope, and the curve becomes increasingly concave until the third region is reached where the cheese fractures (Foegeding et al., 2003).

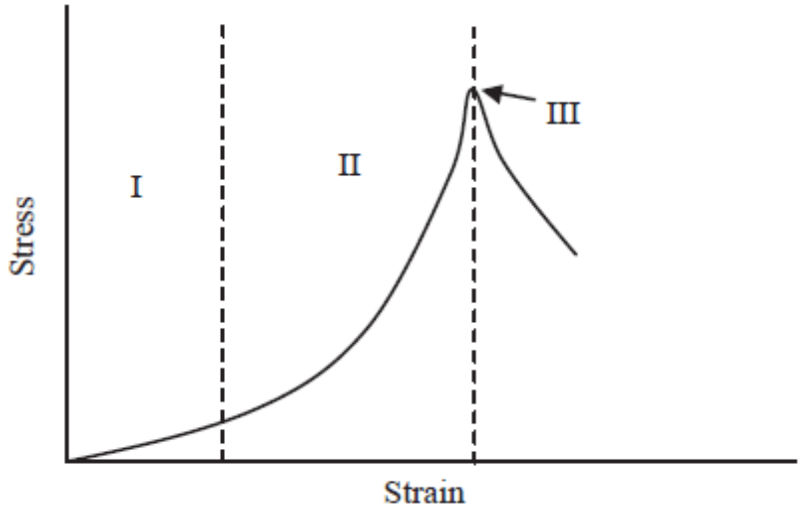


Figure 1.11. Rheological regimes for the viscoelastic behaviour of cheese (Foegeding et al., 2003).

Force compression tests are commonly used for cheese rheology studies. In these tests a cheese sample of defined geometry is subjected to varying stress and strain over time between two parallel plates, with the top plate causing deformation of the sample. Depending on the rheological information required, a cheese rheologist may

carry out large deformation or small deformation tests. In large deformation, non-linear deformation is achieved where structural bonds are broken and do not reform during the experiment, i.e., the stress-strain curve passes through regions 1, 2 and possibly 3 (Figure 1.11). This type of deformation occurs in uniaxial compression tests (fracture tests) and texture profile analysis (TPA). In TPA, the cheese sample is subjected to a double compression cycle, with the sample being compressed by a certain percentage (e.g., 70%) of its initial height for each compression. A typical texture profile of cheese obtained from TPA is shown in Figure 1.12. TPA can be referred to as an imitative test as it is used to attempt to correlate with sensory analysis. Information obtained from TPA compression curves include fracturability, hardness, adhesiveness, cohesiveness, springiness, gumminess and chewiness (Tunick, 2000). Large deformation properties strongly depend on the size of the largest inhomogeneities or 'weak spots' in the cheese matrix (Luyten and Van Vliet, 1996), which may be curd junctions, cracks and eyes, depending on the cheese variety.

The most important small deformation tests are termed dynamic rheological tests where cheese samples are placed between two parallel plates and subjected to small amplitude oscillatory shear and a strain that must be within the linear viscoelastic region (Figure 1.11) for the cheese. In this type of experiment, there exists a type of equilibrium between bond breakage and reformation. Typical frequency and strain regimes applied to Cheddar cheese during dynamic small amplitude oscillatory rheology tests (DSAOR) are $\leq$ 1 Hz and $\leq$ 0.5%, respectively (Gunasekaran and Ak, 2003; Lucey et al., 2005). Parameters derived from DSAOR include the storage modulus (G′), the loss modulus (G″), the complex modulus G* and the loss tangent

(LT) also known as tan δ, which is the ratio of viscous to elastic moduli (G″/G′) (Gunasekaran and Ak, 2003). The information obtained from DSAOR can be related to the molecular interactions occurring in the cheese (Lucey et al., 2003). A common DSAOR test used in cheese rheology studies is the temperature sweep, where the temperature of the cheese sample is usually increased to > 70 °C at a fixed frequency and strain.

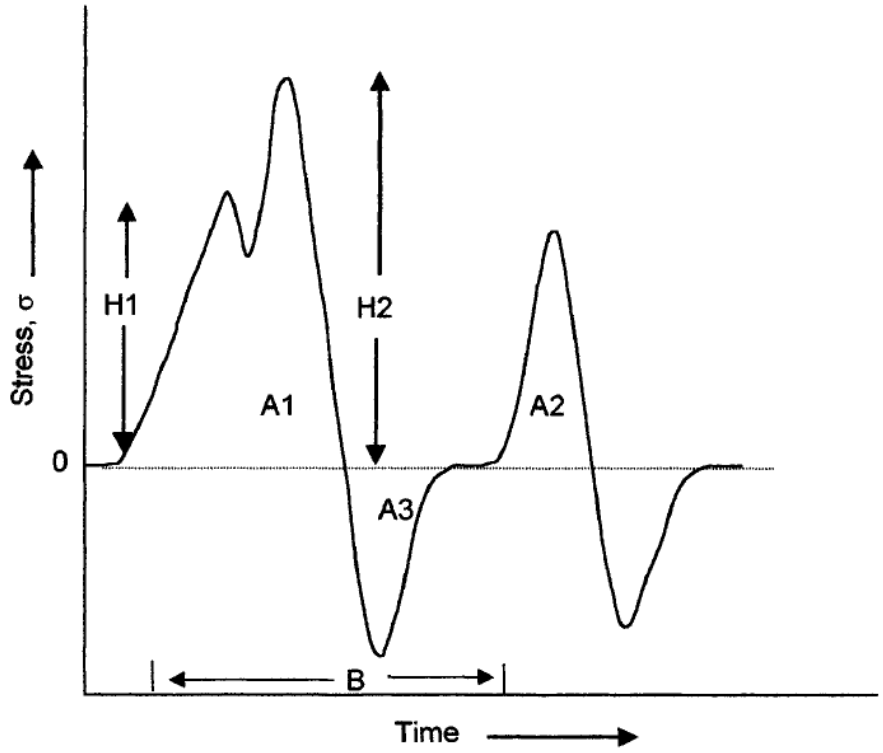


Figure 1.12. A typical texture profile of cheese obtained from texture profile analysis. Areas (A) and heights (H) of the curve used to calculate TPA parameters, e.g., H2 = hardness (Fox et al., 2000).

Cheese is thermorheomorphic and its rheological behaviour changes considerably during heating. Typical changes in G′ and LT as a function of temperature during Cheddar cheese ripening are shown in Figure 1.13. Heating cheese from refrigeration temperatures to 40 °C causes a decrease in the G′ of cheese which is partially attributed to melting of milkfat, but above 40 °C all of the fat in cheese is thought to be liquid (Walstra and Jenness, 1984) and further decrease in G′ > 40 °C is the result

of the interactions between caseins (Lucey et al., 2003). As temperature increases, so do hydrophobic interactions between protein molecules (Bryant and McClements, 1998), which is thought to result in shrinking of casein particles and a decrease in contact area causing a decrease in overall gel strength leading to decreased G′ values in cheese (Lucey et al., 2003). Other interesting parameters that can be derived from temperature sweeps include the temperature at LT = 1, which can be taken as the melting point of cheese (Gunasekaran and Ak, 2003) and maximum loss tangent (LT_{max}) which indicates the point of highest bond mobility and can be used as an index of the maximum meltability of cheese (Lucey et al., 2005). As insoluble calcium is involved in the structural integrity of the *para*-casein matrix of cheese via CCP crosslinks, the level of insoluble calcium in cheese has a major influence on its rheological properties, primarily due to electrostatic interactions (Lucey et al., 2003).

1.4.3. Influence of calcium on rheological properties of unmelted cheese

Most cheese varieties do not exhibit flow at refrigeration temperature to ~25 °C and so large deformation and fracture properties are appropriately measured in this temperature range. Machinability of cheese, i.e., the ability to be cut, sliced or shredded by machines, is also better when cheese displays moderate to high hardness in this lower temperature range (Lucey, 2008). At low temperatures, hydrophobic interactions are weak which allows casein molecules to exist in an expanded form. This increases contact area between caseins, leading to more casein-casein interactions resulting in the firmness observed (Lucey et al., 2005; Lucey, 2008). As calcium phosphate is more soluble at low temperatures (Lucey and Horne, 2009), solubilization of CCP may increase electrostatic repulsion and also contribute to the increase in contact area in the lower temperature range. Originally, it was thought

that chymosin-mediated cleavage of the Phe_{23}-Phe_{24} bond of α_{S1}-casein was responsible for the softening observed during the early ripening stages of Cheddar cheese (Creamer and Olson, 1982). By inhibiting residual chymosin activity in Cheddar cheese, O'Mahony et al. (2005) still observed a significant softening of Cheddar cheese during early ripening and correlated this with the decrease in insoluble calcium content in cheese within the first month of ripening. Changes in Cheddar cheese texture beyond the first month of ripening are principally attributed the proteolytic degradation of the *para*-casein network (Lucey et al., 2005).

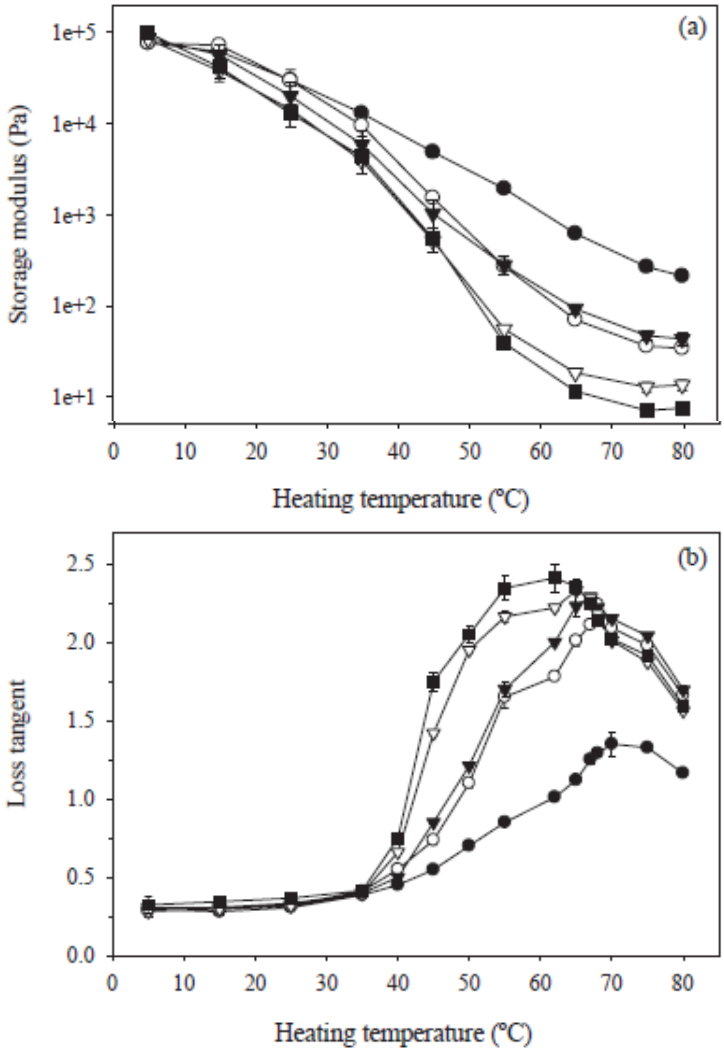


Figure 1.13. Changes in the storage modulus (a) and the loss tangent (b) as a function of temperature for Cheddar cheese ripened for 3 days (●), 1 month (○), 2 months (▼), 3 months (▽), and 9 months (■), obtained from dynamic small amplitude oscillatory rheology (Lucey et al., 2005).

Reduction of total calcium content by manufacturing cheeses with lower pH values can lead to softer cheeses (Sheehan and Guinee, 2004), however, the low pH increases the rate of hydrolysis of α_{S1}-casein during ripening causing reduced cheese firmness (Watkinson et al., 2001), so an indirect effect of lower insoluble calcium may be the reduced attractive forces between caseins making them more susceptible to hydrolysis (Fox, 1970). Watkinson et al. (2001) reported an increase in cheese firmness with increase in pH for directly acidified cheeses with initial pH values ranging from ~5.2 to 6.2, where the compositions of the cheeses were relatively similar. Presumably, as calcium phosphate complexes are less soluble at higher pH values, the increased firmness in this study was attributable to increased CCP crosslinking in the cheeses as pH increased. Ong et al. (2013) observed a significant increase in the TPA hardness values of Cheddar cheeses made with 100 to 600 mg/L $CaCl_2$ added to the cheesemilk. Supplementing Cheddar cheese with $CaCl_2$ at the salting stage, Brickley et al. (2009) observed an increase in TPA hardness values in a calcium-supplemented cheese containing ~1160 mg Ca/100 g cheese compared to a control cheese containing ~828 mg Ca/100 g cheese during ripening. The authors attributed this increase in hardness to increased CCP content in the calcium supplemented cheese. Addition of TSC at the salting stage of cheesemaking can decrease TPA hardness values of cheese (Mizuno and Lucey, 2005; Brickley et al., 2009), due to reduction of CCP crosslinks via calcium sequestration by TSC forming soluble calcium citrate complexes.

1.4.4. Influence of calcium on cheese melt and high temperature cheese rheology

When cheese is heated above 40 °C there is a dramatic reduction in its dynamic moduli (G′ and G″) and an increase in LT, indicating the prevalence of a more

viscous-like behaviour in the cheese. Moisture and liquid fat are the plasticizing agents during cheese melt (Muthukumarappan et al., 1999), facilitating casein particles to flow past each other when heated. Hydrophobic interactions increase with temperature (Bryant and McClements, 1998) and it is proposed that the decreased elasticity of heated cheese is the result of casein particles contracting on themselves causing individual casein particles to shrink leading to a reduction in contact area and subsequent reduction in intermolecular/particle bonding in the *para*-casein network (Lucey et al., 2003). Hydrophobic interactions increase to maximum strength up to 60-70 °C and lose strength thereafter (Bryant and McClements, 1998). As electrostatic repulsion also increases with temperature (Bryant and McClements, 1998), Lucey et al. (2003) proposed that cheese melt occurs when electrostatic repulsion becomes the dominant casein interaction. Increased protein hydration is also thought to increase cheese melt (McMahon and Oberg, 1999; Sheehan and Guinee, 2004) as protein hydration increases repulsion between molecules (Bryant and McClements, 1998). The presence of CCP crosslinks in casein micelles greatly reduces the localized electrostatic repulsion (Horne, 1998), so the insoluble calcium content in cheese plays a key role in determining the melting behaviour of heated cheese (Choi et al., 2008).

The melting of cheese occurs in two stages: softening and flow. As cheese is heated, it always softens before it flows, but some varieties do not flow after softening (Lucey et al., 2003). Softening of cheese refers to a loss of elasticity when cheese is heated. Flowability of cheese on heating may be defined as the displacement of contiguous molten planes of the *para*-casein matrix as mediated by heat-induced stress (Guinee et al., 2000). Flowability and meltability are interchangeable terms in

the context of heated cheese. As mentioned previously, analysis of cheese using DSAOR temperature sweeps can provide invaluable information about cheese melting behaviour. In cheese rheology studies, it is useful to use more than one type of test to evaluate cheese melt. Numerous methods exist for measuring cheese meltability such as the Schreiber test where cheese cylinders are subjected to oven temperatures (e.g., 232 °C for 5 min) (Altan et al., 2005) or the 'squeeze-flow' approach using the UW Meltmeter which carries out 'melt profile analysis' on the cheese, which gives information such as softening temperature and degree of flow (Muthukumarappan et al., 1999; Lucey et al., 2005). However, a lack of correlation between melting tests can occur (Park et al. 1984). It is known that the type of heating system, the rate of heating and sample geometry greatly influence the results of melting analyses (Lucey et al., 2003).

Cheeses with reduced calcium content may soften and melt at lower temperatures and exhibit higher meltability (Joshi et al., 2003). Lucey et al. (2005) found that the increase in melt (as indicated by LT_{max}) during the first few weeks of Cheddar cheese ripening is more significantly correlated with the decrease in levels of insoluble calcium than with extent of proteolysis. Solubilization of CCP crosslinks during early ripening increases electrostatic repulsion between casein particles resulting in weakening of the *para*-casein matrix that is sufficient to increase meltability. Subsequent increases in cheese meltability after the first month of ripening can be attributed to proteolytic breakdown of the matrix (Lucey et al., 2005). When α_{S1}-casein is degraded, the remaining larger peptides no longer interact with other caseins causing a weakening of the matrix that increases melting during ripening (Joshi et al., 2003). A resultant decrease in temperature at which LT_{max} occurs during

ripening suggests that less thermal energy is required to achieve maximum meltability as ripening proceeds (Lucey et al., 2005). Selecting cheese at the appropriate ripening age is essential for its use as a functional ingredient in foods and also for use as an ingredient in the manufacture of processed cheese; in particular, the insoluble calcium content of natural cheese can have a major influence on the rheological properties of processed cheese made therefrom (Guinee and O'Kennedy, 2009).

As discussed in Section 1.3.3, manufacturing protocols can be altered to produce cheeses with lower pH values that result in lower levels of insoluble calcium. Reducing the insoluble calcium content of cheese can lead to increased LT values in cheese heated at pH values > 5.0 (Pastorino et al., 2003a; Lee et al., 2005, 2010; Choi et al., 2008). However, decreasing cheese pH < 4.9 inhibits meltability of cheese even in cheeses with reduced levels of insoluble calcium (Lee et al., 2005, 2010) as electrostatic attraction becomes the dominant casein interaction due to the proximity of the pH to the isoelectric point of casein (pH 4.6) (Lucey et al., 2003). Cheese varieties with pH values < 4.9 such as cottage cheese or Feta exhibit very little flow as a result of the attractive interactions in the cheese matrix (Lucey, 2008). A decrease in cheese pH not only results in solubilization of CCP but also to a decrease in the net charge of caseins. Choi et al. (2008) separated the effects of charge and insoluble calcium level on the rheological properties of cheese by producing cheeses with the same pH values but varying insoluble calcium levels by addition of EDTA to cheesemilk. It was found that cheeses with the same pH (~5.7) and composition exhibited increased LT values (Figure 1.14) and decreased G′

values at high temperatures as insoluble calcium level decreased, indicating a decrease in elastic-like properties with reduction of CCP level.

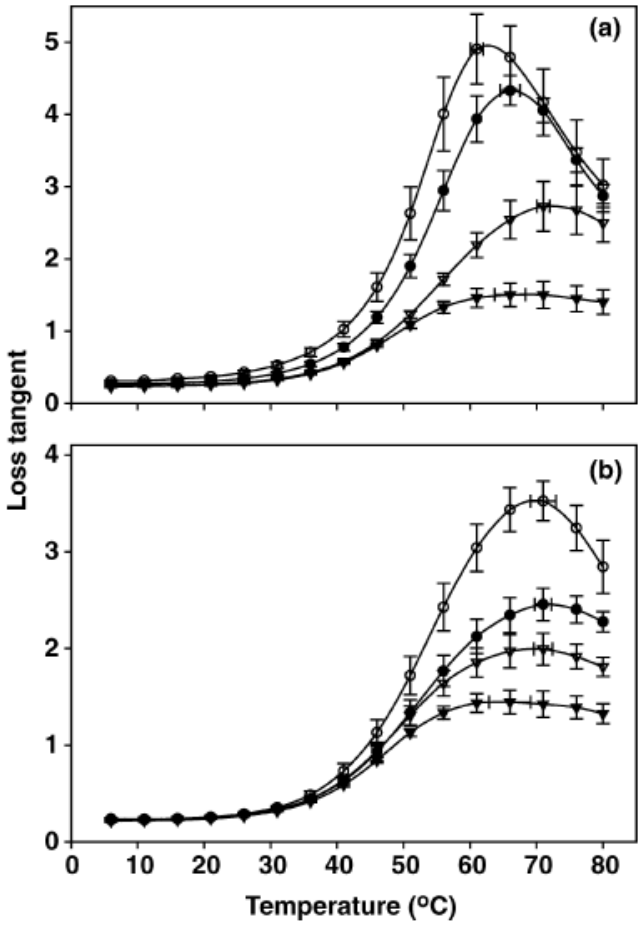


Figure 1.14. Loss tangent as a function of temperature for cheese (a) made from milk acidified to pH 6.0 (▼), 5.8 (▽), 5.6 (●), or 5.4 (○) and cheese (b) made from milk that had 0 (▼), 2 (▽), 4 (●), or 6 mM (○) EDTA added before cheese making (Choi et al., 2008).

By immersing Cheddar cheese slices in SCCAP with varying calcium concentrations, O'Mahony et al. (2006) reported a significant decrease in LT (Figure 1.15) and increase in G′ values at 70 °C with increasing cheese CCP concentration, the opposite effect was observed with decreasing CCP concentration. The holding time of melted cheese at certain temperatures can also influence its flow properties. Kuo et al. (2001) reported that the extent of cheese flow decreased with increasing

holding time of melted cheese at 60 °C. The authors attributed this finding to increased aggregation in the cheese mass due to an increased number of hydrophobic interactions at this temperature, which is in the temperature range where hydrophobic interactions are at their strongest (Bryant and McClements, 1998). It is evident that the types of bonding involved in cheese melting are very sensitive to temperature and pH.

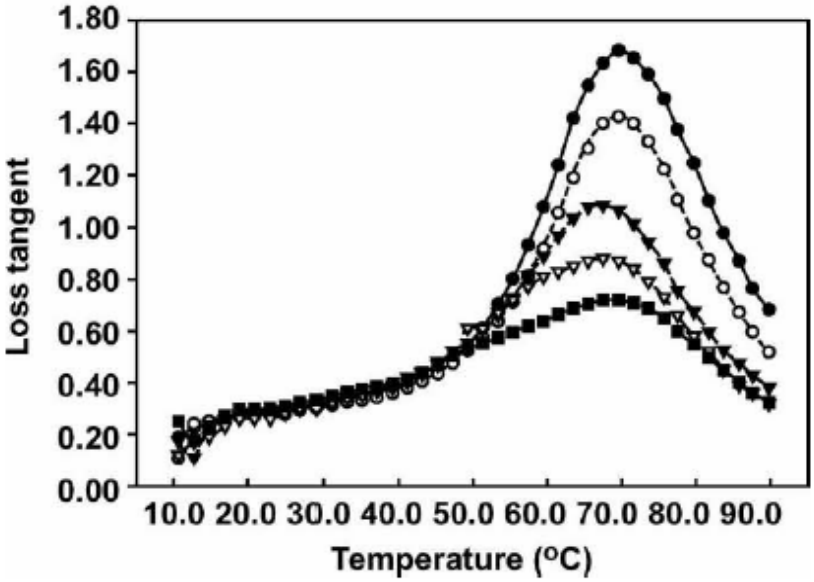


Figure 1.15. Loss tangent as a function of temperature from DSAOR for Cheddar cheese slices incubated in synthetic Cheddar cheese aqueous phase solutions containing 1.39 (●), 2.78 (○), 5.56 (▼), 6.95 (▽), or 8.34 (■) g of calcium/L (O'Mahony et al., 2006).

Cheese stretch is an important functional property at high temperatures in Mozzarella which is commonly used as a pizza topping. Stretchability is the ability of melted cheese to form fibrous strands that elongate without breaking under tension during ripening (Kindstedt, 1993). For cheese to stretch correctly, a low insoluble calcium content is required (Lucey, 2008), as stretch is impaired when casein-casein interactions are too strong (Lucey et al., 2003).

1.5. Conclusions

The concentration and form of calcium in cheesemilk and cheese is of critical importance to the many textural, rheological and functional properties of cheese. By manipulating the calcium equilibrium of cheese, the cheesemaker can modulate the rheological properties of cheese and enhance its functionality. Accelerating the development of cheese texture during ripening may also be useful as mature cheese is more expensive. A detailed understanding of how calcium influences all aspects of cheesemaking is essential for a food technologist developing cheese with tailored functional properties for ingredient food applications.

Further characterization and understanding of the structure of CCP nanoclusters in the casein micelles of milk are of great interest to dairy chemistry. Most of the existing knowledge about calcium equilibrium in cheese simply encompasses the quantity of soluble and insoluble Ca, but there is a lack of information on the soluble forms of calcium, i.e., Ca lactate, Ca phosphate, Ca citrate, etc., and also the form of insoluble Ca in cheese. Studies on the form of CCP nanoclusters in cheese and changes in their structure during ripening are also of interest as they will provide more information about the mechanisms controlling rheological properties in ripened cheese which is important for manipulating the functional properties of cheese.

Development of novel methods for altering the calcium equilibrium in cheese should be pursued in order to determine which methods are the most efficient in terms of cost and flexibility in the extent of alteration. While most of the studies on calcium equilibrium have focused on Cheddar and Mozzarella, it would be of interest for Ca equilibrium studies to be carried out on a wider range of cheese varieties that are

currently used for or have potential as food ingredients. A more precise understanding of the mechanisms involved in cheese melting, i.e., relationship between hydrophobic and electrostatic interactions is required in order to enhance the accuracy and efficiency in modulation of functional properties.

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Thesis experimental objectives

The main objective of the following experimental chapters was to further develop the understanding of the mechanisms that govern rheological and functional properties in Cheddar cheese and rennet-induced skim milk gel systems, and in the case of Cheddar cheese, the mechanism of casein interactions at elevated temperatures was emphasized. These experimental chapters investigated a number of different strategies to explore this topic such as alteration of the mineral equilibria of cheese and rennet-induced skim milk gels and also use of a fat replacement technology using a hydrocolloid.

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- In **Chapters 2** and **3**, chloride salts of the alkaline earth metals strontium (Sr) and magnesium (Mg) were added to Cheddar cheese to evaluate the ability of their cations to influence calcium (Ca) equilibrium, and also to observe their own partition between the serum and casein-bound phases. The influence of these mineral equilibria changes on rheological properties and melting properties (in the case of **Chapter 2**) were evaluated to compare Mg and Sr to Ca as much information is available on Ca behaviour in this context. In theory, it was presumed that as Mg and Sr would exhibit behaviour similar to Ca and increase CCP and network rigidity as their cations are divalent and they are from the same group in the Periodic table. In **Chapter 2**, $MgCl_2$ and $SrCl_2$ were introduced to the system at salting to avoid complicating the cheese making protocol as they would likely interfere with milk pH, gelation time and texture development that would create too many variables in the experiment.

- In **Chapter 4**, the opposite approach of **Chapter 2** was used. It was evaluated if Ca-binding salts would decrease CCP and cheese rigidity. TSC, DSP and EDTA were chosen, as TSC and DSP are commonly used in processed cheese manufacture to disintegrate casein aggregates; and EDTA is a well known Ca-chelator that has been used for this purpose in milk systems for decades. As with **Chapter 2**, the mineral salts were added during salting to avoid complicating the cheese manufacturing protocol. Along with **Chapter 2**, this study would try to develop the known theories available on high temperature casein interactions in cheese and relate the impact of CCP content on rheological properties.
- **In Chapter 5**, a similar approach to **Chapter 2** was used as metal cations that could potentially increase insoluble mineral levels in cheese were added. Cations of iron (II) and (III) (Fe^{2+} and Fe^{3+}); copper (II) (Cu^{2+}); and zinc (Zn^{2+}) were introduced to the cheese system as chloride salts at the salting stage of Cheddar manufacture in order to evaluate their impact on mineral equilibria and their consequent effects on cheese rheological properties. The elements used in Chapter 2 belong to the same group as Ca (alkaline earth metals) and so could be related to Ca behaviour, specifically their interaction with casein which is electrostatic in nature. However, the heavy metals used in **Chapter 5** could potentially bond to casein either electrostatically or via coordination bonding which may generate more useful information about the relationship between mineral-casein interactions and their influence on cheese rheological and functional properties. These metals are often added to cheese milk at low levels for various reasons such as nutritional fortification (Fe and Zn) and also flavour development (Cu addition to Emmental) and

addition at high levels may further develop the knowledge of how the mineral equilibria of cheese governs its physical properties. Lactic acid bacteria populations and redox potential were also determined as these metals are known to be toxic at moderate levels, and a link between redox potential and mineral equilibria has not been established in literature. It should be noted that Na replacement was not an objective of any of these studies with mineral salt addition.

- **Chapter 6** explored the mechanisms involved in cheese rheology and functionality from a different perspective compared to **Chapters 2-5**, by evaluating a fat reduction strategy designed to improve cheese texture, rheological and melting properties. Gum tragacanth (GT) was used as a fat-replacer and its ability to improve half-fat Cheddar cheese was investigated. Similar to **Chapter 2-5**, this study attempted to create a better understanding of high temperature rheological properties of Cheddar cheese due to casein interactions and also in this case, the potential interaction of GT with casein.

Chapter 2

The influence of alkaline earth metal equilibria on the rheological, melting and textural properties of Cheddar cheese

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Abstract

The total calcium content of cheese, along with changes in the equilibrium between soluble and casein (CN)-bound calcium during ripening can have a major impact on its rheological, functional and textural properties; however, little is known about the effect of other alkaline earth metals. NaCl was partially substituted with $MgCl_2$ or $SrCl_2$ (8.7 and 11.4 g/kg curd, respectively) at the salting stage of cheesemaking to study their effects on cheese. Three cheeses were produced: $MgCl_2$ supplemented (+Mg), $SrCl_2$ supplemented (+Sr) and a control Cheddar cheese. Ca, Mg and Sr contents of cheese and expressible serum obtained therefrom were determined by atomic absorption spectroscopy. Addition of Mg^{2+} or Sr^{2+} had no effect on % moisture, protein, fat and extent of proteolysis. A proportion of the added Mg^{2+} and Sr^{2+} became CN-bound. The level of CN-bound Mg was higher in the +Mg cheese than the control throughout ripening. The level of CN-bound Ca and Mg decreased during ripening in all cheeses, as did % CN-bound Sr in the +Sr cheese. The presence of Sr^{2+} increased % CN-bound Ca and Mg at a number of ripening times. Adding Mg^{2+} had no effect on % CN-bound Ca. The +Sr cheese exhibited a higher G′ at 70°C and a lower LT_{max} than the control and +Mg cheeses throughout ripening. The +Sr cheese had significantly lower meltability compared to the control and +Mg cheeses after 2 months of ripening. Hardness values of the +Sr cheese were higher at week 2 than the +Mg and control cheeses. Addition of Mg^{2+} did not influence the physical properties of cheese. Supplementing cheese with Sr appeared to have effects analogous to those previously reported for increasing Ca content. Sr^{2+} may form and/or modify nanocluster crosslinks causing an increase in the strength of the *para*-casein matrix.

2.1. Introduction

It is well recognised that the physical properties of cheese are influenced by numerous factors such as pH, calcium content, composition (fat, moisture, protein and salt) and proteolysis. Physical properties of cheese such as hardness, melt, stretch and sliceability are of great importance to both consumers and industry alike. The calcium present in milk and cheese exists in two primary phases: insoluble casein-bound Ca phosphate (CCP), known in milk as colloidal calcium phosphate, and soluble calcium in the aqueous phase. CCP is one of the primary structural elements of the casein micelle (Horne, 1998). CCP exists as nanoclusters several nanometers in size, consisting of a calcium phosphate core linked to numerous organic phosphates from phosphorylated serine residues of casein molecules, and are distributed throughout the protein matrix of the casein micelle (Holt, 2004). Lucey & Fox (1993) first suggested that the level of CCP in cheese is more important than the total calcium content in relation to influencing cheese texture and functionality. Subsequent studies have shown that during cheese ripening (especially during the first month), there is partial solubilization of CCP and a pseudoequilibrium of calcium phosphate between the soluble and insoluble phases is reached (Hassan et al., 2004; Lucey et al., 2005). Increasing the total calcium content in cheese promotes casein-casein interactions through CCP bridging and charge neutralization leading to increased hardness (Pastorino et al., 2003). O'Mahony et al. (2006) developed a novel model system using a synthetic Cheddar cheese aqueous phase to study the effects of CCP concentration on the rheological properties of Cheddar Cheese independent of proteolysis. In this study, increasing the CCP content of cheese led to an increase in storage modulus (G′) at 70°C and a decrease in maximum loss tangent (LT_{max}). Increasing the total calcium content of cheese by addition of calcium

chloride at the salting stage (Brickley et al., 2009) or by injecting calcium chloride after manufacture (Pastorino et al., 2003) has been found to increase hardness and decrease the meltability of cheese. Thus, an increase in calcium level can enhance the rigidity of cheese.

Calcium is an element in Group 2 of the Periodic Table (alkaline earth metals) and forms divalent cations (Ca^{2+}) in aqueous solution. Magnesium and strontium are also in this group and have similar chemical properties to calcium. Magnesium occurs naturally in milk and is present in bovine milk at a level of 4-6 mmol/kg (Lucey & Horne, 2009). In bovine milk, approximately one-third of the total magnesium and two-thirds of the total calcium are associated with casein micelles (Gaucheron, 2005). There is still uncertainty as to the location and role of casein-bound magnesium. Strontium can occur in milk at trace levels and as with magnesium and calcium, strontium can associate with casein micelles (Zhang & Aoki, 1995; Rosskopfova et al., 2011). The level of soluble calcium throughout ripening has a major influence on physicochemical properties of cheese (Lucey et al., 2005; O'Mahony et al., 2005; Lee et al., 2010); however, little information is known about the effect of magnesium equilibrium throughout ripening on the physical properties of cheese and to the best of our knowledge, there has been no research published on supplementation of Cheddar cheese with strontium and its effect on cheese physical properties. The objective of the present study was to investigate if addition of magnesium or strontium could influence the textural, functional and rheological properties of Cheddar-style cheese in a manner similar to that of calcium by evaluating their partition between the soluble and casein-bound phase and also their

influence on calcium equilibrium. The impact of Na replacement by Mg and Sr was not an objective of this study.

2.2. Materials and Methods

2.2.1. Cheese manufacture

Raw bovine milk was standardized to 3.5% fat and pasteurised (72 °C x 15 s). Three Cheddar-style cheeses were manufactured according to standard protocol on a 50 kg scale in the food processing facilities at University College, Cork. R-604Y (Chr. Hansen Ltd., Little Island, Co. Cork, Ireland) was used as the starter culture at a level of 0.02% (w/v). Chymosin (Maxiren 180; DSM Food Specialities, Delft, Netherlands), at a strength of 180 IMCU·ml^{-1}, was added to the cheesemilk at a level of 0.3 mL·L^{-1}. Coagulum was cut at equal firmness (measured subjectively). Curd was cooked from 31 to 39 °C over 30 min. Whey was drained at pH 6.2. The curd was cheddared until pH 5.4 was reached and was then milled and dry salted. The control curd was salted with NaCl at a level 2.5% w/w. The cheese curd supplemented with magnesium (+Mg) was salted with 0.87% $MgCl_2.6H_2O$ + 1.75% NaCl. The cheese curd supplemented with strontium (+Sr) was salted with 1.14% $SrCl_2.6H_2O$+ 1.75% NaCl. These salt treatments were calculated to ensure the ionic strength of the +Mg and +Sr cheeses were equal to the control cheese and to supplement these cheeses with the same molar quantity (43 mmol/kg) of $MgCl_2$ or $SrCl_2$. The salted curd was transferred to rectangular moulds 25.4 cm x 20.3 cm and pressed overnight at 490 kPa. The cheeses were vacuum packaged and ripened at 8 °C for a period of 8 months. Three independent cheesemaking trials were performed.

2.2.2. Compositional analysis

Compositional analysis was performed on the cheeses at day 14 of ripening. The moisture contents of the cheeses were determined by an oven drying method (IDF, 1982), protein by the macro-Kjeldahl procedure (IDF, 1986), fat by the Gerber method (IIRS, 1955), salt by a titrimetric method using potentiometric end-point determination (Fox, 1963). Cheese pH was determined by measuring the pH of homogenized cheese slurry made from 10 g cheese and 10 g water at room temperature. Proteolysis was assessed by determining the levels of pH 4.6-soluble nitrogen as % of total nitrogen (pH4.6SN%TN) according to O'Mahony et al. (2005) at 8 weeks of ripening. Urea-polyacrylamide gel electrophoresis (PAGE) was carried out directly on the cheeses using the procedure described in O'Mahony et al. (2005).

2.2.3. Extraction of cheese juice and determination of casein-bound Mg, Ca and Sr

This method was based on the method developed by Morris et al. (1988) and Hassan et al. (2004). The extraction apparatus consisted of a stainless steel mould, a perforated stainless steel base plate, a stainless steel top plate on which pressure was exerted by a hydraulic ram. Freshly grated cheese (100 g) was mixed with sea sand (150 g) and placed in the stainless steel mould lined with nylon cheese cloth. The cheese-sand mixture was subjected to high pressure using a hydraulic press at room temperature. Pressure was gradually increased up to a maximum of ~37 MPa over 3 hours. Liquid from the cheese was collected in a vessel below the perforated base plate. The collected juice and liquid fat were centrifuged at 2000g for 10 min at 4°C to separate fat and curd particles from the juice. Three cheese juice extractions were

made from each cheese. The cheeses and their juices were analyzed for Mg and Ca content using flame atomic absorption spectroscopy (Varian SpectrAA-100, Varian Australia Pty Ltd, Mulgrave, Victoria, Australia) according to IDF (2007) and their Sr content was analysed based on the same method for Ca determination according to IDF (2007) except with a wavelength of 460.7 nm and a lamp current of 10 mA. The concentration of each cation in the cheese juice was taken as the percentage of soluble cation in the cheese. Previous studies (Hassan et al., 2004; Lucey et al., 2005) have equated insoluble Ca with CCP; however this is not totally accurate as Ca^{2+} alone can bind directly to caseins (Dickson & Perkins, 1971; Gaucheron et al., 1997). Hence, we refer to the insoluble Mg, Ca and Sr as CN-bound Mg, Ca and Sr. This definition takes into account all possible forms of the bound cation that is associated with casein. Estimation of percentage CN-bound Mg, Ca and Sr of cheese was calculated according to Hassan et al. (2004).

2.2.4. Analysis of salt precipitate from +Sr cheese

Salt precipitates were physically extracted from the +Sr cheese at 8 months of ripening. This material was subsequently ashed and prepared for atomic absorption spectroscopy and analyzed to determine Ca and Sr contents as described in Section 2.2.3.

2.2.5. Texture profile analysis

Texture profile analysis (TPA) was performed using a Texture Analyser TA-XT2i (Stable Micro Systems, Godalming, Surrey, UK) according to the method of O'Mahony et al. (2005), except cylindrical samples of dimensions: height 20 mm,

diameter 20 mm were used. Hardness was defined according to Bourne (1978). Five replicate samples from each cheese were compressed at each ripening time point.

2.2.6. Dynamic small amplitude oscillatory rheology

Rheological properties of the cheese samples were measured using a Carri-Med CSL^2/ 100 Controlled Stress Rheometer (TA Instruments, Leatherhead, UK). Measuring geometry consisted of a 40 mm serrated stainless steel parallel plate above, a flat base plate below and a gap size of 1.8 mm. Cheese discs (40 mm diameter, 2 mm height) were glued to the base plate of the rheometer using cyanoacrylate glue in order to prevent slippage during measurement. The sample was compressed to the gap size and allowed rest for 10 min at 20 °C in order to allow stress relaxation prior to oscillation. Storage modulus (G′), loss modulus (G″) and loss tangent (LT) were recorded continuously at a low amplitude shear strain (0.05) at a frequency of 6.283 $rad \cdot s^{-1}$ over 20 min during which the temperature was increased from 20 to 82 °C at a rate of 3.1 °C/min. The frequency and strain values chosen were found to be within the linear viscoelastic range for the cheese samples. Each sample was analysed in triplicate.

2.2.7. Melt analysis

Melt analysis was carried out using a covered Schreiber test (Altan et al., 2005). Each cheese cylinder (5 mm height, 35 mm diameter) was placed in a covered glass petri dish and then placed in an oven at 232 °C for 5 min. These were then removed and cooled for 30 min at room temperature. Measurements of the melt distance were made using electronic calipers. The diameter of the melted sample was measured at 5 different points and an average diameter was determined. Results were expressed as

percentage increase in cheese diameter. Analysis on each cheese sample was performed in triplicate.

2.2.8. Statistical analysis

ANOVA was carried out using the PASW Statistics Version 18 program (IBM, Armonk, NY, USA). Differences between means were analyzed using Tukey's HSD *post hoc* test. The level of significance was determined at $P < 0.05$.

2.3. Results and Discussion

2.3.1. Chemical composition of cheeses

The composition of the cheeses is shown in Table 2.1. Addition of $MgCl_2$ or $SrCl_2$ at the salting stage had no appreciable effect on the percentage moisture, protein and fat in the cheese. Cl^- levels were slightly lower in the +Mg and +Sr cheeses compared to the control. The pH values of the experimental cheeses throughout ripening are shown in Figure 2.1. Addition of $MgCl_2$ and $SrCl_2$ led to a reduced pH throughout ripening in the +Mg and +Sr cheeses compared to the control cheese. Addition of Ca^{2+} to milk can cause a decrease in pH due to formation of calcium phosphates and calcium citrates and by exchanges between added Ca^{2+} and micellar H^+ (Philippe et al., 2003). Pastorino et al. (2003) observed a decrease in cheese pH after injecting a concentrated $CaCl_2$ solution into the cheese. This effect was attributed to binding of Ca^{2+} to caseins promoting the release of protons, thereby decreasing pH in a similar way to the mechanism that occurs in milk. Based on these studies, it is possible that some of the added Mg^{2+} and Sr^{2+} formed complexes with inorganic phosphates and may have also formed CCP and thereby decreased pH.

There were no consistent differences in levels of pH4.6SN%TN between the three cheeses (Table 2.1), and urea-PAGE electrophoretograms (not shown) displayed no differences in proteolytic patterns throughout ripening. As the cheeses were manufactured with all salting treatments calculated on the basis of equal ionic strength, it is to be expected that no great effect on proteolysis would be observed due to NaCl substitution (Brickley et al., 2009). The level of total calcium generally remained the same in all cheeses at a level of ~800 mg/100 g cheese regardless of salt substitution treatment (Table 2.1). Addition of $MgCl_2$ increased the magnesium

level in the +Mg cheese to more than double that of the control and +Sr cheeses. Addition of $SrCl_2$ led to a residual strontium level of ~200 mg/100 g cheese in the +Sr cheese, whereas the control and +Mg cheeses did not contain detectable levels of strontium.

2.3.2. Calcium equilibrium

The % CN-bound Ca in the cheeses is shown in Figure 2.2. The % CN-bound Ca in the control cheese decreased during ripening from ~62-65% on day 1 to ~56-59% by day 60 and remained relatively constant up to day 224. This is generally in agreement with previous studies (Hassan et al., 2004; Lee et al., 2005; Lucey et al., 2005; O'Mahony et al., 2005). It is thought that this reduction in CN-bound Ca reflects the slow attainment of a pseudoequilibrium between CCP and soluble Ca during the early stages of ripening (Hassan et al., 2004). Added Mg^{2+} appeared to have no impact on % CN-bound Ca. Zhang & Aoki (1995) suggested that Mg^{2+} alone cannot crosslink caseins directly, but could promote calcium crosslinking when forming artificial casein micelles. In the present study, however, increasing the Mg^{2+} level did not appear to influence the ability of calcium to form CCP in cheese curd. In Trials 1 and 2, the % CN-bound Ca was higher at day 1 in the +Sr cheese compared to the control and +Mg cheeses. The presence of Sr^{2+} promoted more Ca to become CN-bound at day 1 in Trials 1 and 2. At day 224 of ripening, the +Sr cheese had higher % CN-bound Ca in Trials 2 and 3. Comparing pH values and % CN-bound Ca (Figures 2.1 and 2.2), suggests that the presence of Sr^{2+} may promote Ca binding to casein at lower pH values. This phenomenon of Sr^{2+} promoting calcium binding to caseins was also reported by Zhang & Aoki (1995), who found that addition of Sr^{2+} could increase the level of micellar calcium in artificial micelles.

Table 2.1. Chemical composition of control Cheddar cheese, cheese supplemented at salting with 43 mmol/kg $MgCl_2$(+Mg) and cheese supplemented at salting with 43 mmol/kg $SrCl_2$(+Sr) during ripening (mean ± standard deviation)

		Treatment		
Trial	Parameter[1]	Control	+Mg	+Sr
1	Moisture (%)	37.37 ± 0.34^{a}	37.68 ± 0.22^{a}	36.76 ± 0.11^{a}
	Fat (%)	31.83 ± 0.29^{a}	31.00 ± 0.50^{a}	31.08 ± 0.14^{a}
	Protein (%)	25.74 ± 0.68^{a}	25.28 ± 0.13^{a}	25.84 ± 0.34^{a}
	Cl^- (%)	0.86 ± 0.01^{a}	0.80 ± 0.01^{b}	0.78 ± 0.01^{b}
	Ash	2.94 ± 0.13^{a}	2.89 ± 0.06^{a}	3.14 ± 0.13^{a}
	pH4.6SN%TN	15.07 ± 0.22^{b}	15.76 ± 0.05^{a}	15.14 ± 0.22^{b}
	Calcium	843.20 ± 7.54^{a}	810.76 ± 13.90^{b}	794.40 ± 8.54^{b}
	Magnesium	32.15 ± 0.71^{b}	79.42 ± 2.05^{a}	31.10 ± 0.13^{b}
	Strontium	NA	NA	197.23 ± 5.68
	Cheese juice moisture at d 1 (%)	87.64 ± 0.45^{aA}	87.87 ± 0.31^{aA}	88.82 ± 0.64^{aA}
	Cheese juice moisture at mo 8 (%)	70.65 ± 0.24^{bB}	72.59 ± 0.46^{aB}	68.52 ± 0.30^{cB}
2	Moisture (%)	37.93 ± 0.12^{a}	37.66 ± 0.19^{a}	38.08 ± 0.20^{a}
	Fat (%)	31.50 ± 0.43^{a}	31.33 ± 0.29^{a}	31.50 ± 0.50^{a}
	Protein (%)	25.40 ± 0.30^{a}	25.49 ± 0.08^{a}	25.67 ± 0.65^{a}
	Cl^- (%)	0.87 ± 0.02^{a}	0.83 ± 0.01^{b}	0.81 ± 0.02^{b}
	Ash	3.45 ± 0.11^{ab}	3.26 ± 0.09^{b}	3.77 ± 0.28^{a}
	pH4.6SN%TN	17.07 ± 0.17^{a}	16.77 ± 0.05^{ab}	16.51 ± 0.19^{b}
	Calcium	838.82 ± 23.34^{a}	809.93 ± 18.32^{a}	803.74 ± 17.15^{a}
	Magnesium	30.62 ± 0.57^{b}	80.24 ± 0.25^{a}	29.7 ± 0.84^{b}
	Strontium	NA	NA	254.67 ± 3.30
	Cheese juice moisture at d 1 (%)	85.81 ± 0.43^{bA}	86.34 ± 0.35^{bA}	89.88 ± 0.19^{aA}
	Cheese juice moisture at mo 8 (%)	73.11 ± 0.28^{aB}	72.40 ± 0.43^{aB}	73.00 ± 0.39^{aB}
3	Moisture (%)	37.93 ± 0.43^{b}	38.96 ± 0.26^{a}	38.21 ± 0.12^{b}
	Fat (%)	32.42 ± 0.72^{a}	32.58 ± 0.63^{a}	31.92 ± 0.38^{a}
	Protein (%)	25.46 ± 0.94^{a}	25.51 ± 0.62^{a}	25.38 ± 0.05^{a}
	Cl^- (%)	0.86 ± 0.01^{a}	0.80 ± 0.01^{b}	0.78 ± 0.01^{c}
	Ash	3.32 ± 0.04^{a}	3.14 ± 0.17^{a}	3.25 ± 0.03^{a}
	pH4.6SN%TN	16.56 ± 0.13^{c}	17.05 ± 0.10^{b}	17.80 ± 0.13^{a}
	Calcium	787.25 ±15.25^{a}	769.06 ± 30.85^{a}	773.72 ± 12.87^{a}
	Magnesium	28.43 ± 1.63^{b}	79.59 ± 0.65^{a}	30.49 ± 1.17^{b}
	Strontium	NA	NA	197.77 ± 3.57
	Cheese juice moisture at d 1 (%)	87.64 ± 0.47^{aA}	86.41 ± 0.62^{aA}	87.91 ± 0.58^{aA}
	Cheese juice moisture at mo 8 (%)	73.72 ± 0.40^{aB}	72.89 ± 0.52^{aB}	74.00 ± 0.38^{aB}

^{a,b,c}Different lower case superscript letters in the same row within a trial indicate that values are significantly different ($P < 0.05$)

^{A,B}Different upper case superscript letters in the same column within a trial indicate that values for the same parameter at different ripening times are significantly different ($P < 0.05$)

[1]pH4.6SN%TN = pH 4.6 soluble nitrogen as a % of total nitrogen. Calcium, magnesium and strontium expressed as mg/100g cheese

d = day; mo = month

NA = cheese not analysed for strontium

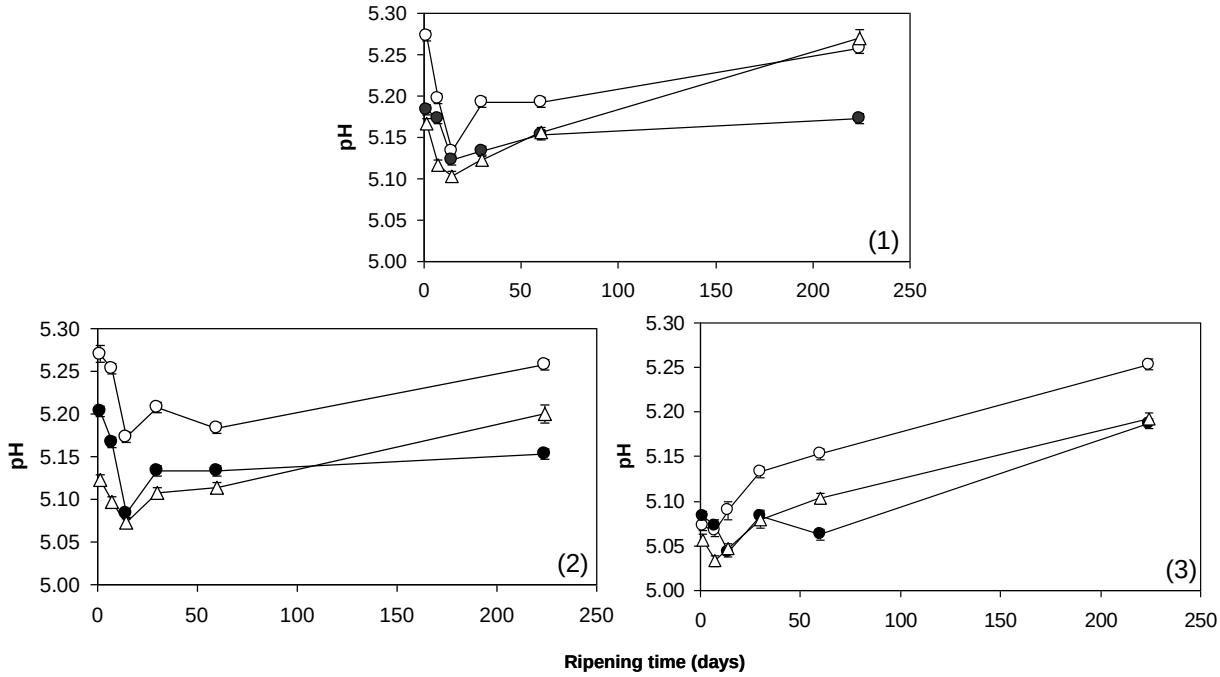


Figure 2.1. pH values of control cheese (○), cheese supplemented at salting with 43 mmol/kg $MgCl_2$ (●), and cheese supplemented at salting with 43 mmol/kg $SrCl_2$ (Δ) during ripening in cheesemaking trials 1-3. Error bars indicate ± 1 standard deviation

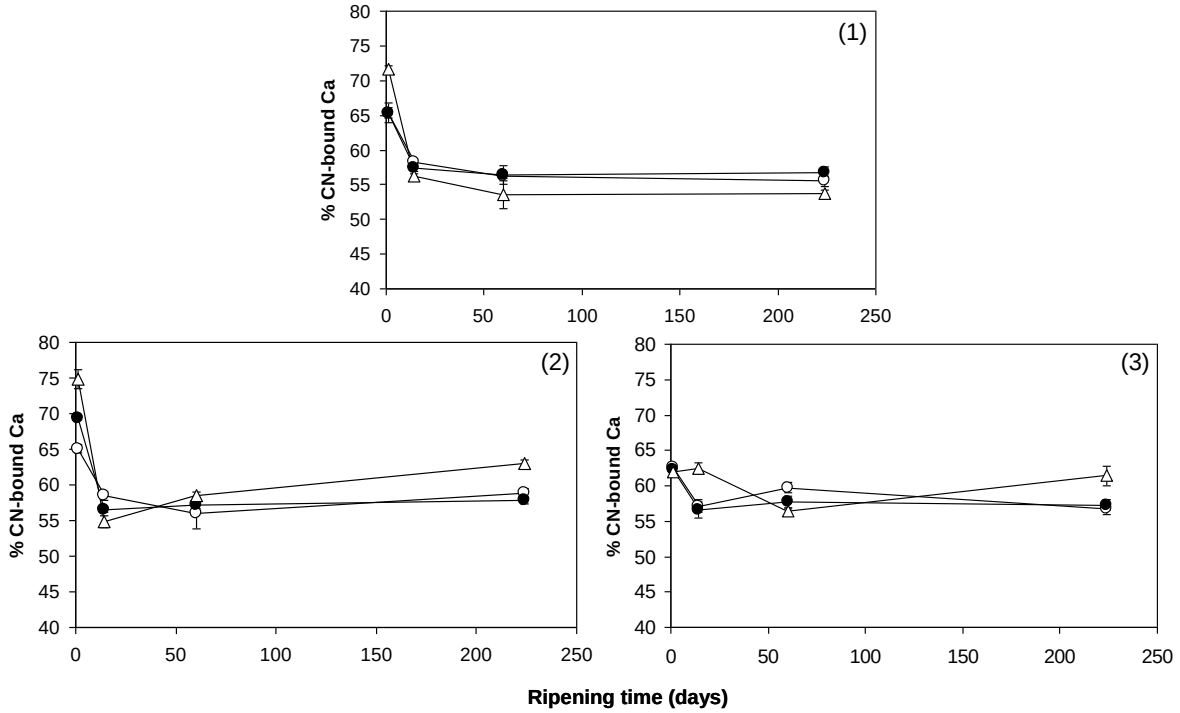


Figure 2.2. % CN-bound Ca in control cheese (○), cheese supplemented at salting with 43 mmol/kg $MgCl_2$ (●), and cheese supplemented at salting with 43 mmol/kg $SrCl_2$ (Δ) in cheesemaking trials 1-3. Error bars indicate ± 1 standard deviation

2.3.3. Magnesium equilibrium

The level of CN-bound Mg decreased in all cheeses during ripening (Figure 2.3). At day 1 of ripening, the % CN-bound Mg in the control cheese was ~20% and decreased to ~10% by day 14. Morris et al. (1988) reported that 23% of the total magnesium was casein-bound in a 1 month old Cheddar cheese. A lower association of magnesium with casein micelles compared with calcium also exists in milk and is thought to be due to magnesium phosphates being more soluble than the corresponding calcium salts and not being at saturation levels in the aqueous phase of milk (Philippe et al., 2005). The +Mg cheese had a higher level of CN-bound Mg than the control cheese up to day 60 of ripening. This indicates that during early ripening, a proportion of the added Mg^{2+} was bound to casein. Zhang & Aoki (1995) suggested that Mg^{2+} alone has no crosslinking ability in casein solutions, i.e., replacing Ca^{2+} with Mg^{2+} when formulating artificial casein micelles does not lead to micelle formation. There are no published data supporting the existence of magnesium phosphate nanoclusters in milk or any other casein solution/gel, and so it would be unwise to assume the CN-bound Mg necessarily exists in this state. Possible binding sites for Mg^{2+} on caseins other than nanoclusters include monoester phosphate groups on serine and threonine residues and carboxylic groups of aspartic acid and glutamic acid (Dickson & Perkins, 1971) and also phenolic, sulfhydryl and imidazole groups (Gaucheron et al., 1997). However, since Mg^{2+} is a constituent of CCP nanoclusters (Holt, 2004; Lucey & Horne, 2009), it is reasonable to assume that some of the added Mg^{2+} will contribute to CCP crosslinks during cheese ripening.

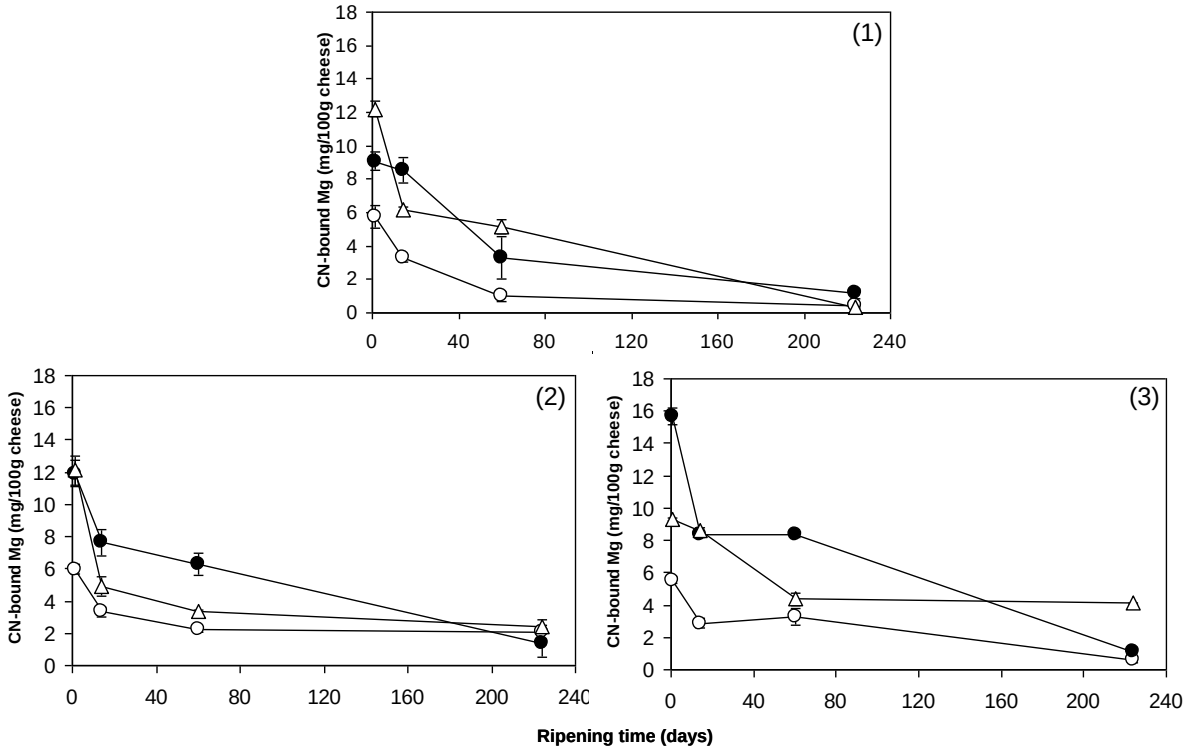


Figure 2.3. CN-bound Mg in control cheese (○), cheese supplemented at salting with 43 mmol/kg $MgCl_2$ (●), and cheese supplemented at salting with 43 mmol/kg $SrCl_2$ (Δ) in cheesemaking trials 1-3. Error bars indicate ± 1 standard deviation.

The binding capacity of divalent cations to either α_{s1}- or β-casein is in the order $Mg^{2+}>Ca^{2+}>Sr^{2+}$ (Dickson & Perkins, 1971). This suggests that more Mg^{2+} can be CN-bound compared to Ca^{2+} and Sr^{2+} without necessarily being involved in nanoclusters. The +Sr cheese also had a higher level of CN-bound Mg than the control cheese up to day 60 of ripening. This suggests that the added Sr^{2+} caused more innate Mg^{2+} to associate with casein than in the control.

2.3.4. Strontium equilibrium

The % CN-bound Sr in the +Sr cheeses in all three trials is shown in Figure 2.4. The % CN-bound Sr decreased rapidly from ~68-78% on day 1 to ~36-45% by day 14. It decreased further during ripening to a value of ~7-32% by day 224. As with the % CN-bound Ca, the CN-bound Sr solubilized during ripening but unlike calcium it did not reach a state of equilibrium during early ripening. Zhang & Aoki (1995)

observed that artificial casein micelles could be formed when Ca^{2+} was replaced by Sr^{2+} during preparation, suggesting that strontium phosphate has casein crosslinking ability. These crosslinks are likely to be strontium phosphate nanoclusters. The high proportion of added Sr^{2+} in the present study that is CN-bound may therefore exist as strontium phosphate nanoclusters or perhaps mixed cation nanoclusters containing both Ca^{2+} and Sr^{2+} in their structure. The most likely form of calcium phosphate in nanoclusters is a basic salt with low solubility such as $Ca_3(PO_4)_2$ (tricalcium phosphate) (Lucey and Horne, 2009). If we compare the solubility of $Ca_3(PO_4)_2$ to that of $Mg_3(PO_4)_2$ and $Sr_3(PO_4)_2$ (Table 2.2), it is evident that the Ca and Sr salts have similar solubilities that are much lower than the Mg salt. So it is likely that Sr^{2+} precipitates to the casein-bound phase as strontium phosphate, in agreement with (Zhang & Aoki, 1995). As outlined previously for magnesium binding (see Section 2.3.3.), Sr^{2+} may also bind directly to caseins. In native casein micelles, an estimated 10% of phosphoserine centres are unreacted, i.e., not involved in stabilizing nanoclusters (Holt, 2004). Interactions between *para*-casein particles in cheese curd during manufacture and ripening should create more possible sites for nanocluster formation. Philippe et al. (2003) speculated that new CCP formed at these unreacted phosphoserine centres would be different to the native CCP form. When Sr^{2+} is added at salting, it is possible that new strontium-based CCP nanoclusters formed due to this availability of nucleation sites.

Table 2.2. Solubilities and molecular weights of phosphate salts of Mg, Ca and Sr

	$Mg_3(PO_4)_2$	$Ca_3(PO_4)_2$	$Sr_3(PO_4)_2$
Solubility index (K_{sp} at 25°C)	1.0×10^{-24}	1.3×10^{-32}	1.0×10^{-31}
Molecular weight (g/mol)	262.85	310.17	452.80

Solubility data obtained from Kelter et al. (2008)

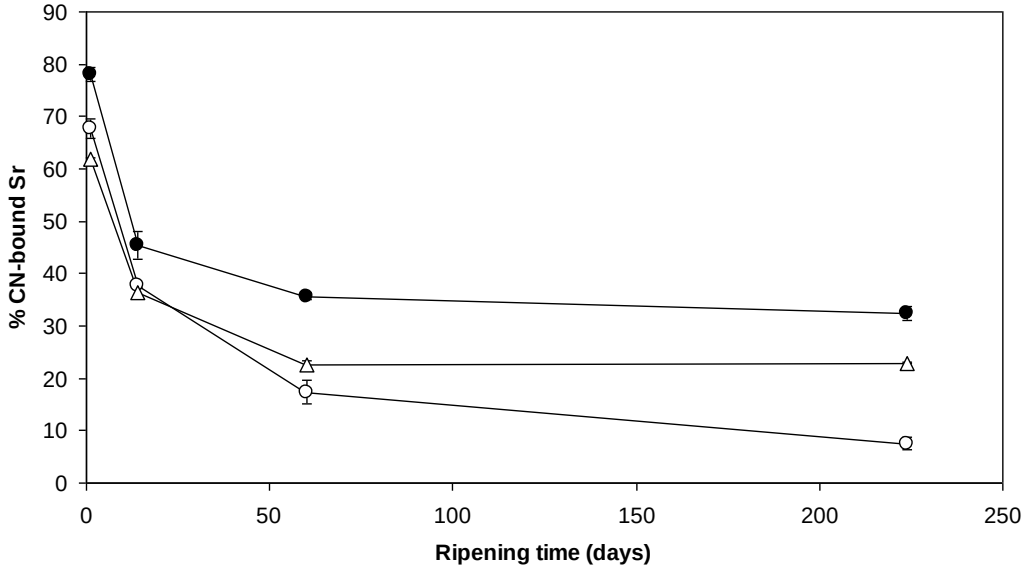


Figure 2.4. % CN-bound Sr in cheese supplemented at salting with 43 mmol/kg $SrCl_2$ in cheesemaking trial 1 (○), trial 2 (●), and trial 3 (Δ). Error bars indicate ± 1 standard deviation.

At day 1 of ripening, the presence of Sr^{2+} in the +Sr cheese caused an increase in the level of CN-bound Ca and Mg compared to the control cheese. Substitution of Ca^{2+} with Sr^{2+} in hydroxyapatite lattice structure has been found to increase the lattice dimensions and volume as Sr^{2+} has a larger ionic radius than Ca^{2+} (Wang and Ye, 2008). Rosskopfova et al. (2011) found that Sr^{2+} could exchange with Ca^{2+} in hydroxyapatite and casein micelles. Cross et al. (2005) proposed a model of the bound calcium phosphate core consisting of two calcium phases based on their Ca:P ratio: a calcium poor phase in the interior and a calcium-rich phase in contact with the phosphoserine groups. The possible existence of strontium phosphate nanoclusters and/or mixed Ca and Sr phosphate-based nanoclusters along with pre-existing Ca phosphate nanoclusters in the +Sr cheese may account for the unusual Ca and Mg equilibria during early ripening as nanocluster ion ratios would likely be different in nanoclusters with Sr^{2+} as a major constituent. Such strontium-based nanoclusters may accommodate more Mg^{2+} in their structure than conventional

nanoclusters in the control cheese and the increase in CN-bound Ca due to Sr^{2+} addition may support the existence of the proposed mixed Ca-Sr nanoclusters. Lucey et al. (2003) suggested that larger CCP nanoclusters may grow at the expense of smaller ones through a type of Ostwald ripening. As the % CN-bound Sr decreased extensively by 8 months of ripening, a situation may arise where the most stable form of CCP in the +Sr cheese at this stage is a Sr^{2+} depleted form.

At 8 months of ripening, there were large, white, amorphous salt precipitates present in the cheese (Figure 2.5). Sr was determined to be the dominant cation in these precipitates (Sr:Ca = ~3:1), which also contained Ca. Therefore, three forms of strontium co-existed in the +Sr cheese by month 8: insoluble CN-bound Sr, soluble Sr in the aqueous phase and insoluble precipitated Sr in the form of macroscopic precipitates that are not associated with casein. It is likely that the continuing solubilization of Sr from the CN-bound phase during ripening led to the nucleation and growth of strontium salt microcrystals in the aqueous phase. These precipitates may have been a combination of precipitated Sr lactate and Ca lactate salts.

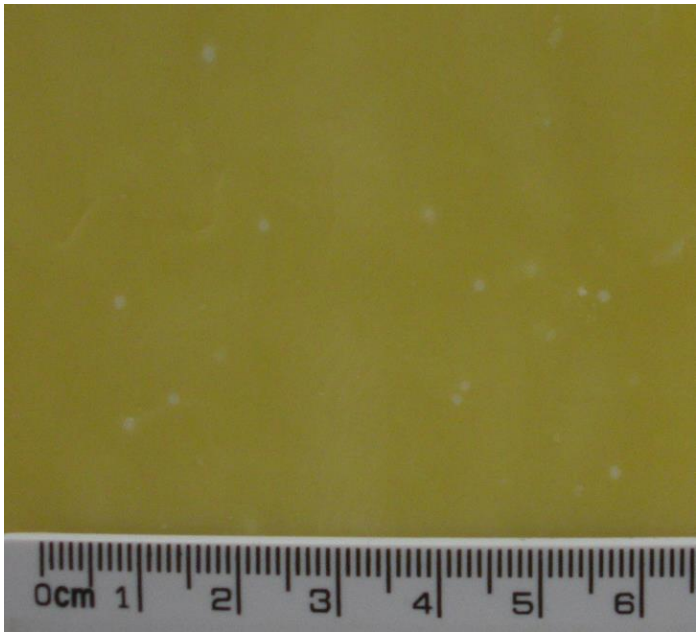


Figure 2.5. Salt precipitates as seen on the surface of a cheese slice taken from the cheese supplemented at salting with 43 mmol/kg $SrCl_2$.

2.3.5. Small amplitude dynamic oscillatory rheology

As can be seen from Figure 2.6, the values for G′ at 70°C were higher in the +Sr cheese compared to the control and +Mg cheeses throughout ripening. The control and +Mg cheeses had statistically similar values ($P > 0.05$) for this parameter throughout ripening. O'Mahony et al. (2006) observed an increase in G′ at 70°C in cheese with increasing CCP concentration and attributed this to increased CCP bridging between casein molecules which increased the rigidity of the cheese matrix. As the presence of Sr^{2+} led to increased G′ at 70°C, it is likely that Sr phosphate nanocluster crosslinks increased the strength and rigidity of the *para*-casein matrix.

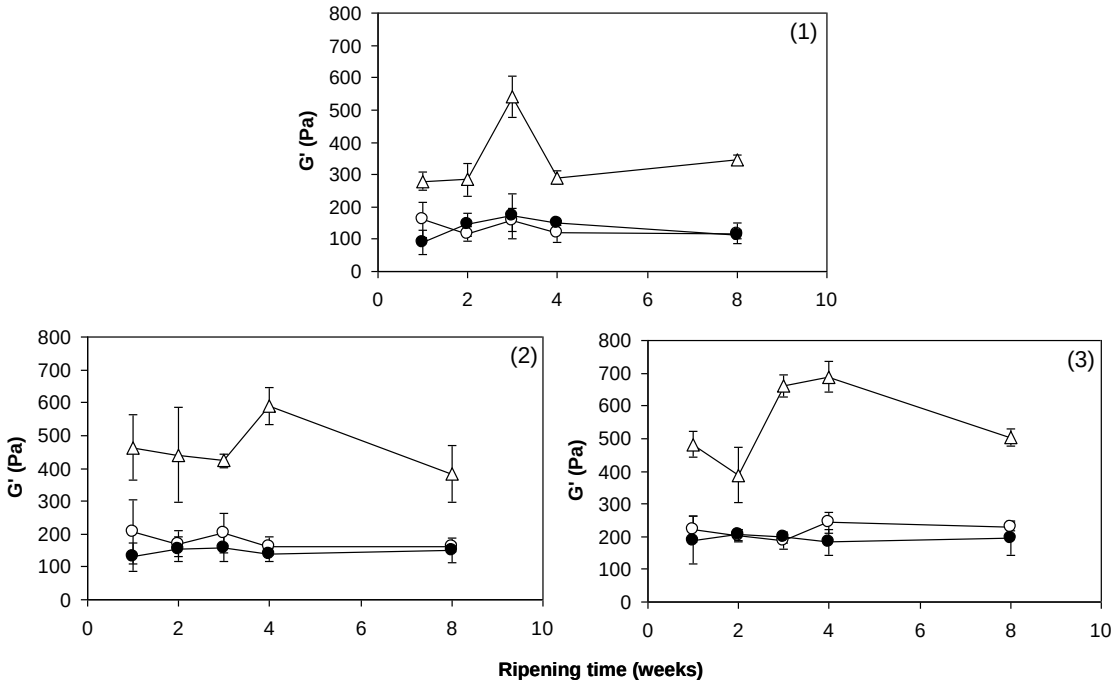


Figure 2.6. Storage modulus (G′) at 70°C of control cheese (○), cheese supplemented with 43 mmol/kg $MgCl_2$ (●), and cheese supplemented with 43 mmol/kg $SrCl_2$ (Δ) during ripening in cheesemaking trials 1-3. Error bars indicate ± 1 standard deviation.

This hypothesis is supported by results shown in Figure 2.4 which indicate that a large proportion of added Sr^{2+} was bound to casein during ripening. A higher level of CN-bound Mg is also present in the +Sr cheese (Figure 2.3) and this may have also contributed to increased rigidity of this cheese.

The LT_{max} values of experimental cheeses throughout ripening are shown in Figure 2.7. The +Sr cheese had lower LT_{max} values than the control and +Mg cheeses throughout ripening. As LT_{max} can be used as an index of melt, these results indicate that the +Sr cheese had the lowest meltabilty. This observation can be explained using the same proposed mechanism as described above for G′ at 70°C. Strontium crosslinks increased the strength of interactions between caseins resulting in less melt. The similarity of the viscoelastic properties between the +Mg cheese and control infer that added Mg^{2+} did not sufficiently form or enhance CCP crosslinks. Figure 2.3 shows that a proportion of added Mg was CN-bound during ripening; however, it is likely that this CN-bound Mg exists at binding sites like carboxylic groups, free phosphoserine groups, etc, rather than forming magnesium phosphate nanocluster crosslinks analogous to conventional CCP. Added Mg^{2+} may contribute in some way to CCP nanoclusters, but not as much as added Sr^{2+}. Zhang & Aoki (1995) speculated that the similarity of the hydrodynamic radii of Ca^{2+} and Sr^{2+} may help explain their similar casein crosslinking abilities.

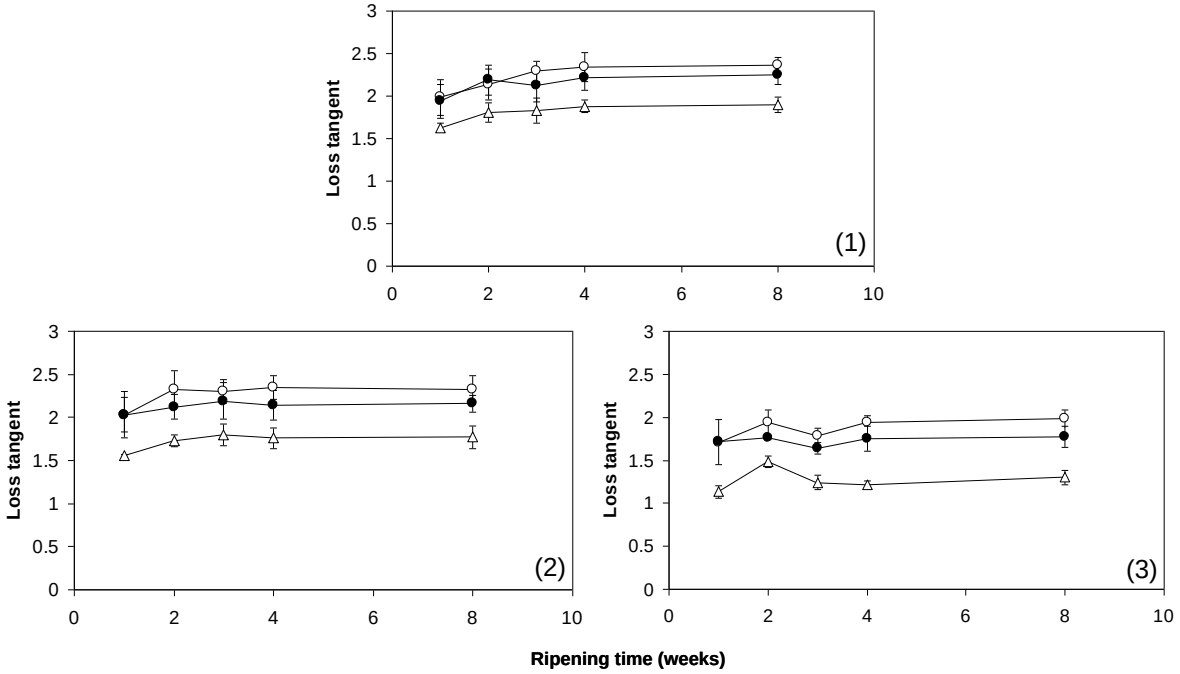


Figure 2.7. Maximum loss tangent (LT_{max}) of control cheese (○), cheese supplemented with 43 mmol/kg $MgCl_2$ (●), and cheese supplemented with 43 mmol/kg $SrCl_2$ (Δ) during ripening in cheesemaking trials 1-3. Error bars indicate ± 1 standard deviation.

2.3.6. Schreiber melting test

Changes in cheese meltability are shown in Figure 2.8. The meltability of all cheeses increased during ripening. During the first few weeks of ripening, solubilization of CCP nanoclusters increased the localised electrostatic repulsion due to exposure of negatively charged phosphoseryl groups which increase melt (Lucey et al., 2003). In conjunction with CCP solubilization, proteolysis will also increase melt by reducing the level of intact casein during ripening. At most time points across all trials, the control and +Mg cheeses had similar meltability. After 2 months of ripening, the +Sr cheese had significantly lower meltability ($P < 0.05$) compared to the control and +Mg cheeses in all trials. This reduction in true meltability is in agreement with the melt index LT_{max} from dynamic small amplitude oscillatory rheology analysis (Figure 2.7). As can be seen in Figures 2.3 and 2.4, the +Sr cheese had CN-bound Sr together with more CN-bound Mg than the control. As discussed above, these results

suggest that a denser *para*-casein matrix was present in the +Sr cheese, inducing less meltability than the control and +Mg cheeses. CCP solubilization slows down and reaches a pseudoequilibrium within the first 2 months of ripening (Hassan et al., 2004; Lucey et al., 2005; O'Mahony et al., 2005), and so it is likely that the increase in melting from week 8 onwards in all cheeses is primarily due to proteolysis. However, the lower meltability of the +Sr cheese compared to the control and +Mg cheeses cannot be attributed to the extent of proteolysis as pH4.6SN%TN levels were similar at week 8 for all cheeses (Table 2.1). When cheese is heated above 70°C, it has been proposed that heat-induced CCP can form (Udayarajan et al., 2005) which may also account for the lower meltability in the +Sr cheese.

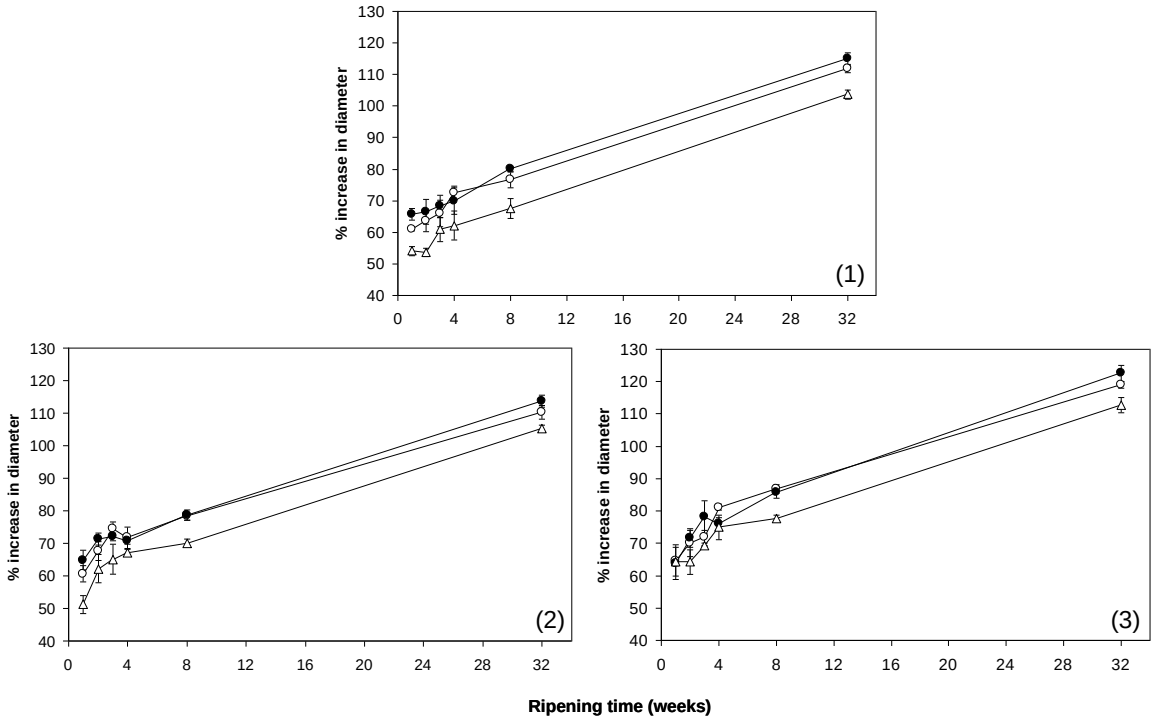


Figure 2.8. Percentage increase in cheese diameter from Schreiber melting test for the control cheese (○), cheese supplemented with 43 mmol/kg $MgCl_2$ (●), and cheese supplemented with 43 mmol/kg $SrCl_2$ (Δ) in cheesemaking trials 1-3. Error bars indicate ± 1 standard deviation.

2.3.7. Texture profile analysis (TPA) hardness

TPA hardness values are shown in Table 2.3. Hardness values generally decreased in all cheeses as ripening time increased. The decrease in hardness during ripening is attributed to solubilization of CCP during early ripening (O'Mahony et al., 2005) and to a lesser extent the proteolytic breakdown of α_{s1}-casein in the protein matrix (Creamer & Olson, 1982). At week 4 of ripening, the +Sr cheese had significantly higher hardness ($P < 0.05$) than both the control and +Mg cheeses in all 3 trials. At weeks 2 and 4 of ripening, the +Sr cheese had greater hardness than +Mg cheese. As Sr^{2+} appears to form CCP crosslinks, the +Sr cheese would be expected to be harder than the control. Brickley et al. (2009) observed increased hardness in cheeses after 28 days of ripening when the same molar quantity of $CaCl_2$ was added as $SrCl_2$ in the present study. In the +Sr cheese, at day 14 the % CN-bound Sr was ~36-45% and the level of CN-bound Mg was higher than the control. This suggests that a higher level of casein association and a denser *para*-casein matrix existed in the +Sr cheese during early ripening, leading to higher hardness values. As can be seen in Figures 2.2, 2.3 and 2.4, the % CN-bound Ca tends to stabilize after day 60, whereas the level of CN-bound Mg and Sr continue to decrease up to day 224 in the +Sr cheese. Therefore, the contribution of Mg^{2+} and/or Sr^{2+} to the structural integrity of the cheese matrix decreased as ripening progressed and may account for the lower influence of Sr on hardness values by late ripening.

Table 2.3. Hardness values (g) as determined by texture profile analysis of control Cheddar cheese, cheese supplemented at salting with 43 mmol/kg $MgCl_2$ (+Mg) and cheese supplemented at salting with 43 mmol/kg $SrCl_2$ (+Sr) during ripening experimental cheeses during ripening (mean ± standard deviation)

		Treatment		
Trial	Ripening time (weeks)	Control	+Mg	+Sr
1	1	12659 ± 1248^{aAB}	12013 ± 1192^{aAB}	12204 ± 1229^{aB}
	2	12925 ± 745^{bA}	12411 ± 804^{bA}	16156 ± 1415^{aA}
	4	11936 ± 396^{bAB}	10728 ± 788^{cB}	14766 ± 652^{aA}
	8	12084 ± 620^{aAB}	10494 ± 710^{bB}	12293 ± 797^{aB}
	32	11337 ± 815^{aB}	10791 ± 1007^{aB}	10823 ± 446^{aB}
2	1	12814 ± 1307^{aB}	12670 ± 912^{aA}	13287 ± 1233^{aA}
	2	14653 ± 738^{aA}	12603 ± 1087^{bA}	14576 ± 1067^{aA}
	4	13077 ± 985^{bAB}	12492 ± 861^{bA}	14810 ± 943^{aA}
	8	11426 ± 1207^{aB}	10626 ± 1058^{aB}	10720 ± 661^{aB}
	32	12611 ± 700^{aB}	10856 ± 874^{bB}	10049 ± 455^{bB}
3	1	14049 ± 1480^{aA}	13150 ± 1095^{aA}	13573 ± 528^{aA}
	2	12993 ± 621^{aAB}	11292 ± 919^{bBC}	13957 ± 896^{aA}
	4	12232 ± 448^{bB}	11893 ± 331^{bAB}	13446 ± 944^{aA}
	8	12204 ± 349^{aB}	10140 ± 737^{bC}	10449 ± 578^{bB}
	32	11700 ± 631^{aB}	10916 ± 626^{aBC}	10020 ± 370^{bB}

[a,b,c]Different lower case superscript letters in the same row within a trial indicate that values are significantly different ($P < 0.05$)
[A,B,C]Different upper case superscript letters in the same column within a trial indicate that values for the same parameter at different ripening times are significantly different ($P < 0.05$)
Cheese samples compressed by 75% of initial height

2.4. Conclusions

Supplementing Cheddar cheese with $SrCl_2$ can dramatically alter its physical properties. A proportion of the added Mg^{2+} and Sr^{2+} became CN-bound. The nature of the binding appeared to be the factor that dictated the ability of these ions to alter the physical properties of cheese. It is suggested that the ability of added Sr^{2+} to form CCP nanoclusters increased the strength and density of the *para*-casein matrix, thereby increasing rigidity and reducing melt. Even though some added Mg^{2+} became CN-bound, this did not alter meltability or rheological parameters. This is

likely due to the inability of Mg^{2+} to form nanoclusters. Strontium appeared to exhibit behaviour analogous to calcium when added to cheese. It is likely that the solubility of the cation salts in the serum phase of cheese is the principal factor determining their ability to form CCP and thereby modulate textural, rheological and functional properties of cheese. A better understanding of the form of CCP in cheese will enhance our ability to manipulate the physical properties of cheese.

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Chapter 3

The influence of alkaline earth metal equilibria on the rheological properties of rennet-induced skim milk gels

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Abstract

The mineral equilibria of milk can have a major influence on the properties of rennet milk gels. The influence of increased Mg, Ca and Sr concentrations on milk and rennet milk gels was investigated. Reconstituted skim milk was supplemented with $MgCl_2$, $CaCl_2$ or $SrCl_2$ at levels from 2.5-10 mmol·L^{-1} and adjusted to pH 6.6. Dynamic rheological properties of the renneted milks at 32 °C were investigated during 6 h time sweeps and by frequency sweeps. Whey was separated from rennet gels by centrifugation at the gelation point and also 6 h after rennet addition. The concentrations of Mg, Ca, Sr and inorganic P in milks and wheys were determined. Levels of casein-bound Mg, Ca and Sr increased with addition of their respective chloride salt. The % CN-bound inorganic P increased with increasing concentration of each divalent cation. Milk gelation time decreased with increasing concentration of each divalent cation salt. Milks containing 10 mmol·L^{-1} $CaCl_2$ or 10 mmol·L^{-1} $SrCl_2$ exhibited buffering capacities significantly higher (P<0.05) than the 10 mmol·L^{-1} $MgCl_2$ and control milks. An increase in G′ and a decrease in tan δ were observed in milk gels supplemented with the divalent cation salts. Milk gels supplemented with either $CaCl_2$ or $SrCl_2$ had superior rennet gel properties in terms of elasticity and gelation time compared with $MgCl_2$ supplemented milk gels (especially at low frequencies). An increase in insoluble divalent cation phosphate nanocluster content appeared to have a greater influence on rennet milk gel properties than an increase in total divalent cation species in milk.

3.1. Introduction

In milk, calcium exists in a dynamic equilibrium between soluble and insoluble forms, namely insoluble casein-bound calcium phosphate nanoclusters (CCP) and soluble Ca salts of citrates and phosphates and free calcium ions (Ca^{2+}). Supplementing milk with mineral salts can change this equilibrium (Mekmene et al., 2009; Philippe et al., 2003; Udabage et al., 2001). In particular, addition of Ca^{2+} can lead to the formation of excess calcium phosphate salts in the aqueous phase and subsequent precipitation to the micellar phase forming CCP nanoclusters and also binding of individual Ca^{2+} ions to the charged groups on caseins, all of which increase the attractive forces within the casein micelle. CCP nanoclusters are ~2.5 nm diameter granules of insoluble Ca phosphate complexes that also contain Mg and citrate in their structure (McGann et al., 1983a,b; Srinivasan and Lucey 2002). These nanoclusters are involved in ionic attraction with numerous phosphoserine centres on casein molecules and represent an important structural component of the casein micelle (Horne, 1998). The addition of salts affects Ca^{2+} activity, CCP concentration, the proportion of casein in the micelle, and the ionic strength of the milk (Udabage et al., 2001), all of which can influence rennet gel properties. Addition of Ca to cheese milk is common practice for many cheese varieties to enhance renneting and yield (Lucey and Fox, 1993; Wolfschoon-Pombo, 1997) but the level of addition is important. McMahon et al. (1984) reported a minimum in rennet coagulation time (RCT) with 0.05 $mol \cdot L^{-1}$ $CaCl_2$ addition to skim milk but a dramatic increase in RCT at 0.4 $mol \cdot L^{-1}$.

Rennet-induced gelation is the primary step in the manufacture of most cheese varieties. The addition of the enzyme chymosin cleaves the Phe_{105}-Met_{106} bond of κ-

CN which destabilizes the casein micelle leading to Ca^{2+}-mediated aggregation of *para*-casein micelles ultimately forming a three-dimensional gel network of partly fused *para*-casein micelles. Addition or removal of Ca does not influence the enzymatic phase if the pH is kept constant, but the aggregation phase is dependent on Ca concentration (Van Hooydonk et al., 1986; Zoon et al., 1988). A certain CCP level is required for proper renneting properties as partial removal of CCP by EDTA increases RCT (Choi et al., 2007; Udabage et al., 2001). Ca addition to milk can lead to increased strength of rennet gels (Sandra et al., 2012; Udabage et al., 2001; Zoon et al., 1988). Rennet-induced milk gels are viscoelastic and can be characterized using dynamic small amplitude oscillatory rheometry (DSAOR). The relationship between DSAOR and aging of gels has been investigated by numerous authors in prolonged gelation experiments to monitor changes due to factors such as proteolysis (Esteves et al., 2002; Srinivasan and Lucey, 2002) and modification of Ca equilibrium (Choi et al., 2007; Zoon et al., 1988).

In the present study, the mineral equilibria at the gelation point and late aging of rennet milk gels supplemented with salts of divalent cations of alkaline earth metals was determined in order to investigate the effects of changes in mineral equilibria during gel aging on their rheological properties. pH and temperature were kept constant to investigate the effects on rennet gels caused solely by addition of divalent salt cations. The overall objective was to relate the behaviour of Mg^{2+} and Sr^{2+} in rennet milk gels to the well documented behaviour of Ca^{2+} in order to generate further knowledge on the mechanisms of mineral-casein interactions in casein gels.

3.2. Materials and methods

3.2.1. Milk sample preparation

Low-heat skim milk powder was reconstituted (10% w/w) in deionized water for 1 h at room temperature. Sodium azide (0.02%) was added at the time of reconstitution to prevent bacterial growth. $MgCl_2$, $CaCl_2$ and $SrCl_2$ were prepared as 1 $mol \cdot L^{-1}$ stock solutions, and added to the milks along with deionized water to give final concentrations of 2.5 $mmol \cdot L^{-1}$, 5.0 $mmol \cdot L^{-1}$ or 10 $mmol \cdot L^{-1}$ of each salt in respective milks. The total volume added was 1% v/v; deionized water was added to the control to compensate for the dilution of the experimental milks. The milks and salt solutions were mixed for 1 h at room temperature. These milks were adjusted to pH 6.6 using 2 N NaOH and held overnight at 4 °C. After storage, the pH of the milks was readjusted to 6.6 if necessary. Three independent batches of each milk type were prepared for analyses.

3.2.2. Mineral analysis

The total Ca and Mg contents of the milks and wheys were determined by atomic absorption spectroscopy (Varian SpectrAA-100, Varian Australia Pty Ltd, Mulgrave, Victoria, Australia) according to IDF (2007) and their Sr content was analysed based on the same method for Ca determination according to IDF (2007) except with a wavelength of 460.7 nm and a lamp current of 10 mA. The total inorganic phosphorus content of the milks and whey was determined using the colorimetric method of Allen (1940).

3.2.3. Dynamic small amplitude oscillatory rheometry

Milk samples were heated to 32 °C and maintained at this temperature for 30 min. An aliquot (100 µL) of diluted chymosin (Maxiren 180; DSM Food Specialities, Delft, Netherlands) (1:10 dilution) was added to 25 g of reconstituted skim milk and stirred using a glass rod for 1 min at 32 °C. This milk was then transferred to a controlled shear stress rheometer equipped with a Peltier concentric cylinder system and a conical rotor (diameter 28 mm x length 42 mm; model AR-G2; TA instruments, Waters LLC, Leatherhead, Surrey, UK). Approximately 5 min elapsed between rennet addition and commencement of oscillation on the rheometer. A 6 h time sweep was performed on samples which were oscillated at a frequency of 0.1 Hz and 0.05% strain. This was found to be within the linear viscoelastic range for these milk gels. After the time sweep, a frequency sweep was performed maintaining a strain of 0.05 % and increasing the frequency from 0.001 to 1.0 Hz. Parameters recorded were the storage modulus (G′) and tan δ, which is the ratio of elastic to viscous properties (G″/G′). Gelation time was taken as the time at which $G' \geq 1$.

3.2.4. Separation of curds and whey

Milk samples were heated to 32 °C and maintained at this temperature for 30 min. An aliquot (80 µL) of diluted chymosin (Maxiren 180; DSM Food Specialities, Delft, Netherlands) (1:10 dilution) was added to 20 g of reconstituted skim milk at 32 °C. Rennet gels were cut at their gelation times which were predetermined from dynamic small amplitude oscillatory rheology results. After a heal time of 5 min, cut curds were centrifuged at 1700 g for 3 min. The supernatant (whey) and the pellet were collected. The moisture contents of pellets were determined by oven drying at 103 °C for 12 h and calculating the difference between pellet mass before and after drying.

This mass difference was expressed as % CN-bound water. Rennet gels were also prepared and allowed to stand for 6 h after rennet addition before being cut and subjected to the same treatment as above.

3.2.5. Buffering capacity

The buffering capacity of the milk samples was determined at 25 °C based the work of Lucey et al. (1993). An automated pH titration system (Metrohm AG 907 Titrando, Herisau, Switzerland) was used for acid-base titrations. The pH electrode was calibrated with the following pH buffers: 4.0, 7.0 and 9.0. The calibration slope of the pH electrode was maintained $\geq$ 98%. Milk samples (40 ml) were titrated from the initial pH of ~6.6 to 3.0 with 0.5 N HCl and back-titrated to pH 9.0 with 0.5 N NaOH. Titrants were added in 0.1 ml increments at 30 s intervals to allow for equilibration of titrant and milk. The change in pH (dpH), resulting from the incremental addition of acid or base, and the volume of titrant used in the titration were recorded by the titrator software and exported to a Microsoft Excel spreadsheet. Buffering indices (dB/dpH) were calculated according to Van Slyke (1922). Buffering curves were prepared by plotting buffering index as a function of pH. The change in total volume of sample due to the addition of acid or alkali during the titration was taken into account in the buffering index calculations. Microsoft Excel was used to calculate the area under the buffering curves. The curves were integrated between the pH limits of 5.8 to 4.1, based on the work of Hassan et al. (2004). The difference in area between the acidification and alkalization buffering curves was then calculated. The magnitude of this area is directly related to the CCP content of milk (Lucey et al., 1993). Buffering capacity of milks was reported as buffering area.

3.2.6. Statistical analysis

ANOVA was carried out using the PASW Statistics Version 18 program (IBM, Armonk, NY, USA). Differences between means were analyzed using Tukey's HSD *post hoc* test. The level of significance was determined at $P<0.05$.

3.3. Results and discussion

3.3.1. Buffering capacity of supplemented milks from acid-base titration

The buffering capacity of the control and supplemented milks are shown in Table 3.1. All milks displayed a buffering peak ~pH 5 during titration with 0.5 N HCl and an absence of this peak during the back-titration with 0.5 N NaOH (results not shown), which is in agreement with Lucey et al. (1993). With the addition of 2.5 $mmol \cdot L^{-1}$ of all salts, there were no significant differences ($P>0.05$) in buffering area between supplemented milks and the control. At 5.0 and 10 $mmol \cdot L^{-1}$ addition levels, the $CaCl_2$ and $SrCl_2$ milks had significantly greater ($P<0.05$) buffering area compared to the control milk. The $MgCl_2$ supplemented milks did not differ significantly ($P>0.05$) from the control milk at any addition level. Both the 10 $mmol \cdot L^{-1}$ $CaCl_2$ and 10 $mmol \cdot L^{-1}$ $SrCl_2$ milks had significantly higher buffering areas ($P<0.05$) than the 10 $mmol \cdot L^{-1}$ $MgCl_2$ milk. The buffering peak at ~ pH 5 is attributed to the solubilization of Ca phosphate nanoclusters and liberation of phosphate ions therefrom that combine with H^+, resulting in buffering (Lucey et al. 1993). Thus, increased buffering around this pH value is indicative of a higher CCP level in milk. The higher buffering area observed for the 10 $mmol \cdot L^{-1}$ $CaCl_2$ and $SrCl_2$ milks may be due to increased formation of nanoclusters.

3.3.2. Mineral partition between curds and whey

Addition of the divalent cation salts to the milks led to an increase in the level of CN-bound cationic component of these salts (Figures 3.1, 3.2 and 3.3). Binding of these cations to caseins may occur via monoester phosphate groups on serine and threonine residues, carboxylic groups of aspartic acid and glutamic acid (Dickson and Perkins, 1971), phenolic, sulfhydryl and imidazole groups (Gaucheron et al., 1997) and/or as

insoluble divalent cation phosphate nanocluster complexes. After ~6 h of aging, an increase in soluble Ca, Mg and Sr was observed in a number of the milk gels. This decrease in casein associated minerals coincided with a slight but significant decrease (P<0.05) in CN-bound water in all gels during aging (Table 3.2). The contraction of the *para*-casein network during aging creates pores which fill with unbound moisture (whey). The decrease in water associated with casein over time could lead to solubilization of minerals due to increased water content in gel pores and permit internal rearrangements of caseins in *para*-casein micelles. It should be noted that CN-bound water decreased independently of addition of divalent cation salts. Addition of the divalent cation salts increased the % CN-bound P_i values at all addition levels compared to the control gel (Figure 3.4). CN-bound P_i level decreased by late aging only in gels with 10 mmol·L^{-1} concentration of any of the three divalent cation salts and also in the gel made from milk containing 5.0 mmol·L^{-1} $SrCl_2$. Van Hooydonk et al. (1986) suggested that complexes of divalent cations and phosphates may bind to caseins when divalent cations are added to milk. All of the inorganic phosphorus associated with the micelles is thought to be present in the CCP (Zoon et al., 1988).

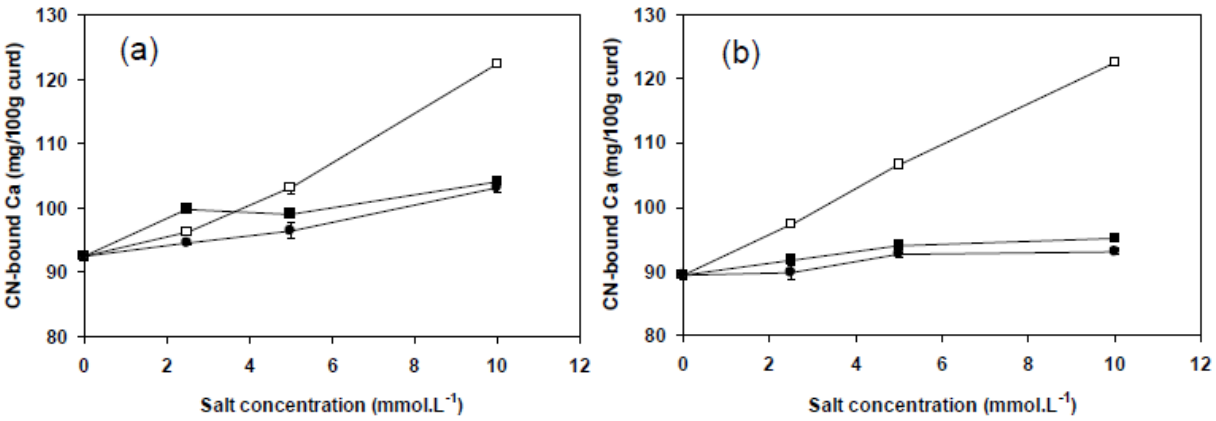


Figure 3.1. Casein-bound calcium in rennet-induced milk gels supplemented with $MgCl_2$ (■), $CaCl_2$ (□) or $SrCl_2$ (●) at (a) gelation point and (b) 6 h after rennet addition. Error bars indicate ± 1 standard deviation.

Table 3.1. Differences in buffering area between acid and alkaline titration curves for milks supplemented with chloride salts of Mg, Ca and Sr (mean ± standard deviation)

Salt addition ($mmol \cdot L^{-1}$)	Area (mm^2)
Control (0)	19.1 ± 0.5^{d}
$MgCl_2$ 2.5	19.2 ± 0.1^{d}
$MgCl_2$ 5.0	20.3 ± 0.5^{bcd}
$MgCl_2$ 10	20.7 ± 0.1^{bcd}
$CaCl_2$ 2.5	19.5 ± 0.8^{cd}
$CaCl_2$ 5.0	21.6 ± 1.4^{abc}
$CaCl_2$ 10	22.3 ± 0.7^{a}
$SrCl_2$ 2.5	20.2 ± 0.5^{bcd}
$SrCl_2$ 5.0	21.8 ± 0.8^{ab}
$SrCl_2$ 10	22.9 ± 0.8^{a}

Means within a column without a common lower case superscript letter are significantly different ($P<0.05$)

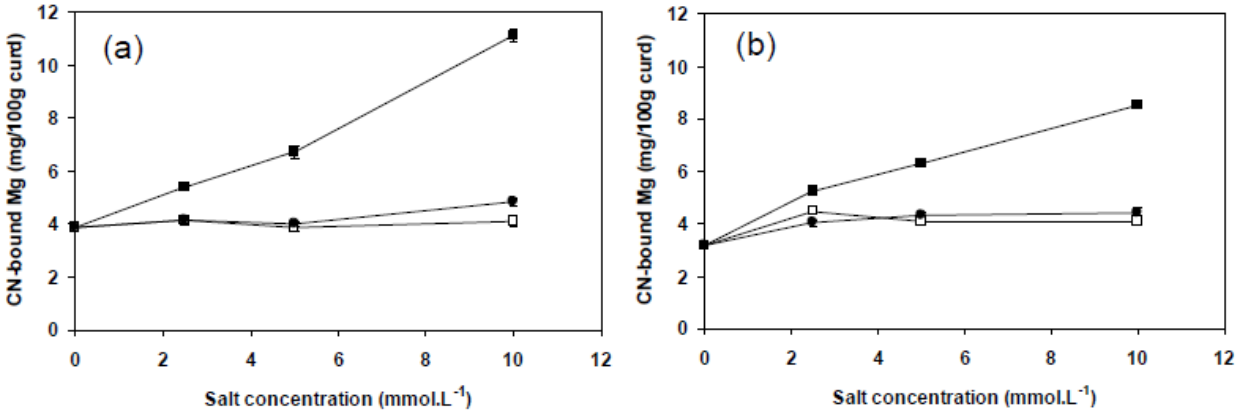


Figure 3.2. CN-bound Mg in rennet-induced milk gels supplemented with $MgCl_2$ (■), $CaCl_2$ (□) or $SrCl_2$ (●) at (a) gelation point and (b) 6 h after rennet addition. Error bars indicate ± 1 standard deviation.

Table 3.2. Percentages of CN-bound Ca, Mg, Sr and H_2O in rennet-induced gels supplemented with chloride salts of divalent metal ions at the gelation point and 6 h after rennet addition (6 h) (mean ± standard deviation)

	% CN-bound Ca		% CN-bound Mg		% CN-bound Sr		% CN-bound H_2O	
Salt addition ($mmol \cdot L^{-1}$)	Gelation point	6 h	Gelation point	6 h	Gelation point	6 h	Gelation point	6 h
Control (0)	68.60 ± 0.48^{deA}	67.87 ± 0.22^{dA}	32.84 ± 1.41^{bcA}	27.81 ± 1.10^{cdB}	—	—	82.14 ± 0.15^{aA}	80.13 ± 0.16^{aB}
$MgCl_2$ 2.5	72.82 ± 0.29^{bA}	69.69 ± 0.06^{cB}	30.17 ± 0.61^{cdA}	29.40 ± 0.75^{cA}	—	—	82.61 ± 0.44^{aA}	80.07 ± 0.16^{aB}
$MgCl_2$ 5.0	73.19 ± 0.33^{bA}	71.00 ± 0.28^{bB}	28.39 ± 0.97^{dA}	26.39 ± 0.25^{dB}	—	—	82.67 ± 0.46^{aA}	79.70 ± 0.35^{aB}
$MgCl_2$ 10	76.64 ± 0.21^{aA}	71.88 ± 0.15^{aB}	29.88 ± 0.61^{dA}	23.63 ± 0.30^{eB}	—	—	81.84 ± 0.42^{aA}	79.75 ± 0.26^{aB}
$CaCl_2$ 2.5	68.00 ± 0.33^{fA}	67.59 ± 0.06^{dA}	34.63 ± 0.55^{bB}	37.65 ± 0.51^{aA}	—	—	81.93 ± 0.22^{aA}	79.77 ± 0.20^{aB}
$CaCl_2$ 5.0	67.89 ± 0.53^{fA}	68.24 ± 0.44^{dA}	33.66 ± 1.18^{bA}	34.55 ± 1.38^{bA}	—	—	82.33 ± 0.31^{aA}	79.88 ± 0.19^{aB}
$CaCl_2$ 10	68.90 ± 0.21^{deA}	68.24 ± 0.60^{dA}	34.38 ± 1.17^{bA}	34.41 ± 0.87^{bA}	—	—	82.16 ± 0.51^{aB}	80.16 ± 0.19^{aB}
$SrCl_2$ 2.5	69.66 ± 0.10^{cdA}	68.24 ± 0.84^{dB}	34.12 ± 1.63^{bA}	33.9 ± 0.96^{bA}	62.80 ± 1.04^{aA}	60.79 ± 0.48^{aB}	82.64 ± 0.44^{aA}	80.40 ± 0.42^{aB}
$SrCl_2$ 5.0	70.06 ± 0.91^{cA}	69.85 ± 0.51^{cA}	33.83 ± 0.90^{bA}	35.85 ± 0.95^{abA}	63.08 ± 0.56^{aA}	58.14 ± 0.36^{bB}	82.18 ± 0.46^{aA}	80.21 ± 0.26^{aB}
$SrCl_2$ 10	73.53 ± 0.53^{bA}	69.87 ± 0.38^{cB}	40.18 ± 1.47^{aA}	37.32 ± 1.41^{aA}	63.59 ± 0.50^{aA}	57.01 ± 0.96^{bB}	82.50 ± 0.54^{aA}	80.00 ± 0.19^{aB}

Means within a column without a common lower case superscript letter are significantly different ($P<0.05$)
Means within a row for the same parameter without a common upper case superscript letter are significantly different ($P<0.05$)
% CN-bound element refers to the percentage of total element in the system that is bound to casein.

3.3.2.1. Effect of $CaCl_2$ addition on mineral partition

The amount of CN-bound Ca increased with level of addition of $CaCl_2$ (Figure 3.1). The % CN-bound Ca (as a % of total Ca) in the $CaCl_2$ supplemented gels did not deviate from the control by more than 1% (Table 3.2). Similarly, Philippe et al. (2005) observed that the percentage of Ca associated remains constant with increased Ca addition to casein micelle suspensions, which indicates that added Ca precipitates to the CN-bound phase in order to maintain an equilibrium ratio due to the aqueous phase being saturated with Ca salts. Philippe et al. (2003) reported that 20% of added Ca remained soluble regardless of $CaCl_2$ addition level (up to 13.5 $mmol \cdot kg^{-1}$) in skim milk. The % CN-bound inorganic phosphorous (P_i) increased with increasing $CaCl_2$ concentration (Figure 3.4). Co-precipitation of added Ca^{2+} with phosphate has been observed in numerous studies (Philippe et al., 2003, 2005; Udabage et al., 2000; Van Hooydonk et al., 1986). The co-precipitation of added Ca with P_i from the aqueous phase strongly indicates nanocluster formation. Zoon et al. (1988) found that the more calcium a sample contained at a certain phosphate concentration, the higher the amount of CCP was present. However, new CCP nanoclusters formed at unreacted phosphoserine centres after Ca^{2+} addition to milk is thought to differ from the natural form of CCP (Philippe et al., 2003). $CaCl_2$ addition had no impact on CN-bound Mg levels at gelation point (Table 3.2 and Figure 3.2) at all concentrations studied.

3.3.2.2. Effect of $MgCl_2$ addition on mineral partition

The amount of CN-bound Mg increased with level of addition of $MgCl_2$ (Figure 3.2). The % CN-bound Mg decreased in the control milk gel after 6 h (Table 3.2). The lower association of Mg with the casein network compared to Ca is likely due to the

fact that Mg phosphate salts are not at saturation in milk unlike Ca phosphates (Philippe et al. 2005). The % CN-bound Mg in the control milk gel was ~33% at gelation point which is close to values previously reported for bovine milk (~35%) (Gaucheron, 2005). This value decreased to ~27% after ~6 h of aging. The % CN-bound Mg was similar (P>0.05) at the gelation point and after 6 h (~30%) for the 2.5 mmol·L^{-1} $MgCl_2$ gel. Van Hooydonk et al. (1986) reported that ~30% of added Mg was CN-bound after adding 3 mmol·L^{-1} $MgCl_2$ to milk. However, the % CN-bound Mg decreased in the 5.0 mmol·L^{-1} $MgCl_2$ milk gel and to a greater extent in the 10 mmol·L^{-1} $MgCl_2$ milk gel after aging (Table 3.2). The decrease in CN-bound Mg during aging may be explained by the decrease in % CN-bound water (Table 3.2) leading to an increase in water available as solvent in the network, causing a decrease in effective Mg salt concentration in the serum. $MgCl_2$ addition appeared to cause more displacement of soluble Ca to the CN-bound phase than $SrCl_2$ addition (Table 3.2). Addition of $MgCl_2$ at all levels increased the % CN-bound Ca, with the 10 mmol·L^{-1} $MgCl_2$ gel having ~8% higher CN-bound Ca than the control milk gel. Van Hooydonk et al. (1986) observed an increase in CN-bound Ca and Ca^{2+} activity due to $MgCl_2$ addition and attributed this to added Mg^{2+} exchanging with Ca^{2+} from soluble citrate complexes. $MgCl_2$ may also cause a displacement of non-nanocluster CN-bound Ca as the caseins have a higher affinity for Mg binding (Dickson and Perkins, 1971). This would increase ionic Ca^{2+} in aqueous phase and, coupled with possible displacement of Ca from soluble Ca salt complexes, this may explain the increase in CN-bound Ca in $MgCl_2$ milks. Zhang and Aoki (1995) reported that Mg phosphate alone has no cross linking ability but increasing the Mg concentration (2.5-10 mmol·L^{-1}) when forming artificial casein micelles leads to increased CN-bound Ca and inorganic phosphate and could thereby indirectly promote calcium

crosslinking of caseins. The increases in CN-bound Ca and P_i at all addition levels of $MgCl_2$ in the present study suggest that levels of CCP nanoclusters may increase in these milks but not sufficiently to change the buffering capacity (Table 3.1). Previous studies have reported that a greater increase in CN-bound inorganic phosphate is observed in casein systems due to $CaCl_2$ compared to $MgCl_2$ added at the same levels (Philippe et al., 2005; Van Hooydonk et al., 1986). This is in agreement with the 5.0 and 10 mmol·L^{-1} levels of both salts in the present study.

3.3.2.3. Effect of $SrCl_2$ addition on mineral partition

The amount of CN-bound Sr increased with level of addition of $SrCl_2$ (Figure 3.3). The % CN-bound Sr at the gelation point was ~63% in all $SrCl_2$ supplemented milks (Table 3.2). This value decreased in these milks during aging with the value of the 10 mmol·L^{-1} $SrCl_2$ milk gel decreasing to the greatest extent. There was a lower % association of Sr with casein (~63%) compared to Ca (~68%) in the control milk gel. The % CN-bound P_i increased with increasing $SrCl_2$ concentration (Figure 3.4). Of all the divalent cation salts, $SrCl_2$ at 5.0 and 10 mmol·L^{-1} increased CN-bound P_i to the greatest extent. Insoluble Sr phosphate complexes have the ability to crosslink caseins and form aggregates, but this crosslinking ability is lower than for Ca phosphates (Zhang and Aoki, 1995). The addition of 5.0 and 10 mmol·L^{-1} $SrCl_2$ caused a significant increase (P<0.05) in CN-bound Ca compared to the control milk gel. Increased CN-binding of Ca due to the presence of Sr has been reported by Zhang and Aoki (1995). The percentage CN-bound Mg was much higher in the 10 mmol·L^{-1} $SrCl_2$ milk gel compared to the control milk gel at both the gelation point and 6 h after rennet addition. It is likely that Sr phosphate nanoclusters formed in the $SrCl_2$ milks. These Sr nanoclusters may have a different stoichiometric ratio of ions

compared to conventional Ca-based CCP nanoclusters (Cooke and McSweeney, 2013) and may have slightly different properties as a result. In the 10 mmol·L^{-1} $CaCl_2$ milk gel, the level of CN-bound Ca remained constant between the gelation point and during aging, whereas the CN-bound Mg and Sr decreased in the 10 mmol·L^{-1} $MgCl_2$ and $SrCl_2$ milks, respectively, during this time period (Figures 3.1, 3.2 and 3.3). This decrease in CN-bound Mg or Sr led to a concurrent decrease in Ca in the respective milks 6 h after rennet addition, indicating that their ability to promote Ca binding decreased. It is also possible that the Sr phosphate nanoclusters are less stable during aging compared with Ca phosphate nanoclusters.

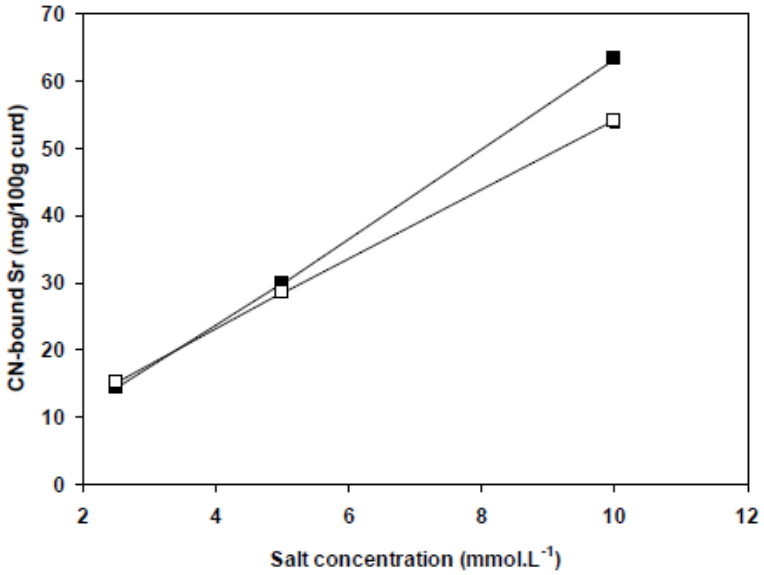


Figure 3.3. CN-bound Sr in rennet-induced milk gels supplemented with $SrCl_2$ at gelation point (■) and 6 h after rennet addition (□). Error bars indicate ± 1 standard deviation.

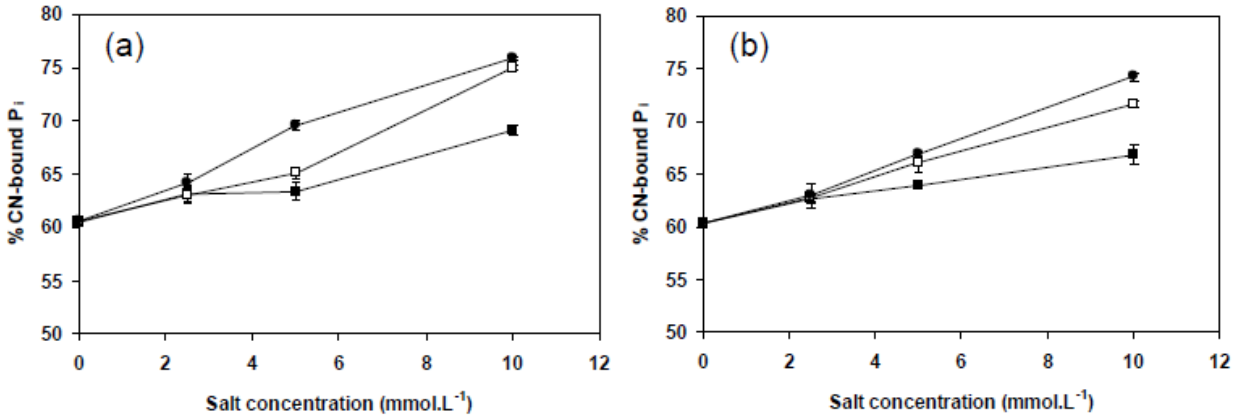


Figure 3.4. % CN-bound inorganic phosphorous (P_i) in rennet-induced milk gels supplemented with $MgCl_2$ (■), $CaCl_2$ (□) or $SrCl_2$ (●) at (a) gelation point and (b) 6 h after rennet addition. Error bars indicate ± 1 standard deviation.

3.3.3. Rennet gelation time of supplemented milks

Rennet gelation time decreased with increasing concentration of all salts (Table 3.3). A reduction in RCT of milk due to $CaCl_2$ addition has also been reported by numerous authors (McMahon et al., 1984; Sandra et al., 2012; Udabage et al., 2001; Van Hooydonk et al., 1986; Zoon et al., 1988). Both $CaCl_2$ and $SrCl_2$ decreased gelation time to a greater extent than $MgCl_2$ at most addition levels investigated. The reduction in gelation time was similar (P>0.05) between $CaCl_2$ and $SrCl_2$ at both 2.5 and 10 mmol·L^{-1} addition levels. The difference in gelation times between the salts, specifically between $MgCl_2$ and the other two may be due to the formation of nanoclusters in the micelles of the $CaCl_2$ and $SrCl_2$ milks but not in the $MgCl_2$ milk. Van Hooydonk et al. (1986) also observed that $CaCl_2$ reduced gelation time more than $MgCl_2$ at the same molar addition level (3 mmol·L^{-1}) and suggested that the total activity of divalent cations may be less important than interactions between the micelle and insoluble salt complexes in the context of rennet coagulation. Changes on the micelle surface due to addition of the cations may also attribute to the differences in gelation time. Van Hooydonk et al. (1986) reported that at the same addition level (3mM), Ca^{2+} and Mg^{2+} reduced the macropeptide layer by 7.14 and 1.78% respectively. In theory, the reduced size of the brush border would allow adjacent micelles to approach each other at a closer distance and therefore facilitate an increased rate of aggregation.

3.3.4. Dynamic rheological properties as a function of renneting time

The G′ values as a function of time for rennet-induced gels are shown in Figure 3.5. Gel properties were studied for 6 h after rennet addition. It is evident that addition of

$MgCl_2$, $CaCl_2$ and $SrCl_2$ at all concentrations caused an increase in gel firming rate. The slow increase in the G′ above 10 Pa probably reflects ongoing fusion of micelles and other structural rearrangements which results in an increase in the contact area between aggregated particles (Lucey, 2002; Mellema et al., 2002), increasing the number of bonds in the network. Supplemented milk gels had much higher G′ values 6 h after rennet addition (G'_{6h}) compared to the control (Table 3.3). Increasing the concentration of all salts from 2.5 to 10 mmol·L^{-1} also increased G'_{6h}. At an addition level of 2.5 mmol·L^{-1}, the $SrCl_2$ and $MgCl_2$ milk gels had similar (P>0.05) G'_{6h} values, whereas at 5.0 and 10 mmol·L^{-1} addition levels, the values for $SrCl_2$ were higher (P<0.05) than those of gels with $MgCl_2$ added. The $CaCl_2$ supplemented milk gels had a higher G'_{6h} than $MgCl_2$ milk gels at all addition levels. Zoon et al. (1988) reported that G′ was ~10% higher in milk gels supplemented with 6 mmol·L^{-1} added $CaCl_2$ after long aging times. Udabage et al. (2001) observed an increase in G′ after ~3 h of aging when $CaCl_2$ was increased from 5 to 10 mmol·kg^{-1}. Differences in G′ between both $CaCl_2$ and $SrCl_2$ gels compared with $MgCl_2$ gels suggest that a higher nanocluster concentration in the former milks had a major effect. Cooke and McSweeney (2013) proposed that because Sr phosphate and Ca phosphate have similar solubilities that are both much lower than Mg phosphate, they are much more likely to precipitate to casein-bound phase than Mg phosphate. Udabage et al. (2001) observed an increase in G′ of rennet milk gels with an increase in CCP content up to ~130% of that in the original micelles. Choi et al. (2007) found that removal of CCP from milk at constant pH led to a decrease in G′. In native casein micelles, it is estimated that ~10% of phosphoserine clusters are unreacted, i.e., not involved in stabilizing nanoclusters (Holt, 2004). An increased number of individual nanoclusters within the casein micelles of the $CaCl_2$ or $SrCl_2$ milks (assuming their

spatial distribution remain similar to those in native micelles) would presumably limit the amount of fusion between contacting micelles. However, partial micellar fusion should increase the number of sites where nanocluster can form. Reduced micellar fusion leads to increased strand thickness and strength and therefore more resistance to endogenous stresses, giving the gel a more elastic-like character and less tendency to rearrange (Mellema et al., 2002). Therefore, an increase in casein-casein affinity without increased particle fusion may explain the increased gel strength.

Table 3.3. Effects of added divalent cation salts on rheological properties of rennet-induced skim milk gels (mean ± standard deviation)

Salt addition ($mmol \cdot L^{-1}$)	Gelation time (min)*	G'_{6h} (Pa)**	Loss tangent***	Slope of log G′ vs log frequency
Control (0)	36.32 ± 0.34^{a}	84.30 ± 1.82^{h}	0.51 ± 0.00^{a}	0.26 ± 0.00^{a}
$MgCl_2$ 2.5	25.35 ± 1.03^{b}	109.59 ± 0.13^{g}	0.49 ± 0.01^{b}	0.25 ± 0.00^{b}
$MgCl_2$ 5.0	20.32 ± 0.01^{cd}	116.43 ± 1.23^{e}	0.49 ± 0.01^{b}	0.25 ± 0.00^{b}
$MgCl_2$ 10	15.60 ± 1.21^{f}	130.58 ± 0.23^{c}	0.47 ± 0.00^{c}	0.24 ± 0.00^{c}
$CaCl_2$ 2.5	19.81 ± 0.21^{cd}	112.70 ± 0.39^{f}	0.47 ± 0.00^{c}	0.24 ± 0.00^{c}
$CaCl_2$ 5.0	17.57 ± 0.69^{e}	120.49 ± 0.45^{d}	0.45 ± 0.00^{d}	0.23 ± 0.00^{d}
$CaCl_2$ 10	11.93 ± 0.86^{g}	137.01 ± 0.39^{a}	0.41 ± 0.01^{f}	0.22 ± 0.00^{f}
$SrCl_2$ 2.5	21.10 ± 0.53^{c}	108.67 ± 1.13^{g}	0.47 ± 0.00^{c}	0.24 ± 0.00^{c}
$SrCl_2$ 5.0	19.29 ± 0.37^{d}	121.18 ± 0.61^{d}	0.44 ± 0.00^{e}	0.23 ± 0.00^{e}
$SrCl_2$ 10	12.99 ± 0.18^{g}	134.14 ± 0.45^{b}	0.38 ± 0.00^{g}	0.21 ± 0.00^{f}

Means within a column without a common lower case superscript letter are significantly different ($P<0.05$)

*Defined as the point when gels had a storage modulus $\geq$ 1 Pa. 5 min added to these values to compensate for time between rennet addition and commencement of oscillation

**Measured ~6 h after rennet addition

***Measured 6 h after rennet addition at a frequency of 0.001 Hz

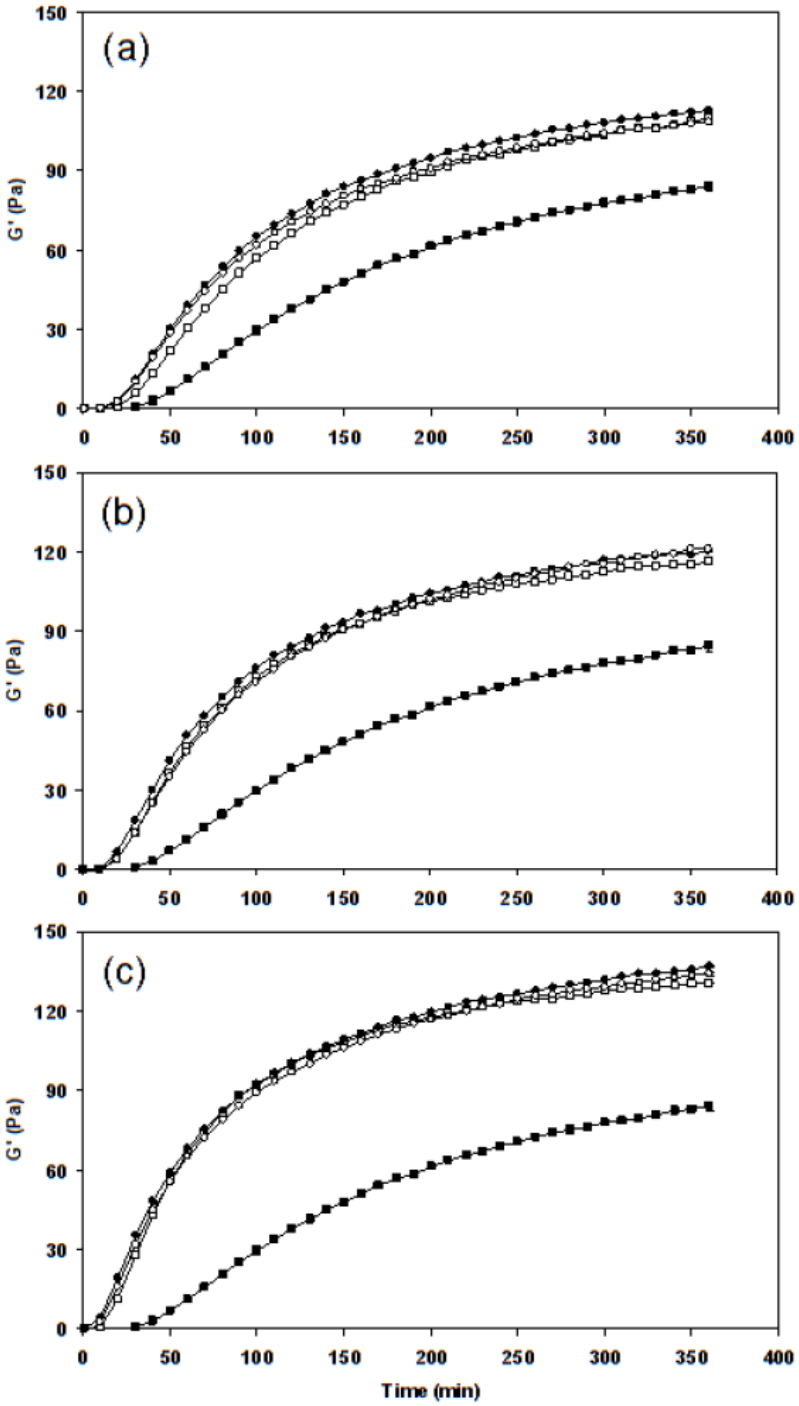


Figure 3.5. Storage modulus (G′) as a function of time for rennet-induced milk gels produced from control milk (■) and milks supplemented with $MgCl_2$ (□), $CaCl_2$ (●) or $SrCl_2$ (○) at concentrations of (a) 2.5 mmol·L^{-1}, (b) 5.0 mmol·L^{-1} and (c) 10 mmol·L^{-1}. Error bars indicate ± 1 standard deviation.

3.3.5. Dynamic rheological properties as a function of frequency

Figure 3.6 displays G′ values as a function of frequency for rennet gels. The G′ values increased with increasing frequency in agreement with Zoon et al. (1988) as bonds in the matrix have less time to relax if the timescale of the applied stress is

shorter. The G′ values of gels at all addition levels of $MgCl_2$, $CaCl_2$ and $SrCl_2$ were higher than the control milk gel at all frequencies analysed. For 2.5 mmol·L^{-1} addition levels, the G′ values of $MgCl_2$, $CaCl_2$ and $SrCl_2$ were similar (P>0.05) at all frequencies. However, at addition levels of 5.0 mmol·L^{-1}, the G′ value of the $MgCl_2$ milk gel was lower than both the $CaCl_2$ and $SrCl_2$ milk gels and this difference increased further at 10 mmol·L^{-1}, especially at low frequencies (<0.01 Hz). Zoon et al. (1988) observed that lower levels of CCP in skim milk can lead to lower G′ values as a function of frequency in rennet-induced gels, whereas higher CCP has the opposite effect. These results indicate that addition of $CaCl_2$ and $SrCl_2$ to milks at 5.0-10 mmol·L^{-1} led to rennet gels with increased elastic properties and rigidity than the gels made from the respective $MgCl_2$ supplemented milks.

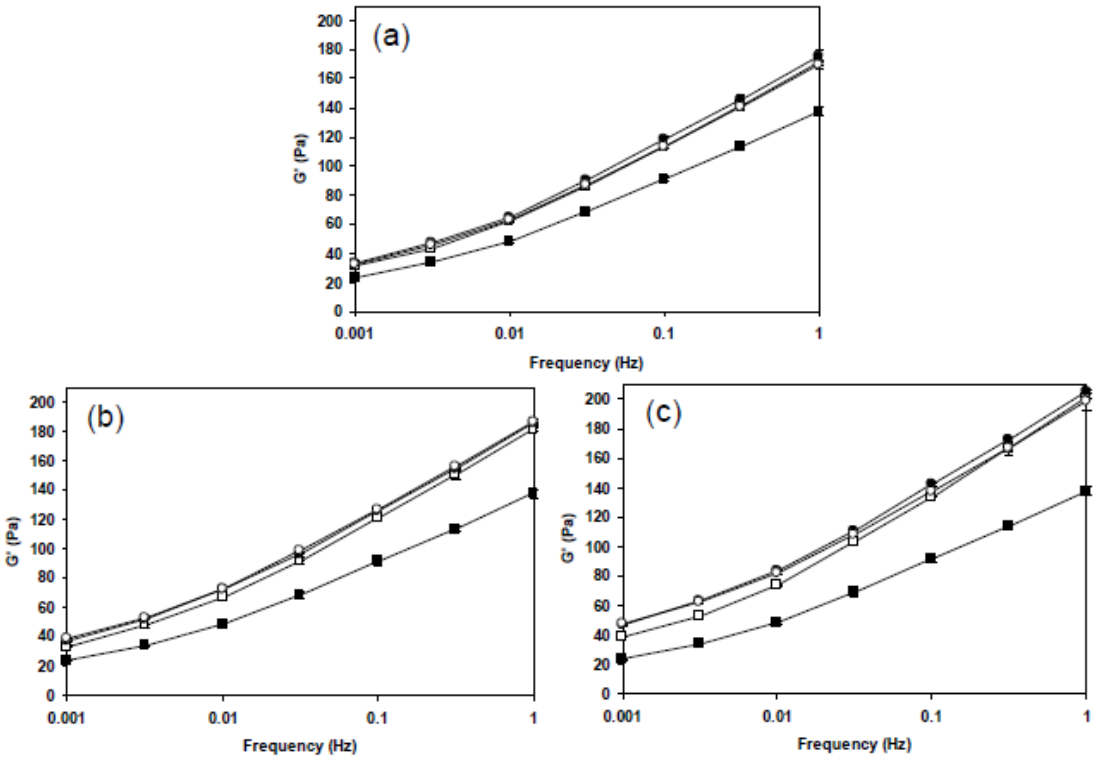


Figure 3.6. Storage modulus (G′) as a function of frequency for rennet-induced milk gels produced from control milk (■) and milks supplemented with $MgCl_2$ (□), $CaCl_2$ (●) or $SrCl_2$ (○) at concentrations of (a) 2.5 mmol·L^{-1}, (b) 5.0 mmol·L^{-1} and (c) 10 mmol·L^{-1}. Frequency sweep was carried out 6 h after rennet addition. Error bars indicate ± 1 standard deviation.

Tan δ as a function of frequency in rennet gels is shown in Figure 3.7. Tan δ values generally decreased with increasing frequency in agreement with Zoon et al. (1988). At low frequencies (below 0.1 Hz) all supplemented milk gels had lower tan δ values than the control milk gel. At 2.5 mmol·L^{-1} addition levels, the tan δ at 0.001 Hz was lower in both the $CaCl_2$ and $SrCl_2$ milk gels compared to the $MgCl_2$ gel. This difference became more marked at 5.0 and 10 mmol·L^{-1} supplementation levels. At 0.1 Hz, the tan δ values of all the $MgCl_2$ gels and the control gel were similar (P>0.05); however, the gels made from milk containing 5.0 or 10 mmol·L^{-1} $CaCl_2$ and $SrCl_2$ were lower than both the equivalent gels containing $MgCl_2$ and the control gel. At low frequencies (below 0.01), tan δ values of 10 mmol·L^{-1} $SrCl_2$ milk gels were lower (P<0.05) than the corresponding $CaCl_2$ gels. The susceptibility of rennet milk gels to syneresis has been associated with a high value (>0.4) for tan δ at long time scales (Van Vliet et al., 1991), which is related to increased network rearrangements after gel formation. The control milk gel had a tan δ value of ~0.51 when measured at 0.001 Hz. Addition of $MgCl_2$, $CaCl_2$ and $SrCl_2$ significantly decreased (P<0.05) this value, with $CaCl_2$ and $SrCl_2$ addition having a much greater effect than $MgCl_2$ at all concentrations added. Mellema et al. (2002) observed that the higher the % Ca in micelles, the lower the value of tan δ measured at 0.001 Hz. This indicates that the divalent cations may have reinforced the internal structure of the casein micelles in the supplemented milks and produced gels with increased resistance to syneresis. As partial particle fusion is related to the liquid-like or viscous behaviour of primary particles in rennet milk gels (Mellema et al. 2002), it can be concluded that a higher tan δ can indicate increased particle fusion in rennet-induced gels. Presumably, an increased rigidity of the internal structure of micelles caused by increased nanocluster levels would retard micellar fusion. Zoon et al.

(1988) found that rennet milk gels displayed higher tan δ values with lower CCP whereas gels with higher CCP had lower tan δ values especially at low frequencies.

For all rennet-induced gels, plotting log G′ versus log (frequency) curves gave straight lines (R^2>0.98 in all cases; results not shown). The power law exponent for the relationship between G′ and frequency in rennet milk gels is in the range ~0.23 to 0.25 (Lucey, 2002). The mean value of the power law exponent for the control rennet gel in the present study was ~0.26, which is in close agreement with previously reported values for skim milk rennet gels (Esteves et al., 2002; Mishra et al., 2005; Srinivasan and Lucey, 2002). Addition of divalent cations decreased the value of this parameter (Table 3.3), indicating a more solid-like character in these gels. $CaCl_2$ and $SrCl_2$ addition had a much greater impact than $MgCl_2$ especially at 10 mmol·L^{-1} addition levels where values of the $CaCl_2$ and $SrCl_2$ gels were ~0.21 compared to ~0.24 for the $MgCl_2$ gel.

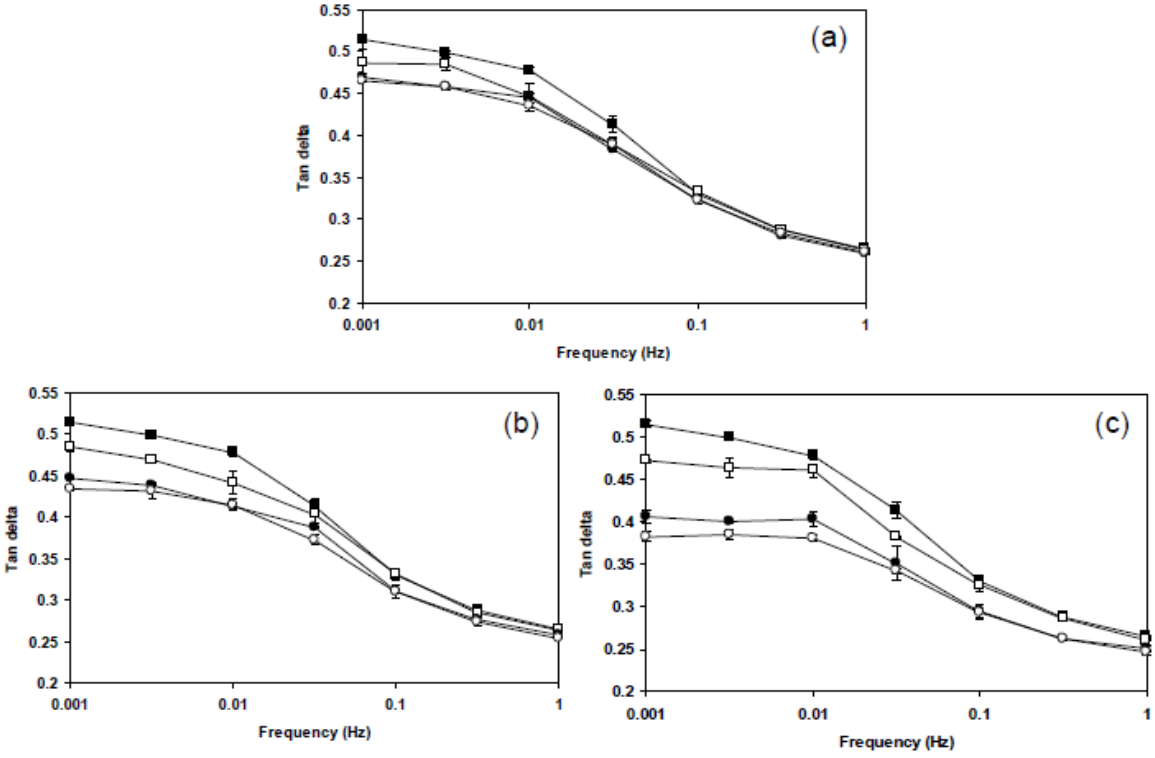


Figure 3.7. Loss tangent (tan δ) as a function of frequency for rennet-induced milk gels produced from control milk (■) and milks supplemented with $MgCl_2$ (□), $CaCl_2$ (●) or $SrCl_2$ (○) at concentrations of (a) 2.5 mmol·L^{-1}, (b) 5.0 mmol·L^{-1} and (c) 10 mmol·L^{-1}. Frequency sweep was carried out 6 h after rennet addition. Error bars indicate ± 1 standard deviation.

3.4. Conclusions

This study demonstrated that the mineral equilibria of rennet milk gels have a major effect on gel properties during aging. It was evident that both the $CaCl_2$ and $SrCl_2$ supplemented milks had superior gel properties compared to the $MgCl_2$ supplemented milks and the control milk in terms of elasticity and gelation time. This was most likely due to increased level of nanoclusters in the micelles of milks supplemented with $CaCl_2$ and $SrCl_2$. An increase in nanocluster level is supported by results reported for buffering capacity and partition of Ca, Mg, Sr and P_i in the milks and rennet gels produced therefrom. An increase in nanocluster level caused micelles to have a denser, less flexible structure and reduced the amount of rearrangements and micelle fusion leading to stronger gel networks with high G′ and low tan δ values. The inability of added $MgCl_2$ to induce rennet gel properties similar to those of $CaCl_2$ and $SrCl_2$ supplemented gels is likely a consequence of higher solubility of Mg phosphate salts compared to Ca and Sr phosphates (Zhang and Aoki, 1995), preventing Mg phosphate nanoclusters from forming. As CN-bound levels of certain minerals decreased during aging, it is possible that a change in the dimensions or density of nanoclusters during gel aging may facilitate rearrangements.

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Chapter 4

The influence of calcium-binding salts on the rheological and melting properties of Cheddar cheese

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Abstract

Cheddar cheese was supplemented with Ca-binding salts in order to modify cheese melting and rheological properties. Experimental cheeses were salted with a combination of NaCl and either trisodium citrate (TSC), disodium phosphate (DSP) or EDTA. The % insoluble (INSOL) Ca of cheeses was determined using an acid-base titration method. Meltability of cheeses was determined using the covered Schreiber melting test. Small deformation rheological properties of cheeses at elevated temperatures were determined by dynamic small amplitude oscillatory rheology. Cheese hardness was determined using texture profile analysis. Addition of the Ca-binding salts did not cause any consistent differences in gross composition, proteolysis and bacterial growth in the cheeses and pH values did not vary greatly between cheeses. Addition of TSC or EDTA decreased % INSOL Ca in cheese during ripening, whereas addition of DSP increased % INSOL Ca of cheese during ripening. Addition of each Ca-binding salt caused an increase in meltability compared to the control cheese by week 10 of ripening. Addition of Ca-binding agents generally caused a decrease in maximum loss tangent of cheeses compared to the control. At 80 °C, the TSC cheese had a significantly higher storage modulus than the other cheeses. The cheeses with added Ca-binding salts had lower hardness values than the control at a number of ripening times especially during early ripening. Alteration of casein interactions in the cheese matrix influenced the melt and rheological properties of the cheeses. Supplementation of cheese with Ca-binding salts may be a viable method for modulating the functional properties of cheese used as a food ingredient.

4.1. Introduction

The use of cheese as a food ingredient has increased over the past few decades due to its numerous functional properties (Lucey, 2008). Cheeses with reduced Ca contents have improved softening, melting, and flow properties (Joshi et al., 2003, 2004; Sheehan and Guinee, 2004). More importantly, the level of soluble Ca in cheese has a major effect on its textural, rheological and functional properties (Lucey and Fox, 1993; Lucey et al., 2003, 2005; O'Mahony et al., 2005; Choi et al., 2008). During cheese ripening, partial solubilization of the insoluble Ca of cheese occurs (Hassan et al., 2004) which is responsible for the initial softening of cheese during early ripening (O'Mahony et al., 2005). The Ca in cheese exists in dynamic equilibrium between the insoluble casein-bound Ca and soluble forms of Ca in the aqueous phase. The insoluble casein-bound Ca phosphate known as colloidal calcium phosphate (CCP) in milk is an important structural element in casein micelles (Horne, 1998) and a decrease in the level of residual CCP in cheese can lead to increased meltability (Lucey et al., 2003). Alteration of the CCP content of cheese has been studied by altering acid development and pH during (Lee et al., 2005) and after manufacture (Pastorino et al., 2003a).

Studies attempting to alter the Ca equilibrium of cheese by use of Ca-binding agents at various stages of cheese manufacture have also been performed (Pastorino et al., 2003b; Mizuno and Lucey, 2005b; Choi et al., 2008; Brickley et al., 2009). Ca-binding agents referred to as 'emulsifying salts' are integral ingredients in the manufacture of processed cheese, where they function by disrupting the CCP crosslinks in natural cheese by sequestering Ca and adjusting the pH of the processed cheese mixture (Kapoor and Metzger, 2008). Various emulsifying salts can alter the

properties of native casein micelles in different ways depending on the concentration of these salts (Mizuno and Lucey, 2005a; De Kort et al., 2011). At sufficient concentrations, some phosphate salts can even crosslink native casein micelles in solution (Gaucher et al., 2007; Mizuno and Lucey, 2007; De Kort et al., 2011). Two emulsifying salts commonly used in the manufacture of processed cheese are trisodium citrate and disodium phosphate. Numerous studies have used EDTA to remove insoluble Ca from casein micelles in milk (e.g., Udabage et al., 2000, 2001; Choi et al., 2007). Addition of citrate, orthophosphate or EDTA to milk can decrease ionic calcium (Ca^{2+}) activity and inhibit rennet coagulation properties (Udabage et al., 2001).

Reducing the Ca content of cheesemilk can have a negative impact on the finished cheese, so a method of decreasing insoluble Ca after the renneting, cooking and milling stages of cheesemaking may provide a more suitable way of altering cheese texture as there is very little change to the cheesemaking procedure. Addition of trisodium citrate at the salting stage of cheesemaking has previously been investigated in Cheddar-style cheese (Brickley et al., 2009) and non-fat *pasta filata* cheese (Mizuno and Lucey, 2005b). The objective of this study was to monitor the changes in Ca equilibrium together with the textural, rheological and melting properties of Cheddar cheese during ripening after the individual addition of several Ca-binding agents at the salting stage of cheese manufacture. Although NaCl was partially replaced, the impact of Na replacement was not an objective of the study. Evaluating the impact of Ca sequestration in cheese by the Ca-binding salts on the rheological and functional properties during ripening was the sole objective of this study.

4.2. Materials and methods

4.2.1. Cheese manufacture

Raw bovine milk was standardized to 3.5% fat and pasteurised (72 °C x 15 s). Three Cheddar-style cheeses were manufactured according to standard protocol on a 50 kg scale in the food processing facilities at University College, Cork. R-604Y (Chr. Hansen Ltd., Little Island, Co. Cork, Ireland) was used as the starter culture at a level of 0.02% (w/v). Chymosin (CHY-MAX^{TM} Plus, Chr. Hansen A/S, Horsholm, Denmark), at a strength of 200 IMCU·ml^{-1}, was added to the cheesemilk at a level of 0.3 mL·L^{-1}. Coagulum was cut at equal firmness (measured subjectively). Curd was cooked from 31 to 39 °C over 30 min. Whey was drained at pH 6.2. The curd was cheddared until pH 5.4 was reached and was then milled and dry salted. The control curd was salted with NaCl at a level 2.5% (w/w). The cheese curd supplemented with trisodium citrate (TSC) was salted with 0.24% (w/w) trisodium citrate dihydrate + 2.21% (w/w) NaCl. The cheese curd supplemented with disodium phosphate (DSP) was salted with 0.145% (w/w) disodium hydrogen phosphate dehydrate + 2.35% (w/w) NaCl. The cheese curd supplemented with EDTA was salted with 0.2% (w/w) disodium EDTA dihydrate + 2.4% (w/w) NaCl. These salt treatments were calculated to ensure the ionic strength of all the cheeses were equal. The salted curd was transferred to rectangular moulds 25.4 cm x 20.3 cm and pressed overnight at 490 kPa. The cheeses were vacuum packaged and ripened at 8 °C for a period of 5 months. Three independent cheesemaking trials were performed.

4.2.2. Compositional analysis

Compositional analysis was performed on the cheeses at day 14 of ripening. The moisture contents of the cheeses were determined by an oven drying method (IDF, 1982), protein by the macro-Kjeldahl procedure (IDF, 1986), fat by the Gerber method (IIRS, 1955), NaCl by a titrimetric method using potentiometric end-point determination (Fox, 1963). Cheese pH was determined by measuring the pH of homogenized cheese slurry made from 10 g cheese and 10 g water at room temperature. The total calcium content of the cheeses was determined using atomic absorption spectroscopy according to IDF (2007). Proteolysis was assessed by determining the levels of pH 4.6-soluble nitrogen as % of total nitrogen (pH4.6SN%TN) according to O'Mahony et al. (2005) at 5 months of ripening. All above mentioned analyses were carried out in triplicate. Urea-polyacrylamide gel electrophoresis (PAGE) was carried out directly on the cheeses using the procedure described by O'Mahony et al. (2005). Contribution of EDTA to nitrogen content was not taken into account for the Kjeldahl or pH4.6SN%TN calculations. Hypothetically, if there was no loss of $Na_2EDTA.2H_2O$ in the salt whey the maximum contribution of EDTA to nitrogen content would be 0.15 g N/kg cheese curd. As a proportion of the $Na_2EDTA.2H_2O$ was probably lost in the salt whey, the contribution of added EDTA to total N was considered negligible.

4.2.3. Determination of cheese insoluble Ca content

The insoluble calcium (INSOL Ca) content of the cheese samples was determined by performing acid-base titrations on aqueous homogenates of cheese based on the work of Lucey et al. (1993a, b) and Hassan et al. (2004). Cheese samples were prepared for titration by homogenizing grated cheese (8 g) with 40 ml of deionized water at 55

°C for 5 min using an Ultra-Turrax homogenizer (T25 with S25N-18G dispersing element, IKA-Werke, Staufen, Germany). This homogenate was then cooled to 25 °C for the titration step. Milk samples (48 ml) were heated to 25 °C before commencement of titration. An automated pH titration system (Metrohm AG 907 Titrando, Herisau, Switzerland) was used for acid-base titrations. The pH electrode was calibrated with buffers at pH 4.0, 7.0 and 9.0. The calibration slope of the pH electrode was maintained $\geq$ 98%. The cheese homogenates and milk samples were titrated at 25 °C from the initial pH of cheese (~5.2) and milk (~6.6) to pH 3.0 with 0.5 N HCl and back-titrated to pH 9.0 with 0.5 N NaOH. Titrants were added in 0.1 ml increments at 30 s intervals to allow for equilibration of titrant and sample. The change in pH (dpH) resulting from the incremental addition of acid or base, and the volume of titrant used in the titration were recorded by the titrator software and exported to a Microsoft Excel spreadsheet. Buffering indices (dB/dpH) were calculated according to Van Slyke (1922). Buffering curves were prepared by plotting buffering index as a function of pH. The change in total volume of sample due to the addition of acid or alkali during the titration was taken into account in the buffering index calculations. Microsoft Excel was used to calculate the area under the buffering curves. The curves were integrated between the pH limits of ~5.8 to 4.1 and ~5.1 and 4.0 for milk and cheese, respectively, based on the work of Hassan et al. (2004). The difference in area between the acidification and alkalization buffering curves was then calculated. The magnitude of this area is directly related to the CCP content of milk (Lucey et al., 1993) and cheese (Lucey et al., 1993; Hassan et al., 2004). The % INSOL Ca content of the cheeses was calculated as described by Hassan et al. (2004). Buffering analyses were carried out in triplicate.

4.2.4. Texture profile analysis

Texture profile analysis (TPA) was performed using a Texture Analyser TA-XT2i (Stable Micro Systems, Godalming, Surrey, UK) according to the method of O'Mahony et al. (2005), except cylindrical samples of dimensions: height 20 mm, diameter 20 mm were used. Hardness was defined according to Bourne (1978). Five replicate samples from each cheese were compressed at each ripening time point.

4.2.5. Dynamic small amplitude oscillatory rheology (DSAOR)

Rheological properties of the cheese samples during a temperature sweep were measured using a controlled shear stress rheometer (model AR-G2; TA instruments, Waters LLC, Leatherhead, Surrey, UK). Measurement geometry consisted of a 40 mm serrated stainless steel parallel plate above and a serrated base plate below. Cheese discs were made with the dimensions: diameter 40 mm and height ~2 mm. The exposed edges of the cheese disc were coated with liquid paraffin to prevent evaporation of moisture from the cheese sample during analysis. The top plate was lowered and an axial force of 0.7 ± 0.1 N was applied to the sample and maintained throughout the procedure. Prior to oscillation, the sample was tempered at 20 °C for 10 min to allow temperature equilibration. The temperature was raised from 20 to 80 °C at a rate of 2 °C/min during which time the storage modulus (G′), loss modulus (G″) and loss tangent (LT) were recorded continuously at a shear strain of 0.5% and a frequency of 1 Hz. Shear strain and stress values chosen were found to be within the linear viscoelastic range for these cheeses. Each sample was analysed in triplicate.

4.2.6. Melt analysis

Melt analysis was carried out using a covered Schreiber test (Altan et al., 2005). Each cheese cylinder (5 mm height, 35 mm diameter) was placed in a covered glass petri dish and then placed in an oven at 232 °C for 5 min. These were then removed and cooled for 30 min at room temperature. Measurements of the melt distance were made using electronic calipers. The diameter of the melted sample was measured at 5 different points and an average diameter was determined. Results were expressed as percentage increase in cheese diameter. Analysis on each cheese sample was performed in triplicate.

4.2.7. Microbiological analysis

Aseptic samples (~10 g) were taken from cheeses using a sterile cheese trier and placed into a stomacher bag. These samples were diluted 1:10 with sterile trisodium citrate (2% w/v) followed by homogenization in a stomacher (Seward Stomacher 400; Seward Ltd. London, UK) for 4 min. Further dilutions were prepared depending on the stage of ripening. Starter bacteria were enumerated on LM17 agar (Terzaghi and Sandine, 1975) incubated aerobically for 3 days at 30°C. Non-starter lactic acid bacteria (NSLAB) were enumerated on Rogosa agar (Rogosa et al., 1951) incubated anaerobically for 5 days at 30°C.

4.2.8. Statistical analysis

ANOVA was carried out using the PASW Statistics Version 18 program (IBM, Armonk, NY, USA). Differences between means were analyzed using Tukey's HSD *post hoc* test. The level of significance was determined at $P < 0.05$.

4.3. Results and discussion

4.3.1. Composition

The composition of the cheeses is shown in Table 4.1. There were no consistent differences between all cheeses for values of percentage moisture, protein, fat, NaCl and ash. The pH values of the cheeses during ripening are shown in Table 4.1. The pH decreased in all cheeses during early ripening up to 4 weeks but then increased by 20 weeks of ripening. The pH values ranged from 5.12-5.31 during ripening and pH values did not differ by more than 0.05 pH units across all cheeses in all trials. The addition of emulsifying salts such as TSC and phosphates in the manufacture of processed cheese influences the pH of the product (Kapoor and Metzger, 2008). However, the levels of these salts added during process cheese manufacture (> 2%) are much higher than in the present study. Previous studies have reported that addition of TSC at the salting stage of cheese manufacture had no significant effect on pH compared to control cheese (Mizuno and Lucey, 2005b; Brickley et al., 2009). Similarly, Pastorino et al. (2003b) observed no change in cheese pH when a 40% TSC solution was injected into Cheddar cheese. However, addition of orthophosphate to skim milk can decrease milk pH (Gaucher et al., 2007). Presumably, the buffering capacity of the cheeses was likely able to resist any major pH changes caused by the salts added in the present study.

Table 4.1. Chemical composition, level of proteolysis and pH values during ripening of control Cheddar cheese and experimental cheeses supplemented with Ca-binding salts (mean ± standard deviation)

		Treatment[2]			
Trial	Parameter[1]	Control	TSC	DSP	EDTA
1	Moisture (%)	35.6 ± 0.4^{a}	35.3 ± 0.4^{a}	35.5 ± 0.4^{a}	35.1 ± 0.3^{a}
	Fat (%)	32.7 ± 0.7^{a}	32.3 ± 0.8^{a}	32.3 ± 0.6^{a}	32.1 ± 0.4^{a}
	Protein (%)	26.4 ± 0.4^{a}	26.8 ± 0.2^{a}	26.6 ± 0.4^{a}	26.6 ± 0.2^{a}
	NaCl (%)	1.50 ± 0.01^{a}	1.46 ± 0.04^{a}	1.47 ± 0.03^{a}	1.50 ± 0.02^{a}
	Ash (%)	3.54 ± 0.07^{b}	3.84 ± 0.16^{a}	3.50 ± 0.07^{b}	3.74 ± 0.05^{ab}
	pH4.6SN%TN	19.68 ± 0.25^{a}	19.34 ± 0.18^{a}	19.51 ± 0.16^{a}	19.63 ± 0.09^{a}
	Total Ca	832 ± 7^{a}	834 ± 9^{a}	836 ± 9^{a}	834 ± 5^{a}
	pH at 1 d	5.20 ± 0.01^{bB}	5.23 ± 0.01^{aB}	5.22 ± 0.01^{abB}	5.20 ± 0.01^{bB}
	pH at 4 wk	5.15 ± 0.01^{aC}	5.16 ± 0.01^{aC}	5.17 ± 0.01^{aC}	5.12 ± 0.05^{aC}
	pH at 20 wk	5.26 ± 0.01^{aA}	5.27 ± 0.01^{aA}	5.27 ± 0.01^{aA}	5.24 ± 0.01^{bA}
2	Moisture (%)	35.8 ± 0.2^{a}	36.3 ± 0.4^{a}	35.8 ± 0.1^{a}	36.0 ± 0.4^{a}
	Fat (%)	31.5 ± 1.3^{a}	31.8 ± 0.6^{a}	32.3 ± 0.8^{a}	32.2 ± 0.8^{a}
	Protein (%)	27.0 ± 0.3^{a}	26.3 ± 0.4^{a}	26.6 ± 0.6^{a}	26.4 ± 0.2^{a}
	NaCl (%)	1.49 ± 0.01^{a}	1.47 ± 0.02^{a}	1.47 ± 0.01^{a}	1.47 ± 0.02^{a}
	Ash (%)	4.05 ± 0.25^{a}	4.06 ± 0.06^{a}	3.68 ± 0.1^{a}	3.72 ± 0.15^{a}
	pH4.6SN%TN	18.82 ± 0.47^{a}	19.26 ± 0.08^{a}	18.53 ± 0.42^{a}	19.22 ± 0.23^{a}
	Total Ca	892 ± 20^{a}	896 ± 25^{a}	888 ± 6^{a}	891 ± 8^{a}
	pH at 1 d	5.25 ± 0.01^{aA}	5.27 ± 0.01^{aA}	5.27 ± 0.01^{aA}	5.27 ± 0.01^{aA}
	pH at 4 wk	5.14 ± 0.00^{aC}	5.13 ± 0.01^{aC}	5.14 ± 0.01^{aC}	5.14 ± 0.01^{aC}
	pH at 20 wk	5.23 ± 0.01^{abB}	5.23 ± 0.01^{abB}	5.24 ± 0.01^{aB}	5.22 ± 0.01^{bB}
3	Moisture (%)	35.3 ± 0.2^{c}	35.8 ± 0.1^{b}	36.2 ± 0.1^{a}	35.5 ± 0.2^{c}
	Fat (%)	33.7 ± 0.1^{a}	32.3 ± 0.7^{a}	33.1 ± 0.1^{a}	33.2 ± 0.2^{a}
	Protein (%)	27.4 ± 0.7^{a}	26.4 ± 0.3^{a}	26.7 ± 0.4^{a}	26.8 ± 0.3^{a}
	NaCl (%)	1.63 ± 0.05^{ab}	1.68 ± 0.05^{a}	1.63 ± 0.03^{ab}	1.58 ± 0.03^{b}
	Ash (%)	3.84 ± 0.14^{a}	3.46 ± 0.14^{b}	3.85 ± 0.11^{a}	3.66 ± 0.07^{ab}
	pH4.6SN%TN	16.71 ± 0.36^{a}	17.18 ± 0.33^{a}	17.18 ± 0.35^{a}	17.53 ± 0.40^{a}
	Total Ca	850 ± 20^{a}	855 ± 20^{a}	862 ± 29^{a}	861 ± 28^{a}
	pH at 1 d	5.28 ± 0.01^{bB}	5.29 ± 0.01^{bB}	5.30 ± 0.01^{aA}	5.25 ± 0.01^{cB}
	pH at 4 wk	5.20 ± 0.01^{aC}	5.21 ± 0.05^{aC}	5.20 ± 0.01^{aB}	5.16 ± 0.01^{bC}
	pH at 20 wk	5.31 ± 0.01^{aA}	5.30 ± 0.01^{aA}	5.31 ± 0.01^{aA}	5.28 ± 0.01^{bA}

[a,b,c]Different lower case superscript letters in the same row within a trial indicate that values are significantly different ($P < 0.05$)

[A,B,C]Different upper case superscript letters in the same column within a trial indicate that values for the same parameter at different ripening times are significantly different ($P < 0.05$)

[1]pH4.6SN%TN = pH 4.6 soluble nitrogen as a % of total nitrogen. Total Ca expressed as mg/100 g cheese

[2]TSC = cheese supplemented at salting with 0.24% w/w trisodium citrate dihydrate; DSP = cheese supplemented at salting with 0.145% w/w disodium hydrogen phosphate dihydrate; EDTA = cheese supplemented at salting with 0.2% w/w disodium EDTA dihydrate

d = day; wk = week

There were no consistent differences in levels of pH4.6SN%TN between the four cheeses (Table 4.1), and urea-PAGE electrophoretograms (not shown) displayed no differences in proteolytic patterns between cheeses throughout ripening. As the cheeses were manufactured with all salting treatments calculated on the basis of equal ionic strength, it is to be expected that no great effect on proteolysis would be observed due to NaCl substitution. Brickley et al. (2009) also observed no differences in proteolysis between a control Cheddar cheese and a cheese salted with an identical TSC/NaCl mixture as used in the present study. Starter bacteria and NSLAB counts were similar in all cheeses of all three trials during ripening (results not shown).

4.3.2. Calcium equilibrium of cheeses

Changes in % INSOL Ca during ripening are shown in Figure 4.1. The % INSOL Ca decreased during ripening in all cheeses, especially during early ripening, in agreement with Hassan et al. (2004), Lee et al. (2005) and O'Mahony et al. (2005). The % INSOL Ca in the control cheese decreased from a maximum of ~76% to a minimum of ~56% after 5 months of ripening; these results are in agreement with the range of values previously reported for Cheddar cheese (Hassan et al., 2004; Lucey et al., 2005). It is known that pH influences the solubility of the Ca phosphate in the CCP of cheese (Lucey et al., 2003). As the pH values were generally similar between all cheeses (Table 4.1), it appears that Ca equilibrium differences observed between the cheeses is due solely to the Ca-binding agents and was not pH-induced.

4.3.3. Effects of TSC addition

The TSC cheese had a significantly lower ($P < 0.05$) % INSOL Ca compared to the control cheese throughout ripening (Figure 4.1). Mizuno and Lucey (2005b) reported a decrease in insoluble Ca and inorganic phosphate (P_i) content in non-fat *pasta filata* cheese salted with a mixture of NaCl and TSC. Similarly, Brickley et al. (2009) observed an increase in soluble Ca levels in cheeses salted with a TSC/NaCl mixture, especially during the first 28 days of ripening. Pastorino et al. (2003b) injected sodium citrate solution into 1 week-old Cheddar cheese blocks and observed no change in the level of CN-bound Ca, but a decrease in CN-bound P_i which indicated a reduction in CCP. TSC is known to decrease Ca^{2+} activity and CCP concentration when added to milk (Udabage et al., 2000, 2001). The addition of TSC at salting most likely caused a displacement of Ca from CCP to aqueous phase due to its Ca-sequestering ability. Studies on the interaction of citrate with the Ca-phosphate mineral hydroxyapetite (HA) have found that citrate can exchange with phosphate at the surface of HA and that citrate may form complexes with Ca on the surface of HA (Lopez-Macipe et al., 1998) and that this interaction is pH dependent. This exchange mechanism would explain the results of Pastorino et al. (2003b) who postulated that their observed decrease in CN-bound P_i may be the result of added citrate exchanging with P_i and forming CN-bound Ca-citrate complexes. Thus, citrate may displace P_i from nanoclusters and modify the nanocluster composition and properties. In a one-month-old Cheddar cheese, Morris et al. (1988) reported that 55% of the total citrate was CN-bound. This value is much higher than that in milk (~10%). The high CN-bound level of citrate probably results from the low solubilities of its salt complexes in cheese compared to milk due to the much lower moisture content in cheese. It is therefore probable that a proportion of added citrate

may precipitate in some form of salt during ripening such as calcium citrate tetrahydrate (Mizuno and Lucey, 2005b).

4.3.4. Effects of DSP addition

The DSP cheese had a significantly higher ($P < 0.05$) % INSOL Ca during the first 4 weeks of ripening (Figure 4.1). By week 20 of ripening, the control and DSP cheeses had statistically similar % INSOL Ca ($P > 0.05$). It appears that the calcium phosphate pseudoequilibrium was achieved after more than 4 weeks of ripening. A number of authors have reported that the addition of orthophosphate to milk can increase the level of casein-bound Ca and P_i (Udabage et al., 2000, 2001; Gaucher et al., 2007). In particular, Udabage et al. (2001) reported an increase in CCP content of milk upon addition of >10 mM orthophosphate. Kaliappan and Lucey (2011) found that high proportions of DSP in mixtures of Ca-binding salts could increase CN-bound Ca and inorganic phosphate in milk protein concentrate solutions. At the pH of the DSP cheese (~5.3), the dominant ionic forms of orthophosphate are HPO_4^{2-} and $H_2PO_4^-$ with the former having a much higher affinity for Ca^{2+} than the latter (Mekmene et al., 2009). The increased CCP content in the DSP cheese during early ripening compared to the control is likely the result of added HPO_4^{2-} combining with Ca^{2+} in the aqueous phase and forming $CaHPO_4$ which has low aqueous solubility (Mekmene et al., 2009). Precipitation of the salt to the CN-bound phase resulted in the increase in % INSOL Ca observed (Figure 4.1).

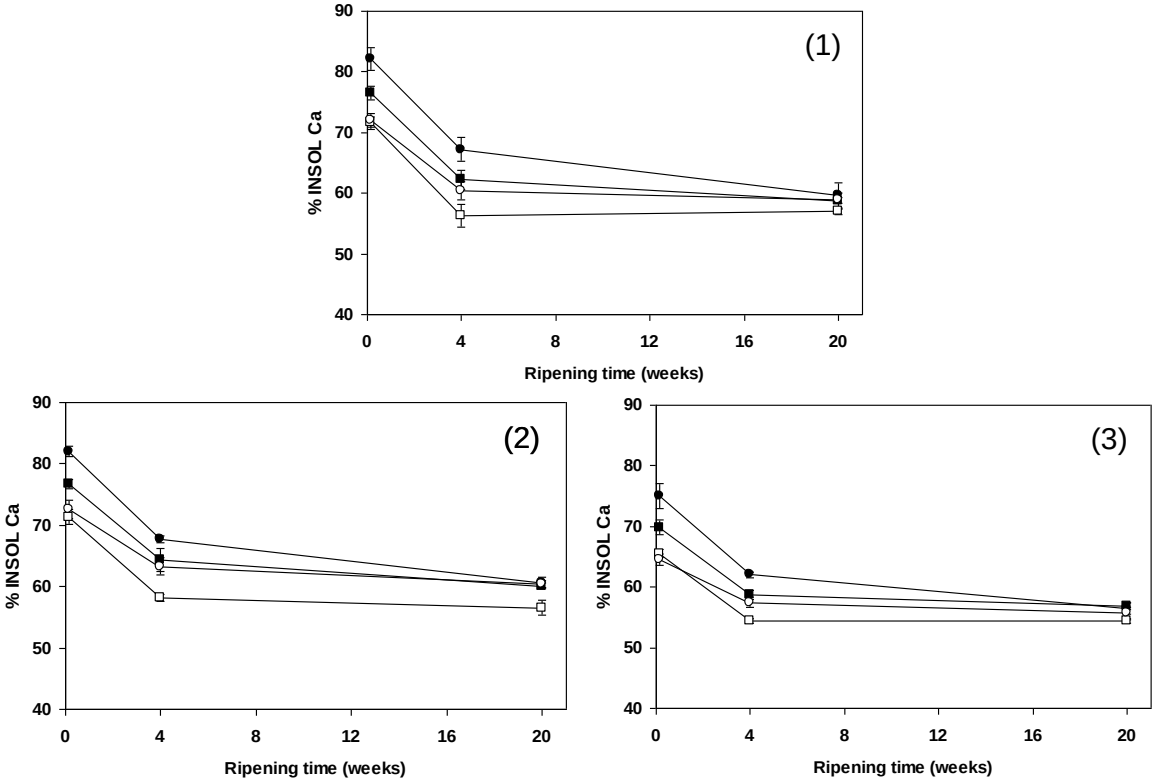


Figure 4.1. Percentage insoluble (INSOL) Ca in control cheese (■), cheese supplemented at salting with 0.24% w/w trisodium citrate dehydrate (TSC) (□), cheese supplemented at salting with 0.145% w/w disodium hydrogen phosphate dehydrate (DSP) (●) and cheese supplemented at salting with 0.2 % disodium EDTA dihydrate (EDTA) (○) during ripening in cheesemaking trials 1-3. Error bars indicate ± 1 standard deviation.

The maximum concentration of $CaHPO_4$ that is soluble in milk is thought to be 0.6 mM (Mekmene et al., 2009), so the amount of this salt that is soluble in cheese would be much lower due to the lower water content in cheese. Gradual protonation of HPO_4^{2-} to $H_2PO_4^-$ may have also occurred during ripening as the pH decreased (Table 4.1) and thereby caused solubilization of the CCP formed by DSP addition, which may account for the statistically similar % INSOL Ca values ($P > 0.05$) between the DSP and control cheeses by late ripening. As it is likely that new CCP forms at unreacted phosphoserine clusters (Holt, 2004), it is also possible that the proteolytic breakdown of caseins during ripening reduced the number of available sites for excess CCP to stabilize leading to a decrease in CN-bound Ca. Gaucher et al. (2007) reported the formation of phosphate salt crystals several microns in

diameter in the aqueous phase of milk supplemented with high levels of KH_2PO_4. It is also possible that non-CCP phosphate salt micro-crystals formed in the DSP cheese during ripening that may influence interactions in the system; however, this was not investigated.

4.3.5. Effects of EDTA addition

At day 1 of ripening, the EDTA cheese had a significantly lower % INSOL Ca level ($P < 0.05$) compared to the control (Figure 4.1). By 4 weeks of ripening, the control and EDTA cheeses had statistically similar levels of % INSOL Ca ($P > 0.05$). Many studies have used EDTA to reduce the CCP content of milk (e.g., Udabage et al., 2001; Choi et al., 2007, 2008). This reduction in CCP is followed by dissociation of casein molecules from micelles. As EDTA can reduce both Ca^{2+} activity and CCP in casein solutions (Udabage et al., 2000, 2001) it is likely that the EDTA chelated Ca directly from CCP in the cheese causing the decrease in % INSOL Ca observed during early ripening (Figure 4.1). EDTA is a very strong Ca-chelating agent and may also displace Ca^{2+} from phosphate and citrate complexes. It is worth noting that, unlike inorganic phosphate and citrate, EDTA is not a natural constituent of milk. Ca-phosphate and Ca-citrate salt complexes exist in a dynamic equilibrium in milk and cheese, allowing exchange of Ca^{2+} between the aqueous and casein-bound phase. When EDTA binds Ca^{2+}, it may prevent Ca^{2+} from interacting with other components of the casein/mineral system in the cheese.

As addition of phosphate, citrate or EDTA to milk is known to decrease Ca^{2+} activity (Udabage et al., 2000, 2001), it is likely that the initial source of Ca that is sequestered by these anions is the free Ca^{2+} ions available in the aqueous phase.

Subsequent displacement of Ca^{2+} from soluble Ca-phosphate or Ca-citrate complexes, Ca caseinate and/or CCP may occur depending on the affinity of the particular Ca-binding agent for Ca^{2+}. Solubilization of CCP from the casein micelle is dependent on the degree of saturation of the Ca complexes formed in the aqueous phase of casein solutions (Holt, 1985). Ca-citrate complexes are much more soluble than Ca-phosphate complexes and this may help explain the decrease in % INSOL Ca caused by TSC and the increase caused by DSP addition. Based on this theory, poor solubility of the Ca-EDTA complex formed in the EDTA cheese during early ripening may explain why this cheese no longer had a lower % INSOL Ca than the control cheese by week 4, and suggests that Ca-EDTA salts may have precipitated in the aqueous phase of EDTA cheese.

4.3.6. Schreiber melting test

The meltability of the cheeses derived from Schreiber melting tests is presented in Figure 4.2. The meltability of all cheeses increased during ripening. During the first 4 weeks of ripening there were no consistent differences in meltability between the cheeses which infers that the decreased % INSOL Ca level in the TSC and EDTA cheeses (Figure 4.1) did not increase meltability early in ripening. Cheeses with lower insoluble Ca are known to have increased melt (Choi et al., 2008), but this effect was not observed during early ripening. After 10 weeks of ripening the TSC, DSP and EDTA cheeses had significantly higher meltability ($P < 0.05$) than the control cheese. From the results shown in Figure 4.2 it is probable that a critical level of proteolysis must occur before melting differences between cheeses become apparent. Proteolysis leads to increased hydration of casein molecules in the *para*-casein matrix and increased protein hydration promotes repulsion between casein

molecules leading to increased melting. For good meltability, a strong interaction between protein and moisture in the cheese structure is required (Joshi et al., 2003). It is thought that cheese melts upon heating due to electrostatic repulsion becoming the dominant interaction between casein molecules (Lucey et al., 2003).

Mizuno and Lucey (2005b) observed an increase in meltability of non-fat *pasta filata* cheeses salted with a TSC/NaCl mixture. Lower % INSOL Ca levels during early ripening of the TSC and EDTA cheeses may have caused higher hydration of the casein molecules in the *para*-casein matrix. Increased hydration of caseins due to reduction in CCP can increase their susceptibility to proteolysis (Fox, 1970), thus critical casein fibers in casein strands of the TSC and EDTA cheeses may have been degraded more than in the denser control cheese matrix without any differences in overall net proteolysis level. Increased hydration of protein molecules increases intermolecular repulsion between them (Bryant and McClements, 1998) and these interactions would have complemented the electrostatic repulsion at the high temperature of analysis during the Schreiber test.

In the case of the DSP cheese, the % INSOL Ca content was initially higher than the control but became similar to the control by late ripening. The increase in melting may arise due to the high level of negatively charged phosphate ions that solubilized from CCP during ripening causing a reduction in the concentration of soluble ionic Ca^{2+} and individual Ca^{2+} counter-ions loosely bound to various side chain functional groups of casein molecules (Ca caseinate). This may have increased electrostatic repulsion between caseins and enhanced meltability.

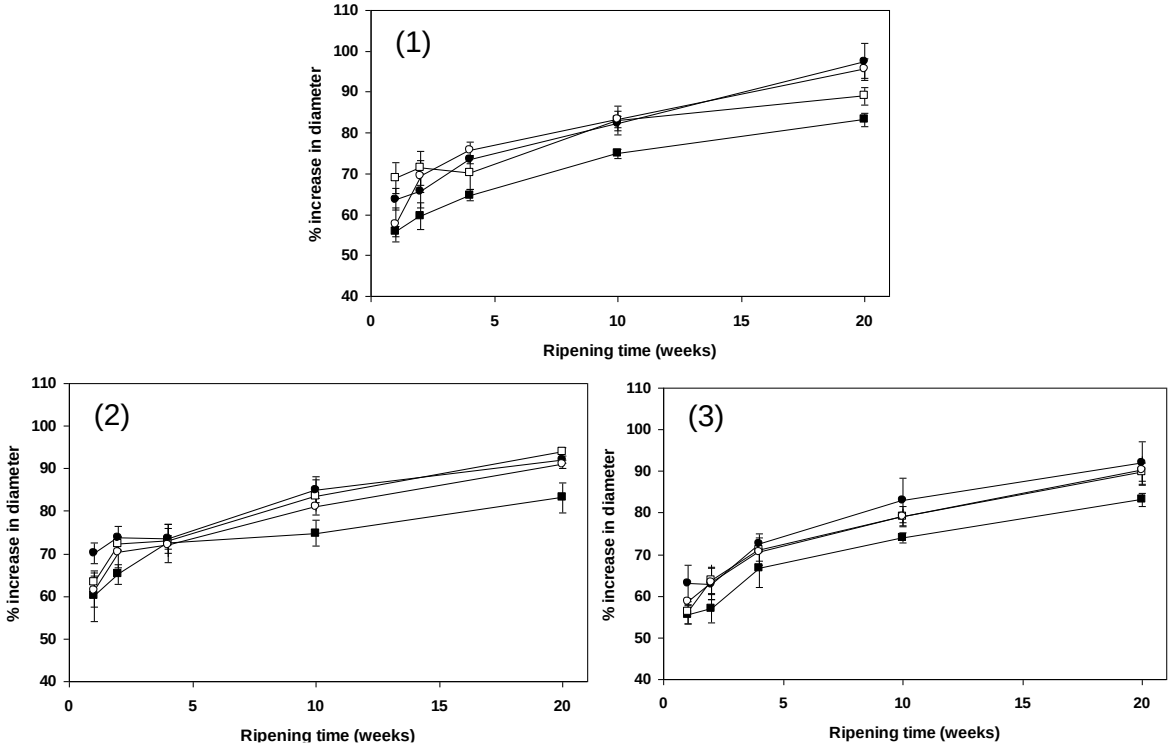


Figure 4.2. Percentage increase in cheese diameter from Schreiber melting test for the control cheese (■), cheese supplemented at salting with 0.24% w/w trisodium citrate dehydrate (TSC) (□), cheese supplemented at salting with 0.145% w/w disodium hydrogen phosphate dehydrate (DSP) (●) and cheese supplemented at salting with 0.2 % disodium EDTA dihydrate (EDTA) (○) during ripening in cheesemaking trials 1-3. Error bars indicate ± 1 standard deviation.

4.3.7. Dynamic small amplitude oscillatory rheometry (DSAOR)

There was a considerable increase in maximum loss tangent (LT_{max}) in all cheeses during the first 4 weeks of ripening (Figure 4.3) in agreement with Lucey et al. (2005). The TSC cheese had a significantly lower LT_{max} than the control cheese after 4 weeks of ripening. The TSC cheese had the lowest LT_{max} value of all the cheeses after 10 weeks of ripening. Similarly, Brickley et al. (2009) observed a lower maximum phase angle in cheeses with identical TSC/NaCl salting treatment used in the present study, especially after the first 28 days of ripening. In contrast, Choi et al. (2008) reported that cheeses with identical composition and pH but lower CCP had increased LT values and decreased G′ values at 70 °C compared to a control cheese

by day 1 of ripening. Mizuno and Lucey (2005b) reported that the ability of TSC to increase LT_{max} in non-fat *pasta filata* cheese was concentration dependent, as only high addition levels (5%) significantly increased LT_{max} to values higher than that of the control. The DSP and EDTA cheeses also had lower LT_{max} values than the control cheese at a number of ripening times. At 20 weeks of ripening, the DSP had a higher LT_{max} value than the control cheese in trials 1 and 2, and similar ($P > 0.05$) to the control cheese in trial 3.

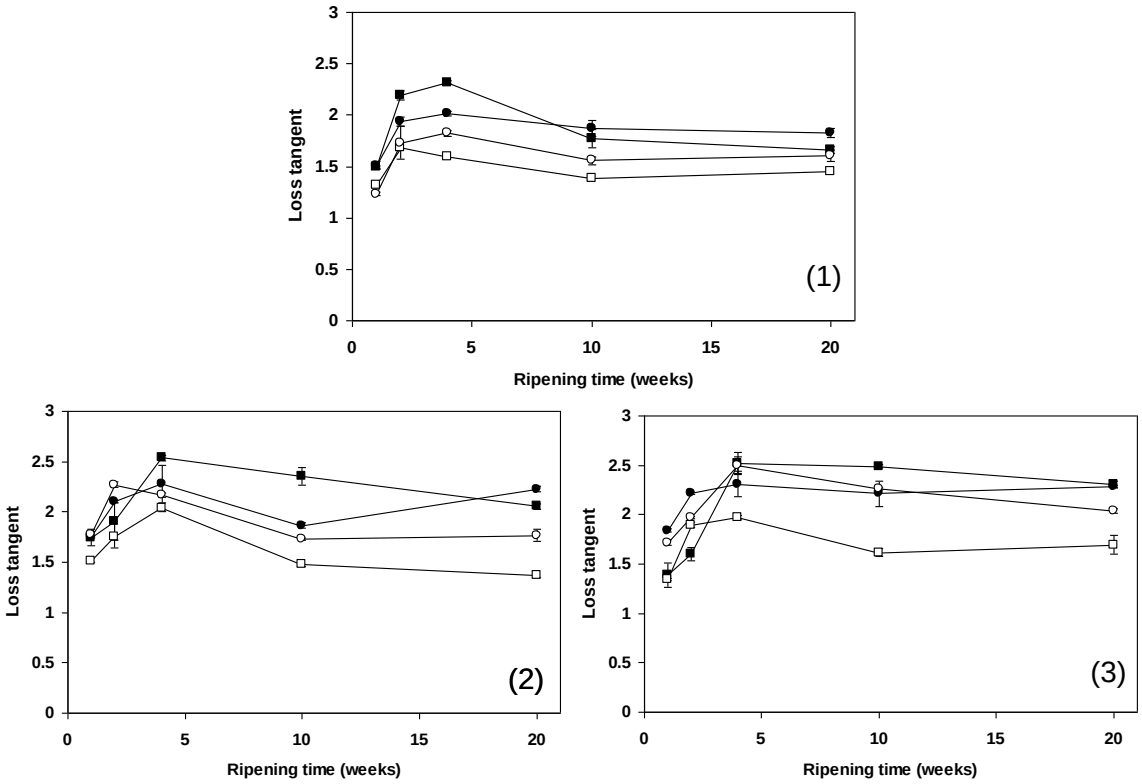


Figure 4.3. Maximum loss tangent (LT_{max}) values of control cheese (■), cheese supplemented at salting with 0.24% w/w trisodium citrate dehydrate (TSC) (□), cheese supplemented at salting with 0.145% w/w disodium hydrogen phosphate dehydrate (DSP) (●) and cheese supplemented at salting with 0.2 % disodium EDTA dihydrate (EDTA) (○) during ripening in cheesemaking trials 1-3. Error bars indicate ± 1 standard deviation.

As LT_{max} is used as an index of meltability, it is clear that the Schreiber melting test results (Figure 4.2) do not correlate well with LT_{max} values in this study. It is known that the type of heating system, the rate of heating and sample geometry greatly influence the results of melting analyses (Lucey et al., 2003). Lack of correlation

between melting tests has also been previously reported by Park et al. (1984). Kuo et al. (2001) observed a decrease in Cheddar cheese meltability the longer the cheese was held at 60 °C before being allowed to flow and this effect was more pronounced after 6 weeks of ripening. The authors attributed this decrease in meltability to an increased level of hydrophobic attractions between caseins during holding time at 60 °C and a subsequent increase in aggregation of casein molecules in the melted cheese. The slow increase in temperature through the region where hydrophobic attractions increase in strength and number and reach a maximum (~60-70 °C) may have altered the microstructure in a different way during the DSAOR analysis compared to the Schreiber melting test before electrostatic repulsion forces became dominant (>70 °C).

The storage modulus values at 80 °C (G'_{80}) of the cheeses during ripening are displayed in Figure 4.4. The G'_{80} values of all cheeses decreased rapidly during the first 4 weeks of ripening and generally remained unchanged after 10 weeks of ripening. The G'_{80} of the TSC cheese was significantly higher ($P < 0.05$) than other cheeses after 10 weeks of ripening, indicating that the TSC cheese had the most elastic character of all the cheeses at the highest temperature of analysis during DSAOR. The increased elasticity of the TSC cheese may be due to interactions between Ca-citrate complexes and casein molecules or perhaps heat-induced precipitation of Ca-phosphate at this temperature (Udayarajan et al., 2005). O'Mahony et al. (2006) suggested that a higher soluble Ca level in cheese may have a higher propensity to form heat-induced CCP. The higher level of soluble Ca in the TSC cheese may have caused this phenomenon. Froehlich-Wyder et al. (2009) found that Raclette cheese with a large reduction in both total and insoluble Ca due to citric

acid addition to wash water had much higher G′ values at elevated temperatures and much lower LT_{max} values compared to the control. The authors attributed these results to increased hydration and viscosity of the *para*-casein matrix in these cheeses.

The temperature at which the loss tangent equals 1 ($T_{LT=1}$) decreased during ripening in all cheeses (Figure 4.5). The point where LT = 1 is known as the crossover modulus, where the cheese is equally liquid and solid and the temperature at this point can be used as an index of cheese melting temperature (Gunasekaran and Ak, 2003). Less thermal energy is required to induce melt in aged cheese due to CCP solubilization and proteolysis (Lucey et al., 2005). A marked decrease in melting temperature occurs during the first 4 weeks of ripening in all cheeses; however, no consistent differences between cheeses were observed during this time across all three trials. After 10 weeks of ripening, the TSC cheese had a significantly higher $T_{LT=1}$ than the control cheese. The $T_{LT=1}$ at week 1 of ripening is between 64-68 °C for all cheeses, whereas the melting point after 10 weeks of ripening is between 54-57 °C for all cheeses. This temperature range is below the range where hydrophobic interactions exhibit maximum strength (60-70 °C) (Bryant and McClements, 1998). It is possible that differences between the cheeses for all parameters of DSAOR may be attributed to interactions dependent on the melting temperature. Microcrystals of the added salts along with crystals of Ca complexes of the anionic component of these Ca-binding salts may have also influenced casein interactions.

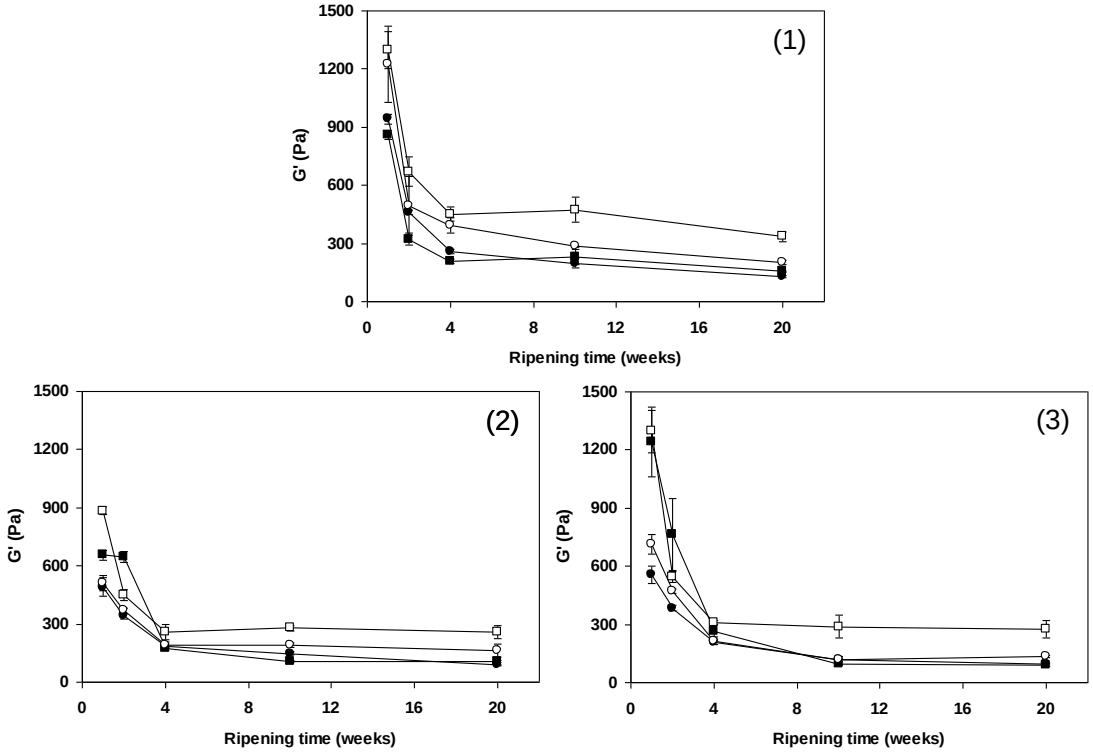


Figure 4.4. Storage modulus (G′) at 80 °C of control cheese (■), cheese supplemented at salting with 0.24% w/w trisodium citrate dehydrate (TSC) (□), cheese supplemented at salting with 0.145% w/w disodium hydrogen phosphate dehydrate (DSP) (●) and cheese supplemented at salting with 0.2 % disodium EDTA dihydrate (EDTA) (○) during ripening in cheesemaking trials 1-3. Error bars indicate ± 1 standard deviation.

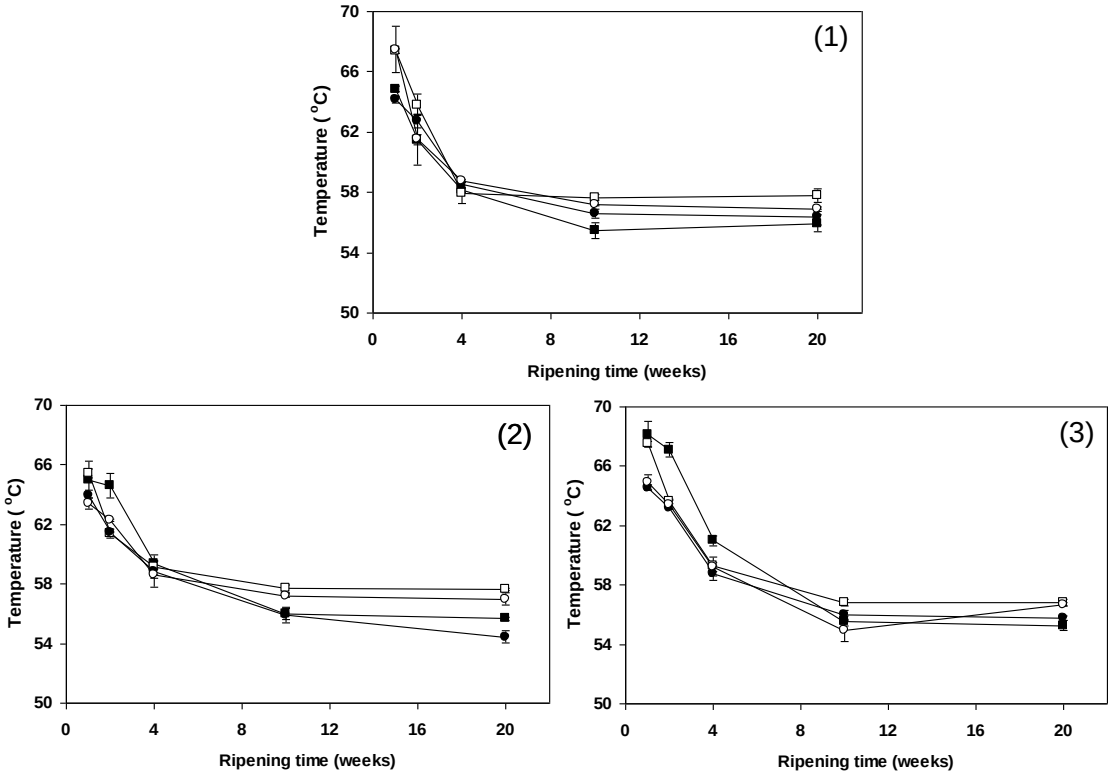


Figure 4.5. Temperature at loss tangent equals 1 ($T_{LT=1}$) of control cheese (■), cheese supplemented at salting with 0.24% w/w trisodium citrate dehydrate (TSC) (□), cheese supplemented at salting with 0.145% w/w disodium hydrogen phosphate dehydrate (DSP) (●) and cheese supplemented at salting with 0.2 % disodium EDTA dihydrate (EDTA) (○) during ripening in cheesemaking trials 1-3. Error bars indicate ± 1 standard deviation.

4.3.8. Texture profile analysis hardness

Hardness values of all cheeses decreased with ripening time (Table 4.2). Partial solubilzation of CCP occurs during the first month of ripening of Cheddar cheese (Hassan et al., 2004; Lucey et al., 2005) which is thought to be responsible for the initial softening of Cheddar cheese during early ripening (O'Mahony et al., 2005). Changes in the hardness of cheese beyond the first month of ripening are likely a consequence of proteolysis of casein molecules (Lucey et al., 2003), giving cheese a much 'shorter' texture due to breakdown of primary structural elements of the *para*-casein matrix. It can be seen from Table 4.2 that the TSC, DSP and EDTA cheeses had significantly lower hardness values ($P < 0.05$) than the control cheese at a number of ripening times. The EDTA cheese had significantly lower hardness compared to the control cheese in all trials up to 10 weeks of ripening. Both the TSC and DSP cheeses had lower hardness values ($P < 0.05$) than the control cheese at 10 weeks of ripening across all trials. Employing a TSC/NaCl salting treatment identical to that used in the present study, Brickley et al. (2009) observed lower hardness values in cheese compared with the control Cheddar cheese throughout ripening from 7 to 202 days. Mizuno and Lucey (2005b) reported a decrease in hardness in non-fat *pasta filata* cheeses dry salted with a combination of TSC/NaCl. These authors attributed the increased softness of these cheeses to sequestration of Ca from CCP crosslinks by the added TSC. Decreased hardness at various ripening times in the TSC and EDTA cheeses are likely due to decreased % INSOL Ca especially during early ripening (Figure 4.1).

Table 4.2. Changes in hardness values (g) as determined by texture profile analysis during ripening of control Cheddar cheese and experimental cheeses supplemented with Ca-binding salts (mean ± standard deviation)

		Treatment[1]			
Trial	Ripening time (weeks)	Control	TSC	DSP	EDTA
1	1	15358 ± 180^{aA}	15715 ± 608^{aA}	16294 ± 987^{aA}	12475 ± 887^{bA}
	4	14144 ± 426^{aB}	11373 ± 654^{bB}	11521 ± 685^{bB}	10386 ± 836^{bB}
	10	11856 ± 122^{aC}	10120 ± 737^{bC}	10044 ± 383^{bC}	10933 ± 517^{bB}
	20	9902 ± 354^{aD}	9587 ± 326^{aC}	9825 ± 95^{aC}	10077 ± 308^{aB}
2	1	17056 ± 939^{aA}	16235 ± 469^{abA}	15259 ± 813^{bcA}	14887 ± 816^{cA}
	4	14941 ± 783^{aB}	12348 ± 468^{cB}	11726 ± 724^{cB}	13542 ± 387^{bB}
	10	12140 ± 188^{aC}	10951 ± 312^{bC}	10136 ± 595^{cC}	10454 ± 379^{bcC}
	20	10895 ± 515^{aD}	9582 ± 301^{bD}	9835 ± 671^{bC}	10385 ± 464^{abC}
3	1	23446 ± 876^{aA}	19495 ± 906^{bA}	17943 ± 878^{cA}	14750 ± 583^{dA}
	4	15759 ± 496^{aB}	14916 ± 886^{abB}	14884 ± 624^{abB}	14182 ± 406^{bA}
	10	14258 ± 657^{aC}	12117 ± 425^{bC}	12003 ± 963^{bC}	11799 ± 744^{bB}
	20	13674 ± 648^{aC}	11520 ± 629^{bC}	11120 ± 569^{bC}	11411 ± 219^{bB}

^{a,b,c,d}Means within a row without a common superscript letter are significantly different ($P < 0.05$).

^{A,B,C,D}Means within a column within a trial without a common superscript letter are significantly different ($P < 0.05$).

[1]TSC = cheese supplemented at salting with 0.24% w/w trisodium citrate dihydrate; DSP = cheese supplemented at salting with 0.145% w/w disodium hydrogen phosphate dihydrate; EDTA = cheese supplemented at salting with 0.2% w/w disodium EDTA dihydrate.

4.4. Conclusion

The Ca-binding salts had a major influence on Ca equilibrium during early ripening of Cheddar cheese. Reduction of % INSOL Ca in the TSC and EDTA cheeses appeared to be a consequence of the Ca-binding abilities of TSC and EDTA, respectively, and the solubility of their Ca complexes. The increase in % INSOL Ca in the DSP cheese was most likely a consequence of precipitation of Ca-phosphate complexes from an aqueous phase already saturated with these salts. The increased meltability after 10 weeks of ripening and decreased hardness at a number of ripening times in the experimental cheeses compared to the control suggest that they may be suitable for use as functional ingredients in foods. The thermorheological properties of the experimental cheeses determined from DSAOR did not correlate well with the Schreiber melting test probably due to differences in heating rate. Although an interesting tool to predict mechanical properties of heated cheese, DSAOR should be used with caution for this purpose as functional melting tests where cheese is subjected to conditions analogous to oven cooking are much more accurate when determining true melt properties. Partial substitution of NaCl with these Ca-binding agents may have potential in the cheese industry as a method of producing cheeses tailored for use as functional ingredients in food products.

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Chapter 5

Effects of iron, copper and zinc chlorides on the mineral equilibria, rheological and microbiological properties of Cheddar-style cheese

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Abstract

Cheddar cheese was supplemented with chloride salts of Fe^{3+}, Fe^{2+}, Cu^{2+} and Zn^{2+} in order to investigate their effect on the physicochemical properties of cheese. Experimental cheeses were salted with a combination of NaCl and either $FeCl_3$ (FE3), $FeCl_2$ (FE2), $CuCl_2$ (CU2) or $ZnCl_2$ (ZN2). Cheeses were ripened at 8 °C for 20 weeks. The % insoluble (INSOL) Ca, Fe, Cu and Zn of cheeses was determined using the cheese juice method. Meltability of cheeses was determined using the covered Schreiber melting test. Small deformation rheological properties of cheeses were determined by dynamic small amplitude oscillatory rheology. Cheese hardness was determined using texture profile analysis. Starter and non-starter bacterial populations were monitored during ripening. Redox potential (E_h) of the cheeses was measured at week 20 of ripening. Supplementation of cheeses with the metal cation salts did not cause any consistent differences in gross composition and levels of proteolysis. A decrease in pH was observed in the FE3 and ZN2 cheeses during ripening. A slight, but significant, increase in % INSOL Ca was observed in the supplemented cheeses ($P < 0.05$). A high proportion of the added metal cations were in the insoluble phase of cheese. There was a higher % INSOL Fe in the FE3 cheese compared to the FE2 cheese ($P < 0.05$). The ZN2 cheese had a higher storage modulus at 80 °C (G'_{80}) and lower maximum loss tangent (LT_{max}) and Schreiber meltability than the control and the other supplemented cheeses during ripening. Supplementation of cheese with the metal cations had no effect on rheological properties $\leq$ 25 °C. The CU2 cheese had abnormally low starter and non-starter LAB counts during ripening. The CU2 cheese had a highly positive E_h after 20 weeks of ripening, which is abnormal for Cheddar cheese. There were no correlations found between redox and both % INSOL metal and rheological properties. The type of interaction between metal cations and caseins may have a major influence on rheological properties of cheese at elevated temperatures.

5.1. Introduction

The mineral composition of cheese has a major influence on its rheological and functional properties (Lucey and Fox, 1993; Lucey et al., 2003). Cheese variety and manufacturing steps (i.e., acid development, pH at drainage, curd washing) are known to influence greatly the mineral composition of cheese (Lucey and Fox, 1993; Lee et al., 2005, 2010). In recent decades, studies on the relationship between the mineral composition and the rheological, textural and functional properties of cheese have primarily focused on calcium equilibrium, which is related to both the calcium and phosphate levels in cheese (Lee et al., 2005; O'Mahony et al., 2005, 2006; Choi et al., 2008). The insoluble calcium phosphate associated with casein micelles is commonly referred to as colloidal calcium phosphate and has a major influence on the density and rigidity of the *para*-casein matrix in cheese (Lucey et al., 2003). It has been shown that the level of casein-bound calcium phosphate (CCP) decreases during the first few weeks of cheese ripening (Hassan et al., 2004; Lucey et al., 2005) and this is thought to be responsible for the decrease in elastic character of cheese during early ripening (Lucey et al., 2005; O'Mahony et al., 2005).

A number of studies have also attempted to alter mineral equilibria and physicochemical properties of cheese by addition of various salts containing ions intrinsic to milk either during or post-manufacture such as calcium chloride, magnesium chloride and trisodium citrate (Pastorino et al., 2003; Mizuno and Lucey, 2005; O'Mahony et al., 2006; Brickley et al., 2009; Cooke and McSweeney, 2013) and also salts containing ions not normally present in milk or cheese above trace levels, i.e., strontium chloride and sodium thiocyanate and tetrasodium pyrophosphate (Mizuno and Lucey, 2005; Stankey et al., 2011; Cooke and

McSweeney, 2013). Altering the mineral equilibria of cheese by such means can produce invaluable information regarding mechanisms of casein interactions involved in physicochemical properties of cheese that cannot be studied by merely altering pH or temperature during manufacture.

Iron, copper and zinc are present in milk at trace levels. Typical concentration ranges of Fe, Cu and Zn in bovine milk are 0.40-0.59 μg/ml, 0.06-0.09 μg/ml and 3.23-5.15 μg/ml, respectively (Fransson and Lonnerdal, 1983). Iron cations exist in two possible oxidation states namely ferrous (Fe^{2+}) or ferric (Fe^{3+}) cations, both of which may have different mechanisms of interaction with casein molecules (Raouche et al., 2009a). The majority of Fe added to whole or skim milk binds to casein micelles (Hegenauer et al., 1979). Demott and Dincer (1976) reported that of the ~85% of added Fe^{3+} that binds to caseins in skim milk, approximately 72, 21 and 4% of this Fe^{3+} was bound to α_S-, β- and κ-casein, respectively. Zinc cations exist as Zn^{2+} in milk. Singh et al. (1989b) reported that ~32% of the total Zn in skim milk is directly bound to caseins and ~63% is associated with CCP. Addition of zinc salts to casein solutions results in a high association of Zn with casein micelles (Philippe et al., 2005). Copper cations may also exist in two different oxidation states (i.e., cuprous, Cu^{+} or cupric, Cu^{2+}, cations). Addition of Cu salts to casein solutions leads to association of a proportion of Cu with casein micelles (Philippe et al., 2005). The phosphoseryl clusters on α_S- and β-caseins are thought to be the primary binding sites for Fe, Cu and Zn ions (Manson and Cannon, 1978; Hegenauer et al., 1979), but these cations may also bind at carboxyl, phenolic, sulfhydryl and imidazole groups (Gaucheron et al., 1997a). It is thought that Fe and Cu form coordinate bonds with

casein molecules, whereas, Zn associates with casein molecules via electrostatic binding and possibly with CCP (Gaucheron et al., 1997b; Silva et al., 2001).

Previous studies on the supplementation of cheese with Fe, Cu or Zn have been undertaken for the purpose of nutritional fortification, sensory or microbiological evaluation (Zhang and Mahoney, 1989a,b, 1990; Mato-Rodriguez et al., 2011; Kahraman and Ustunol, 2012). In these studies, supplementation without substantial alteration of physicochemical properties of cheese was desirable. In the present study, Fe, Cu and Zn were added to cheese at levels far in excess of those found naturally in milk/cheese in order to investigate their effects on the physicochemical properties, growth of lactic acid bacteria and oxidation-reduction (redox) potential of cheese. The influence of redox on mineral equilibria, rheological properties and LAB was also evaluated.

5.2. Materials and methods

5.2.1. Cheese manufacture

Raw bovine milk was standardized to 3.5% fat and pasteurised (72 °C x 15 s). Three Cheddar-style cheeses were manufactured according to standard protocol on a 50 kg scale in the food processing facilities at University College, Cork. R-604Y (Chr. Hansen Ltd., Little Island, Co. Cork, Ireland) was used as the starter culture at a level of 0.02% (w/v). Chymosin (CHY-MAX^{TM} Plus, Chr. Hansen A/S, Horsholm, Denmark), at a strength of 200 IMCU·ml^{-1}, was added to the cheesemilk at a level of 0.3 mL·L^{-1}. Coagulum was cut at equal firmness (measured subjectively). Curd was cooked from 31 to 39 °C over 30 min. Whey was drained at pH 6.2. The curd was cheddared until pH 5.4 was reached and was then milled and dry salted. The control curd was salted with NaCl at a level of 25 g/kg curd. The cheese supplemented with ferric iron (FE3 cheese) was salted with 2.704 g $FeCl_3.6H_2O$ + 21.49 g NaCl/kg curd. The cheese supplemented with ferrous iron (FE2 cheese) was salted with 1.988 g $FeCl_2.4H_2O$ + 23.25 g NaCl/kg curd. The cheese supplemented with cupric copper (CU2 cheese) was salted with 1.704 g $CuCl_2.2H_2O$ + 23.25g NaCl/kg curd. The cheese supplemented with zinc (ZN2 cheese) was salted with 1.362 g $ZnCl_2$ + 23.25 g NaCl/kg curd. These salt treatments were calculated to ensure the ionic strengths of the supplemented cheeses were equal to that of the control and that 10 mmol of each supplementation salt was added per kg of curd in the experimental cheeses. The salted curd was transferred to rectangular moulds 25.4 cm x 20.3 cm and pressed overnight at 490 kPa. The cheeses were vacuum packaged and ripened at 8 °C for a period of 20 weeks. Three independent cheesemaking trials were performed.

5.2.2. Compositional analysis

Compositional analysis was performed on the cheeses at week 5 of ripening. The moisture contents of the cheeses were determined by an oven drying method (IDF, 1982), protein by the macro-Kjeldahl procedure (IDF, 1986), fat by the Gerber method (IIRS, 1955), salt by a titrimetric method using potentiometric end-point determination (Fox, 1963). Cheese pH was determined by measuring the pH of homogenized cheese slurry made from 10 g cheese and 10 g water at room temperature. Proteolysis was assessed by determining the levels of pH 4.6-soluble nitrogen as % of total nitrogen (pH4.6SN%TN) according to O'Mahony et al. (2005) at 20 weeks of ripening. All above mentioned analyses were carried out in triplicate. Urea-polyacrylamide gel electrophoresis (PAGE) was carried out directly on the cheeses using the procedure described by O'Mahony et al. (2005).

5.2.3. Determination of insoluble minerals in cheese

Cheese juice was extracted from cheeses in triplicate as described by Cooke and McSweeney (2013). The Ca content of the cheeses and their juices were determined by atomic absorption spectroscopy (Varian SpectrAA-100, Varian Australia Pty Ltd, Mulgrave, Victoria, Australia) according to IDF (2007) and their Fe, Cu and Zn contents were analysed based on the same method for Ca determination according to IDF (2007) except that a wavelength and lamp current of 248.3 nm and 5 mA, 324.8 nm and 4 mA, and 213.9 nm and 5 mA were used for Fe, Cu and Zn, respectively. The concentration of each cation in the cheese juice was taken as the percentage of soluble cation in the cheese. Estimation of percentage INSOL Ca, Fe, Cu and Zn of cheese was calculated based on the work of Morris et al. (1988) and Hassan et al. (2004). For the present study, the INSOL percentage took into account all possible

forms of the minerals that are not in the aqueous phase, i.e., CCP nanoclusters, ions directly bound to casein molecules and/or associated with the fat phase.

5.2.4. Texture profile analysis

Texture profile analysis (TPA) was performed using a Texture Analyser TA-XT2i (Stable Micro Systems, Godalming, Surrey, UK) according to the method of O'Mahony et al. (2005), except cylindrical samples of dimensions: height 20 mm, diameter 20 mm were used. Hardness was defined according to Bourne (1978). Five replicate samples from each cheese were compressed at each ripening time point.

5.2.5. Dynamic small amplitude oscillatory rheology (DSAOR)

Rheological properties of the cheese samples during a temperature sweep were measured using a controlled shear stress rheometer (model AR-G2; TA instruments, Waters LLC, Leatherhead, Surrey, UK). Measuring geometry consisted of a 40 mm serrated stainless steel parallel plate above and a serrated base plate below. Cheese discs were made with the dimensions: diameter 40 mm and height ~2 mm. The exposed edges of the cheese disc were coated with liquid paraffin to prevent evaporation in the cheese sample during analysis. The top plate was lowered and an axial force of 0.7 ± 0.1 N was applied to the sample and maintained throughout the procedure. Prior to oscillation, the sample was tempered at 20 °C for 10 min to allow temperature equilibration. The temperature was raised from 20 to 80 °C at a rate of 2 °C/min during which time the storage modulus (G′), loss modulus (G″) and loss tangent (LT) were recorded continuously at a shear strain of 0.5 and a frequency of 1 Hz. Shear strain and stress values chosen were found to be within the linear viscoelastic range for these cheeses. Each sample was analysed in triplicate.

5.2.6. Melt analysis

Melt analysis was carried out using a covered Schreiber test (Altan et al., 2005). Each cheese cylinder (5 mm height, 35 mm diameter) was placed in a covered glass petri dish and then placed in an oven at 232 °C for 5 min. These were then removed and cooled for 30 min at room temperature. Measurements of the melt distance were made using electronic calipers. The diameter of the melted sample was measured at 5 different points and an average diameter was determined. Results were expressed as percentage increase in cheese diameter. Analysis on each cheese sample was performed in triplicate.

5.2.7. Microbiological analysis

Aseptic samples (~10 g) were taken from cheeses using a sterile cheese trier and placed into a stomacher bag. These samples were diluted 1:10 with sterile trisodium citrate (2% w/v) followed by homogenization in a stomacher (Seward Stomacher 400; Seward Ltd. London, UK) for 4 min. Further dilutions were prepared depending on the stage of ripening. Starter bacteria were enumerated on LM17 agar (Terzaghi and Sandine, 1975) incubated aerobically for 3 days at 30°C. Non-starter lactic acid bacteria (NSLAB) were enumerated on Rogosa agar (Rogosa et al., 1951) incubated anaerobically for 5 days at 30°C.

5.2.8. Measurements of oxidation-reduction potential

Redox potential was measured using a platinum working electrode (XM120) and a calomel reference electrode (REF 421, both from Radiometer Analytical, Villeurbanne Cedex, Lyon, France) connected to a pH meter (PHM210 Standard pH

Meter, Radiometer Copenhagen, Denmark). Before measurement, the reference electrode was cleaned according to the manufacturer's instructions and filled with saturated KCl solution (Sigma-Aldrich, St. Louis, Missouri, USA). The surface of the platinum working electrode was cleaned by polishing with fine aluminum oxide powder (Sigma-Aldrich) for 2 min; after polishing, the electrode surface was rinsed thoroughly with distilled water and allowed to dry in air. The accuracy of electrodes was checked against a 3 M KCl solution (Topcu et al., 2008) and against tap water (Abraham et al., 2007) at the beginning of the measurements and between samples. In general, redox potentials are referred to the hydrogen reference electrode and expressed as E_h at a defined pH and temperature. The redox potential readings (ORP) were converted to E_h according to Caldeo and McSweeney (2012) with temperature compensation using the following equation:

$$E_h = ORP + E_r \quad [1]$$

where E_r is the potential of the reference electrode versus the standard hydrogen electrode at a certain temperature. In the present study, for the saturated calomel electrode, the E_r value was +248 mV at 20°C (Skoog et al., 2004). To measure redox potential, a block (about 8 x 8 cm) of cheese was covered with plastic film to reduce surface dehydration and the electrodes were inserted into the cheese following the method of Topcu et al. (2008). The equilibrium ORP value after 3 days of measurement in each cheese was used for E_h calculation. For each cheese, the measurement of the equilibrium ORP value was taken at 20 weeks of ripening.

5.2.9. Statistical analysis

ANOVA was carried out using the PASW Statistics Version 18 program (IBM, Armonk, NY, USA). Differences between means were analyzed using Tukey's HSD *post hoc* test. The level of significance was determined at $P < 0.05$.

5.3. Results and discussion

5.3.1. Composition

The gross composition of the cheeses made with added metal chlorides is shown in Table 5.1. There were no significant differences ($P < 0.05$) between all cheeses for values of percentage protein, fat and ash. No consistent differences were observed for percentage moisture between cheeses in all trials. The pH values of the cheeses during ripening are displayed in Figure 5.1. Generally, the pH of all cheeses decreased during ripening to various extents up to week 10 of ripening and an increase in pH was observed by week 20. The pH values of the FE3 cheese was significantly lower ($P < 0.05$) than the control cheese throughout ripening; however, the FE2 cheese had similar pH values to the control cheese after 5 weeks of ripening. The ZN2 cheese also had a significantly lower pH than the control throughout ripening ($P < 0.05$). The pH values of 10 mM aqueous solutions of $FeCl_3$, $FeCl_2$, $CuCl_2$ and $ZnCl_2$ salts used were 2.12, 3.51, 4.40 and 6.50, respectively. The pH decreases observed in some of the supplemented cheeses may be attributed to the acidities of the salts in aqueous solution and also due to exchanges between added metal cations and micellar H^+ (Gaucheron et al., 1997a). As the pH of $ZnCl_2$ solution (6.5) was higher than the pH of cheese curd at salting (~5.4), the pH decrease in the ZN2 cheese is likely attributed to liberation of H^+ due to exchange with Zn^{2+} and possibly CCP formation. The pH of the CU2 cheese was $\geq$ control cheese during ripening after 5 weeks of ripening and was significantly higher than the control after 10 weeks of ripening ($P < 0.05$) in all trials. The pH of the CU2 cheese did not decrease significantly ($P > 0.05$) during the first 5 weeks of ripening.

Table 5.1. Chemical composition and levels of proteolysis in control cheese, cheese supplemented with 10 mmol $FeCl_3.6H_2O$/kg curd (FE3), cheese supplemented with 10 mmol $FeCl_2.4H_2O$/kg curd (FE2), cheese supplemented with 10 mmol $CuCl_2.2H_2O$/kg curd (CU2) and cheese supplemented with 10 mmol $ZnCl_2$/kg curd (ZN2) (mean ± standard deviation)

		Cheese treatment				
Trial	Parameter[1]	Control	FE3	FE2	CU2	ZN2
1	% Moisture	34.3 ± 0.5^{a}	34.6 ± 0.3^{a}	35.0 ± 0.2^{a}	34.2 ± 0.2^{a}	34.2 ± 0.4^{a}
	% Protein	25.4 ± 0.6^{a}	25.5 ± 0.8^{a}	25.5 ± 0.1^{a}	25.9 ± 0.3^{a}	25.8 ± 0.3^{a}
	% Fat	32.0 ± 0.9^{a}	31.9 ± 0.1^{a}	32.2 ± 0.8^{a}	32.2 ± 0.3^{a}	31.9 ± 0.4^{a}
	% Cl^-	1.5 ± 0.02^{a}	1.4 ± 0.01^{b}	1.4 ± 0.01^{bc}	1.3 ± 0.03^{d}	1.4 ± 0.05^{cd}
	% Ash	4.0 ± 0.10^{a}	3.8 ± 0.11^{a}	3.8 ± 0.10^{a}	3.7 ± 0.21^{a}	3.8 ± 0.14^{a}
	pH4.6SN%TN	18.7 ± 0.3^{ab}	18.9 ± 0.3^{a}	18.6 ± 0.1^{ab}	18.7 ± 0.3^{ab}	18.1 ± 0.4^{b}
	% moisture in cheese juice	81.3 ± 0.2^{c}	82.1 ± 0.1^{b}	81.5 ± 0.2^{c}	81.2 ± 0.2^{c}	82.7 ± 0.1^{a}
2	% Moisture	36.4 ± 0.2^{a}	35.8 ± 0.2^{a}	36.6 ± 0.3^{a}	35.8 ± 0.4^{a}	36.4 ± 0.3^{a}
	% Protein	24.9 ± 0.5^{a}	24.2 ± 0.4^{a}	24.4 ± 0.8^{a}	24.4 ± 0.5^{a}	23.9 ± 0.3^{a}
	% Fat	32.4 ± 0.5^{a}	32.3 ± 0.3^{a}	32.4 ± 0.5^{a}	32.2 ± 0.3^{a}	32.1 ± 0.4^{a}
	% Cl^-	1.6 ± 0.03^{a}	1.6 ± 0.04^{a}	1.5 ± 0.04^{a}	1.5 ± 0.07^{a}	1.3 ± 0.05^{b}
	% Ash	3.4 ± 0.06^{a}	3.4 ± 0.15^{a}	3.3 ± 0.20^{a}	3.3 ± 0.11^{a}	3.1 ± 0.17^{a}
	pH4.6SN%TN	22.7 ± 0.3^{a}	22.3 ± 0.1^{a}	22.4 ± 0.3^{a}	21.4 ± 0.2^{b}	22.2 ± 0.5^{a}
	% moisture in cheese juice	79.7 ± 0.4^{c}	81.0 ± 0.2^{b}	81.0 ± 0.1^{b}	80.2 ± 0.3^{c}	82.1 ± 0.2^{a}
3	% Moisture	37.2 ± 0.3^{a}	37.3 ± 0.3^{a}	36.6 ± 0.2^{b}	36.5 ± 0.1^{b}	36.3 ± 0.2^{b}
	% Protein	24.5 ± 0.4^{a}	24.5 ± 0.3^{a}	24.3 ± 0.2^{a}	24.3 ± 0.2^{a}	24.3 ± 0.3^{a}
	% Fat	31.5 ± 0.5^{a}	31.3 ± 0.6^{a}	31.7 ± 0.3^{a}	31.5 ± 0.5^{a}	32.0 ± 0.5^{a}
	% Cl^-	1.6 ± 0.09^{a}	1.6 ± 0.02^{a}	1.4 ± 0.01^{b}	1.3 ± 0.05^{b}	1.3 ± 0.09^{b}
	% Ash	3.5 ± 0.15^{a}	3.7 ± 0.42^{a}	3.4 ± 0.12^{a}	3.2 ± 0.07^{a}	3.4 ± 0.28^{a}
	pH4.6SN%TN	22.2 ± 0.4^{a}	21.2 ± 0.4^{ab}	21.6 ± 0.4^{ab}	21.0 ± 0.6^{ab}	20.6 ± 0.7^{b}
	% moisture in cheese juice	80.9 ± 0.1^{c}	82.1 ± 0.7^{b}	81.4 ± 0.3^{b}	81.0 ± 0.2^{c}	83.0 ± 0.0^{a}

[a,b,c,d]Different lower case superscript letters in the same row within a trial indicate that values are significantly different ($P < 0.05$)
[1]pH4.6SN%TN = pH 4.6 soluble nitrogen as a % of total nitrogen

There were no consistent differences in levels of pH4.6SN%TN between the four cheeses after 20 weeks of ripening (Table 5.1), and urea-PAGE electrophoretograms (not shown) displayed no consistent differences in proteolytic patterns between cheeses throughout ripening. As the cheeses were manufactured with all salting treatments calculated on the basis of equal ionic strength, it is to be expected that no great effect on proteolysis would be observed due to NaCl substitution (Brickley et

al., 2009). There was no significant difference in total Ca level between all cheeses within each trial ($P > 0.05$) (Table 5.2). Mean total Fe contents in the FE3 and FE2 cheeses ranged from ~36-39 mg/100 g cheese and there was no significant difference in total Fe content between these cheeses ($P > 0.05$) (Table 5.2). This indicates that the oxidation state of Fe did not affect Fe uptake by the curd during salting. In the study of Zhang and Mahoney (1989), Cheddar cheese fortified with $FeCl_3$ contained 9.1 mg Fe/100 g cheese. Mean total Cu content in the CU2 cheeses ranged from ~39-47 mg/100 g cheese (Table 5.2). In a study where Emmental cheese was supplemented with copper sulfate, Mato-Rodriguez et al. (2011) produced cheeses with ~1.5 mg Cu/100 g cheese. Mean total Zn content in the ZN2 cheese ranged from ~57-67 mg/100g cheese (Table 5.2). Fortifying Cheddar cheese with zinc sulphate, Kahraman and Ustunol (2012) produced cheeses containing ~22.8 mg Zn/100 g cheese. The Ca concentration in the juice of the control cheese ranged from ~743 to 819 mg/100 g juice at week 5 of ripening (Table 5.3). There was a significantly lower Ca concentration ($P < 0.05$) in the juices of the FE3, FE2, CU2 and ZN2 cheeses compared to the control.

Table 5.2. Total Ca, Fe, Cu and Zn (mg metal/100 g cheese) in control cheese, cheese supplemented with 10 mmol $FeCl_3.6H_2O$/kg curd (FE3), cheese supplemented with 10 mmol $FeCl_2.4H_2O$/kg curd (FE2), cheese supplemented with 10 mmol $CuCl_2.2H_2O$/kg curd (CU2) and cheese supplemented with 10 mmol $ZnCl_2$/kg curd (ZN2) (mean ± standard deviation)

		Cheese treatment				
Trial	Metal	Control	FE3	FE2	CU2	ZN2
1	Ca	819 ± 7[a]	771 ± 5[b]	769 ± 8[b]	751 ± 5[c]	779 ± 7[b]
	Fe	1.16 ± 0.07[c]	19.68 ± 1.35[b]	23.79 ± 0.90[a]	NA	NA
	Cu	2.62 ± 0.07[b]	NA	NA	31.70 ± 1.21[a]	NA
	Zn	4.22 ± 0.04[b]	NA	NA	NA	35.74 ± 0.92[a]
2	Ca	780 ± 3[a]	718 ± 6[b]	716 ± 5[b]	668 ± 2[c]	718 ± 2[b]
	Fe	1.25 ± 0.13[c]	20.51 ± 1.24[b]	24.06 ± 0.56[a]	NA	NA
	Cu	2.70 ± 0.17[b]	NA	NA	28.29 ± 1.75[a]	NA
	Zn	4.15 ± 0.16[b]	NA	NA	NA	35.22 ± 0.95[a]
3	Ca	743 ± 5[a]	706 ± 20[b]	704 ± 3[b]	604 ± 9[c]	713 ± 10[b]
	Fe	1.11 ± 0.12[c]	18.51 ± 0.43[b]	25.66 ± 0.67[a]	NA	NA
	Cu	2.37 ± 0.12[b]	NA	NA	NA	25.28 ± 1.19[a]
	Zn	4.43 ± 0.16[b]	NA	NA	NA	35.70 ± 2.39[a]

[a,b,c]Means within a row without a common superscript letter are significantly different ($P < 0.05$)
NA = cheese not analysed for particular metal

Table 5.3. Total Ca, Fe, Cu and Zn in cheese juices (mg metal/100 g juice) extracted at week 5 of ripening from control cheese, cheese supplemented with 10 mmol $FeCl_3.6H_2O$/kg curd (FE3), cheese supplemented with 10 mmol $FeCl_2.4H_2O$/kg curd (FE2), cheese supplemented with 10 mmol $CuCl_2.2H_2O$/kg curd (CU2) and cheese supplemented with 10 mmol $ZnCl_2$/kg curd (ZN2) (mean ± standard deviation)

		Treatment				
Trial	Metal species	Control	FE3	FE2	CU2	ZN2
1	Ca	841 ± 4[a]	844 ± 2[a]	838 ± 3[a]	840 ± 7[a]	848 ± 9[a]
	Fe	1.42 ± 0.20[b]	38.91 ± 0.73[a]	38.60 ± 3.11[a]	NA	NA
	Cu	1.81 ± 0.18[b]	NA	NA	47.39 ± 1.20[a]	NA
	Zn	4.89 ± 0.26[b]	NA	NA	NA	57.76 ± 7.47[a]
2	Ca	737 ± 12[a]	734 ± 2[a]	742 ± 7[a]	724 ± 5[a]	744 ± 9[a]
	Fe	1.53 ± 0.14[b]	39.53 ± 5.96[a]	39.97 ± 1.53[a]	NA	NA
	Cu	1.87 ± 0.29[b]	NA	NA	45.97 ± 2.43[a]	NA
	Zn	5.28 ± 0.18[b]	NA	NA	NA	64.29 ± 1.92[a]
3	Ca	753 ± 5[a]	760 ± 9[a]	761 ± 4[a]	760 ± 5[a]	761 ± 8[a]
	Fe	1.67 ± 0.12[b]	37.88 ± 4.51[a]	36.12 ± 1.80[a]	NA	NA
	Cu	1.80 ± 0.21[b]	NA	NA	39.06 ± 1.59[a]	NA
	Zn	4.94 ± 0.19[b]	NA	NA	NA	62.49 ± 1.46[a]

[a,b]Means within a row without a common superscript letter are significantly different ($P < 0.05$)
NA = cheese not analysed for particular metal

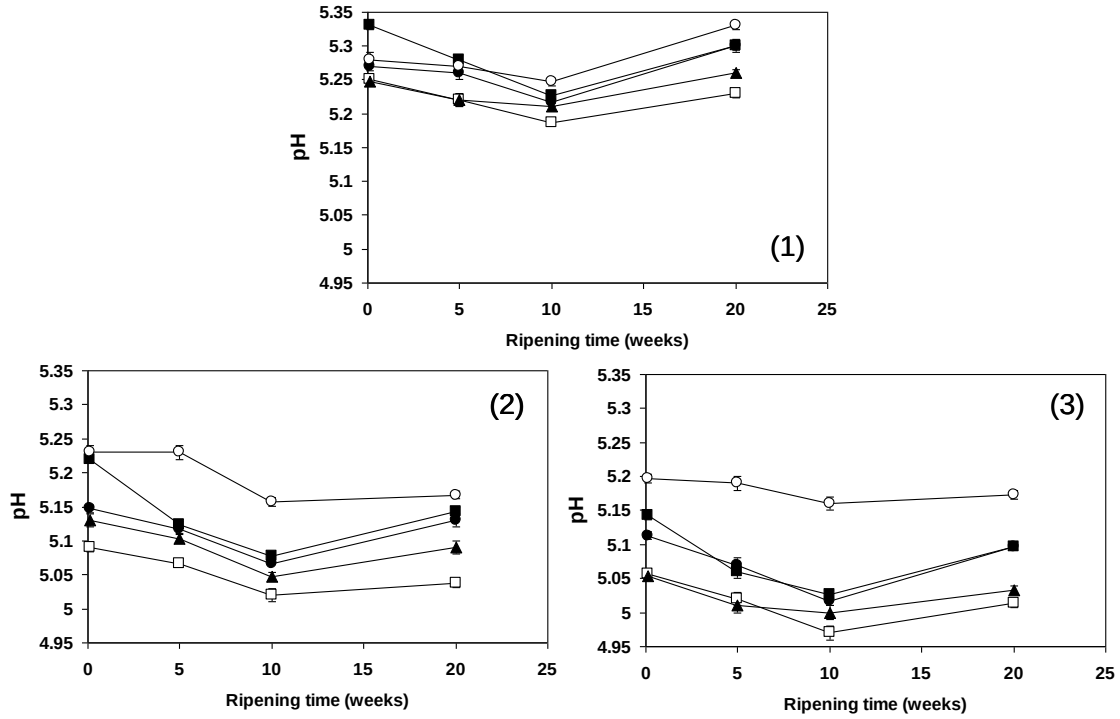


Figure 5.1. pH values of control cheese (■), cheese supplemented with 10 mmol $FeCl_3.6H_2O$/kg curd (FE3) (□), cheese supplemented with 10 mmol $FeCl_2.4H_2O$/kg curd (FE2) (●), cheese supplemented with 10 mmol $CuCl_2.2H_2O$/kg curd (CU2) (○) and cheese supplemented with 10 mmol $ZnCl_2$/kg curd (ZN2) (▲) during ripening in cheesemaking trials 1-3. Error bars indicate ± 1 standard deviation.

5.3.2. Mineral equilibria in cheeses

5.3.2.1. Insoluble calcium

The % INSOL Ca in the control cheese at 5 weeks of ripening ranged from 58-62% (Table 5.4). This value is in agreement with previous studies for this stage of ripening (Hassan et al., 2004; Lucey et al., 2005). The FE3, FE2, CU2 and ZN2 cheeses had significantly higher % INSOL Ca than the control at 5 weeks of ripening ($P < 0.05$). Gaucheron et al. (1997b) reported that $FeCl_2$ increased casein-bound Ca in skim milk whereas added $FeCl_3$ had little effect when added up to 1.5 mM. However, higher concentrations of $FeCl_3$ (8 mmol/kg) have been found to increase

casein-bound Ca in casein solutions (Philippe et al., 2005). Although addition of Fe caused a significant increase in % INSOL Ca compared to the control, the oxidation state of Fe had no influence. The thermodynamic stability constants (representing the affinity between anions and cations) for salts of Cit^{3-} and HPO_4^{2-} with Fe^{3+}, Zn^{2+} and Cu^{2+} are greater than those with Ca^{2+} (Philippe et al., 2005), which likely caused a displacement of Ca^{2+} from soluble citrate and phosphate complexes. Casein molecules also have a much stronger affinity for Zn^{2+}, Cu^{2+} and Fe^{3+} ions compared to Ca^{2+} (Gaucheron et al., 1997a). Hegenauer et al. (1979) proposed that Fe^{2+} may initially displace Ca^{2+} from casein molecules and then undergo *in situ* oxidation forming a Fe^{3+}-casein complex. Displacement of Ca^{2+} directly bound to casein would increase the Ca^{2+} concentration in the aqueous phase, possibly resulting in complexation of Ca^{2+} with soluble P_i and subsequent precipitation to form CCP (Gaucheron et al., 1997b). It is probable that Cu^{2+} and Zn^{2+} addition also induced displacement of Ca^{2+} to the CN-bound phase in this manner. Philippe et al. (2005) reported that increasing concentrations of $FeCl_3$, $CuCl_2$ and $ZnCl_2$ up to 8 mmol/kg slightly increased levels of casein-bound Ca. Fortification of milk with $FeCl_3$ has been found to make CCP more resistant to solubilization during acidification (Hekmat and McMahon, 1998), which may have retarded the partial solubilzation of CCP that occurs during early ripening of cheese.

Table 5.4. Percentage of insoluble (% INSOL) Ca, Fe, Cu and Zn in control cheese, cheese supplemented with 10 mmol $FeCl_3.6H_2O$/kg curd (FE3), cheese supplemented with 10 mmol $FeCl_2.4H_2O$/kg curd (FE2), cheese supplemented with 10 mmol $CuCl_2.2H_2O$/kg curd (CU2) and cheese supplemented with 10 mmol $ZnCl_2$/kg curd (ZN2) (mean ± standard deviation)

		Treatment				
Trial	Metal	Control	FE3	FE2	CU	ZN
1	Ca	62.65 ± 0.33^{d}	64.92 ± 0.22^{b}	64.15 ± 0.37^{c}	66.39 ± 0.25^{a}	65.47 ± 0.30^{b}
	Fe	68.64 ± 1.96^{c}	80.56 ± 1.34^{a}	75.93 ± 0.91^{b}	NA	NA
	Cu	44.46 ± 1.39^{b}	NA	NA	74.39 ± 0.98^{a}	NA
	Zn	66.93 ± 0.33^{b}	NA	NA	NA	76.72 ± 0.60^{a}
2	Ca	58.56 ± 0.14^{c}	60.38 ± 0.31^{b}	60.64 ± 0.26^{b}	62.25 ± 0.13^{a}	60.72 ± 0.13^{b}
	Fe	65.82 ± 3.65^{c}	78.96 ± 1.27^{a}	75.07 ± 0.58^{a}	NA	NA
	Cu	39.48 ± 3.83^{b}	NA	NA	74.82 ± 1.56^{a}	NA
	Zn	67.10 ± 1.31^{b}	NA	NA	NA	77.69 ± 0.60^{a}
3	Ca	58.24 ± 0.31^{c}	61.18 ± 1.10^{b}	61.89 ± 0.18^{b}	67.03 ± 0.59^{c}	62.32 ± 0.54^{b}
	Fe	71.68 ± 3.12^{b}	79.59 ± 0.47^{a}	70.67 ± 0.76^{b}	NA	NA
	Cu	41.99 ± 2.05^{b}	NA	NA	73.22 ± 1.26^{a}	NA
	Zn	62.05 ± 1.40^{b}	NA	NA	NA	77.07 ± 1.54^{a}

^{a,b,c}Different lower case superscript letters in the same row within a trial indicate that values are significantly different ($P < 0.05$)
NA = cheese not analysed for particular metal

5.3.2.2. Insoluble iron

The % INSOL Fe in cheeses after 5 weeks of ripening is shown in Table 5.4. The % INSOL Fe in the control cheese ranged from ~65-71%. The % INSOL Fe in the FE3 and FE2 cheeses ranged from ~78-80% and 70-75%, respectively. These values are lower than those previously reported for association of added Fe with casein in casein solutions (>85%) (Demott and Dincer, 1976; Gaucheron et al., 1996; Raouche

et al., 2009b). Philippe et al. (2005) reported >98% association of added Fe^{3+} with caseins in whey protein-free casein micelle suspensions with $FeCl_3$ concentrations of 2.5-8.0 mmol/kg. Lower % INSOL Fe in the FE3 and FE2 cheeses compared to values reported for milk may also be attributed to solubilzation of Fe-casein complexes due to proteolytic breakdown of α_{s1}- and β-casein during ripening, rendering soluble peptides with bound Fe. Cheese supplemented with Fe^{3+} had a significantly higher % INSOL Fe ($P < 0.05$) than that of the Fe^{2+} supplemented cheese. Raouche et al. (2009b) reported that less added Fe associated with casein in skim milk when added in the form of $FeCl_2$ compared to milk supplemented with $FeCl_3$. When Fe salts are added to milk, some of the added Fe may associate with the milk fat phase, especially when added as Fe^{2+} salts (Hegenauer et al., 1979). Fe cations that interact with the fat phase are thought to bind to the outer milk fat globule membrane (Fransson and Lonnerdal 1983). Phosphoseryl residues present in α_{s1}- and β-casein rapidly catalyse the oxidation of iron from the Fe^{2+} to Fe^{3+} in the presence of dissolved O_2, forming highly stable Fe-phosphoprotein complexes (Manson and Cannon, 1978; Emery, 1992), with the oxidation rate of the Fe^{2+} to Fe^{3+} being proportional to casein concentration (Emery, 1992). Binding of Fe^{2+} by phosphoserine clusters may also be envisaged as an antioxidant mechanism of the caseins (Kitts, 2005).

As binding of Fe to casein is pH independent in the pH range encountered during processing of dairy products (Gaucheron et al.,1996, 1997a), it is likely that Fe is not bound to caseins by ionic bonds but probably by covalent coordinate bonds via the oxygen of phosphoseryl residues (Hegenauer et al., 1979; Gaucheron et al., 1996), existing in a distorted octahedral coordination (Raouche et al., 2009a). Comparing

the effect of TCA precipitation and enzymatic hydrolysis of caseins on Fe partition in milk, Silva et al. (2001) also proposed that Fe is bound mainly to the peptide backbone of caseins and is not associated with CCP. Binding of Fe to groups other than phosphoseryls of caseins may also occur, i.e., to carboxyl, phenolic, sulfhydryl and imidazole groups (Gaucheron et al., 1997a). Addition of Fe causes displacement of inorganic phosphate from the soluble to the colloidal phase, indicating that CN-bound Fe may also be in the form of Fe-P_i complexes (Philippe et al., 2005; Raouche et al., 2009b). Addition of ferric chloride to casein micelle suspensions can also cause a large decrease in soluble citrate (Philippe et al., 2005). Therefore, Fe may form casein-bound P_i and citrate complexes but it is unlikely that Fe associates with the CCP nanoclusters.

5.3.2.3. Insoluble zinc

The % INSOL Zn in the control cheese ranged from ~62-67% (Table 5.4). The majority of innate Zn in milk is most likely associated with CCP (McGann et al., 1983; Singh et al., 1989b; Silva et al., 2001).Singh et al. (1989b) reported that ~32% of the total Zn in skim milk is directly bound to caseins and ~63% is associated with CCP. The % INSOL Zn in the ZN2 cheese was ~77% (Table 5.4). When $ZnCl_2$ is added to milk, large amounts of Zn^{2+} are thought to be incorporated into the CCP (Singh et al., 1989b). Philippe et al. (2005) reported an association of Zn with casein of $\geq$ 95% when $ZnCl_2$ was added to casein micelle suspensions in concentrations up to 8 mmol/kg. Zn^{2+} can displace Ca^{2+} from casein binding sites, indicating caseins have a greater affinity for Zn (Singh et al., 1989a). Along with association with CCP, Zn may also directly bind to casein via carboxyl, phenolic, sulfhydryl and imidazole groups (Gaucheron et al., 1997a). The Zn-binding capacities of individual caseins are

α_{s1}-> β- > κ-casein, and are in the same order as their phosphoserine contents (Singh et al., 1989a). Proteolytic breakdown of caseins during ripening, specifically α_{s1}- and β-casein may have liberated phosphopeptides associated with Zn and contributed to the lower % INSOL Zn in the ZN2 cheese compared to Zn associations with casein systems reported in literature. Unlike Fe, the association of Zn to caseins is pH sensitive (Singh et al., 1989b; Pabon and Lonnerdal, 2000); thus, the pH decrease in the ZN2 cheese during ripening caused by addition of $ZnCl_2$ may have decreased the association of Zn with casein during the first 5 weeks of ripening.

5.3.2.4. Insoluble copper

The % INSOL Cu in the control cheese ranged from ~39-44% (Table 5.4). Lonnerdal (1985) reported that 44% of innate Cu is bound to casein. This indicates that the majority of the innate Cu is in the soluble phase of cheese after 5 weeks of ripening. The % INSOL Cu in the CU2 cheese ranged from ~73-74%. Philippe et al. (2005) reported an association of Cu^{2+} with casein of 52% when 8 mmol/kg $CuCl_2$ was added to casein micelle suspensions. As copper can associate with milkfat via the milk fat globule membrane (Lonnerdal, 1985), it is possible that a proportion of the % INSOL Cu is associated with the fat phase of cheese as the calculation for % INSOL Cu is based on soluble and total Cu concentration in the cheese; so, the insoluble Cu in the cheese may be casein-bound or associated with the fat phase. Philippe et al. (2005) observed a much lower increase in CN-bound P_i when up to 8.0 mmol/kg $CuCl_2$ was added to casein solutions compared with similar addition of $FeCl_3$ or $ZnCl_2$. This may indicate low precipitation of Cu^{2+}-phosphate complexes to the casein-bound phase in casein systems. Mannino et al. (1987) found that increasing ionic strength has no effect on the Cu^{2+}-binding ability of casein,

indicating that the binding mechanism of Cu^{2+} to casein is unlikely to be electrostatic. Although Cu is thought to associate with caseins via coordinate bonds (Formicka-Kozlowska et al., 1984; Gaucheron et al., 1997a), Cu binding of caseins is pH sensitive (Gaucheron et al., 1997a), indicating that the coordinate bonds that Cu^{2+} can form with casein molecules are not as stable as those formed with Fe.

5.3.3. Dynamic small amplitude oscillatory rheology

5.3.3.1. Maximum Loss tangent (LT_{max})

The LT_{max} values of the cheeses are shown in Figure 5.2. LT_{max} values generally showed no change during ripening for individual cheeses after 5 weeks of ripening, in agreement with Lucey et al. (2005). The ZN2 and FE3 cheeses had significantly lower ($P < 0.05$) LT_{max} values compared with the control cheese from 5 to 20 weeks of ripening in all trials, and the ZN2 cheese had the lowest LT_{max} values of all the cheeses at 5 and 10 weeks of ripening. In trials 2 and 3, the CU2 cheese had a higher LT_{max} than the control, which may be attributed to the higher pH values of this cheese compared to the control. LT_{max} can be used as an index of cheese meltability. Melt is thought to occur when electrostatic repulsion becomes the dominant interaction between casein molecules (Lucey et al., 2003) and therefore, increasing electrostatic attraction in cheese may induce an inhibition of melt.

Fe is not thought to associate with CCP (Gaucheron et al., 1997a; Silva et al., 2001), but the addition of both Fe^{2+} and Fe^{3+} caused a slight but significant increase ($P < 0.05$) in % INSOL Ca in the FE2 and FE3 cheeses, respectively, which may represent an increase in CCP content in these cheeses. Increased CCP concentration has been reported to decrease loss tangent values of cheese at elevated temperatures

(O'Mahony et al., 2006). However, the FE3 cheese had a significantly lower LT_{max} throughout ripening compared to the control, whereas the FE2 and control cheeses had similar values. Fe^{3+} binds strongly to caseins via coordinate bonds (Raouche et al., 2009a) thereby eliminating the negative charge of phosphate on phosphoseryl residues. Binding of Fe to caseins induces neutralization of their negative charge and an enhancement of hydrophobic interactions (Gaucheron, 2000) which modifies the conformation of casein molecules (Gaucheron et al., 1997b). As Fe^{3+} remains strongly bound to casein at relatively high temperatures (~90 °C) (Gaucheron et al., 1996, 1997b), it is likely that a reduction in electrostatic repulsion will remain in the FE3 and FE2 cheeses throughout the temperature sweep in DSAOR due to Fe binding. Presumably, the differences in rheological values observed between the FE3 and FE2 cheese may be due to the higher % insoluble level of Fe (Table 5.4) and lower pH values (Figure 5.1) of the FE3 cheese which may both contribute to further reduction in electrostatic repulsion.

Unlike Fe, a large amount of Zn is thought to associate with CCP when added to bovine milk systems (Singh et al., 1989b). CCP allows crosslinking of many casein molecules creating areas of high attractive interactions between casein molecules and cause a much greater reduction in electrostatic repulsion than direct binding of cations with casein molecules. As increased CCP content decreases melting of cheese, it is likely that the combined effect of increased % INSOL Ca and Zn (Table 5.4) in the ZN2 cheese increased CCP causing the greatest decrease in loss tangent of all the cheeses compared with the control. Cooke and McSweeney (2013) proposed that strontium added to Cheddar cheese precipitated to form Sr phosphate

nanoclusters analogous to CCP. It is also possible that Zn phosphate nanoclusters formed in the ZN2 cheese in the present study.

In trials 2 and 3, the CU2 cheese had a greater LT_{max} than the control and was similar to the control in trial 1. The CU2 cheese had higher pH values in trials 2 and 3 compared to the control. The lower pH of the control cheese may have reduced electrostatic repulsion and influenced cheese melt. As >70% of the added Cu^{2+} is insoluble (Table 5.4), it would be expected that casein-binding of Cu^{2+} would reduce electrostatic repulsion. An explanation for the rheological behaviour of the CU2 and FE2 cheese may be that a proportion of the INSOL Cu^{2+} or Fe^{2+} may be associated with the fat phase in cheese rather than casein, as previous studies have shown that Cu^{2+} and Fe^{2+} can associate with fat in milk (Hegenauer et al., 1979; Lonnerdal, 1985).

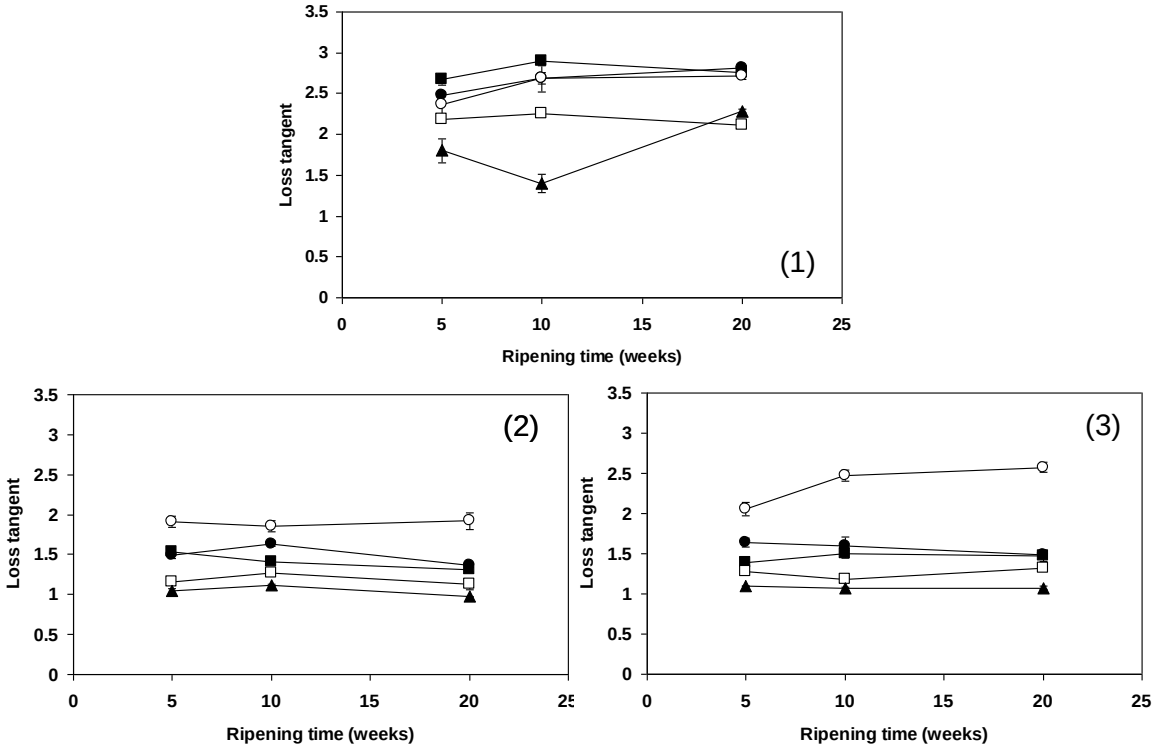


Figure 5.2. Maximum loss tangent (LT_{max}) values of control cheese (■), cheese supplemented with 10 mmol $FeCl_3.6H_2O$/kg curd (FE3) (□), cheese supplemented with 10 mmol $FeCl_2.4H_2O$/kg curd (FE2) (●), cheese supplemented with 10 mmol $CuCl_2.2H_2O$/kg curd (CU2) (○) and cheese supplemented with 10 mmol $ZnCl_2$/kg curd (ZN2) (▲) during ripening in cheesemaking trials 1-3. Error bars indicate ± 1 standard deviation.

5.3.3.2. Storage modulus (G′)

The G′ at two representative temperatures, 25 and 80 °C, were chosen to compare the thermorheological behaviour of cheeses at low and high temperatures (Table 5.5 and Figure 5.3). There were no consistent differences between cheeses for values of storage modulus at 25 °C (G'_{25}) throughout ripening in agreement with Venugopal and Muthukumarappan (2003). It appears that the increased % INSOL Ca content in the supplemented cheeses (Table 5.4) had no influence on this parameter compared to the control cheese. The G'_{80} values of all cheeses decreased during ripening (Figure 5.3) which is in agreement with results reported by Lucey et al. (2005). The decrease in G'_{80} in all cheeses during ripening is likely the consequence of proteolytic breakdown of the *para*-casein matrix and also further solubilization of CCP which reduces the number of bonds between the casein molecules (Lucey et al., 2005). The ZN2 cheese had a much higher G'_{80} value than the control cheese throughout ripening in all trials (Figure 5.3) and was also significantly higher ($P<$ 0.05) than the other supplemented cheeses. As discussed previously, a large amount of the INSOL Zn in the ZN2 cheese is likely associated with the CCP or possible Zn phosphate nanoclusters, whereas, the Fe and Cu is thought to be bound directly to caseins via coordinate bonds. A higher CCP content in cheese induces a decrease in G′ at elevated temperatures (O'Mahony et al., 2006).

5.3.4. Schreiber meltability

Meltability of cheeses as determined by the Schreiber melting test is shown in Table 5.6. The meltability of all cheeses increased during ripening. The increase in cheese meltability during ripening has been attributed to solubilization of CCP and an increase in proteolytic breakdown of the *para*-casein matrix (Lucey et al., 2003,

2005). The ZN2 cheese had a significantly lower meltabilty ($P < 0.05$) than the control cheese throughout ripening in all trials. As discussed above for LT_{max}, it is likely that increased formation of CCP and possibly Zn-phosphate nanoclusters in the ZN2 cheese decreased electrostatic repulsion leading to decreased melt. This result is in agreement with the LT_{max} values (Figure 5.2) which are an index of meltability.

Table 5.5. Storage modulus (G′) values (kPa) at 25 °C during dynamic small amplitude oscillatory rheology analysis in control cheese, cheese supplemented with 10 mmol $FeCl_3.6H_2O$/kg curd (FE3), cheese supplemented with 10 mmol $FeCl_2.4H_2O$/kg curd (FE2), cheese supplemented with 10 mmol $CuCl_2.2H_2O$/kg curd (CU2) and cheese supplemented with 10 mmol $ZnCl_2$/kg curd (ZN2) (mean ± standard deviation)

		Cheese treatment				
Trial	Ripening time (weeks)	Control	FE3	FE2	CU2	ZN2
1	5	55.44 ± 5.10^{aA}	54.53 ± 4.00^{aAB}	59.81 ± 3.56^{aA}	57.99 ± 5.54^{aA}	58.87 ± 4.41^{aA}
	10	57.42 ± 4.16^{aA}	49.81 ± 4.09^{aB}	47.49 ± 5.21^{aA}	54.79 ± 5.84^{aA}	59.38 ± 2.13^{aA}
	20	61.28 ± 6.72^{aA}	60.98 ± 1.66^{aA}	53.98 ± 5.99^{aA}	55.21 ± 5.89^{aA}	58.59 ± 3.41^{aA}
2	5	47.62 ± 1.18^{aB}	39.30 ± 1.40^{bC}	51.15 ± 2.04^{aAB}	41.85 ± 2.81^{bB}	51.73 ± 0.33^{aAB}
	10	58.77 ± 1.68^{aA}	51.69 ± 1.91^{abB}	46.07 ± 5.32^{bB}	51.07 ± 3.88^{abA}	49.19 ± 2.51^{bB}
	20	54.94 ± 4.37^{aA}	62.26 ± 3.73^{aA}	56.16 ± 3.73^{aA}	52.20 ± 1.32^{aA}	58.02 ± 5.08^{aA}
3	5	53.03 ± 3.44^{aA}	41.76 ± 2.82^{bB}	42.51 ± 2.15^{bB}	37.94 ± 3.21^{bB}	38.84 ± 3.28^{bB}
	10	52.97 ± 2.63^{bA}	53.88 ± 1.59^{bA}	53.21 ± 1.98^{bA}	42.60 ± 1.33^{cAB}	61.89 ± 3.36^{aA}
	20	58.36 ± 5.72^{aA}	56.98 ± 4.50^{aA}	59.68 ± 6.05^{aA}	44.38 ± 1.65^{bA}	58.99 ± 4.22^{aA}

^{a,b,c}Means within a row without a common lower case superscript letter are significantly different ($P < 0.05$)
^{A,B}Means within a column within a trial without a common upper case superscript letter are significantly different ($P < 0.05$)

The FE3, FE2 and CU2 cheeses generally displayed meltability similar to the control during ripening in all three trials. Rice and McMahon (1998) found that Mozzarella cheese fortified with $FeCl_3$ which contained 50 mg iron/kg had decreased melt by 28 days of ripening. These authors suggested that this may have been a result of higher levels of casein-bound Ca due to Fe binding with the caseins. There was a significantly higher % INSOL Ca in both FE2 and FE3 cheeses compared to the control (Table 5.4); however, this did not appear to reduce meltability of these cheeses as determined by the Schreiber melting test. The binding of Fe to caseins induces neutralization of their negative charges, enhances hydrophobic attractions (Gaucheron, 2000) and increases the possible formation of intermolecular Fe bridges between casein molecules (Gaucheron et al., 1996). However, the inability of Fe^{3+} to match inhibition of cheese melt caused by Zn^{2+} infers that the reduction of electrostatic repulsion caused by nanoclusters is much more important than the reduction caused by direct metal ion binding to caseins.

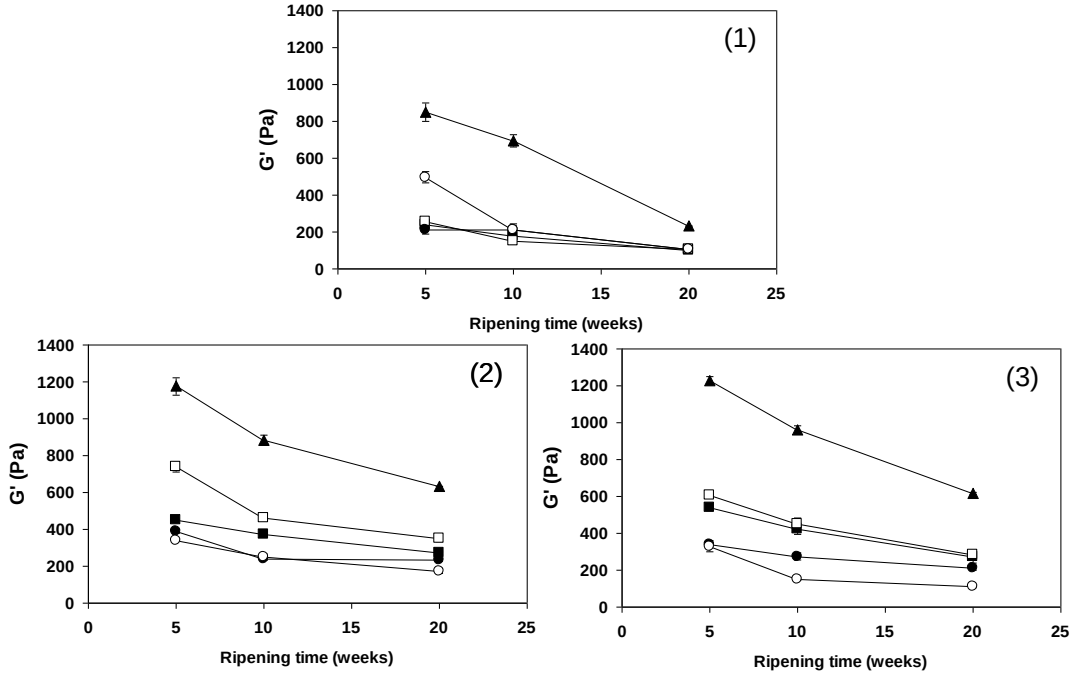


Figure 5.3. Storage modulus (G′) at 80 °C of control cheese (■), cheese supplemented with 10 mmol $FeCl_3.6H_2O$/kg curd (FE3) (□), cheese supplemented with 10 mmol $FeCl_2.4H_2O$/kg curd (FE2) (●), cheese supplemented with 10 mmol $CuCl_2.2H_2O$/kg curd (CU2) (○) and cheese supplemented with 10 mmol $ZnCl_2$/kg curd (ZN2) (▲) during ripening in cheesemaking trials 1-3. Error bars indicate ± 1 standard deviation.

Table 5.6. Percentage increase in cheese diameter during ripening from Schreiber melting test for control cheese, cheese supplemented with 10 mmol $FeCl_3.6H_2O$/kg curd (FE3), cheese supplemented with 10 mmol $FeCl_2.4H_2O$/kg curd (FE2), cheese supplemented with 10 mmol $CuCl_2.2H_2O$/kg curd (CU2) and cheese supplemented with 10 mmol $ZnCl_2$/kg curd (ZN2) (mean ± standard deviation)

Trial	Ripening time (weeks)	Cheese treatment				
		Control	FE3	FE2	CU2	ZN2
1	5	71.52 ± 2.25^{aB}	70.20 ± 2.43^{abB}	69.96 ± 0.21^{abA}	65.07 ± 4.26^{abB}	62.95 ± 3.61^{bB}
	10	76.36 ± 0.82^{aAB}	80.91 ± 1.04^{aA}	75.80 ± 1.58^{aA}	75.67 ± 4.64^{aA}	64.0 ± 3.08^{bB}
	20	80.99 ± 5.56^{aA}	82.06 ± 4.91^{aA}	73.71 ± 5.93^{aA}	79.91 ± 1.77^{aA}	71.62 ± 1.99^{aA}
2	5	75.43 ± 3.06^{aC}	77.11 ± 2.44^{aC}	80.21 ± 3.43^{aB}	74.00 ± 1.16^{aB}	66.38 ± 3.23^{bC}
	10	87.14 ± 1.78^{aB}	88.00 ± 1.03^{aB}	82.14 ± 1.19^{aB}	85.04 ± 3.45^{aA}	74.47 ± 3.00^{bB}
	20	93.65 ± 2.59^{aA}	93.19 ± 1.96^{aA}	90.27 ± 1.51^{abA}	87.74 ± 3.27^{abA}	84.95 ± 2.87^{bA}
3	5	82.18 ± 3.21^{aB}	75.54 ± 4.51^{abC}	70.12 ± 0.43^{bC}	76.90 ± 3.17^{abA}	60.08 ± 2.58^{cC}
	10	83.28 ± 0.40^{aB}	84.81 ± 1.52^{aB}	82.77 ± 2.60^{aB}	76.68 ± 6.23^{bA}	70.72 ± 4.35^{bB}
	20	97.95 ± 1.12^{aA}	99.00 ± 2.60^{aA}	94.17 ± 1.62^{aA}	83.08 ± 1.04^{bA}	81.47 ± 4.39^{bA}

^{a,b,c}Means within a row without a common lower case superscript letter are significantly different ($P < 0.05$)
^{A,B,C}Means within a column within a trial without a common upper case superscript letter are significantly different ($P < 0.05$)

5.3.5. Texture profile analysis hardness

Hardness values of all cheeses decreased during ripening (Table 5.7). It is thought that partial solubilzation of CCP during the first month of Cheddar cheese ripening is principally responsible for the initial softening of the cheese during early ripening (O'Mahony et al., 2005). Subsequent changes in cheese hardness during ripening are likely the result of proteolytic breakdown of the *para*-casein matrix (Lucey et al., 2003). No consistent differences in hardness were observed between the cheeses in all three trials during ripening. This observation, along with the G'_{25} values reported

in Table 5.5, suggests that addition of the metal cations had no effect on the elastic character of cheese at ambient and lower temperatures (i.e., 8 to 25 °C). The slight but significant increase in % INSOL Ca in the FE3, FE2, CU2 and ZN2 cheeses compared to the control cheese observed in all trials did not have any consistent effect on hardness in the three trials. Brickley et al. (2009) reported an increase in hardness values during ripening of Cheddar cheese supplemented at salting with $CaCl_2$ and attributed this to increased CCP levels in the cheese. Any influences observed in rheological properties of the cheeses due to addition of metal cations seemed to occur only at high temperatures (Figures 5.2 and 5.3).

Table 5.7. Hardness values (g) as determined by texture profile analysis of control cheese, cheese supplemented with 10 mmol $FeCl_3.6H_2O$/kg curd (FE3), cheese supplemented with 10 mmol $FeCl_2.4H_2O$/kg curd (FE2), cheese supplemented with 10 mmol $CuCl_2.2H_2O$/kg curd (CU2) and cheese supplemented with 10 mmol $ZnCl_2$/kg curd (ZN2) during ripening (mean ± standard deviation)

		Cheese treatment				
Trial	Ripening time (weeks)	Control	FE3	FE2	CU2	ZN2
1	5	21719 ± 1153^{aA}	20955 ± 1355^{aA}	20458 ± 1639^{aA}	17138 ± 839^{bA}	20200 ± 1849^{aA}
	10	21258 ± 1303^{aA}	15625 ± 1012^{bB}	16588 ± 1065^{bB}	14775 ± 1069^{bB}	19895 ± 1184^{aA}
	20	17147 ± 779^{aB}	12464 ± 1166^{bC}	13715 ± 1002^{bC}	13265 ± 1089^{bB}	17588 ± 1036^{aB}
2	5	14529 ± 623^{aA}	14561 ± 893^{aA}	13024 ± 294^{bA}	13386 ± 917^{abA}	13438 ± 974^{abA}
	10	11291 ± 288^{bB}	12538 ± 627^{aB}	11981 ± 747^{abB}	13063 ± 971^{aA}	11917 ± 562^{abB}
	20	10279 ± 790^{aC}	9865 ± 240^{aC}	10271 ± 363^{aC}	10246 ± 731^{aB}	10954 ± 739^{aB}
3	5	11956 ± 587^{bA}	12082 ± 586^{bA}	13851 ± 745^{aA}	13874 ± 754^{aA}	13989 ± 384^{aA}
	10	10425 ± 545^{cB}	10659 ± 761^{cB}	11841 ± 780^{bB}	12284 ± 476^{bB}	13901 ± 763^{aA}
	20	10469 ± 386^{aB}	10548 ± 578^{aB}	10378 ± 860^{aC}	10232 ± 323^{aC}	11219 ± 950^{aB}

^{a,b,c}Means within a row without a common lower case superscript letter are significantly different ($P < 0.05$)

^{A,B,C}Means within a column within a trial without a common upper case superscript letter are significantly different ($P < 0.05$)

5.3.6. Redox potential

The equilibrium redox potential values (E_h) of the cheeses after 20 weeks of ripening are shown in Table 5.8. In all three trials, the control cheese had the most negative E_h value of all cheeses, ranging from -107 to -62 mV. These values are close to those previously reported for Cheddar cheese by Topcu et al. (2008), i.e., -118 to -126 mV. The negative redox potential value of cheese is mainly due to lactic acid starter bacteria, as they ferment residual lactose to lactic acid (Fox et al., 2000). The FE3 cheese had E_h values ranging from -57 to +14 mV, whereas the FE2 cheese had a negative E_h value in all trials, ranging from -60 to -46 mV. Binding of Fe by caseins may restrict change in the oxidation state between Fe^{2+} and Fe^{3+} (Rice and McMahon, 1998). Raouche et al. (2009a) reported that 5 mmol/kg of added Fe^{2+} was completely oxidized to Fe^{3+} in milk stored for 1 day, and proposed that the oxidation reaction was dependent on both time and protein concentration. The Fe^{2+} ions added at the salting stage of cheese would likely have been rapidly oxidized. It is therefore likely that the form of Fe in both the FE2 and FE3 cheeses by week 20 of ripening is mainly Fe^{3+}. Also, it is known that casein phosphopeptides can exhibit both primary and secondary antioxidant activity via Fe^{2+} sequestration and direct free radical quenching (Kitts, 2005), which may have a large influence on the redox state of the FE cheeses. The ZN2 cheese had an E_h ranging from -82 to +42 mV, and was more negative than the control in trial 3. Variation of the E_h observed for ZN2 between trials may be due to slight compositional differences between the trials. The CU2 cheese had a highly positive E_h value, ranging from +210 to +264 mV. This E_h range is abnormal for Cheddar cheese (Fox et al., 2000; Topcu et al., 2008) and indicates a highly oxidizing environment in this cheese. Overall, the differences in E_h observed between the cheeses at week 20 likely reflect the influence of the added metal cations

on bacterial growth during ripening (see Section 5.3.7). There was no correlation between level of % INSOL metals (Table 5.4) and E_h (Table 5.8). E_h did not appear to have any effect on rheological, melting or textural properties (Figures 5.2, 5.3 and Tables 5.5, 5.6, 5.7). However, as E_h was determined only at week 20 of ripening, the possibility of a link during early ripening cannot be eliminated.

5.3.7. Microbiological counts

5.3.7.1. Starter bacteria

Starter lactic acid bacteria (LAB) counts during ripening are shown in Figure 5.4. Starter counts in the control cheese decreased from ~10^{10} to ~10^{8} cfu/g during ripening in agreement with Fox et al. (2000). The starter counts in the ZN2 cheese were similar to the control throughout ripening. Kahraman and Ustunol (2012) reported no differences in starter culture activity between a control Cheddar cheese and a Cheddar cheese containing 22.8 mg Zn/100 g cheese during 2 months of ripening. However, it should be noted that the ZN2 cheese in this study contained more than 2.5 times this amount of Zn (Table 5.2). The starter bacteria counts decreased in the FE3 and FE2 cheeses at a faster rate compared to the control after 5 weeks of ripening. Compared to the control, starter counts decreased quickly in the CU2 cheese, especially beyond 5 weeks and by week 10 the starter counts were ~5 log lower than the control cheese. Starter counts were similar in all cheeses in all trials at day 3 of ripening. No pH drop from day 1 to week 5 was observed in the CU2 cheese (Figure 5.1). This may indicate inhibited starter activity during this period as a slight pH drop in early ripening is attributed to metabolism of residual lactose to lactic acid by starter bacteria (Fox et al., 2000). The slight pH decrease in the CU2 cheese after week 5 could be due to NSLAB growth. These results indicate

an inhibitory effect of Fe^{2+} and Fe^{3+} on starter bacteria and a more pronounced toxicity of Cu^{2+} ions toward starter LAB bacteria during ripening at the added concentrations of their chloride salts. It is noteworthy that no function for Cu has been identified in LAB (Solioz et al., 2011). Fe and Cu ions can stimulate the Fenton reaction causing oxidative damage to LAB cells (Solioz et al., 2011). Mato-Rodriguez and Alatossava (2008) suggested that decreased survival of LAB exposed to high concentrations of Cu could be a result of redox cycling between Cu^{2+} and Cu^{+} under anaerobic conditions catalysing the production of highly toxic hydroxyl radicals leading to oxidative damage to cell components. Intracellular Cu is always in the form of Cu^{+} due to the reducing environment of the cytoplasm (Solioz et al., 2011) so, presumably, LAB species with insufficient Cu exclusion mechanisms will be most at risk to this damage. As mentioned previously, it is possible that a proportion of added Cu^{2+} associated with the fat phase in the CU2 cheese. It has been proposed that starter LAB are localized at the fat-water interface of cheese adhered to the milk fat globule membrane (Laloy et al., 1996), therefore, the bacteria may have been exposed to high localized Cu^{2+} concentration due to association of Cu^{2+} with the fat fraction.

Table 5.8. Equilibrium redox potential (E_h) values of control cheese, cheese supplemented with 10 mmol $FeCl_3.6H_2O$/kg curd (FE3), cheese supplemented with 10 mmol $FeCl_2.4H_2O$/kg curd (FE2), cheese supplemented with 10 mmol $CuCl_2.2H_2O$/kg curd (CU2) and cheese supplemented with 10 mmol $ZnCl_2$/kg curd (ZN2) at week 20 of ripening

	Cheese treatment				
Trial	Control	FE3	FE2	CU2	ZN2
1	-107	-57	-60	+210	+42
2	-90	+14	-60	+264	-82
3	-62	+3	-46	+251	-73

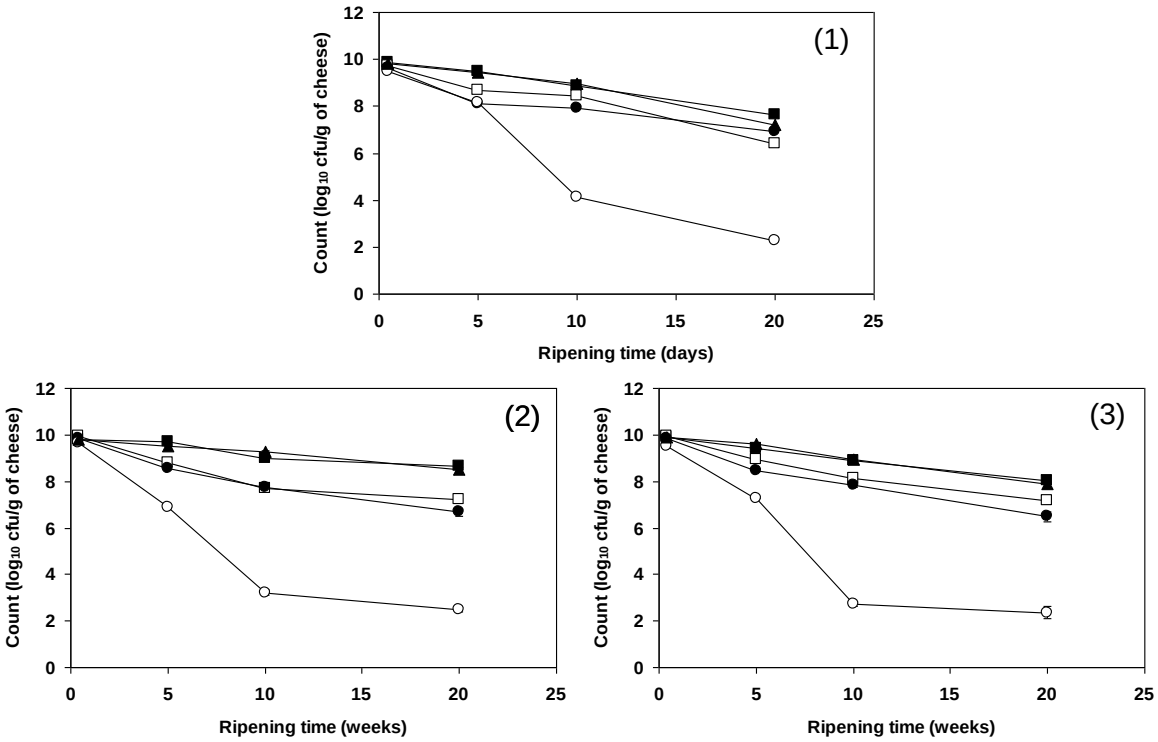


Figure 5.4. Starter counts in control cheese (■), cheese supplemented with 10 mmol $FeCl_3.6H_2O$/kg curd (FE3) (□), cheese supplemented with 10 mmol $FeCl_2.4H_2O$/kg curd (FE2) (●), cheese supplemented with 10 mmol $CuCl_2.2H_2O$/kg curd (CU2) (○) and cheese supplemented with 10 mmol $ZnCl_2$/kg curd (ZN2) (▲) during ripening in cheesemaking trials 1-3. Error bars indicate ± 1 standard deviation.

5.3.7.2. Non-starter lactic acid bacteria (NSLAB)

NSLAB counts during ripening are shown in Figure 5.5. NSLAB counts increased from 0 to 10^5-10^7 cfu/g in the control cheese during ripening. These counts are typical of NSLAB populations in Cheddar cheese where populations increase up to 10^6-10^8 cfu/g after 3 months of ripening and beyond (Banks, 2004). The control and ZN2 cheeses had similar NSLAB counts in all 3 trials. After 10 weeks of ripening, the FE2 cheese had significantly lower NSLAB counts ($P < 0.05$) than the control cheese. There was no growth of NSLAB in the CU2 cheese up to 5 weeks of ripening. After 5 weeks of ripening, the CU2 cheese had the lowest NSLAB counts of all cheeses. These results indicate an inhibitory effect of Cu^{2+} ions on NSLAB growth during ripening. The species of NSLAB were not identified in this study; however, *Lactobacillus paracasei* and *Lactobacillus plantarum* are the most common NSLAB found in Cheddar cheese (Beresford and Williams, 2004).

Traditionally, the production of Swiss Emmental cheese in copper cheese vats leads to increased levels of Cu ions in the milk/cheese that are thought to be essential for correct ripening, and even addition of Cu as $CuSO_4$ is used in some countries for the production of Emmental where stainless steel cheese vats are used (Mato-Rodriguez et al., 2011). A number of studies on LAB species and strains (especially *Lactobacillus* spp.) used as starters for Emmental have found that their tolerance of Cu^{2+} ions depends on both species and strain (Mueller et al., 1952; Maurer et al., 1975; Mato-Rodriguez and Alatossava, 2008). The retarded growth of NSLAB in the CU2 cheese is likely due to a low Cu tolerance and possible oxidative damage to cells as discussed above for the starter bacteria in that cheese.

The relationship between redox potential and bacterial metabolism can be considered from the two points of view: redox potential influences bacterial growth or the growth of bacteria influences redox potential (Davis, 1932). The low E_h in cheese is thought to be related to the fermentation of lactose to lactic acid by starter LAB (Fox et al., 2000). Caldeo et al. (2012) observed acute toxicity to starter LAB and retarded growth of NSLAB related to highly positive E_h values in Cheddar cheese supplemented with 1% KIO_3 at salting. The marked difference in starter and NSLAB counts between the control and CU2 cheeses (Figures 5.4 and 5.5) may be related to the large differences in E_h observed for both cheeses (Table 5.8). A negative E_h in cheese is required for the proper ripening of the cheese, i.e., bacterial metabolism and production of volatile compounds (Kristoffersen, 1985). The highly positive E_h and abnormally low LAB counts in the CU2 cheese after 20 weeks of ripening may be viewed as interrelated. A combination of Cu toxicity and high positive E_h is the likely cause of the abnormal LAB growth patterns observed in the CU2 cheese.

There was no correlation between level of % INSOL metals (Table 5.4) and LAB populations (Figures 5.4 and 5.5). Adding the same molar addition level of Zn and Cu led to very similar % INSOL of each metal but a huge contrast for impact on LAB populations during ripening.

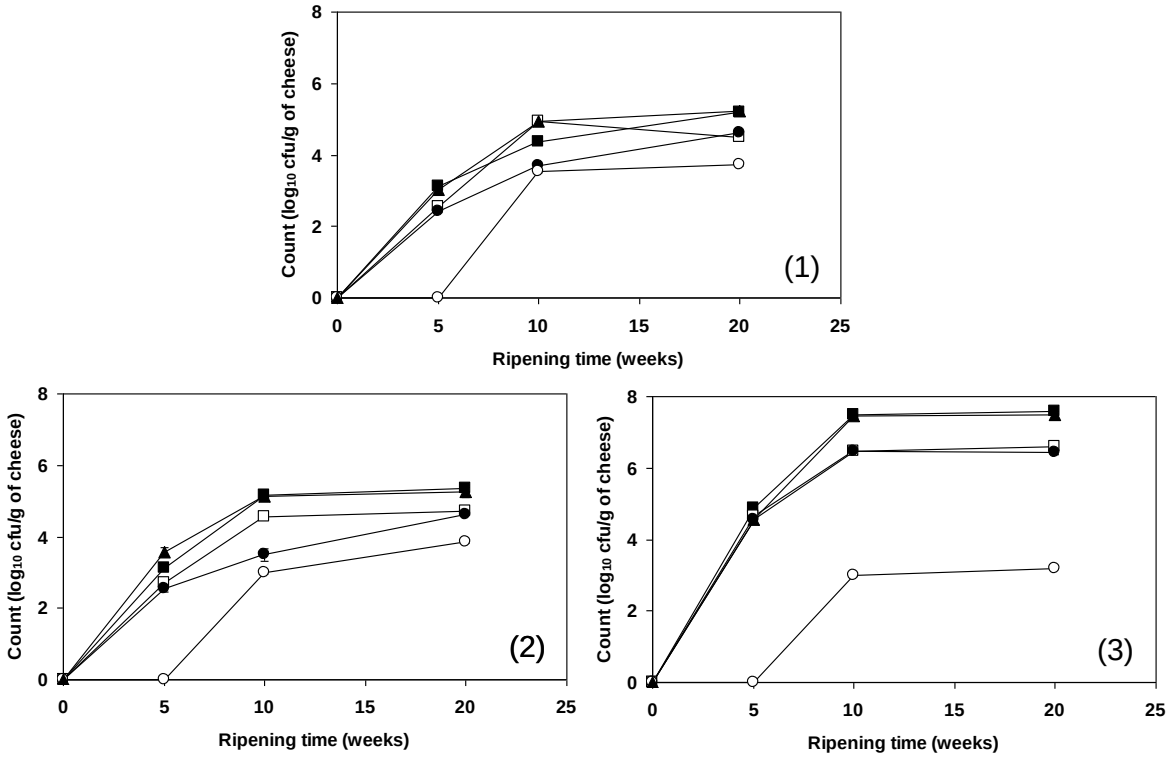


Figure 5.5. Non-starter lactic acid bacteria counts in control cheese (■), cheese supplemented with 10 mmol $FeCl_3.6H_2O$/kg curd (FE3) (□), cheese supplemented with 10 mmol $FeCl_2.4H_2O$/kg curd (FE2) (●), cheese supplemented with 10 mmol $CuCl_2.2H_2O$/kg curd (CU2) (○) and cheese supplemented with 10 mmol $ZnCl_2$/kg curd (ZN2) (▲) during ripening in cheesemaking trials 1-3. Error bars indicate ± 1 standard deviation.

5.4. Conclusions

Addition of Fe^{3+}, Fe^{2+}, Cu^{2+} and Zn^{2+} to Cheddar cheese at salting had numerous effects on its physicochemical and microbiological properties. All the supplemented cheeses had increased % INSOL Ca and high insoluble levels of the added cation at 5 weeks of ripening, but only Zn^{2+} had a substantial impact on rheological properties. The ZN2 cheese displayed the highest elasticity and lowest meltability of all the cheeses at elevated temperatures. This was likely due to the high association of Zn^{2+}

with CCP, as Zn^{2+} is thought to associate with caseins by electrostatic interactions (Singh et al., 1989b; Silva et al., 2001). Binding of Fe and Cu to caseins occurs via coordinate bonds and not association with CCP (Hegenauer et al., 1979; Gaucheron et al., 1996, 1997a). A much greater reduction in electrostatic repulsion due to CCP formation compared to coordinate bonding is probably responsible for the more solid-like character of the ZN2 cheese compared to the other supplemented cheeses. However, Zn^{2+} addition or addition of any of the other metal cations had no consistent effect on large or small deformation rheology properties of cheese at temperatures $\leq$ 25 °C. The FE3 and FE2 cheeses exhibited different pH, % INSOL Fe and LT_{max} values, demonstrating that the oxidation state of Fe can affect its behaviour in cheese systems. Cu^{2+} addition had a pronounced toxic and inhibitory effect on starter and non-starter LAB, respectively, in the CU2 cheese. The abnormal positive redox potential and the low LAB counts in the CU2 cheese can be viewed as interrelated. The type of interaction between metal cations and caseins may have major influence on the thermorheological properties of cheese. This should be considered in studies where cheese is supplemented with metal cations, when the functional properties of the cheese are important.

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Chapter 6

Effect of gum tragacanth on the rheological and functional properties of full-fat and half-fat Cheddar cheese

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Abstract

Fat replacers can be used to improve the sensory and functional properties of reduced-fat cheeses. The effect of gum tragacanth (GT) on the rheological, functional and sensory properties of half-fat and full-fat Cheddar cheese during ripening was investigated. Four Cheddar-style cheeses were made in triplicate: full-fat control (FFC); half-fat control (HFC); full-fat + GT (FFGum) and half-fat + GT (HFGum). Cheesemilk for the latter two cheeses was supplemented with GT at a level of 0.05% (w/v); all cheeses were ripened at 8 °C for 10 months. Moisture and moisture-to-protein ratio were increased by GT addition. GT addition resulted in decreased pH in both FFGum and HFGum cheeses during ripening, especially the FFGum cheese. GT appeared to affect proteolysis only in the FF cheeses. GT was successful in decreasing hardness and springiness values during ripening. GT increased meltability in the FFGum cheese and to a lesser extent in the HFGum cheese in late ripening. An increase in opaqueness was also observed due to GT addition regardless of fat level. Dynamic small amplitude oscillatory rheology showed a depression in LT_{max} and an increase in G′ at 75 °C caused by GT at 7 months of ripening. GT did not appear to have any impact on starter bacteria and NSLAB counts. Results from a consumer ranking preference test showed that the GT was not successful in fully mimicking the sensory properties of the FFC cheese. These results suggest that GT appears more suited to enhancing textural and functional properties than sensory properties in half-fat Cheddar cheese.

6.1. Introduction

Health-conscious consumers now demand foods with lower fat contents that have sensory properties similar to their full-fat counterparts. However, most consumers are not willing to sacrifice flavour or texture for fat reduction in cheese (Childs and Drake, 2009). Fat has an important role in the development of flavour, texture and appearance of cheese. Removal of fat from cheese can cause textural, functional and sensory defects such as rubbery texture, lack of flavour, bitterness, off-flavour, poor meltability and undesirable colour (Banks, 2004; Johnson et al., 2009; Mistry, 2001). The different composition of reduced-fat cheeses compared to full-fat variants alters the biochemical and microbiological changes during ripening that influence flavour and texture development (Fox and Wallace, 1997).

One of the most important strategies for improving the properties of reduced fat cheese is to increase its moisture content sufficiently to provide a moisture-to-protein ratio (M:P) that is equal to or greater than its full-fat counterpart (Broadbent et al., 2001). The addition of fat replacers to cheesemilk is one method used to achieve this objective. Fat replacers are ingredients used to take the place of milk fat and to mimic its characteristics in cheese. One of the most important functional properties of ingredient cheese is meltability. Along with improving the sensory and textural properties of unmelted cheese, an ideal fat replacer should increase meltability in a reduced-fat cheese. These ingredients can be composed of protein, carbohydrate (fat mimetics) or fat (fat substitutes). Carbohydrate-based fat mimetics are polar, water soluble compounds that act mainly by immobilising water thereby increasing the moisture content of the cheese. Commercially available carbohydrate-based fat mimetics such as Novagel™ and Stellar™ (McMahon et al., 1996); Perfectamyl gel

and Satiagel (Kavas et al., 2004); Raftiline® (Koca and Metin, 2004) along with β-glucan hydrocolloid suspensions (Konuklar et al., 2004) have had mixed success in altering both rheological and sensory properties of reduced and low-fat cheeses.

Gum tragacanth (GT) is a dried gummy exudate which is obtained from the stems of Asiatic species of *Astragalus* (Leguminosae). It consists of water-soluble tragacanthin, water-swellable bassorin (60-70% of polymer) and up to 4% of hydroxyproline-rich protein (Whistler, 1993). GT is stable over a wide range of pH and has a molecular weight of 840 $kg \cdot mol^{-1}$. It is widely used as a stabilizer, emulsifier and thickener in the food, pharmaceutical and cosmetic industries. Tragacanthin is a highly branched arabinogalactan consisting of a core of D-galactose residues to which highly ramified chains of L-arabinofuranose are attached. The backbone also contains a small quantity of galacturonic acid residues. (Glicksman, 1983). Bassorin is a highly branched arabinogalactan comprising a core of D-galactose residues containing attached side chains of L-arabinofuranose residues (Glicksman, 1983). It is believed that when GT is added to an aqueous system of casein micelles, the tragacanthin fraction adsorbs onto the micelle surface via carboxylic groups on the galacturonic residues of its backbone, modifying the electrostatic repulsion and enhancing steric repulsion between micelles, while the non-adsorbing bassorin fraction can increase the viscosity of the aqueous phase (Azarikia and Abbasi, 2010).

Nasirian et al. (2010) used GT successfully to mimic the sensory properties of fat in reduced-fat dairy cream due to its favourable emulsification properties. Rahimi et al. (2007) found that GT can be used to improve the rheological and sensory properties

of low-fat Iranian White Cheese when added at levels up to 0.75 $g \cdot kg^{-1}$ milk. However, a level of 1 $g \cdot kg^{-1}$ milk caused a negative effect on sensory texture scores. Aziznia et al. (2009) speculated that increasing GT level above 0.5 $g \cdot L^{-1}$ in non-fat yoghurt led to an increased distance between casein micelles due to increased steric stabilization and therefore less contact and structure in the yoghurt gel. Evidently, the level of GT addition to dairy products is of critical importance.

The objective of this study was to evaluate the effects of supplementing cheesemilk with gum tragacanth on the rheological and sensory properties of half-fat and full-fat Cheddar cheeses during ripening and determine its effectiveness as a fat replacement strategy for reduced-fat Cheddar cheese manufacture.

6.2. Materials and methods

6.2.1. Cheese manufacture

Raw bovine milk was standardized with cream to a fat content of 3.5% for full-fat control (FFC) milk and 1.5% for half-fat control (HFC) milk. Milks were batch pasteurised at 63 °C for 30 min. A 2 kg quantity of each milk type was heated to 45 °C and supplemented with 25 g food grade gum tragacanth (Cake-stuff, Lesmahagow, Lanarkshire, Scotland) by mixing at 3200 rpm using a Silverson L4RT blender (Silverson Machines Ltd, Chesham, Buckinghamshire, UK). This mixture was then flash pasteurised at 75 °C for 30 s. The supplemented milks were recombined with bulk milk in cheese vats containing the respective milk type. Four Cheddar-style cheeses were manufactured according to standard protocol on a 50 kg scale in the food processing facilities at University College, Cork. Starter culture (R-604Y; Chr. Hansen Ltd., Little Island, Co. Cork, Ireland) was used as the starter culture at a level of 0.02% (w/v). Chymosin (Maxiren 180; DSM Food Specialities, Delft, Netherlands), at a strength of 180 IMCU·ml^{-1}, was added to the cheesemilk at a level of 0.3 mL·L^{-1}. Coagulum was cut at equal firmness (measured subjectively). Curd was cooked from 31-39 °C over 30 min. Whey was drained at pH 6.2. The curd was Cheddared until pH 5.4 was reached and was then milled and dry salted at a level 2.5% w/w NaCl. The salted curd was transferred to rectangular moulds 25.4 cm x 20.3 cm and pressed overnight at 5 g·cm^{-2}. The cheeses were vacuum packaged and ripened at 8 °C for a period of 10 months. Three independent cheesemaking trials were performed.

6.2.2. Compositional analysis

Compositional analysis was performed on the cheeses at day 14 of ripening. The moisture contents of the cheeses were determined by an oven drying method (IDF, 1982), protein by the macro-Kjeldahl procedure (IDF, 1986), fat by the Gerber method (IIRS, 1955), salt by a titrimetric method using potentiometric end-point determination (Fox, 1963). Cheese pH was determined by measuring the pH of homogenized cheese slurry made from 10 g cheese and 10 g water at room temperature. Proteolysis was assessed by determining the levels of pH 4.6-soluble nitrogen as % of total nitrogen (pH4.6SN%TN) according to O'Mahony et al. (2005) at 1 and 6 months of ripening. Urea-polyacrylamide electrophoresis (PAGE) was carried out directly on the cheeses using the procedure described by O'Mahony et al. (2005). Urea-PAGE gels were loaded on an equal protein basis for all cheeses.

6.2.3. Texture Profile Analysis

Texture profile analysis (TPA) was performed using a Texture Analyser TA-XT2i (Stable Micro Systems, Godalming, Surrey, UK) according to the method of O'Mahony et al. (2005), except cylindrical samples of dimensions: height 20 mm, diameter 20 mm were used. Hardness, cohesiveness and springiness were defined according to Bourne (1978). Five replicate samples from each cheese were compressed at each ripening time point.

6.2.4. Dynamic small amplitude oscillatory rheology

Rheological properties of the cheese samples were measured using a Carri-Med CSL^2/ 100 Controlled Stress Rheometer (TA Instruments, Leatherhead, UK). Measuring geometry consisted of a 40 mm serrated stainless steel parallel plate

above, a flat base plate below and a gap size of 1.8 mm. Cheese discs (40 mm diameter, 2 mm height) were glued to the base plate of the rheometer using cyanoacrylate glue in order to prevent slippage during measurement. The sample was compressed to the gap size and allowed rest for 10 min at 20 °C in order to allow stress relaxation prior to oscillation. Storage modulus (G′), loss modulus (G″) and loss tangent (LT) were recorded continuously at a low amplitude shear strain (0.05) at a frequency of 6.283 $rad \cdot s^{-1}$ over 20 min during which the temperature was increased from 20 to 82 °C. Each sample was analysed in triplicate.

6.2.5. Melt analysis

Melt analysis was carried out using a covered Schreiber test (Altan et al., 2005). Each cheese cylinder (5 mm height, 35 mm diameter) was placed in a covered glass petri dish and then placed in an oven at 232 °C for 5 min. These were then removed and cooled for 30 min at room temperature. Measurements of the melt distance were made using electronic calipers. The diameter of the melted sample was measured at 5 different points and an average diameter was determined. Results were expressed as percentage increase in cheese diameter. Analysis on each cheese sample was performed in triplicate.

6.2.6. Colour analysis

Hunter L*-values of cheese samples were measured at room temperature using a Minolta Colorimeter CR-300 (Minolta Camera Co., Osaka, Japan). Five different locations on the freshly cut surface of a cheese slice were measured for each cheese.

6.2.7. Consumer preference ranking test

A consumer acceptance sensory panel evaluated randomly coded samples of the full-fat control (FFC), half-fat control (HFC) and half-fat gum (HFGum) cheeses. The acceptance panel consisted of 70 members (34% males and 66% females) ranging from 18 to 59 years old. Consumer panellists were students and staff members of University College Cork. Cheese blocks were cut into pieces (2.5 x 1 x 1 cm) and evaluated at room temperature. Evaluations were carried out in a panel room with individual booths. Normal lighting conditions were used, allowing the panellists also to discriminate on the basis of colour. Panellists completed a questionnaire asking their gender, age, and frequency of Cheddar cheese consumption (never, 1 time/month, 1 time/week, >3 times/week). Panellists ranked the cheeses according to preference. Panellists (n = 5) who selected 'never' as their frequency of consumption were eliminated from data analysis. Sensory evaluation was carried out at 10 months of ripening.

6.2.8. Microbiological analysis

Samples (~10 g) were taken aseptically from cheeses using a sterile cheese trier and placed into a stomacher bag. These samples were diluted 1:10 with sterile trisodium citrate (2% w/v) followed by homogenization in a stomacher (Seward Stomacher 400; Seward Ltd., London, UK) for 4 min. Further dilutions were prepared depending on the stage of ripening. Starter bacteria were enumerated on LM17 agar (Terzaghi and Sandine, 1975) incubated aerobically for 3 days at 30 °C. Non-starter lactic acid bacteria (NSLAB) were enumerated on Rogosa agar (Rogosa et al., 1951) incubated anaerobically for 5 days at 30 °C. Enumeration of starter bacteria and NSLAB were carried out in duplicate on all cheeses at month 3 and 6 of ripening.

6.2.9. Statistical analysis

ANOVA was carried out using the PASW Statistics Version 18 program (IBM, Armonk, NY, USA). Differences between means were analyzed using Tukey's HSD *post hoc* test. The level of significance was determined at $P < 0.05$. For the consumer preference ranking test, a critical difference value was obtained from Basker tables (Resurreccion, 1998).

6.3. Results and discussion

6.3.1. Chemical composition of cheeses

The composition of the cheeses at day 14 of ripening is shown in Table 6.1. The half-fat (HF) cheeses had a significantly higher ($P < 0.05$) moisture content compared to their full-fat (FF) counterparts. Addition of GT caused a significant ($P < 0.05$) increase in moisture content in both the FF and HF cheeses, which can be attributed to its water binding properties. The negatively charged GT may interact with positively charged groups on caseins, resulting in bridging flocculation and/or steric stabilization due to the low level of the polysaccharide present (Dickinson, 1998). This situation may partially reduce coagulation during the renneting stage due to the increased distance between casein micelles and therefore reduction in hydrophobic association between *para*-casein micelles. An increased viscosity in the aqueous phase and immobilization of water by GT may induce greater retention of whey in the initial *para*-casein gel matrix and reduce syneresis, conferring a more open protein matrix in the final cheese structure. Supplementing cheesemilk with 0.1% GT, Khosrowshahi (unpublished) observed an unsightly level of syneresis throughout ripening in both half-fat and full-fat Cheddar cheeses. Based on these findings, a GT supplementation level of 0.05% was chosen for the cheesemilk in the current study. In agreement with Guinee et al. (2000a), the reduction in fat content of the HF cheeses led to increases in protein and moisture contents, and a decrease % MNFS compared to their FF counterparts. Moisture replaces the fat in the protein matrix but not on an equal basis.

Table 6.1. Chemical composition, pH and levels of proteolysis in experimental Cheddar-style cheeses during ripening (mean ± standard deviation)

		Treatment			
Trial	Parameter[2]	FFC[1]	FFGum[1]	HFC[1]	HFGum[1]
1	Moisture (%)	38.84 ± 0.29^{c}	41.67 ± 0.76^{b}	42.89 ± 0.31^{b}	44.4 ± 0.4^{a}
	Fat (%)	29.25 ± 0.43^{a}	26.25 ± 0.25^{b}	16.92 ± 0.38^{d}	15.42 ± 0.38^{c}
	Protein (%)	26.45 ± 0.45^{b}	26.61 ± 0.72^{b}	33.03 ± 0.43^{a}	32.5 ± 0.12^{a}
	NaCl (%)	1.44 ± 0.01^{d}	1.66 ± 0.01^{b}	1.55 ± 0.01^{c}	1.74 ± 0.01^{a}
	S/M (%)	3.72 ± 0.01^{c}	3.98 ± 0.02^{a}	3.61 ± 0.01^{d}	3.92 ± 0.01^{b}
	F/DM (%)	47.82 ± 0.7^{a}	45.03 ± 0.43^{b}	29.62 ± 0.67^{c}	27.73 ± 0.69^{d}
	M:P	1.47 ± 0.02^{b}	1.57 ± 0.04^{a}	1.3 ± 0.02^{d}	1.37 ± 0.01^{c}
	MNFS (%)	54.9 ± 0.75^{a}	56.5 ± 1.22^{a}	51.62 ± 0.41^{b}	52.49 ± 0.69^{b}
	pH at wk[3] 2	$5.2 \pm 0.01^{b,A}$	$5.16 \pm 0.01^{c,A}$	$5.23 \pm 0.01^{a,A}$	$5.19 \pm 0.01^{b,A}$
	pH at mo 2	$5.11 \pm 0.01^{b,B}$	$5.02 \pm 0.01^{c,B}$	$5.17 \pm 0.01^{a,B}$	$5.10 \pm 0.01^{b,B}$
	pH at mo 6	$4.98 \pm 0.01^{c,C}$	$4.94 \pm 0.01^{d,C}$	$5.17 \pm 0.01^{a,B}$	$5.10 \pm 0.01^{b,B}$
	pH4.6SN%TN at mo 1	$9.84 \pm 0.12^{b,B}$	$10.53 \pm 0.19^{a,B}$	$9.87 \pm 0.21^{b,B}$	$9.76 \pm 0.03^{b,B}$
	pH4.6SN%TN at mo 6	$22.45 \pm 0.12^{ab,A}$	$23.12 \pm 0.08^{a,A}$	$21.92 \pm 0.73^{b,A}$	$20.87 \pm 0.27^{c,A}$
2	Moisture (%)	38.87 ± 0.31^{d}	40.7 ± 0.4^{c}	42.16 ± 0.47^{b}	43.88 ± 0.21^{a}
	Fat (%)	30.33 ± 0.29^{a}	27.0 ± 0.5^{b}	16.92 ± 0.14^{c}	15.17 ± 0.14^{d}
	Protein (%)	26.05 ± 0.63^{b}	26.39 ± 0.69^{b}	33.94 ± 0.78^{a}	32.77 ± 0.36^{a}
	NaCl (%)	1.48 ± 0.01^{d}	1.65 ± 0.01^{b}	1.57 ± 0.01^{c}	1.75 ± 0.01^{a}
	S/M (5)	3.8 ± 0.03^{c}	4.06 ± 0.01^{a}	3.71 ± 0.01^{d}	3.98 ± 0.01^{b}
	F/DM (%)	49.62 ± 0.47^{a}	45.5 ± 0.79^{b}	29.25 ± 0.25^{c}	27.02 ± 0.25^{d}
	M:P	1.50 ± 0.01^{b}	1.55 ± 0.01^{a}	1.24 ± 0.03^{d}	1.34 ± 0.01^{c}
	MNFS (%)	55.8 ± 0.53^{a}	55.9 ± 0.11^{a}	50.74 ± 0.6^{b}	51.73 ± 0.29^{b}
	pH at wk 2	$5.2 \pm 0.01^{b,A}$	$5.15 \pm 0.01^{c,A}$	$5.24 \pm 0.01^{a,A}$	$5.19 \pm 0.01^{b,A}$
	pH at mo 2	$5.16 \pm 0.01^{b,B}$	$5.04 \pm 0.01^{d,B}$	$5.22 \pm 0.01^{a,AB}$	$5.11 \pm 0.01^{c,B}$
	pH at mo 6	$5.08 \pm 0.01^{b,C}$	$4.95 \pm 0.01^{c,C}$	$5.21 \pm 0.01^{a,B}$	$5.08 \pm 0.01^{b,C}$
	pH4.6SN%TN at mo 1	$10.89 \pm 0.36^{ab,B}$	$11.49 \pm 0.27^{a,B}$	$10.45 \pm 0.36^{b,B}$	$10.82 \pm 0.19^{b,B}$
	pH4.6SN%TN at mo 6	$22.53 \pm 0.37^{a,A}$	$22.74 \pm 0.31^{a,A}$	$19.77 \pm 0.38^{c,A}$	$20.83 \pm 0.23^{b,A}$
3	Moisture (%)	38.47 ± 0.61^{c}	40.77 ± 0.64^{b}	42.32 ± 0.64^{b}	43.89 ± 0.48^{a}
	Fat (%)	29.92 ± 0.38^{a}	26.17 ± 0.29^{b}	16.67 ± 0.29^{c}	14.83 ± 0.14^{d}
	Protein (%)	26.26 ± 0.26^{b}	26.17 ± 0.11^{b}	33.52 ± 0.53^{a}	32.86 ± 0.24^{a}
	NaCl (%)	1.45 ± 0.01^{d}	1.64 ± 0.01^{b}	1.56 ± 0.01^{c}	1.74 ± 0.01^{a}
	S/M (%)	3.76 ± 0.02^{c}	4.03 ± 0.02^{a}	3.68 ± 0.01^{d}	3.95 ± 0.01^{b}
	F/DM (%)	48.62 ± 0.62^{a}	44.18 ± 0.48^{b}	28.9 ± 0.5^{c}	26.44 ± 0.25^{d}
	M:P	1.47 ± 0.02^{b}	1.56 ± 0.01^{a}	1.25 ± 0.02^{d}	1.35 ± 0.01^{c}
	MNFS (%)	54.89 ± 0.87^{a}	55.22 ± 1.09^{a}	50.78 ± 0.61^{b}	51.54 ± 0.49^{b}
	pH at wk 2	$5.21 \pm 0.01^{b,A}$	$5.17 \pm 0.01^{c,A}$	$5.23 \pm 0.01^{a,A}$	$5.21 \pm 0.01^{b,A}$
	pH at mo 2	$5.13 \pm 0.01^{b,B}$	$5.04 \pm 0.01^{c,B}$	$5.19 \pm 0.01^{a,B}$	$5.14 \pm 0.01^{b,B}$
	pH at mo 6	$5.11 \pm 0.01^{c,C}$	$4.98 \pm 0.01^{d,C}$	$5.23 \pm 0.01^{a,B}$	$5.21 \pm 0.01^{b,B}$
	pH4.6SN%TN at mo 1	$11.72 \pm 0.49^{ab,B}$	$12.41 \pm 0.16^{a,B}$	$11.27 \pm 0.07^{b,B}$	$11.68 \pm 0.04^{b,B}$
	pH4.6SN%TN at mo 6	$23.21 \pm 0.15^{a,A}$	$23.46 \pm 0.15^{a,A}$	$22.12 \pm 0.13^{b,A}$	$23.22 \pm 0.23^{a,A}$

[a,b,c,d]Different lower case superscript letters in the same row within a trial indicate that values are significantly different ($P < 0.05$)

[A,B,C]Different upper case superscript letters in the same column within a trial indicate that values for the same parameter at different ripening times are significantly different ($P < 0.05$)

[1]FFC = Full fat control; FFGum = Full fat + gum tragacanth; HFC = Half fat control; HFGum = Half fat + gum tragacanth

[2]S/M = salt in moisture; F/DM = fat in dry matter; M:P = ratio of moisture to protein; MNFS = moisture in non-fat solids; pH4.6SN%TN = pH 4.6 soluble nitrogen as a % of total nitrogen

[3]wk = week: mo = month

The increase in moisture caused by GT coincided with a significant ($P < 0.05$) decrease in fat content in both the FF and HF cheeses. The GT did not cause a significant change in protein content in either the FF or HF cheeses. As % MNFS did not change significantly due to addition of GT, it appears that the fat is replaced by water on a seemingly equal basis. However, GT addition increased the moisture-to-protein ratio (M:P) in both HF and FF cheeses. The pH of the HFC cheese was significantly higher than the FFC cheese during ripening, in agreement with Fenelon and Guinee (2000). These authors speculated that an increase in cheese pH with decreasing fat content may be attributed to the concomitant decrease in % MNFS, which has been found to lower the lactate-to-protein ratio. The higher protein content and lower M:P in the HF cheeses appear to give them a greater buffering capacity. The addition of GT generally causes a slight decrease in pH during ripening compared to the FF and HF control cheeses. This may be attributed to the increase in M:P caused by GT addition, which may reduce the buffering capacity. The FFGum cheese exhibited the largest decrease in pH. The lower pH in the FFGum cheese may be due in part to a higher retention of lactose post manufacture due to increased moisture retention and therefore, a higher level of lactate production in the cheese early during ripening.

6.3.2. Microbiological analysis

Starter cell counts (LM17 agar) decreased in all cheeses from 3 to 6 months (Table 6.2). Starter counts were generally higher in the HF cheeses possibly due to their increased moisture content. This is contrary to results reported by Laloy et al. (1996) who demonstrated that 50% reduced fat Cheddar cheeses had fewer starter cells than full fat cheeses. The presence of GT appears to have no effect on the number of

Table 6.2. Numbers (log cfu) of starter and non-starter lactic acid bacteria (NSLAB) in cheeses during ripening

		Treatment			
Trial	Bacteria	FFC[1]	FFGum[1]	HFC[1]	HFGum[1]
1	Starter bacteria mo[2] 3	$8.69^{b,A}$	$8.84^{b,A}$	$9.07^{a,A}$	$9.03^{a,A}$
	Starter bacteria mo 6	$6.71^{c,B}$	$6.57^{c,B}$	$7.31^{a,B}$	$6.96^{b,B}$
	NSLAB mo 3	$5.77^{a,B}$	$4.70^{c,B}$	$5.41^{b,B}$	$5.70^{a,B}$
	NSLAB mo 6	$6.37^{c,A}$	$6.35^{c,A}$	$6.73^{b,A}$	$7.14^{a,A}$
2	Starter bacteria mo 3	$8.66^{b,A}$	$9.06^{a,A}$	$9.09^{a,A}$	$9.00^{a,A}$
	Starter bacteria mo 6	$6.65^{b,B}$	$6.62^{b,B}$	$7.23^{a,B}$	$7.14^{a,B}$
	NSLAB mo 3	$6.01^{b,A}$	$5.26^{b,B}$	$5.45^{b,B}$	$5.97^{a,B}$
	NSLAB mo 6	$6.30^{b,A}$	$6.76^{a,A}$	$6.78^{a,A}$	$6.53^{b,A}$
3	Starter bacteria mo 3	$8.89^{b,A}$	$9.07^{a,A}$	$9.12^{a,A}$	$8.82^{b,A}$
	Starter bacteria mo 6	$6.58^{c,B}$	$6.41^{c,B}$	$6.84^{b,B}$	$7.02^{a,B}$
	NSLAB mo 3	$6.25^{a,B}$	$5.97^{b,B}$	$4.74^{d,B}$	$5.32^{c,B}$
	NSLAB mo 6	$7.02^{b,A}$	$7.28^{a,A}$	$6.11^{c,A}$	$5.81^{c,A}$

[a,b,c,d]Different lower case superscript letters in the same row within a trial indicate that values are significantly different ($P < 0.05$).
[A,B]Different upper case superscript letters in the same column within a trial indicate that values for the same parameter at different ripening times are significantly different ($P < 0.05$).
[1]FFC = Full fat control; FFGum = Full fat + gum tragacanth; HFC = Half fat control; HFGum = Half fat + gum tragacanth.
[2]mo = month.

starter bacteria. NSLAB counts increased from 3 to 6 months. Fat level and GT appeared to have no effect on NSLAB.

6.3.3. pH 4.6-soluble nitrogen as a percentage of total nitrogen

The level of pH4.6SN%TN significantly increased ($P < 0.05$) in all cheeses from 1 to 6 months during ripening (Table 6.1). In Trials 2 and 3, there was a significant difference ($P < 0.05$) in pH4.6SN%TN between the FFC and HFC cheeses at 6 months of ripening. A number of studies have observed a decrease in pH4.6SN%TN with decreasing fat content throughout ripening (Fenelon et al., 2000; Guinee et al., 2000a,b; Rudan et al., 1999). The same relationship of fat level with % MNFS occurred in the present study. pH4.6SN%TN values of the FFGum cheese were

generally higher than the values of the HFC cheese at 1 and 6 months of ripening. As the pH of the FFGum cheese was much lower than that of the HFC cheese, increased residual chymosin activity at the lower pH cheese may be responsible.

6.3.4. Urea-PAGE

From the urea-PAGE electrophoretograms (Figure 6.1), it appeared that fat reduction had an effect on the breakdown of α_{s1}- and β-casein. The rate of degradation of α_{s1}-casein was higher in the FF cheeses, whereas the rate of β-casein degradation appeared higher in the HF cheeses. Fenelon and Guinee (2000) reported a similar effect when fat level was reduced in Cheddar cheese. The FFGum cheese appeared to have increased hydrolysis ofα_{s1}-casein compared to the FFC cheese. The level of α_{s1}-CN(f24-199) in the FFGum cheese was lower than that of the FFC cheese at 24 weeks of ripening. This may be attributed to increased cleavage of α_{s1}-CN(f24-199) to (f102-199) due to increased residual chymosin activity at the lower pH of the FFGum cheese. There was a higher concentration of γ-caseins in the FFC cheese at 16 weeks in comparison to the FFGum cheese. In cheese, β-casein is hydrolyzed principally by plasmin (Sousa et al., 2001); therefore, the higher pH in the FFC cheese caused increased plasmin activity compared to the FFGum cheese. It appeared that the decrease in pH caused by addition of GT caused an increase in α_{s1}-casein breakdown via enhanced chymosin activity and a reduction in β-casein breakdown via reduced plasmin activity. This effect of pH on proteolysis is in agreement with Watkinson et al. (2001). The slightly higher S/M in the FFGum cheese compared to the FFC cheese may have also caused a reduction in chymosin hydrolysis of β-casein (Kelly et al., 1996). GT did not appear to cause a difference in the breakdown of α_{s1}- or β-casein in the HF cheeses.

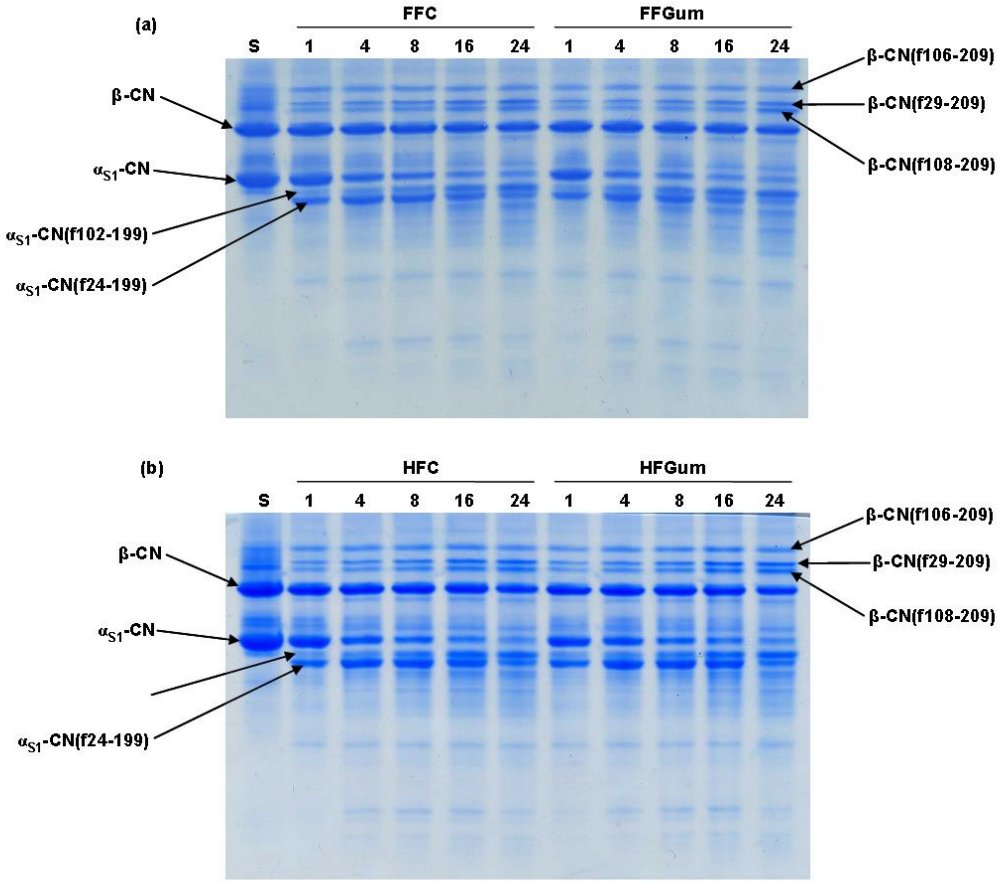


Figure 6.1. Urea-polyacrylamide gel electrophoretograms of sodium caseinate standard (S) and cheese for (a) full-fat and (b) half-fat cheeses at 1, 4, 8, 16 and 24 weeks of ripening. FFC = Full fat control; FFGum = Full fat + gum tragacanth; HFC = Half fat control; HFGum = Half fat + gum tragacanth.

6.3.5. Texture profile analysis

Hardness, cohesiveness and springiness values decreased during ripening for all cheeses. The decrease in hardness during ripening is widely attributed to a significant level of proteolytic breakdown of α_{s1}-casein in the protein matrix (Creamer and Olson, 1982). The decrease in hardness in early ripening may be largely due to solubilization of colloidal calcium phosphate (CCP) leading to a decrease in the number of CCP crosslinks between caseins and a decrease in structural integrity of the *para*-casein matrix (Lucey et al., 2003). The HFC cheese was generally the

hardest cheese throughout ripening and was significantly harder ($P < 0.05$) than the FFC cheese at most time points (Figure 6.2). These results are in agreement with many studies which have shown that decreasing fat content in cheese increases hardness/firmness (e.g., Fenelon and Guinee, 2000; Guinee et al., 2000a; Lteif et al., 2009). This is due to the reduction in filler volume and hence, a denser protein structure in this cheese. At most time points during ripening, there was no significant difference ($P > 0.05$) between the FFC and the HFGum cheeses. The increase in moisture content, M:P and physical disruption of the protein network caused by GT, create more inhomogeneities in the overall structure of the cheese, leading to a weaker protein structure. GT appeared to reduce hardness values in the HFGum cheese to a greater extent than in the FFGum cheese. Konuklar et al. (2004) also reported that carbohydrate-based fat mimetics can successfully decrease the hardness of reduced-fat Cheddar cheese. pH may have also had a notable contribution to the hardness values as HFC cheese has the highest hardness and pH values, while the opposite was true for the FFGum cheese. Decreasing the pH of cheese causes solubilization of CCP crosslinks, which may have contributed to the difference in hardness values observed between the cheeses during ripening.

The HFC cheese had significantly higher cohesiveness values compared to the FFC cheese. A decrease in cohesiveness with fat level has also been reported by Awad et al. (2005). At 1 week of ripening there was no significant difference between the FFC, HFC and HFGum cheeses, but a divergence was seen throughout ripening (Figure 6.3) resulting in the HFC and HFGum cheeses having significantly higher ($P < 0.05$) cohesiveness than the FFC cheese in all trials at 6 months. In contrast to the effect of GT on cohesiveness during ripening, Koca and Metin (2004) observed an

increase in cohesiveness in low-fat Kashar cheese supplemented with the carbohydrate (inulin) based fat mimetic Raftiline® after 60 days of ripening. The HF cheeses had higher springiness than the FF cheeses at 1 week of ripening, but as the cheeses ripened, a convergence was seen (Figure 6.4), which ultimately led to no significant difference ($P < 0.05$) at 6 months between the FFC and HFGum cheeses. The decrease in cohesiveness and springiness as in the case of hardness, is likely attributable to the proteolytic breakdown of the *para*-casein matrix during ripening.

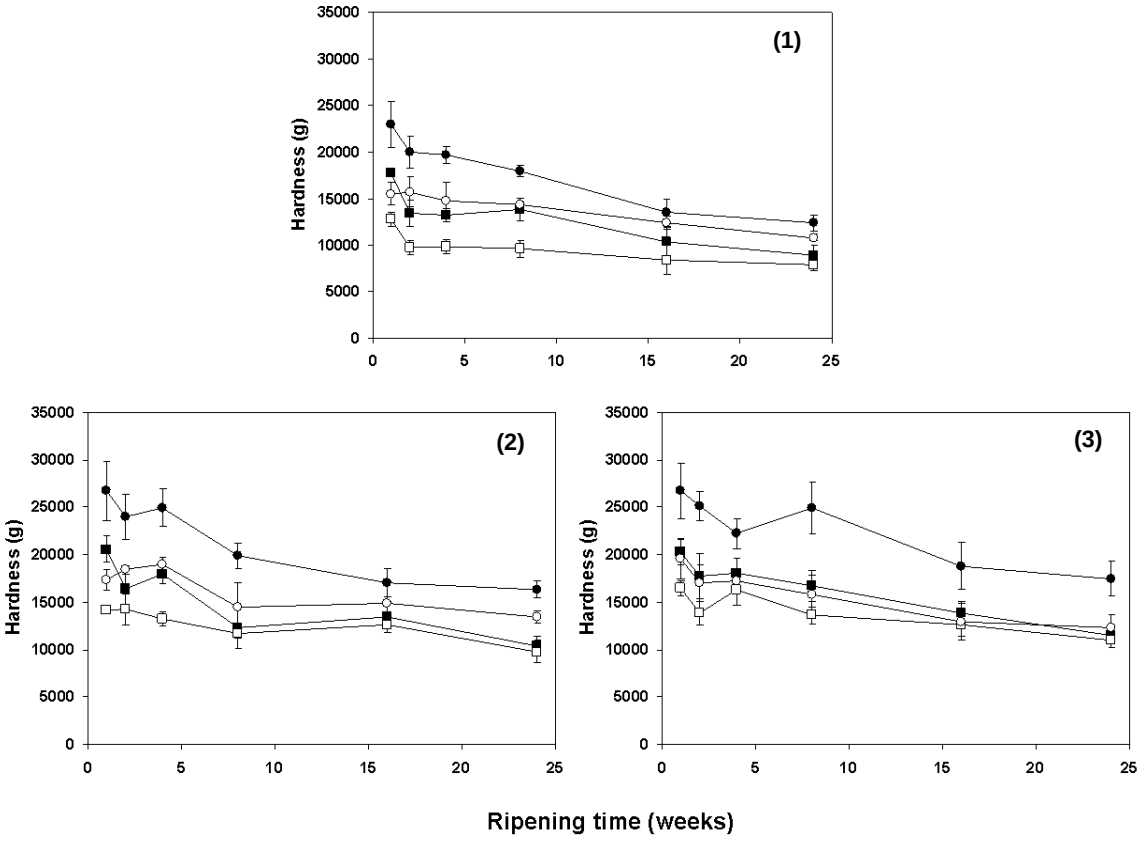


Figure 6.2. Hardness values as determined by texture profile analysis for the full-fat control (■), full-fat + gum tragacanth (□), half-fat control (●) and half-fat + gum tragacanth (○) during ripening in cheese making trials 1-3. Error bars indicate ± 1 standard deviation.

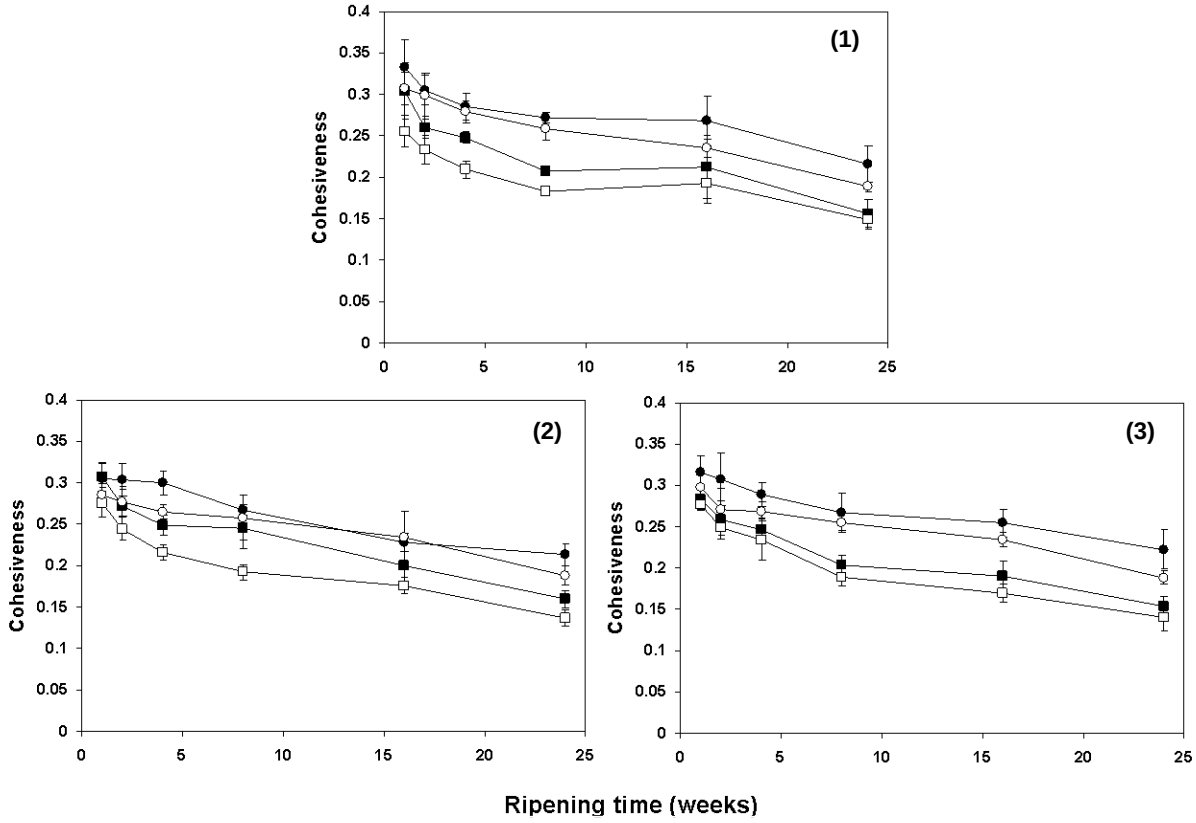


Figure 6.3. Cohesiveness values as determined by texture profile analysis for the full-fat control (■), full-fat + gum tragacanth (□), half-fat control (●) and half-fat + gum tragacanth (○) during ripening in cheese making trials 1-3. Error bars indicate ± 1 standard deviation.

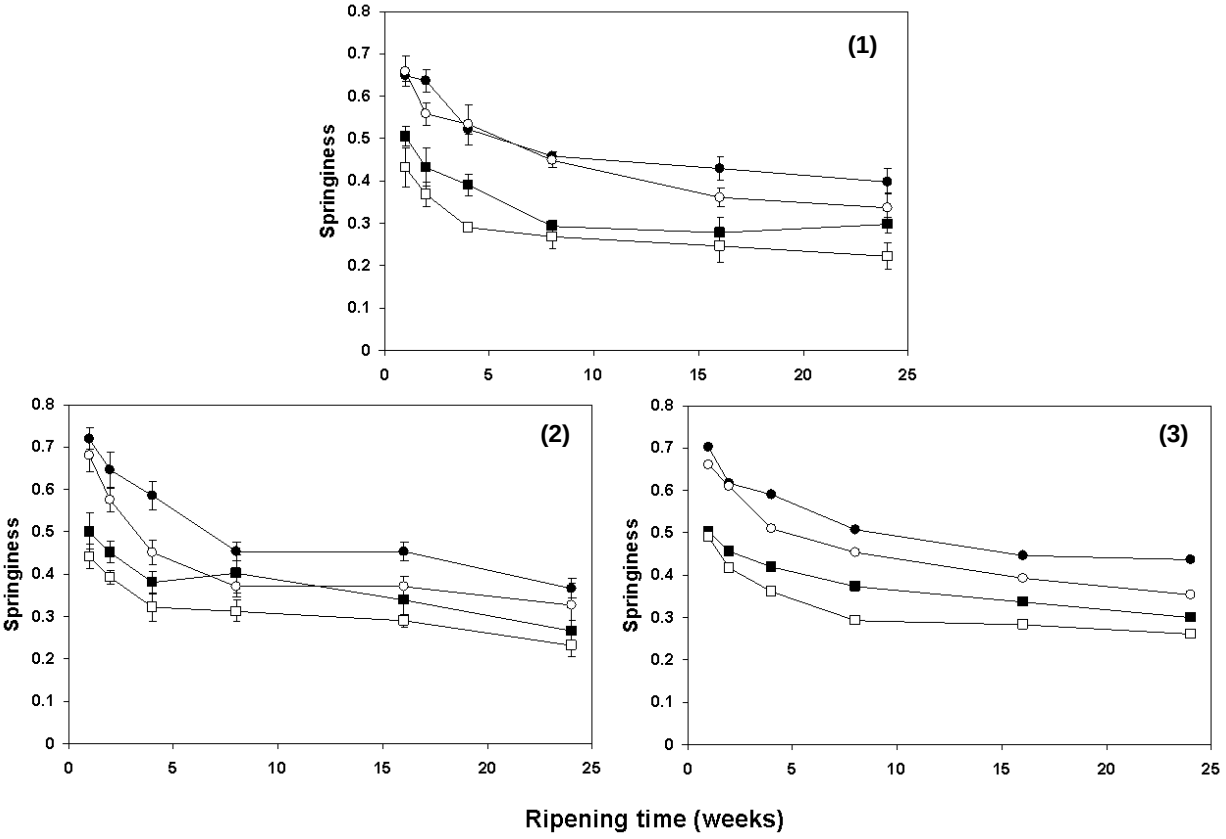


Figure 6.4. Springiness values as determined by texture profile analysis for the full-fat control (■), full-fat + gum tragacanth (□), half-fat control (●) and half-fat + gum tragacanth (○) during ripening in cheese making trials 1-3. Error bars indicate ± 1 standard deviation.

6.3.6. Melt analysis

Results from the Schreiber melting test indicated that the HF cheeses melt much less than the FF cheeses at most ripening times (Figure 6.5). These results are consistent with numerous studies that reported reducing the fat content of Cheddar cheese leads to a decrease in meltability (e.g., Guinee et al., 2000a; Konuklar et al., 2004; Rudan et al., 1999), with the main factors involved thought to be the higher protein content and lower M:P in the HF cheeses leading to an increased density of the protein matrix. The increase in meltability in all cheeses throughout ripening is likely to be due to degradation of the *para*-casein matrix. GT addition caused a large increase in the meltability of the FFGum cheese after 16 weeks of ripening. There appeared to be no difference in meltability between the HFC and HFGum cheeses at most time points during ripening. However, at 6 months in Trial 2, there was no significant difference ($P < 0.05$) between the FFC and HFGum cheeses. In Trial 3, there was a significant difference ($P < 0.05$) between the HFC and HFGum cheeses, with the HFGum cheese having a higher meltability. Koca and Metin (2004) observed an increase in meltability of low-fat Kashar cheese with Raftiline® compared to a low fat control at 90 days of ripening. McMahon et al. (1996) also reported an increase in meltability of reduced-fat cheeses during ripening with carbohydrate-based fat mimetics. Increases in meltability due to GT appear to be attributed mainly to the increase in moisture content, M:P and physical disruption of the protein network in the cheese by GT and decrease in pH. The low pH of the FFGum cheese led to increased proteolysis, and more extensive degradation of the *para*-casein matrix and consequently higher meltability. Increased solubilization of CCP cross-links in the *para*-casein matrix leading to decreased structural integrity in the cheese may have also contributed to increased meltability.

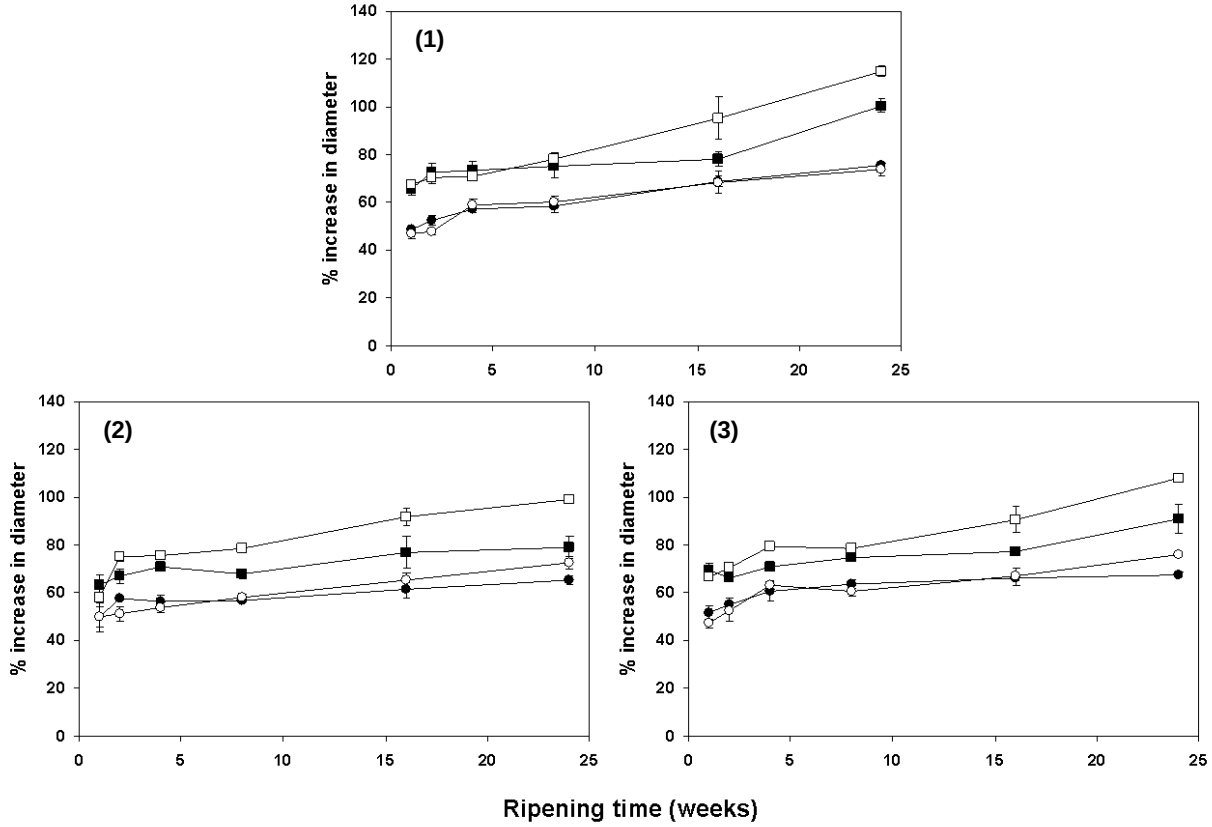


Figure 6.5. Percentage increase in cheese diameter from Schreiber melting test for the full-fat control (■), full-fat + gum tragacanth (□), half-fat control (●) and half-fat + gum tragacanth (○) during ripening in cheese making trials 1-3. Error bars indicate ± 1 standard deviation.

In reduced-fat cheeses, the increased evaporation at the surface of melting cheese due to lack of free oil causes more extensive protein aggregation, forming a thicker 'skin' on the cheese surface (Kim et al., 2011), leading to a reduction in melt area.

6.3.7. Dynamic small amplitude oscillatory rheology

Results from dynamic small amplitude oscillatory rheometry (DSAOR) at 7 months of ripening are shown in Table 6.3. The HF cheeses had a higher G′ at 40°C, possibly reflecting the presence of a stronger protein matrix due to the increased protein content in these cheeses. The HFC cheese had lower LT values than the FFC cheese at 7 months. As a higher LT indicates a higher meltability (Lucey et al., 2003), the results for the FFC and HFC cheeses correlate well with the Schreiber meltability

values. The temperatures at LT_{max} of the HF cheeses were ~10°C higher than those of the FF cheeses. GT caused a significant decrease ($P < 0.05$) in the LT_{max} and an increase in G′ at 75°C in both the FF and HF cheeses. If intact GT still remains at 7 months (i.e. not completely degraded by bacteria), it may retard the phase separation of moisture from protein at high temperature. The protein component of GT may also have a role in depressing the LT. As GT can contain up to 4% protein, there may be interactions between the caseins and this protein fraction. The decrease in pH caused by GT may have also contributed to these results. Lee et al. (2010) also reported that cheese with low pH (< 5.0), had a higher G′ at 40°C and lower LT_{max} than cheeses with higher pH.

Table 6.3. Storage modulus (G′) at different temperatures, maximum loss tangent (LT_{max}) and temperature at maximum loss tangent (Temp at LT_{max}) of Cheddar-style cheeses at month 7 of ripening from the dynamic small amplitude oscillatory rheology test (mean ± standard deviation)

		Treatment			
Trial	Parameter	FFC[2]	FFGum[2]	HFC[2]	HFGum[2]
1	G′ at 40°C (kPa)	8.1 ± 1.0^{b}	6.9 ± 0.9^{b}	34.7 ± 1.5^{a}	37.5 ± 3.6^{a}
	G′ at 75°C (Pa)	38.3 ± 3.2^{d}	78.0 ± 4.6^{c}	515.6 ± 21.4^{b}	627.6 ± 15.1^{a}
	LT_{max}	2.11 ± 0.05^{a}	1.62 ± 0.02^{c}	1.76 ± 0.02^{b}	1.35 ± 0.03^{d}
	Temp at LT_{max} (°C)	63.7 ± 0.8^{b}	63.2 ± 1.3^{b}	72.0 ± 2.5^{a}	72.5 ± 1.2^{a}
2	G′ at 40°C (kPa)	27.8 ± 2.7^{b}	32.3 ± 2.3^{b}	53.5 ± 3.7^{a}	57.5 ± 3.2^{a}
	G′ at 75°C (Pa)	40.5 ± 3.9^{d}	75.6 ± 6.6^{c}	395.0 ± 21.4^{b}	717.0 ± 46.2^{a}
	LT_{max}	2.13 ± 0.05^{a}	1.70 ± 0.05^{c}	1.86 ± 0.07^{b}	1.38 ± 0.08^{a}
	Temp at LT_{max} (°C)	63.9 ± 1.0^{b}	63.8 ± 0.3^{b}	74.7 ± 2.4^{a}	73.2 ± 2.4^{a}
3	G′ at 40°C (kPa)	37.3 ± 3.3^{b}	35.2 ± 4.2^{b}	51.1 ± 3.6^{a}	48.3 ± 4.0^{a}
	G′ at 75°C (Pa)	53.3 ± 2.0^{d}	118.3 ± 9.3^{c}	505.3 ± 50.2^{b}	1098.0 ± 54.8^{a}
	LT_{max}	2.02 ± 0.06^{a}	1.56 ± 0.07^{c}	1.87 ± 0.03^{b}	1.18 ± 0.01^{d}
	Temp at LT_{max} (°C)	64.0 ± 0.4^{b}	63.2 ± 1.1^{b}	74.5 ± 1.6^{a}	72.3 ± 0.7^{a}

[a,b,c,d]Different lower case superscript letters in the same row within a trial indicate that values are significantly different ($P < 0.05$).
[1]Note that the G′ values at 40°C and 75°C are reported in kPa and Pa, respectively
[2]FFC = Full fat control; FFGum = Full fat + gum tragacanth; HFC = Half fat control; HFGum = Half fat + gum tragacanth

When comparing this decrease in 'meltability' caused by GT to the Schreiber meltability (Figure 6.5) it should be noted that the gradual partitioning of the fat, moisture and protein occurring at the temperatures of the DSAOR test occur much faster during the Schreiber test due to rapid liquefaction of fat due to the instant exposure to 232°C. So the timescale during which GT can interact with cheese matrix components at temperatures below cheese melting point differs between the methods. Therefore, it is possible that interactions between GT and caseins at the temperature of the DSAOR may partially inhibit melting.

6.3.8. Colour analysis

L*-values of cheeses during ripening are shown in Figure 6.6. The FF cheeses had significantly higher ($P < 0.05$) L*-values than the HF cheeses throughout ripening, which is in agreement with Rudan et al. (1999). The HF cheeses had much higher protein and lower M:P, MNFS and fat than the FF cheeses, which gives them a much denser protein matrix and less light-scattering centres (i.e., fat droplets and serum pockets). After 16 weeks of ripening, addition of GT appeared to increase significantly the L*-values in both the FF and HF cheeses. Fat replacers, because of their particulate nature can act as light-scattering centres and increase the opaqueness of reduced fat cheese (McMahon et al., 1996). The increased moisture content and interruption of the *para*-casein matrix in cheeses containing GT gave the cheese a less dense protein network and an increased surface area occupied by scattering centres. This effect of GT is in agreement with Rahimi et al. (2007), who observed an increase in the L*-values of low-fat Iranian White Cheese supplemented with GT.

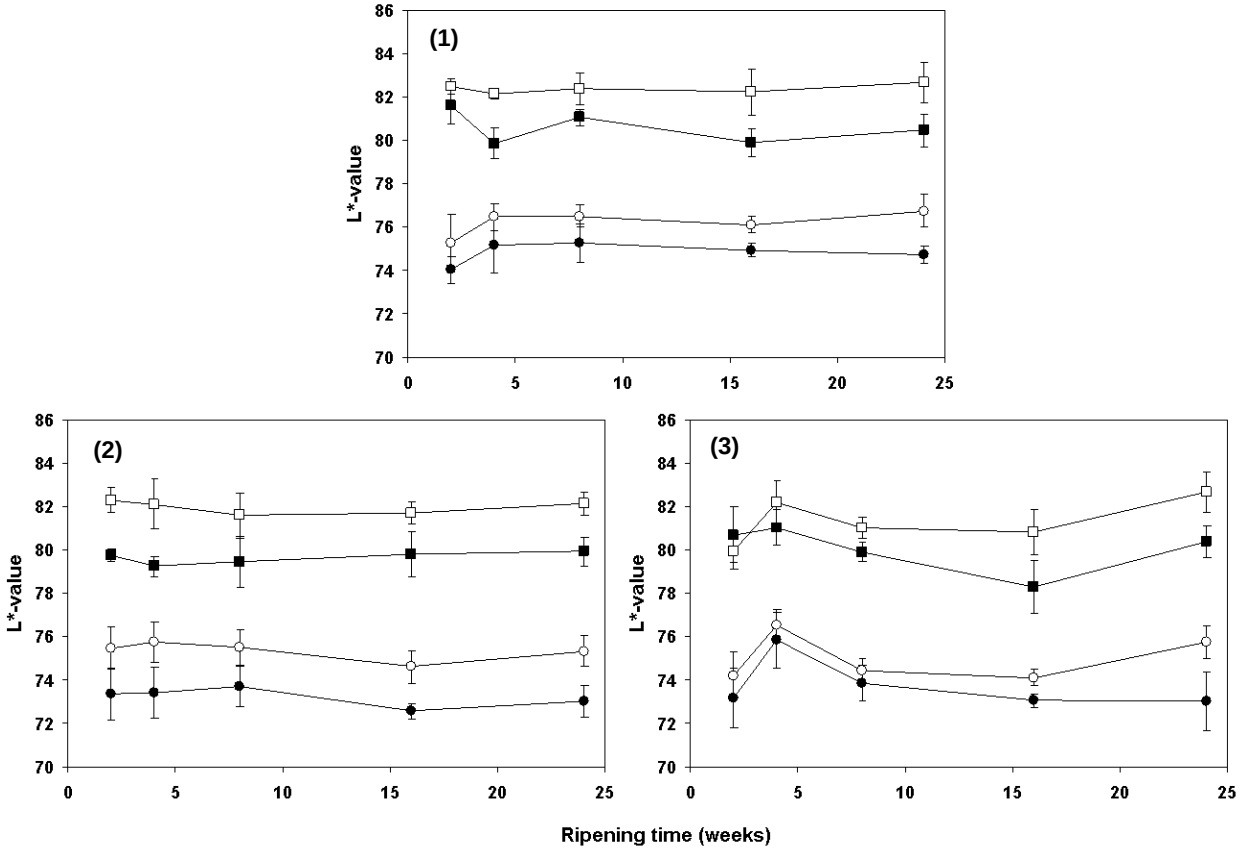


Figure 6.6. L*-values of full-fat control (■), full-fat + gum tragacanth (□), half-fat control (●) and half-fat + gum tragacanth (○) during ripening in cheese making trials 1-3. Error bars indicate ± 1 standard deviation.

6.3.9. Consumer preference ranking test

Table 6.4 shows the consumer preference ranking test scores for three of the experimental cheeses: FFC, HFC and HFGum at month 7 of ripening. The FFC cheese received the highest rank of the 3 cheeses. Results show that the HFGum cheese was overall the lowest ranked cheese when compared to the FFC and HFC cheeses. Rheological parameters obtained from TPA would suggest that the texture of the HFGum cheese should be more favourable than the HFC cheese, indicating flavour may have played a greater role than textural attributes. Rahimi et al. (2007) observed that the ability of GT to improve sensory properties of low-fat Iranian

White cheese is highly dependent on the level of addition. The flavour of full-fat cheese varieties is one of the hardest attributes of cheese to mimic when producing reduced-fat cheeses. Flavour differences between full-fat and reduced-fat cheese is thought to be due to differences in composition, flavour release, structure and ripening biochemistry (Drake et al., 2010). A lower concentration of fat derived volatile flavour compounds typical of full-fat Cheddar along with bitterness have been identified as reasons for atypical flavour in reduced-fat cheeses (Banks, 2004; Drake et al., 2010; Mistry, 2001). In addition, many panellists noticed an off-flavour in the HFGum cheese (not shown). Previous studies have observed unfavourable flavours in reduced-fat cheeses during ripening due to addition of carbohydrate-based fat mimetics (Koca and Metin, 2004; Konuklar et al., 2004). Some fat replacers have been found to form volatile compounds on decomposition that give off-flavours in cheese during ripening (Suriyaphan et al., 1999).

Table 6.4. Results from consumer preference ranking test (rank sum)

Trial	Treatment	Rank sum*
2	FFC[1]	109[b]
	HFC[1]	132[ab]
	HFGum[1]	149[a]
3	FFC[1]	100[b]
	HFC[1]	129[a]
	HFGum[1]	161[a]

[a,b]Different lower case superscript letters in the same column within a trial indicate that values are significantly different. Significance level determined as critical difference of 26.7 from Basker tables
[1]FFC = Full fat control; HFC = Half fat control; HFGum = Half fat + gum tragacanth
*Note that a lower rank sum indicates higher preference

6.4. Conclusions

This study indicates that GT can have a major effect on many of the rheological, sensory and functional properties of Cheddar cheese. The major effect of GT appears to be its ability to alter the composition and indirectly lower the pH of cheeses. The significant increases in moisture ($P < 0.05$) and M:P caused a decrease in TPA hardness and springiness in the HFGum cheese to values similar to the FFC. The increase in proteolysis in the FFGum cheese due to pH reduction may also be related to the increased meltability and lower values for TPA parameters in this cheese. Overall, the TPA parameters indicated that addition of GT to HF cheese at this level is successful in mimicking the hardness and springiness of the FFC cheese, which is a desired textural property. GT appeared to increase the meltability of the HFGum cheese at 6 months of ripening, which is highly desirable in the context of functionality. The effect of GT on the DSAOR parameters G′ and LT may show evidence of modification of casein interactions via the polysaccharide or protein component of GT at increased temperatures. From the consumer preference ranking test it was evident that supplementing half-fat Cheddar cheese with this level of GT may not be suitable for use as a table cheese, but may be suitable as an ingredient cheese due to its favourable functional properties. However, an obstacle for application of this strategy on an industrial scale would be the utilization of the whey produced, as it would contain some GT. This would cause processing issues due to increased viscosity and membrane blockage in ultrafiltration units. A possible avenue for further investigation could involve the use of GT in low-fat Cheddar cheese.

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Chapter 7

Conclusions and recommendations

From our experiments on partial substitution of NaCl at the salting stage with either $MgCl_2$ or $SrCl_2$ in order to evaluate their effects on the mineral equilibria of cheese and cheese rheological and melting properties (Chapter 2) we can conclude that:

1. Addition of $SrCl_2$ increased % CN-bound Ca and Mg to levels greater than in the control cheese, and also caused a high % CN-bound Sr in the cheese supplemented with $SrCl_2$ (+Sr),
2. Addition of $MgCl_2$ had no influence on calcium equilibrium, but a proportion of added Mg^{2+} did become CN-bound,
3. Addition of $SrCl_2$ increased the elastic character of the cheese at elevated temperatures (>60 °C) as observed from dynamic small amplitude oscillatory rheology tests and it also reduced Schreiber meltability; whereas addition of $MgCl_2$ did not alter any rheological or melting properties of cheese,
4. The contrasting influences of Sr^{2+} and Mg^{2+} on cheese mineral equilibria, rheological and melting properties is likely a consequence of the different solubilities of phosphate complexes of either Sr or Mg. It is proposed that Sr^{2+} addition leads to precipitation of Sr phosphate to either enhance or form nanoclusters causing an increased matrix density, whereas Mg^{2+} addition has a negligible contribution to nanocluster formation or matrix integrity.

Based on the findings of this study, the following recommendations for future work are suggested:

1. As $MgCl_2$ addition up to 8.7 g/kg cheese curd had no effect on the rheological or melting properties of cheese, it is suggested that partial substitution of NaCl with $MgCl_2$ may be effective in sodium reduction studies in cheese which is used as a functional ingredient,

2. The proposed formation of Sr phosphate nanoclusters is an interesting concept which may provide new information on the form and structure of CCP nanoclusters and even the casein micelle. Analysis of such nanoclusters using techniques such as small-angle X-ray scattering may generate useful information about the insoluble minerals in cheese,
3. Studying the formation of the macroscopic salt precipitates in the +Sr cheese during ripening may be useful to studies examining the defect of calcium lactate crystals in cheese. Isolation and purification of such crystals followed by analysis by X-ray diffraction analysis is recommended.

From our experiments on $MgCl_2$, $CaCl_2$ and $SrCl_2$ supplementation of reconstituted skim milk and their influences on mineral equilibrium and rheological properties of rennet-induced skim milk gels (Chapter 3) we conclude that:

1. The milks containing 10 $mmol \cdot L^{-1}$ $CaCl_2$ or 10 $mmol \cdot L^{-1}$ $SrCl_2$ exhibited buffering capacities significantly higher than the 10 $mmol \cdot L^{-1}$ $MgCl_2$ and control milks, likely due to the formation of nanoclusters in the former milks,
2. Rennet-induced milk gels formed from milk supplemented with either $CaCl_2$ or $SrCl_2$ had shorter gelation times, higher storage moduli and lower loss tangent values than gels formed from $MgCl_2$ supplemented milks. This indicates greater elastic character in the former gels possibly due to nanocluster formation causing an increased number of bonds within these gel systems,
3. During aging of rennet-induced gels at constant temperature (32 °C), the mineral equilibria changes due to rearrangements in the *para*-casein matrix of the gel.

Based on the findings of this study, the following recommendations for future work are suggested:

1. It would be interesting to compare cheeses produced with these supplemented milks to the cheeses reported in Chapter 2, to evaluate the importance of stage of production at which Mg^{2+} or Sr^{2+} are added to cheese,
2. It would be interesting to analyze by small-angle X-ray scattering the proposed Sr phosphate nanoclusters in the rennet-induced gels made from milks supplemented with $SrCl_2$ as this may provide useful information about the mechanism of CCP formation in milk at pH 6.6. Comparisons with the proposed Sr phosphate nanoclusters from Chapter 2 may also be made on this topic,
3. This study may have implications for the production of fresh, renneted cast cheese (whey not removed).

From our experiments on the addition of the calcium-binding salts trisodium citrate (TSC), disodium EDTA or disodium phosphate (DSP) to Cheddar cheese at the salting stage and observing their influence on Ca equilibrium in cheese and the rheological and melting properties of cheese (Chapter 4) we can conclude that:

1. Addition of TSC or EDTA caused an initial reduction in % insoluble Ca compared to the control cheese by sequestering Ca and forming soluble Ca complexes, inducing displacement of Ca from CCP to soluble phase,
2. Addition of DSP caused an initial increase in % insoluble Ca compared to the control due to precipitation of newly formed Ca phosphate complexes to the CN-bound phase, increasing CCP content,

3. All of the added calcium-binding salts increased cheese meltability as determined by the Schreiber melting test; however, cheese meltability as determined by maximum loss tangent in DSAOR did not correlate well with the Schreiber tests,
4. In studies determining cheese meltability, it is strongly recommended that more than one type of melting test is carried out on the cheese,
5. Addition of the Ca-binding salts caused a decrease in cheese hardness as determined by texture profile analysis at a number of ripening times, possibly due to decreased CCP in cheese caused by the Ca binding salts at 8 °C,
6. From the Schreiber melting tests, it may concluded that supplementation of cheese with Ca binding salts may be a viable method for modulating the functional properties of cheese used as a food ingredient.

Based on the findings of this study, the following recommendations for future work are suggested:

1. Partial substitution of NaCl with these Ca-binding agents may have potential in the cheese industry as a method of producing cheeses tailored for use as functional ingredients in food products,
2. Production of Cheddar cheese with food grade TSC and EDTA may be investigated to determine their influences on sensory properties of 'table' cheese,
3. Addition of Ca-binding salts at different stages of cheese manufacture would be of interest to determine the most efficient stage for addition.

From our experiments on the addition of $FeCl_3$, $FeCl_2$, $CuCl_2$ or $ZnCl_2$ to Cheddar cheese at the salting stage and observing their influences on cheese mineral equilibria along with rheological, melting, microbiological properties, and redox potential of cheese (Chapter 5) we can conclude that:

1. The addition of $ZnCl_2$ increased the elastic character of cheese at elevated temperatures (> 70 °C) compared to the other cheeses, and also decreased meltability of cheese according to the Schreiber melting tests, possibly due to interaction of added Zn^{2+} with CCP,
2. Addition of these metal cations had no influence on the rheological properties of cheeses measured ≤ 25 °C,
3. The oxidation state of iron (Fe^{3+} or Fe^{2+}) affects its behaviour in cheese as the cheeses supplemented with $FeCl_3$ (FE3) and $FeCl_2$ (FE2) exhibited different pH, % insoluble Fe and maximum loss tangent values,
4. Addition of $CuCl_2$ induced a toxic effect on starter LAB and an inhibitory effect on NSLAB growth, which can be related to the abnormal positive redox potential of that cheese.

Based on the findings of this study, the following recommendations for future work are suggested:

1. Fortification of Cheddar cheese with $ZnCl_2$ at the salting stage for studies on nutritional supplementation of cheese may be viable as high levels of $ZnCl_2$ do not affect starter or NSLAB; however, the addition level is important as Zn^{2+} can increase the elasticity of the cheese,

2. Ferrous iron (Fe^{2+}) may be better for cheese supplementation than ferric iron (Fe^{3+}) as the FE2 cheese was more similar to the control cheese for rheological properties,
3. As the metal ions affected starter and NSLAB to varying degrees, it would be interesting to investigate the volatile compound profiles of such cheeses,
4. A detailed examination of the forms of the insoluble cations in the cheese, especially the proposed interaction of Zn^{2+} with CCP by small-angle X-ray scattering, may provide invaluable information about the mechanism of casein-mineral interactions.

From our experiments on the use of gum tragacanth (GT) as a fat-replacer in the manufacture of half-fat Cheddar cheese and its impact on cheese rheological, melting, sensory and microbiological properties (Chapter 6) we can conclude that:

1. GT addition was successful in decreasing TPA hardness and springiness values to values similar to or closer to the full-fat control,
2. Use of GT as a fat replacer in half-fat Cheddar cheese was not successful in improving sensory properties of the cheese as determined from the consumer ranking test, possibly due to an off-flavour,
3. GT improved the colour of half-fat Cheddar cheese,
4. GT improved the Schreiber meltability of half-fat cheese in two out of three trials.

Based on the findings of this study, the following recommendations for future work are suggested:

1. Use of GT as a fat-replacer in the production of low-fat Cheddar cheese may be investigated,
2. Using a combination of GT and other fat-replacers such as microparticulated whey, inulin, etc., to improve the textural and sensory properties of reduced-fat Cheddar cheese may be more successful than use of GT alone,
3. As an off-flavour may have been responsible for consumer sensory panel rejection of the half-fat cheese supplemented with GT, a detailed analysis of the volatile compound profile of cheese by GC-MS is recommended when using this hydrocolloid as a fat-replacer.

Overall Conclusion

These studies have provided insight and developed hypotheses about the relationships between mineral equilibria of cheese and the proposed mechanisms of rheological properties of Cheddar cheese. Manipulating the mineral equilibrium of Cheddar cheese, specifically the calcium equilibrium is an important method of modulating its textural, rheological and functional properties. Today, the structure of CCP nanoclusters in milk is still not fully elucidated and much less information is known about the form and structural changes of nanoclusters in cheese during ripening. In several of the studies featured in this work, it has been hypothesized that CCP nanoclusters that are not exclusively composed of Ca phosphate may be formed when sufficient concentrations of salts such as $SrCl_2$ or $ZnCl_2$ are added to cheese or milk. In-depth characterization of such nanoclusters may provide invaluable

information regarding the mineral sequestering ability of casein micelles and mechanisms of nanocluster formation and stabilization.

Use of model systems using synthetic Cheddar cheese aqueous phase solutions to assess the effects of mineral equilibria on cheese rheological properties is a possible avenue for future research on the topic of mineral-casein interactions. The study of O'Mahony et al. (2006) highlighted the usefulness of this strategy by immersing Cheddar cheese slices in SCCAP solutions of increasing Ca concentration and then evaluating rheological properties. It would be interesting to use this strategy for further work based on Chapters 2, 4 and 5. Preparing SCCAP solutions with various concentrations of Mg, Sr, Fe, Cu, Zn or Ca-binding salts and evaluating the relationship between metal ion solution equilibrium and cheese rheological properties may give a clearer insight into the mechanisms of mineral-casein interactions without interference from proteolysis and cheese compositional variability.

There also exists a lack of information about the exact form and proportion of Ca and Mg salt complexes in the aqueous phase of cheese. An interesting strategy to explore this issue would be to measure the Ca and Mg activity in the cheese juice. Monitoring ion activity throughout ripening would also be useful, especially during late ripening to evaluate behaviour of soluble Ca i.e. precipitation of Ca salts, binding of ionic Ca to hydrolyzed casein fragments, etc.

Supplementation of cheese with high concentrations of elements not normally found in milk or cheese above trace amounts (e.g., Sr, Fe, Cu) can give crucial insight into mechanisms governing its physicochemical properties. The interaction of cations with caseins may occur via electrostatic or coordination bonding depending on their valencies and precipitation of the phosphate or citrate salts of such cations to the

casein-bound phase strongly depends on the solubility of these salt complexes. As most techniques that reduce insoluble Ca of cheese also reduce total Ca content or decrease Ca bioavailability, perhaps microencapsulation of Ca could be useful in producing cheeses with high total Ca content but lower CN-bound Ca and therefore improved functional properties.

Most studies involving manipulation of cheese rheological properties via Ca equilibrium alteration give purely instrumental data results. It would be interesting to add some type of sensory evaluation to these studies (where food grade additives are used), i.e., linking meltability, stretch, browning, etc, to sensory analysis of pizza or other prepared foods which could be potential end use products for the cheese under investigation. Chapter 4 attempted to shorten the ripening time required for softer texture development to occur in Cheddar cheese. As there are studies that attempt to accelerate the ripening of cheese from a flavour point of view, it would be interesting to combine these ideas and formulate strategies for shortening overall ripening time of cheese for use in both functional ingredients and as 'table' cheese.

Although not in the scope of this thesis, the learnings generated may be useful for studies involving sodium substitution and/or mineral fortification in cheese, especially if rheological properties are also under investigation. As reduction of the sodium content of cheese is now a focus in the cheese industry, the substitution of NaCl with mineral salts that positively influence texture and functionality while simultaneously replacing Na is of particular interest as an alternative to KCl, a common substitute for NaCl.

Demand for fat reduction and finding more cost effective ways to modulate cheese textural and functional properties are challenges for the cheese industry. It is the

author's hope that the knowledge generated in this thesis can help build strategies to address these issues.

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Appendix

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