Control of Calcium-Protein Interactions in Designing Casein-Based Food Structures with Novel Functionality

Thesis presented by

Irene McIntyre B.Agr.Sc. (Food Science)

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DECLARATION BY THE CANDIDATE

I hereby declare that the work described in this thesis is the result of my own investigation except where reference is made to published literature. I also certify that this thesis or any part of it has not been previously submitted for any other degree, either in University College Dublin, or elsewhere.

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Irene McIntyre

June 2017
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<tr>
<td>AA</td>
<td>amino acids</td>
</tr>
<tr>
<td>AAS</td>
<td>atomic absorption spectroscopy</td>
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<tr>
<td>$A_{Ca^{++}}$</td>
<td>calcium-ion activity</td>
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<tr>
<td>Ca$^{2+}$</td>
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<td>CCP</td>
<td>colloidal calcium phosphate</td>
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<td>calcium chelating salts</td>
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<td>DPP</td>
<td>dipotassium phosphate</td>
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<tr>
<td>DSP</td>
<td>disodium phosphate</td>
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<td>EMC</td>
<td>enzyme modified cheese</td>
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<td>ESEM</td>
<td>environmental scanning electron microscopy</td>
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<td>FA</td>
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Abstract

This thesis evaluated the basis of calcium-protein interactions in casein systems in order to develop novel casein-based food matrices with different structures and functionalities. Initially, in Chapter 2 the effects of calcium chelating salts (CCS) (disodium phosphate and trisodium citrate) on calcium-ion activity ($A_{Ca^{++}}$), calcium distribution and protein solubility were investigated in model CaCl$_2$ solutions (50 mmol L$^{-1}$) and rennet casein dispersions (12 % w/w). In both model systems, adding trisodium citrate either alone or as part of a mixed chelating salt system resulted in high levels of dispersed “chelated” calcium; conversely, disodium phosphate addition resulted in lower levels, while the $A_{Ca^{++}}$ decreased with increasing concentration of both CCS. Addition of either salt resulted in only a modest increase in soluble protein. Hence, the results suggested that CCS may play a more subtle role in modulating hydration during manufacture of casein-based matrices than simply solubilising calcium or protein. Therefore, despite the fact that the rennet casein dispersion used was relatively concentrated, an even more concentrated protein system was required to clarify the role of calcium chelators in casein hydration and matrix formation during manufacture of concentrated casein-based food matrices e.g. processed and analogue cheese.

Hence, this work was further built upon in Chapter 3, where a small-scale manufacturing protocol for concentrated casein-based food matrices was developed using a Thermomix. Manufacture was stopped at various time-points, and the matrix formed and any free liquid still remaining were collected. The dispersed and insoluble phases were separated by centrifugation and the calcium (total calcium and $A_{Ca^{++}}$) and protein contents of the dispersed phase analysed. The levels of calcium in the dispersed phase increased and $A_{Ca^{++}}$ decreased during manufacture of casein matrices formulated with CCS; ~23 % of the total calcium was solubilised by the end of manufacture. In the absence of CCS, the levels of calcium solubilised were significantly ($P < 0.05$) lower at equivalent processing times and remained...
unchanged as did $A_{Ca}^{+++}$, throughout manufacture. The level of protein in the dispersed phase was low ($\leq 3\%$ of total protein), but was significantly ($P < 0.05$) higher for the matrices containing CCS. The results obtained strongly suggested that the role of CCS was mainly to deplete colloidal calcium by partial solubilisation, which reduced calcium-mediated cross-linking sufficiently to allow adequate protein solubilisation to occur for fat emulsification. All of which contributed to successful matrix formation and stabilisation. The results generated suggested that only $\sim 23\%$ of colloidal calcium needed to be solubilised to form a hydrated and functional casein-based food matrix. This lead us to believe that a similar attenuation of calcium mediated cross-links might possibly be achieved by simply reducing the total calcium concentration of these matrices by a similar proportion.

Therefore in Chapter 4 casein-based food matrices with different calcium levels (1080-37 mg.100 g$^{-1}$) were manufactured by mixing rennet and acid casein. At intermediate calcium levels (673-358 mg.100 g$^{-1}$) homogeneous matrices were formed without CCS after relatively short processing times. However, homogeneous matrices with high ($\geq 775$ mg.100 g$^{-1}$) or low ($\leq 37$ mg.100 g$^{-1}$) calcium levels could not be produced without CCS. On cooling the CCS-free matrices with intermediate calcium levels formed functional cheese-like structures although they had lower hardness, flow on melting and $G'$ values at 25 °C than conventionally formulated high-calcium matrices made from rennet casein with CCS. The results obtained demonstrated that by manipulating total calcium concentration, it was possible to form hydrated casein matrices to meet a range of functional and compositional requirements suitable for different end-use applications, without the use of CCS. The elimination of CCS provided a means of reducing the sodium content of these casein matrices by up to 60 % and also presented the opportunity to formulate end-products with cleaner labels.

The sensory acceptability of casein matrices with low levels of calcium (357 mg.100 g$^{-1}$) manufactured without CCS was investigated in Chapter 5. These matrices were flavoured
with different types of enzyme modified cheeses (EMCs) and of those examined; Emmental was the most preferred by sensory panellists. The sensory acceptability of EMC flavoured casein matrices with different fat levels was also investigated. Firstly, the results of this study demonstrated that it was possible to produce full, half and reduced fat casein matrices without CCS. Furthermore, the results showed that although full-fat flavoured matrices were most preferred by sensory panellists and were rated higher in terms of their texture, flavour and overall liking; there were no significant differences (P > 0.05) detected between matrices containing half or reduced fat levels for any of these attributes.

In Chapter 6 CCS-free casein-based matrices prepared with different types of lipid (i.e. milk fat or rapeseed oil) were formulated with high (774 mg.100 g⁻¹) or low (357 mg.100 g⁻¹) calcium levels again by blending rennet and acid casein. Their physico-chemical characteristics (i.e. composition, texture, microstructure and water mobility) and in vitro digestibility were compared to conventionally formulated high-calcium (723 mg.100 g⁻¹) casein matrices made from rennet casein with CCS. The CCS-free, high-calcium matrices were significantly (P < 0.05) softer than those with low calcium levels and showed the highest rates of disintegration during simulated gastric digestion. Despite having a higher moisture-to-protein ratio, the high-calcium matrices containing CCS had broadly similar hardness values to those of high-calcium concentration prepared without CCS, but had higher cohesiveness. The high-calcium matrices containing CCS had quite a different microstructure and increased water mobility compared to those made without CCS and showed the slowest rate (P < 0.05) of disintegration in the gastric environment. Gastric resistance was shown to be unaffected by the type of lipid phase present in the matrix. Conversely, fatty acid release was similar for all casein matrices prepared from milk fat, however, high-calcium matrices (CCS-free) prepared from rapeseed oil showed higher lipolysis. The results demonstrated that modifying the composition (i.e. calcium concentration, moisture to protein ratio, inclusion of CCS) and
consequently the physical characteristics (e.g. texture, microstructure and water mobility) of casein-based food matrices affects their behavior during in vitro digestion. In particular, the results obtained showed that the physical properties of such matrices could be modified to alter resistance to gastric degradation which may have consequences for the kinetics of nutrient release and delivery of bioactives sensitive to the gastric environment.
Chapter 1

Literature Review
Chapter 1: Literature Review

1.1 Introduction

Global food demand is expected to rise as a result of population growth (estimated to reach ~9.1 billion by 2050), urbanisation and projected increases in per capita disposable income (FAO, 2009). This will have major implications for the international dairy sector and will lead to a growth in demand for dairy products. The abolition of the EU milk quota regime means the Irish dairy sector is well positioned to contribute to meeting this increasing demand, with this sector targeting a 50 % increase in output by 2020 to over 7.5 bn litres (Department of Agriculture Food and Marine (DAFM), 2011). Currently, approximately 85 % of Ireland’s dairy produce is exported to over 130 markets worldwide, the majority of which consists of commodity products e.g. butter, cheese, and milk powders (Bord Bia, 2015). However, in order to fulfil the diverse demands of global markets, the Irish dairy sector must consider the specific needs and requirements of consumers to identify markets for potential growth opportunities.

Recently, changing consumer demands have influenced the market for all types of food. There is now a growing cohort of informed consumers focussed on health and vitality, disease prevention, healthy ageing and performance, and clean product labelling. Consequently, food products with additional health and nutritional benefits e.g. functional foods (i.e. food products that provide health benefits beyond those of normal nutrition), high-protein foods, foods formulated with reduced levels of salt, fat or sugar, have all proven to be important growth vehicles (Norton et al., 2015). The dairy industry is in an excellent position to take advantage of this trend towards consumption of foods believed to positively influence health. Naturally derived from milk, dairy proteins are an excellent platform for the development of such innovative foods. These proteins have a high nutritional value, good sensory properties and are of great importance in dairy technology, as their processing functionality is key to
several dairy processes. The physico-chemical properties of milk proteins facilitate their functionality (e.g. emulsification, water-binding capacity, gelation, structure formation) in food systems. The mineral composition, especially the calcium concentration, is a well-known parameter that influences the structure i.e. degree of aggregation and functional properties of casein, the principal protein in bovine milk. A detailed understanding of this calcium-casein relationship is essential for developing innovative food products in which casein is a major component. There is a need to understand the basis of calcium-protein interactions which alter casein properties by affecting the extent and nature of protein-protein interactions, in order to develop novel casein-based food systems with different structures and functionalities and this is the focus of the research completed in this thesis.

In order to set the scene for this work, this review will begin by introducing casein, and describes its properties, structure, functionality and industrial preparation. Some of the unique physico-chemical properties of casein will be discussed in greater detail, with particular emphasis on changes induced to casein structure and functionality as influenced by calcium chelators. The next section reviews the basic principles and practices underlying the formation and functionality of semi-solid foods in which casein is a major functional ingredient. The final portion of this review will focus on the impact of food structure on digestion, with particular emphasis on understanding and controlling the digestibility of lipids.
1.2 Casein Properties, Functionality and Structure

1.2.1 Classification of milk proteins

Due to their many unique properties which contribute to their technological and commercial importance, the milk proteins are one of the most extensively characterised food protein systems (Fox and McSweeney, 2003; Fox and Brodkorb, 2008) and have been comprehensively reviewed in the scientific literature (Fox and McSweeney, 2003; Thompson et al., 2009). Bovine milk contains on average 87.3 % water and 12.7 % solids, including 3.4 % protein, 3.7 % fat, 4.8 % lactose and 0.7 % ash (Fox and McSweeney, 2003). The protein fraction of milk consists of two major types of proteins: casein proteins which account for approximately 76-86 % of the total protein present and whey proteins which make up the remainder. The whey proteins constitute a heterogeneous group of proteins which remain soluble in milk upon enzymatic or isoelectric precipitation of casein (Hambraeus, 1982). The major whey protein components in milk are: β-lactoglobulin (β-lg), α-lactalbumin (α-la), immunoglobulins and serum albumin (Fox and McSweeney, 2003).

1.2.2 Casein properties and functionality

The casein component in bovine milk is composed of four different casein molecules, namely \( \alpha_s1 \), \( \alpha_s2 \), \( \beta \) and \( \kappa \)-casein, in proportions 40, 10, 35 and 15 % (w/w), respectively (Broyard and Gaucheron, 2015). These casein molecules are well characterised in terms of their amino acid composition, molecular weight, concentrations in milk and physico-chemical properties (Table 1.1). All the caseins are negatively charged (Table 1.1), amphipathic molecules i.e. containing hydrophilic and hydrophobic regions (Horne, 2006). The caseins contain considerable amounts of hydrophobic amino acids and some (especially \( \beta \)- and \( \kappa \)-caseins) have large hydrophobic blocks in their sequences (Swaisgood, 2003). As a result, the caseins can exhibit self-association via hydrophobic interactions, depending on the physico-chemical conditions (Holt, 1992; Horne, 1998; Panouillé et al., 2005). The self-association properties
(Dalgleish, 1997; Dickinson, 2006) of the caseins make them difficult to separate as individual proteins (Holt, 2013) and also render them excellent food emulsifiers (McClements, 2004).

Table 1.1 General properties of individual casein molecules (Broyard and Gaucheron, 2015).

<table>
<thead>
<tr>
<th></th>
<th>$\alpha_{s1}$-casein</th>
<th>$\alpha_{s2}$-casein</th>
<th>$\beta$-casein</th>
<th>$\kappa$-casein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight (Da)</td>
<td>23,614</td>
<td>25,230</td>
<td>23,983</td>
<td>19,023</td>
</tr>
<tr>
<td>Concentration (g. L$^{-1}$)</td>
<td>10</td>
<td>2.6</td>
<td>9.3</td>
<td>3.3</td>
</tr>
<tr>
<td>No of amino acids</td>
<td>199</td>
<td>207</td>
<td>209</td>
<td>169</td>
</tr>
<tr>
<td>Proline</td>
<td>17</td>
<td>10</td>
<td>35</td>
<td>20</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>24</td>
<td>25</td>
<td>18</td>
<td>13</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>8</td>
<td>11</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Phosphoserine</td>
<td>8</td>
<td>11</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Apolar residues</td>
<td>36</td>
<td>40</td>
<td>33</td>
<td>33</td>
</tr>
<tr>
<td>Glycosylation</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td>Charge at pH 6.6</td>
<td>-21</td>
<td>-15</td>
<td>-12</td>
<td>-3</td>
</tr>
</tbody>
</table>

Another important property is the glycosylation of $\kappa$-casein (Table 1.1). Due to this glycosylation, its C-terminal region is hydrophilic. This characteristic is important for casein micelle structure and stability (Bijl et al., 2014) and will be discussed further in Section 1.2.3. The hydrophilic and hydrophobic residues on the casein micelles are not uniformly distributed, so this gives them good surface activity. Functional properties such as emulsification, foaming, and film formation are related to these surface-active properties of the caseins (Singh, 2011).

The caseins are also phosphorylated, the degree of which varies greatly among the individual caseins (Table 1.1). Due to their high content of phosphoseryl residues, both the $\alpha_s$- and $\beta$-
caseins bind polyvalent cations, the phosphorylated serine residues confer negative charge on the proteins. This negative charge causes them to interact strongly with the Ca$^{2+}$ ions (Parker and Dalgleish, 1981) and colloidal calcium phosphate (CCP) (Holt, 1998; Dalgleish and Corredig, 2012), which can cause their precipitation in the presence of sufficient Ca$^{2+}$ (Dalgleish and Parker, 1980; Parker and Dalgleish, 1981). Those caseins ($\alpha_{s2}$-, $\alpha_{s1}$- and $\beta$-casein) with the highest degree of phosphorylation are most Ca$^{2+}$-sensitive and precipitate at concentrations of $< 2$ mM, 3-8 mM, and 8-15 mM Ca$^{2+}$, respectively (Dalgleish and Parker, 1980). In contrast, $\kappa$-casein contains just one phosphoseryl residue and therefore is affected little by the presence of calcium (Farrell and Thompson, 1988) and thus stabilises the former groups of caseins against calcium-induced precipitation (Guo et al., 2003) in the micelle environment.

The caseins possess a high content of the structure-breaking amino acid proline, which accounts for 17% of all residues in $\beta$-casein, they also lack cysteine residues (only $\alpha_{s2}$- and $\kappa$-caseins have cysteine residues), which form disulphide bonds (Farrell, 2011). Both of these features contribute to open and flexible conformations, and consequently the caseins do not have extensive amounts of secondary structure, nor do they possess well-defined permanent tertiary structures. As a result they have been described as ‘‘rheomorphic’’ (Holt and Sawyer, 1993) or ‘‘natively disordered’’ proteins (Gaspar et al., 2008), implying that they may adapt their structures to changes in environmental conditions. Due to their high flexibility, casein molecules have excellent surface-active and emulsion stabilising properties (Mulvihill and Murphy, 1991; Dickinson, 1999; McClements, 2004).

1.2.3 Casein micelle structure

Native milk casein exists as particles of colloidal dimensions known as micelles, which are polydisperse, irregular and roughly spherical in shape (de Kruif et al., 2012). The casein micelles consist of 94 % (dry matter basis) protein and contain ~6 % minerals, which are
collectively referred to as CCP (de Kruif and Holt, 2003). The micelles also contain 76 % of water in volume, giving them a natural sponge-like colloidal structure (Bouchoux et al., 2010) (Figure 1.1) and can be considered hydrated, dynamic structures with a loose packing and high porosity (Walstra, 1979). Some of the physico-chemical characteristics of casein micelles are reported in Table 1.2.

![Figure 1.1](image.jpg)

**Figure 1.1** Electron micrographs of an individual casein micelle captured using the technique of field-emission scanning electron microscopy (Dalgleish et al., 2004).

The casein micelles are essentially complex association colloids, which are formed by the highly phosphorylated caseins (αs- and β-caseins) as core polymers interacting and aggregating with calcium phosphate and whose sizes are controlled by a layer of κ-casein bound to their surfaces (Horne, 2006; Dalgleish, 2011). Although the nature and structure of the casein micelle has been extensively studied and different models have been proposed and reviewed in the literature (Phadungath, 2005; Horne, 2006; Fox and Brodkorb, 2008; Dalgleish, 2011; Holt, 2013), the exact structure of the casein micelle is still not fully understood. Based on the physico-chemical properties of casein micelles, the proposed models fit into three categories, which are: i) the sub-micelle model (Slattery and Evard, 1973; Slattery, 1977, Schmidt, 1980), ii) the nanoclusters model (Holt, 1992; Holt and Horne, 1996; de Kruif and Holt, 2003) and iii) the dual-binding model proposed by Horne (1998, 2006).
Table 1.2 Main physico-chemical characteristics of casein micelles (Fox and Brodkorb, 2008).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Average value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter</td>
<td>120 nm (range 50-500nm)</td>
</tr>
<tr>
<td>Surface area</td>
<td>$8 \times 10^{-10}$ cm$^2$</td>
</tr>
<tr>
<td>Volume</td>
<td>$2.1 \times 10^{-15}$ cm$^3$</td>
</tr>
<tr>
<td>Density (hydrated)</td>
<td>1.0632 g.cm$^{-3}$</td>
</tr>
<tr>
<td>Mass</td>
<td>$2.2 \times 10^{-15}$ g</td>
</tr>
<tr>
<td>Water content</td>
<td>76% w/v</td>
</tr>
<tr>
<td>Hydration</td>
<td>$3.7$ g H$_2$O.g$^{-1}$</td>
</tr>
<tr>
<td>Voluminosity</td>
<td>$4.4$ cm$^3$.g$^{-1}$</td>
</tr>
<tr>
<td>Molecular weight (hydrated)</td>
<td>$1.3 \times 10^9$ g.mol$^{-1}$</td>
</tr>
<tr>
<td>Molecular weight (dehydrated)</td>
<td>$5 \times 10^8$ g.mol$^{-1}$</td>
</tr>
<tr>
<td>Number of peptide chains / micelle</td>
<td>$5 \times 10^3$</td>
</tr>
<tr>
<td>Number of particles per ml of milk</td>
<td>$10^{14}$-$10^{16}$</td>
</tr>
<tr>
<td>Surface area of micelles per ml of milk</td>
<td>$5 \times 10^4$ cm$^2$</td>
</tr>
<tr>
<td>Mean free distance</td>
<td>240 nm</td>
</tr>
</tbody>
</table>

In the sub-micelle model, the caseins aggregate via hydrophobic interactions into sub-units of 15-20 molecules and have an average diameter that ranges from 12 to 15 nanometers (Horne, 2006). The pattern of interaction is such that it causes a variation in the κ-casein content of these sub-micelles. Those rich in κ-casein congregate on the micelle surface, whereas those sub-micelles poor or totally deficient in κ-casein are located in the interior of the micelle as shown in Figure 1.2. In this model the micelle is pushed together mostly by the presence of κ-casein. However, if the casein micelle is depleted of κ-casein, or lacks external sub-units rich in κ-casein, then the CCP is regarded as the cement which links these interior sub-units together (Schmidt, 1980).
Whereas the sub-micelle model emphasised the role of hydrophobic interactions in giving rise to sub-micelles, the nanoclusters model relies solely on the interactions between the caseins and calcium phosphate to hold the micelle together. In the nanoclusters model (Figure 1.3) the $\alpha_s$- and $\beta$-caseins interact with CCP crystallites via their phosphoserine groups until they have completely surrounded CCP and created protein stabilised nanoclusters (de Kruif and Holt, 2003). The formation of nanoclusters would drive micelle formation by randomly binding phosphoproteins causing an ‘inverted’ micelle. More proteins could then coat this new hydrophobic surface and in turn, bind more calcium phosphate until a size limited colloid is formed (Farrell et al., 2006). $\kappa$-casein with its single phosphorylated serine residue interacts weakly or not at all with CCP (Dalgleish, 2011) and thus remains on the surface of the micelle.
Figure 1.3 The nanocluster model of a casein micelle presented by Holt and Horne (1996). Casein molecules ($\alpha_s^1$, $\alpha_s^2$, $\beta$, and $\kappa$-caseins) are thread-like, while the dark circles represent the colloidal calcium phosphate (CCP) nanoclusters.

This approach is broadly similar to the dual-binding model of Horne (1998) (Figure 1.4) which considers the individual caseins as units with different numbers of reactive sites, because they contain phosphorylated domains (charged, hydrophilic) and hydrophobic domains (mainly uncharged, water repellent). The phosphoserine regions of the casein interact with the CCP, leaving the more hydrophobic parts of the caseins exposed on the surfaces of the nanoclusters. This would lead to aggregation of the nanoclusters via hydrophobic interactions, and so the core of the micelles would be formed (Dalgleish, 2011). As the hydrophobically-driven aggregation occurs, the size of the growing aggregates are limited by the binding of the “free” $\kappa$-casein to their surfaces, so that the size of the final micelles depends on the ratio of the $\kappa$-casein to the other caseins. Where the dual-binding and nanocluster models disagree is in the size of the nanocluster and in the number of phosphate clusters (or casein molecules) that the surface of the nanocluster can accommodate (Horne, 2006).
Figure 1.4 A schematic diagram of the dual-binding model for casein micelle structure developed by Horne (1998). Rectangular bars and black lines represent hydrophobic and hydrophilic segments, respectively of the protein sequence.

The controversy and debate over the correct model of the casein micelle is continuously evolving, as new research uncovers further information into the nature of the casein micelle. In recent years, developments in electron microscopy (McMahon and Oommen, 2008), X-ray and neutron scattering techniques (Bouchoux et al., 2010; de Kruijff et al., 2012) have all helped clarify some aspects of the micelle structure. The sub-micelle model was effectively disregarded by the electron microscopy studies of McMahon and McManus (1998), Marchin et al. (2007) and McMahon and Oommen (2008). These authors suggest that the internal micelle structure is quite uniform and that the smaller sub-structures detected are composed of CCP nanoclusters rather than sub-micelles. Therefore, the nanoclusters model generally concurs with the transmission electron micrographs captured and the theories presented by these authors. This knowledge has led to the development of a more refined model for the casein micelle (McMahon and Oommen, 2008). These recent studies suggest that the casein micelle is composed of a highly-hydrated “sponge-like” supramolecular structure, where the
casein molecules form an interlocked lattice (Figure 1.5) where they are associated through calcium phosphate nanoclusters, but also through casein-casein interactions forming linear and branched chains (McMahon and Oommen, 2008; Dalgleish and Corredig, 2012). The micelle has an irregular structure and the proteins have a large diversity of linkages among themselves. Like all the other models, the κ-caseins extend outwards from the main micelle structure. However, the number of these extensions is thought to be much less than has been previously suggested.

![Diagram of the interlocking lattice model of the casein micelle](image)

Figure 1.5 Schematic diagram of the interlocking lattice model of the casein micelle with casein-calcium phosphate aggregates throughout the entire supramolecule and chains of proteins extending between them (McMahon and Oommen, 2008).

Dalgleish (2011) has also proposed a possible new model for the casein micelle (Figure 1.6). This author elucidates that the micelle consists of tubules, presumably of caseins, the ends of which protrude from the bulk of the micelle structure. These tubules are about 20 nm in diameter, which is consistent with the dimensions of the calcium phosphate/casein nanoclusters proposed by Holt et al. (1998). However, the micelle is still protected from close
approach of large particles (such as other micelles) by the protruding tubules. On the other hand, there are large amount of spaces, where individual protein molecules, or even small aggregates of proteins, can approach the micelle (Dalgleish et al., 2004) altering the dynamic equilibrium observed.

In summary, in all the proposed models of casein micelle structure, the glycosylated forms of κ-casein are located at the surface of casein micelles, conferring on them a negative charge and stability due to electrostatic repulsion and steric hindrance. The casein molecules are associated together by a range of protein-protein interactions (e.g. hydrophobic interactions, hydrogen bonding and electrostatic attraction) and also by the presence of CCP (Dalgleish, 2011). There is strong evidence supporting the fact that CCP plays an integral role in the structure of the casein micelle and consequently its contribution to casein micelle structure and stability will be discussed in greater detail in Section 1.3.2. It is concluded that a model based on calcium phosphate nanoclusters is the most appropriate in view of electron microscopic and scattering experiments reported in the literature.

Figure 1.6 Schematic structure of the casein micelle, incorporating calcium phosphate (grey) with their attached caseins (red) and the surface-located κ-casein (green), other caseins are shown in blue (Dalgleish, 2011).
1.2.4 Casein preparation

Casein can be derived from the coagulum formed by treating skim milk with a food-grade acid (acid casein), enzyme (rennet casein), or other food-grade precipitating agents (O’Regan and Mulvihill, 2011). Casein is very sparingly soluble in water and in non-polar organic solvents, but acid casein is soluble in aqueous solutions of alkalis and rennet casein in solutions of calcium chelating salts. The water-soluble salts of casein (produced by reacting acid casein with food-grade alkalis, neutralising agents, enzymes, buffers or chelatants) are known as caseinates (Fox, 2001). For the production of casein products, the fat is firstly removed from raw milk by centrifugal separation to yield skim milk which is then heat treated prior to manufacture of the casein (Figure 1.7). The focus of this discussion will be on acid and rennet casein manufacture, composition and properties.

1.2.4.1 Acid casein

Acidification

Following heat treatment (72 °C x 15s) the skim milk (pH ~6.7) is acidified, the calcium and inorganic phosphate are removed from the casein micelles, the net charge on the micelles decreases, and the micelles become less and less stable until the casein precipitates. Complete precipitation of the casein occurs at the isoelectric point, pH ~4.6. Precipitation can be achieved by acidification of the milk by microbiological (i.e. inoculation with lactic acid producing bacteria), chemical (i.e. direct addition of dilute mineral acid) or indirect acidification processes (i.e. ion exchange resin) (O’Regan and Mulvihill, 2011).
Figure 1.7 Protocol for casein manufacture (Fox, 1982).
**Cooking / Acidulation**

Heating of the acidified milk is typically carried out in the temperature range 45 - 60 °C using direct steam injection, indirect heating (i.e. by means of a heat exchanger), or a combination of both heating mechanisms. Heating promotes agglomeration of the casein curd particles which subsequently synerese to expel whey. Consequently, the curd becomes firmer and is able to withstand the mechanical processing that follows. Both the cooked curd and whey are held in a ‘cooking pipe’ for a period of ~10-60 s before being transferred into an ‘acidulation’ vat. The curd and whey may remain here for a period varying from 30 s to about 15 min, during which time the curd is gently agitated in the whey until acidulation or equilibrium (between the calcium in the curd and that in the whey) is attained.

**Dewheying and washing**

Following acidulation, the curd is separated from the whey using vibratory, moving, or stationary inclined nylon or stainless-steel mesh screens, or polyester fabric screens in a cascade arrangement, or using mechanical devices such as a horizontal bowl centrifuge (decanter) or a roller press. After separation of the curd from the whey, the curd is washed with potable water to remove residual whey constituents such as lactose, salts, and whey proteins.

**Dewatering**

After washing, the curd is mechanically ‘dewatered’ to minimise the quantity of water to be removed during the subsequent drying process (Figure 1.7). The dewatering process typically reduces the moisture content of the curd to ~45-55 % depending on the type of equipment used e.g. roller or belt presses or decanting centrifuges. The texture of the curd is affected by the temperature; therefore, it is necessary to regulate carefully the temperature at the end of washing to optimise the conflicting requirements of minimum water content and maximum friability of the curd.
**Drying**

In accordance with internationally recognised compositional standards for edible casein products, the casein curd is dried to a final moisture content of < 12 % (US Department of Agriculture, 1968). The dry casein particles are then conventionally milled. The casein may then be ground and sieved, using multideck, grating screens, into various particle sizes, usually < 600 µm. Blending after sifting is often used to achieve uniform particle size of the product. Dried acid casein is usually classified using 30-, 60-, 90- and 120-mesh sieves which correspond to mesh openings of 595, 250, 175 and 125 µm, respectively. Casein is then packed into 25 kg multiwall paper bags with plastic liners and stored (Figure 1.7). A typical composition of acid casein is shown in Table 1.3.

**Table 1.3 Typical composition and properties of commercial acid and rennet casein products (adapted from O’Regan and Mulvihill, 2011).**

<table>
<thead>
<tr>
<th>Composition (Amount per 100g)</th>
<th>Acid casein</th>
<th>Rennet casein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture (g)</td>
<td>11.4</td>
<td>11.4</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>1.3</td>
<td>0.8</td>
</tr>
<tr>
<td>Protein (g) dry basis</td>
<td>85.4</td>
<td>79.9</td>
</tr>
<tr>
<td>Lactose (g)</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Ash (g)</td>
<td>1.8</td>
<td>7.8</td>
</tr>
<tr>
<td>Sodium (g)</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Calcium (g)</td>
<td>0.1</td>
<td>3</td>
</tr>
<tr>
<td>pH</td>
<td>4.6 - 5.4</td>
<td>7.3 - 7.7</td>
</tr>
</tbody>
</table>

**Physical properties**

<table>
<thead>
<tr>
<th></th>
<th>Acid casein</th>
<th>Rennet casein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colour</td>
<td>Creamy white</td>
<td>Creamy white</td>
</tr>
<tr>
<td>Solubility in water</td>
<td>Insoluble</td>
<td>Insoluble</td>
</tr>
</tbody>
</table>
1.2.4.2 Rennet casein

In the manufacture of rennet casein, heat-treated skim milk at its natural pH (~6.7) and at 30 °C (or lower) is mixed with a proteolytic enzyme preparation such as calf rennet or, increasingly, genetically engineered chymosin or rennet substitutes such as microbial (aspartic) proteinases in the approximate ratio (by volume) of 1:7500 and held for 20–40 min to induce clotting of the casein (O’Regan and Mulvihill, 2011). Renneting results in cleavage of the κ-casein on the surface of the micelles to form para-κ-casein and soluble glycomacropeptide (GMP); consequently, the modified casein micelles become susceptible to the calcium present in the serum phase of the milk and coagulate at temperatures above 20 °C.

The usual technique for the cooking of rennet casein involves the injection of steam into a cooking line of coagulum pumped from a vat. The cooking temperature employed in manufacturing rennet casein usually varies in the range ~50-60 °C (Carr and Golding, 2015). Other processing steps are similar to those described above for acid casein (Figure 1.7); however, no acidulation step occurs after cooking rennet casein. A typical composition of rennet casein is shown in Table 1.3.

Although the general processing steps for acid and rennet casein are the same, the method employed for precipitation does confer unique properties on the resultant curd, subsequent processing and the final casein composition and properties (Carr and Golding, 2015). As no acid is added during the manufacture of rennet casein, the calcium and inorganic phosphate associated with the casein in the micelles are retained in the casein. Consequently, rennet casein has a much higher ash content (consisting of the oxides of calcium and phosphorus), and a correspondingly lower protein content, than acid casein (Table 1.3). Rennet casein is also devoid of GMP which is lost in the whey resulting in a reduction in yield by ~4 %. Owing to the differences in casein and mineral composition, the potential of acid casein to hydrate and bind water is much higher than that of rennet casein when the pH is reversed.
(from 4.6) to that of native milk (6.7), because of its low calcium level and the presence of the highly hydrated GMP (Guinee, 2011).

1.3 Calcium Equilibria in Bovine Milk

1.3.1 Forms and distribution of calcium in milk

The mineral fraction of milk is relatively small (approximately 8-9 g L⁻¹) (Gaucheron, 2005) and consists of cations (calcium, magnesium, sodium and potassium) and anions (inorganic phosphate, citrate and chloride). Depending on the type of ion, they are present in the aqueous phase or partially associated with casein molecules in the colloidal phase to form casein micelles (Table 1.4). The distribution of the ions between the aqueous and colloidal phases is called salt equilibria.

Table 1.4 Distribution of milk salts (Fox et al., 2015).

<table>
<thead>
<tr>
<th>Species</th>
<th>Concentration mg/l</th>
<th>Soluble %</th>
<th>Form</th>
<th>Colloidal %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium</td>
<td>500</td>
<td>92</td>
<td>Completely ionized</td>
<td>8</td>
</tr>
<tr>
<td>Potassium</td>
<td>1,450</td>
<td>92</td>
<td>Completely ionized</td>
<td>8</td>
</tr>
<tr>
<td>Chloride</td>
<td>1,200</td>
<td>100</td>
<td>Completely ionized</td>
<td></td>
</tr>
<tr>
<td>Sulphate</td>
<td>100</td>
<td>100</td>
<td>Completely ionized</td>
<td></td>
</tr>
<tr>
<td>Phosphate</td>
<td>750</td>
<td>43</td>
<td>10 % bound to Ca and Mg</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>51 % H₂PO₄⁻</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>39 % HPO₄²⁻</td>
<td></td>
</tr>
<tr>
<td>Citrate</td>
<td>1,750</td>
<td>94</td>
<td>85 % bound to Ca and Mg</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>14 % Citr³⁻</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 % HCitr²⁻</td>
<td></td>
</tr>
<tr>
<td>Calcium</td>
<td>1,200</td>
<td>34</td>
<td>35 % Ca²⁺</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>55 % bound to citrate</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10 % bound to phosphate</td>
<td></td>
</tr>
<tr>
<td>Magnesium</td>
<td>130</td>
<td>67</td>
<td>Similar to calcium</td>
<td>33</td>
</tr>
</tbody>
</table>

Among these salts, calcium and phosphate are of importance in maintaining the casein micelle structure (Walstra, 1999). There is approximately 29 mM of calcium in milk, of which 18.8
mM is colloidal and 10.2 mM is in the aqueous phase. Of the latter, free ionic calcium is ~2 mM (Tsioulpas et al., 2007) and 6.97 mM is co-ordinated to citrate (as Cit\textsuperscript{3–}) and 0.67 mM to phosphate (as a mixture of H\textsubscript{2}PO\textsubscript{4} and HPO\textsubscript{4}\textsuperscript{2–}) (Holt, 2004). Similarly, there is approximately 20.6 mM of phosphate in milk. Of this, about 8.2 mM is colloidal, 0.67 mM is bound to calcium in the aqueous phase and 10.15 mM is free inorganic phosphate present as H\textsubscript{2}PO\textsubscript{4} and HPO\textsubscript{4}\textsuperscript{2–} (Holt, 2004). The solubility of calcium phosphate is very low and consequently its concentration in the aqueous phase is less than 1 mmol L\textsuperscript{–1}. Thus, at pH 6.6-6.7, the milk aqueous phase is supersaturated with calcium phosphate and its ionic strength is ~80 mmol L\textsuperscript{–1} (Holt, 2004).

The colloidal calcium in milk can be defined as a mixture of calcium caseinate (containing organic phosphate) and calcium phosphate (which is an inorganic phosphate) (Gaucheron, 2005). Calcium caseinate is the proportion of colloidal calcium in milk that is directly attached to the negatively charged organic phosphate and carboxylic acid groups of the protein (Holt, 2013). Experimentally, these two types of colloidal calcium are not separable, and consequently it is difficult to calculate the composition of calcium phosphate alone (McGann and Pyne, 1960). Holt (2013) helped distinguish between calcium caseinate and calcium phosphate, by suggesting the former referred to the structure in the native micelle, whereas calcium phosphate to only the small ions in the structure and not including the interacting polypeptide chains.

1.3.2 Colloidal calcium phosphate (CCP)

The casein micelle contains approximately 3 x 10\textsuperscript{3} nanoclusters of CCP in an amorphous state (Holt, 1992; Gaucheron, 2005). CCP is composed of calcium and organic and inorganic phosphates (P\textsubscript{o} and P\textsubscript{i}, respectively) at concentrations of about 20, 10 and 7.5 mM, respectively (Holt, 1985). Along with amorphous calcium phosphate, it is also reported that CCP contains sodium, potassium, magnesium and citrate (McGann et al., 1983; Lyster et al.,
1984; Gaucheron, 2005; Walstra et al., 2006). The exact composition of CCP depends on the ionic environment, indicating that it has dynamic ion-exchange properties (Gaucheron, 2005; Walstra et al., 2006).

As described in Section 1.2.3 the internal structure of the casein micelle consists of a protein matrix (\(\alpha_{s1}\), \(\alpha_{s2}\) and \(\beta\)-caseins) in which CCP nanoclusters of \(\sim 2.5\) nm radius are dispersed (McGann et al., 1983; Holt, 1992; Gaucheron, 2005; Walstra et al., 2006; Marchin et al., 2007). The CCP nanoclusters are of critical importance to the structure of the casein micelle as they can cross-link numerous casein molecules and reduce electrostatic repulsion, allowing formation of the casein micelle (Horne, 1998). The CCP is suggested to be bound to phosphorylated serine residues of casein micelles, neutralising their negative charge and giving them stability and structure (Tuinier and de Kruif, 2002; Horne, 2006; Dalgleish and Corredig, 2012). However, whether the CCP particles themselves are charged or not is uncertain. On removal of CCP (by acidification, dialysis or a calcium chelator), the micelles disperse into particles of \(\sim 0.5 \times 10^6\) Da, further confirming that CCP plays an integrating role in the micelles.

**1.3.3 Modification of calcium equilibria**

Casein micelles are in dynamic equilibrium with the aqueous phase. According to the physico-chemical conditions, they can exchange casein molecules, calcium, inorganic phosphate and water with the aqueous phase (Figure 1.8). In some cases, ions and casein molecules can be transferred from the colloidal to the aqueous phase. In other cases, these constituents can be integrated in casein micelles (Broyard and Gaucheron, 2015).
Figure 1.8 Schematic representation of exchanges of minerals, water, and casein molecules as a function of different physico-chemical conditions (Broyard and Gaucheron, 2015).

Lowering the pH decreases the stability of the casein micelle through demineralisation. During acidification, CCP is solubilised (Dalgleish and Law, 1989) and the κ-casein micellar tails collapse (Vasbinder and de Kruif, 2003). The subsequent aggregation of casein has been proposed to be driven by a complex interaction including hydrophobic, van der Waals and calcium-bridging attractions (Lucey, 2002). Removal of a substantial amount of calcium upon acidification without disruption of the casein micelle can be achieved, possibly because of neutralisation of charged residues due to protonation upon acidification, leading to association of casein micelles (Dalgleish and Law, 1989; Lucey et al., 1997).

The solubility of calcium phosphate is markedly temperature-dependent; unlike most compounds, the solubility of calcium phosphate decreases with increasing temperature –
therefore heating causes precipitation of calcium phosphate (Gaucheron, 2004) while cooling increases the concentrations of soluble calcium and phosphate at the expense of CCP (Pierre and Brule, 1981). Added calcium reacts with soluble phosphate and results in precipitation of CCP, an increase in ionised calcium, a decrease in the concentration of soluble phosphate and a decrease in pH. Philippe et al. (2003) studied the physico-chemical properties of skim milk enriched with CaCl₂ and reported that this caused an increase in micellar density and resulted in a more compact micellar core. The increase in ionic strength by adding NaCl, also has a significant effect on ion speciation such as a decrease in pH, an increase in Ca²⁺ concentration, a decrease in the activity co-efficient of ionic species and an increase in the hydration of casein micelles (Gaucheron, 2005; Huppertz and Fox, 2006).

All of these changes induced to calcium equilibria can thus have a significant impact on the physico-chemical properties of the casein micelles and the effects of the aforementioned processes have been previously reviewed in more detail in the literature (Holt et al., 1986; Dalgleish and Law, 1988, 1989; Griffin et al., 1988; Ward et al., 1997; Choi et al., 2007; Augustin and Clarke, 2008; Kaliappan and Lucey, 2011). The focus of this discussion will be on changes induced to casein micelle structure and functionality as influenced by addition of calcium chelators.

1.3.3.1 Effects of addition of calcium chelators on casein micelle structure and functionality

As previously mentioned, calcium in the aqueous and colloidal phases of milk and dairy products are in equilibria (Figure 1.9). Addition of calcium chelators (e.g. ortho-, poly-, and pyrophosphate, citrate, EDTA etc.), to casein micelles shifts the casein-mineral equilibria (Figure 1.9), resulting in a decrease in concentration of free Ca²⁺ ions in the aqueous phase, solubilisation of CCP (as the degree of calcium phosphate saturation of the aqueous phase is reduced) and the release of specific caseins from the micelle (Griffin et al., 1988; Holt, 1992; Panouillé et al., 2004; Gaucheron, 2005). The free Ca²⁺ ions can influence the environment of
the negatively charged casein micelles, leading to enhancement or reduction of the repelling forces among them (Horne and Parker, 1981). Chelation of free Ca\(^{2+}\) ions by calcium chelators increases the repulsion between caseins within the negatively charged casein micelles, resulting in an increase in hydration and voluminosity of the micelles and a decrease in turbidity of milk solutions (Mizuno and Lucey, 2005; Kaliappan and Lucey, 2011). This decrease in turbidity corresponds to a destruction of the casein micelle structure with a release of small aggregates of non-sedimentable casein molecules in the aqueous phase (Broyard and Gaucheron, 2015).

Figure 1.9 Equilibria between free calcium ions, calcium chelator complexes, and casein micelles in dairy systems (de Kort, 2012).

Panouillé et al. (2004) reported that these small micellar particles appeared to be similar to the aggregates present in sodium caseinate solution. Pitkowski et al. (2008) demonstrated that these small aggregates contained 10-15 casein molecules with hydrodynamic radius of ~10 nm. Furthermore, it has been shown that casein micelles may eventually dissociate into even smaller clusters and dispersed proteins at greater chelator concentrations (Vujicic et al., 1968; Morr et al., 1971; Lin et al., 1972; Munyua and Larsson-Raznikiewicz, 1980; Griffin et al., 1980).
However, the extent to which calcium chelators affect the micellar structure depends on their calcium-binding capacity (van Wazer and Callis, 1958; de Kort et al., 2009), the type of ionic species forming the calcium chelator, pH, ionic strength, temperature (Lucey et al., 2011) and their interaction with the calcium ions and amino acids in the casein micelle (Zittle, 1966; Vuijicic et al., 1968; Holt et al., 1981; Holt, 2004; Gaucheron, 2005; Mekmeme et al., 2009; Gao, 2010).

As a result of the physico-chemical modifications of casein micelle structure outlined above, calcium chelators alter the functional properties of casein. de Kort et al. (2011) reported that the presence of calcium chelators induced an increase in viscosity of concentrated micellar casein dispersions. The increase in viscosity was attributed to swelling of the casein micelles (i.e. increased voluminosity) at decreasing calcium-ion activity. The major decrease in turbidity was reportedly due to dissociation of the casein micelles into smaller structures. Besides their generic property of calcium-binding, calcium chelators are reported to generate specific effects e.g. disodium phosphate (DSP) and trisodium citrate (TSC) both chelate calcium from the casein micelle, but calcium phosphate precipitates in the micelle (Guo, 2003), whereas calcium citrate remains as stable, soluble complexes in the aqueous phase (Mizuno and Lucey, 2007; Tsioulpas et al., 2007). Phosphates not only bind to free Ca²⁺ ions in the aqueous phase, but can also bind to the positively charged amino acids of casein residues (de Kort et al., 2011; Anema, 2015) and can remain attached to the casein micelle. Phosphates associated with casein might also have potential to act as a cross-linking agent by bridging within or between casein molecules (Lucey et al., 2003); therefore under certain conditions some types of phosphates can induce gelation or aggregation by the formation of some type of caseinate-Ca phosphate complexes (Lauck and Tucker, 1962; Leviton, 1964; Zittle, 1966; Panouillé et al., 2004). In contrast, TSC does not bind to the casein molecules; and thus, no gel formation has been reported within systems containing TSC because this
calcium chelator does not cause caseins to cross-link upon dissociation (Mizuno and Lucey, 2005).

Calcium chelators are used in the dairy industry to increase the heat stability and storage life of concentrated or sterilised milk products or to retard age gelation in dairy products (Leviton and Pallansch, 1962; Harwalker, 1982; Holt, 1985; Kocak and Zadow, 1985; Augustin and Clarke, 1990; Singh et al., 1995). They are also commonly used to help disrupt the calcium-phosphate linked para-casein network present in natural cheese or insoluble milk protein ingredients such as rennet casein during manufacture of processed and analogue cheese (Kapoor and Metzger, 2008). The following section will outline the impact of calcium chelators on the structure and functionality of the para-casein network in rennet casein during manufacture of such products.

1.4 Food Structure and Functionality

1.4.1 Casein-based food structures

Underlying the structures of most dairy-based foods (e.g. cheese, yoghurt and other fermented milk products) are the casein micelles (Dalgleish, 2014). The self-assembly and surface-active properties of the casein polymers makes them highly suitable for the formulation of food structures of varying consistency, such as, gels (e.g. yoghurt and cheeses), foams (e.g. whipped cream or whipped desserts) and emulsions (e.g. milk, cream, butter) (Dickinson, 2006). Milk is a colloidal suspension in which the solid components dissolve or disperse in a continuous water phase. From a rheological perspective, milk can be simply categorised as a fluid because if deforms and flows immediately when subjected to a stress (Vélez-Ruiz et al., 1997). However, casein-based systems with a higher level of structural complexity, such as yoghurt and cheeses are considered semi- or soft-solid, viscoelastic food materials (Foegeding et al., 2011; Dickinson, 2012).
From the perspective of states of matter, van Vliet et al. (2009) classified food materials as fitting into three broad categories: fluids, semi-solids (or soft-solids) and hard-solids. Bourne and Szczesniak (2003) describe solid food (which breaks) and fluid food (which flows) as two extremes of one continuum, with semi-solid food in the middle. Semi-solids are fluid-like, have a high yield stress, and deform or break without fracturing into pieces (Campbell et al., 2016). However, the definition of semi-solids can be difficult as many of these types of food materials display both viscous and/or elastic properties (McKenna, 2003).

The structure of semi- or soft-solid casein-based food materials is dependent on the nature of the interactions between casein particles involving calcium. In making yoghurt and other fermented dairy products, the action of lactic acid bacteria lowers the pH from ~6.7 to 4.5, this initiates the release of CCP from the micelle which subsequently diffuses in the serum phase (Laligant et al., 2003; Le Graët and Gaucheron, 1999). This process converts a liquid-like dispersion of casein particles into a soft solid-like aggregated network (Horne, 1999). Rennet-induced gelation is the primary step in the manufacture of most cheese varieties. The addition of the enzyme chymosin cleaves the Phe\textsubscript{105}–Met\textsubscript{106} bond of κ-casein which destabilises the casein micelle leading to the Ca\textsuperscript{2+}-mediated aggregation of para-casein micelles, ultimately forming a three-dimensional gel network of partly fused para-micelles (Lucey, 2002). If natural cheese is used for further processing e.g. in processed cheese manufacture, the three dimensional calcium para-casein network is partially disintegrated by the action of calcium chelating salts, which largely contributes to the formation of a smooth, homogeneous and stable, semi-solid casein-based food matrix i.e. processed cheese. The following section is focussed on the basic principles and practices underlying the structure formation and functionality of semi-solid foods containing milk protein (i.e. casein) as the major functional ingredient e.g. processed cheese and analogues. These casein-based systems were chosen in order to gain a greater understanding of the basis of calcium-casein
interactions as affected by calcium chelators, which is of critical importance in order to be able to influence the final structure / texture of concentrated casein-based food matrices.

1.4.2 Processed cheese and analogues

Composite high protein emulsion gels e.g. processed and analogue cheese are semi-solid proteinaceous food materials containing dispersed oil droplets. These matrices can be formulated by blending selected ingredients (e.g. natural cheese and/or milk proteins, water, fat, calcium chelators and optional ingredients) together and heating to produce a stable, molten, oil-in-water emulsion which sets on cooling and acquires texture characteristics similar to those of cheese. An important advantage of these food materials over natural cheese is the amenability of their formulations to be easily altered to yield products with customised nutritional, compositional and functional attributes (e.g. high melt, firm texture, low-fat, low-salt etc.) Other advantages include; their lower production costs (owing to replacement of milk fat with cheaper vegetable oils, absence of a ripening period, reduced capital costs of manufacturing equipment) and simplicity of manufacture from readily available raw materials (Bachmann, 2001). There is a wide range of processed cheese products (PCPs), i.e. natural products made from blending different cheeses to form a range of semi-solid and spreadable products, and cheese analogues (made not from cheese, but from dairy and non-dairy ingredients) available to the food market worldwide. Depending on their composition and the ingredients used in their manufacture PCPs and cheese analogues can be further divided into different sub-categories, these have been discussed in detail in the literature (Bachmann, 2001; Guinee et al., 2004; Noronha et al., 2011) and therefore will not form part of this review. PCPs and cheese analogues are extensively used as ingredients in the convenience food sector (e.g. as a topping on pizza, filling in appetizers, slices on hamburgers and sauces in pasta dishes), and are of increasing economic value in industrialised and developing countries (Lucey, 2008a).
1.4.3 Principles of manufacture

The manufacturing technology for cheese analogues is very similar to that used for PCPs. Cheese analogues differ from PCPs in that the protein source, in the form of a dry powder, is rennet casein, rather than the natural cheese varieties (rennet or acid curd) typically used in PCPs. However, in both cases the protein (e.g. isoelectric casein in acid curd cheeses or acid casein powder; or para-casein in rennet curd cheeses or rennet casein powder) hydrates poorly and is effectively insoluble in water, so adequate hydration and solubility is achieved by the addition of calcium chelating salts (CCS) (e.g. disodium phosphate and trisodium citrate) along with heat (80 °C) and agitation applied during manufacture. The effects of CCS on this process will be discussed in greater detail in Section 1.4.4.

The manufacturing process for rennet casein-based products tends to be quick and uncomplicated as described previously (Noronha et al., 2008a; El-Bakry et al., 2010a) and will not be the focus of this discussion. Briefly, in manufacturing rennet casein-based products, typically batch cookers are used, two common types of cookers use single/twin-screw augers e.g. Blentech (Noronha et al., 2008a) or high speed cutting blades e.g. Stephan (Noronha et al., 2008a). Some products have also been manufactured on a semi-micro scale using a Farinograph batch mixer (Noronha et al., 2008b; El-Bakry et al., 2010a). Figure 1.10 provides a schematic diagram of the typical steps involved during their manufacture. Generally, the water, lipid and rennet casein are blended at ~50 °C in the presence of CCS. The CCS promote, with the aid of heat and shear, the conversion of the insoluble rennet casein to a hydrated sodium para-caseinate. The para-caseinate emulsifies the dispersed oil droplets during processing and thereby contributes to the formation of a stable-oil-in-water emulsion. The addition of CCS also causes the pH of the mixture to increase, which further aids in protein dispersion and hydration. When the casein and lipid are mixed, the temperature of the mixture is increased to ~80 °C (this can vary, but is usually in the range 70-95 °C),
using direct or indirect steam injection until the desired temperature is reached, which usually takes 4-6 min. Cooking and agitation continues until a homogeneous smooth plastic mass is obtained, with no free oil or water present in the cooker. At this stage acidulants, such as citric acid, can be added to adjust the pH to the final desired value, typically between pH 5.9-6.1 (Noronha *et al.*, 2008c). Once cooked the cheese product is discharged from the cooker, is cooled rapidly and stored at 4 °C until required.
Figure 1.10 Schematic illustration of the typical manufacturing procedure for rennet casein-based food matrices.
1.4.4 Role of calcium chelators during and post manufacture

Similar to rennet curd cheese, the protein in rennet casein (i.e. para-casein) has limited hydration capacity and solubility in water due to extensive calcium-mediated intermolecular cross-linking among caseins (Ennis et al., 1998). Added CCS can chelate calcium and disrupt the calcium phosphate-linked para-casein network of rennet casein through their ion-exchange properties; the divalent calcium (Ca$^{2+}$) of the para-casein matrix is exchanged with the monovalent sodium (Na$^+$) or potassium (K$^+$) of the chelating salt (Figure 1.11). The calcium-binding anion of the CCS ultimately competes with the phosphoseryl residues and CCP (de Kort et al., 2011) for Ca$^{2+}$ in the para-casein. This reduces the extent of calcium-mediated protein aggregation, causing partial breakdown and dispersion of the para-casein network and facilitates its conversion into sodium para-caseinate (Figure 1.11) and thus allows increased opportunity for protein-water interactions to occur.

Figure 1.11 Simple model showing the effect of calcium chelating salts on the para-casein network of rennet casein. The cross-linking of casein chains by means of divalent calcium ions (Ca$^{2+}$) in conjunction with calcium phosphate is broken. Water soluble caseinates of monovalent cations (Na$^+$) are formed.

The dispersed and hydrated sodium para-caseinate subsequently acts as an emulsifier for the oil phase dispersed in the aqueous protein phase of the cheese system (Ennis et al., 1998). The
protein component stabilises the oil-in-water emulsion by reducing the interfacial tension at the aqueous phase-oil droplet interface and by increasing the viscosity of the aqueous phase thereby decreasing the frequency of collisions between oil droplets (Ennis and Mulvihill, 1999). The increase in pH initiated by CCS addition also affects the para-casein configuration, solubility and extent to which the CCS bind Ca$^{2+}$ (van Wazer and Callis, 1958; de Kort et al., 2009). The change in charge (i.e. increase in net negative charge) on the proteins during emulsification and their hydration throughout mixing has an impact on the water-binding capacity of casein (Zehren and Nusbaum, 2000), which further aids in protein dispersion and hydration (El-Bakry et al., 2011a).

The nature and extent of hydration of the para-casein (primarily governed by interactions between the casein protein, calcium and CCS) is critical in determining its functional performance (solubility, emulsifying properties, viscosity, structure forming ability) during manufacture of processed and analogue cheese. However, few researchers have studied the dynamics of calcium-ion exchange processes as influenced by CCS, which clearly has a modulating effect on the structure and subsequent hydration of the para-casein. Ennis et al. (1998) investigated the effects of CCS on casein hydration and reported that this process was dependent on the concentration of CCS and occurred in a number of stages: casein swelling followed by clumping of swollen particles, network formation and subsequent breakdown of the protein network. These stages in the hydration process were highly dependent on the presence of CCS, i.e. in the absence of CCS the casein was only slightly swollen and there was no network formation observed. In similar systems that contained fat, it was further demonstrated that fat emulsification only occurred after adequate casein hydration, as in the absence of CCS the poorly hydrated casein was not capable of stabilising the fat phase (Ennis and Mulvihill, 1999). Ennis et al. (1998) also reported that rennet casein particles swelled only slightly in water at 55 °C with little increase in viscosity, however, when dispersed in
solutions of CCS a highly viscous pseudoplastic dispersion resulted. These, and other similar studies were carried out using dilute model systems (Ennis et al., 1998; Ennis and Mulvihill, 1999; Ennis et al., 2000; O’Sullivan and Mulvihill, 2001) which can make extrapolation to real semi-solid food matrices such as processed and analogue cheese difficult. In addition, the interactive effects of the type and concentration of CCS, temperature and pH were not investigated; therefore, it might be argued that these studies did not truly simulate the processing conditions and the more concentrated and mixed CCS systems encountered during industrial processing of processed and analogue cheese. Consequently, the interaction of CCS with the para-casein during manufacture of these dense protein systems is still not clearly understood (Kapoor and Metzger, 2008). Currently in the scientific literature, there are many discrepancies between studies reporting the role of CCS in changing physico-chemical parameters of casein, most likely due to differences in casein concentration, the ratio of casein to CCS applied, the type of CCS used, along with varying processing conditions. Hence, there is scope to follow on from the aforementioned preliminary studies and investigate the hydration of casein during matrix development of real concentrated semi-solid food matrices, with a greater focus on how calcium concentration as affected by addition of CCS has a modulating effect on this process. More definitive conclusions could then be made regarding the role of CCS in product manufacture if this fundamental knowledge of calcium-casein interactions was available.

In addition to their role during manufacture, CCS also help control melting, texture and free oil formation in the final processed or analogue cheese product (Fox et al., 2000). In fact, the focus of most research work on the role of CCS has been largely on the post-manufacture properties rather than the dynamics of calcium-ion exchange processes leading to protein hydration and functionality during manufacture. There have been many studies concerning the effects of CCS on processed cheese functionality (Templeton and Sommer, 1936; Gupta et
al., 1984; Cavalier-Salou and Cheftel, 1991; Swenson et al., 2000; Zehren and Nusbaum, 2000); however, such investigations have been rather limited in the context of rennet casein-based systems e.g. cheese analogues (El-Bakry et al., 2010b; El-Bakry et al., 2011b). The most common types of CCS used in processed and analogue cheese manufacture are sodium phosphates and citrates, the detailed physico-chemical properties of which are well reported (Berger et al., 1989; Zehren and Nusbaum, 2000; Kapoor and Metzger, 2008; Lucey et al., 2011).

There has been mixed interpretation regarding the influence of CCS on processed and analogue cheese functionality, mainly due to different processing conditions employed i.e. different manufacturing times and pH values combined with the use of different types of proteins and post-manufacture functional tests (Cavalier-Salou and Cheftel, 1991). It has previously been reported that cheeses manufactured using disodium phosphate (DSP) as the sole CCS result in a weak body, grainy texture and larger fat globules compared to those made with trisodium citrate (TSC) as the sole CCS (Purna et al., 2006). Processed cheese products manufactured with phosphates have been previously reported to have a lower level of casein dissociation, as determined using the so-called ‘peptidisation co-efficient’ method, with values of 84-86 % obtained; whereas those prepared using citrate had a higher value of 100 % (Dimitreli et al., 2005; Dimitreli and Thomareis, 2008). Savello et al. (1989), Cavalier-Salou and Cheftel (1991) and El-Bakry et al. (2011b) reported increased melting of processed and analogue cheese with increased TSC content, in comparison to products containing DSP at equivalent concentrations. The hardness of these products increased however when greater proportions of DSP were used. This could be due to the more effective calcium chelation and casein dissociation effected by TSC than by DSP or a mixture of TSC and DSP, as has been reported previously (Cavalier-Salou and Cheftel, 1991; Shirashoji et al., 2006). In industrial applications e.g. in processed and analogue cheese manufacture, a combination of both DSP
and TSC are commonly used, firstly to provide desired functional properties, e.g. a good melting cheese with moderate hardness, and secondly phosphates are also added for their antimicrobial characteristics (Guinee et al., 2004; El-Bakry et al., 2011b).

1.4.5 Challenges associated with removal of calcium chelators

The effects of CCS on the properties of processed and analogue cheese have been described in the previous section. However, CCS contribute to a relatively high sodium content in the end products and lead to what is considered an ‘unclean’ label from a consumer perspective, particularly in respect to phosphates. In recent years there has been an increase in consumer demand for food products formulated with clean label ingredients (Brockman and Beeren, 2011; Lähteenmäki, 2015). This has translated into the “clean label trend” currently driving product development within the food industry and consequently formulating products with simple labels using recognisable ingredients is a growing commercial necessity (Boothroyd, 2014). In addition, the global high prevalence of hypertension and cardiovascular disease has raised concerns regarding the sodium content of the foods which we consume (Dötsch et al., 2009). CCS contribute significantly to the sodium content of processed and analogue cheese formulations (Johnson et al., 2009). Hence, inclusion of CCS is construed as negative by consumers and their elimination could bring about a number of innovations to increase the nutritional value of the end-products, e.g. low-sodium cheeses, which could improve the negative perception of processed and analogue cheese products, along with the added benefit of formulating end-products with cleaner labels. In addition, alternative mechanisms to facilitate the formation of hydrated matrices from rennet casein without the need for CCS could result in the development of casein-based products with novel structures and functionalities which may have applications towards tailoring these food products for specific end-uses.
Reducing or eliminating CCS, however, presents a significant challenge, given their influence on critical events during manufacture and end-product functional properties. During manufacture of processed and analogue cheese without CCS, the application of heat and shear to the mixture typically results in a heterogeneous mass, excessive viscosity, with exudation of oil and separation of water in cooking (Guinee and O’Kennedy, 2007; Cruz et al., 2011). Therefore, currently it is not possible to form these types of matrices without the aid of CCS.

Previous research efforts for elimination or reduction of CCS have been focussed on substitution of sodium-based CCS with their potassium equivalents (Metzger and Kapoor, 2007; El-Bakry et al., 2011c), various salt replacers (Schäffer et al., 1999; Schäffer et al., 2001; Černíková et al., 2010), traditional emulsifiers (Lucey, 2008b; Paulus, 2008) or casein hydrolysates (Kwak et al., 2002), often having a negative impact on the functionality or sensory acceptance of the final cheese product.

The major concern with the use of potassium-based CCS is their effect on flavour and sensory properties such as a decrease in the overall saltiness and development of bitter and chemical–metallic off-flavours in the final products (Karahadian and Lindsay, 1984; El-Bakry et al., 2011c). Casein hydrolysates have been suggested as possible substitutes for CCS in processed cheesemaking (Kwak et al., 2002) and while they are capable of emulsifying the fat (i.e. reduce free oil formation) just like the solubilised caseins do in the sodium para-caseinate network created by the action of CCS, they do not exhibit the other functions of CCS (e.g. calcium binding, protein hydration). Moreover, casein hydrolysates can impart a bitter flavour to processed cheese products (Kwak et al., 2002). Černíková et al. (2010) reported that hydrocolloids e.g. locust bean gum, modified starch and low methoxyl pectin cannot be recommended as replacements for phosphate- and citrate- based CCS in processed cheese production as in these cases the oil and aqueous phases cannot be combined in a homogeneous mix in the absence of CCS. In contrast, the same authors reported that when κ- and τ-
carrageenan were incorporated as replacements for CCS a homogeneous matrix was formed, however, the resulting products were very hard with fracturable texture.

Approaches for the reduction of CCS have included reducing the actual level of CCS in the formulation and altering the ratio of CCS used, both of which had a major influence on cheese manufacture and subsequent functional properties (El-Bakry et al., 2010b; 2011a, b). In addition, neither of these approaches could yield a CCS reduction of more than 20 %. El-Bakry et al. (2010b) reported that reducing the concentration of CCS during manufacture of cheese analogues increased processing time and hardness and decreased flowability, viscoelasticity and fat globule diameter compared to products made at standard CCS concentrations. The same author reported in another study (El-Bakry et al., 2011b) that increasing the DSP:TSC ratio from 0:1 to 1:0 caused a decrease in processing time during manufacture and post manufacture led to a decrease in hardness and G’ at 25 °C and increased fat globule size. Therefore reducing the concentration or altering the ratio of CCS could not be achieved without altering the functional properties of the formed matrices. Therefore, further research is required to achieve greater levels of CCS reduction or even total elimination without adversely affecting the sensory or functional properties of these products.

1.5 Food Structure and Digestibility

1.5.1 Structural aspects of food digestion

Recently there has been growing interest in understanding how food structure and matrix design influences the rates of nutrient digestion and bioavailability (i.e. the amount of ingested nutrient that is absorbed and available for physiological functions). This research is being undertaken with a view to developing novel foods that may help regulate calorie intake, provide increased satiety responses, provide controlled digestion, and/or deliver bioactive molecules to specific locations in the gastrointestinal tract (GIT) (Singh and Sarkar, 2011).
Individual foods differ considerably in their nutrient composition and also in terms of the matrix materials within which nutrients are embedded. Digestion is the process of breaking down these materials to allow the release of individual nutrients that can then be absorbed through the wall of the human GIT (Singh and Gallier, 2014).

Digestion starts in the mouth, where the ingested food is exposed to temperature change, lubrication with saliva, mechanical breakdown via mastication and enzymatic breakdown through the action of salivary α-amylase. The generated bolus is swallowed and enters the stomach where gastric acid and digestive enzymes are released and the food is mixed by muscle contraction and peristaltic movement of the stomach wall (Norton et al., 2015). From the stomach this “chyme” enters the small intestine the major site of digestion and nutrient absorption. Here, nutrients are emulsified by bile salts and hydrolysed by pancreatic enzymes, and then absorbed across the intestinal epithelium into the blood stream. The “chyme” then enters the large intestine, where fermentation by bacterial microflora occurs, water is absorbed, and waste is stored before exiting the body (Norton et al., 2015). A more detailed overview of this process has been covered in recent publications (Kong and Singh, 2008; Lentle and Janssen, 2008; Boland, 2016). The ingested food will experience different shear and forces, temperatures, enzymatic behaviour and pH during its transit through the GIT (Figure 1.12), which will affect the breakdown and uptake of nutrients (Norton et al., 2014). The gastric residence time and the rate and extent of digestion is influenced by the physical form of the food material consumed (Bornhorst and Singh, 2014).
1.5.2 Assessment of digestibility

*In vitro* digestion studies are commonly used to simulate the digestion and bioaccessibility of nutrients (i.e. the amount of nutrient potentially available for absorption). These studies have been applied in an attempt to more fully understand how the major structuring units of food materials (i.e. carbohydrates, proteins and fats) are digested, and also the influence that different food microstructures have on the accessibility and availability of these macronutrients. The rate of digestion and absorption of dietary carbohydrates (simple sugars and complex carbohydrates) have attracted a lot of interest because an excess of digestible carbohydrate in the diet is associated with obesity, metabolic syndrome and diabetes (Jenkins *et al.*, 2002; Cummings *et al.*, 2004; Opperman *et al.*, 2004). Food matrix organisation has previously been reported to impact the rate of carbohydrate digestion and absorption (Englyst...
and Englyst, 2005) and the effects of different food microstructures on these processes has been covered in review articles by Turgeon and Rioux (2011) and Norton et al. (2015). A dense food matrix organisation has been shown to delay the enzymatic degradation of starch, as observed for pasta (Fardet et al., 1998). Slowly digestible starch is the starch fraction with slow but complete hydrolysis in the small intestine. Its physiological advantage compared to rapidly digestible starch lies in that it is a source of sustained glucose and thus has a stabilising effect on the blood glucose level; the benefits of which may be linked to diabetes management and effects on satiety/food intake (Lehmann and Robin, 2007). Foods that are rich in soluble fiber and have high fat content create a more viscous chyme and have been associated with longer gastric emptying. This also contributes to the prolonged absorption of glucose. The fiber effect on glycemia depends on specific characteristics such as the solubility and structure of the food. Their action can be the result of increasing viscosity, interfering with enzymatic action or forming electrostatic interactions between polysaccharides, proteins and peptides, limiting access to the cleavage site for the enzyme.

Recently, food structure and its associated changes during digestion have been reported to play an important role on the kinetics of protein digestion (Fang et al., 2016). The rate of protein digestion influences amino acids (AAs) release and their subsequent absorption into plasma. This may affect the postprandial utilisations of proteins, for example, the protein anabolic response and satiating effect (Dangin et al., 2003; Lacroix et al., 2006; Breen and Philips, 2011). Caseins, for example, coagulate in the stomach acidic environment, while whey proteins remain soluble (Fang et al., 2016). As a result, overall gastric emptying time for casein is longer and there is a smaller postprandial increase in plasma AA compared with the non-coagulating whey protein (Hall et al., 2003). Therefore, the postprandial protein anabolic response is more effective for caseins than for whey proteins, as the release of AAs from ingested whey proteins is too fast to sustain the anabolic requirement during the
postprandial period (Lacroix et al., 2006). Gelled protein structures are reported to slow down the kinetics of absorption of AAs (by limiting the accessibility of enzymes to proteins) and are thought to be potentially advantageous in weight-reducing diets as they could have more satiating effects (Barbé et al., 2013). The bioavailability of AAs during in vivo and in vitro digestion of a semi-solid (yoghurt) food material was reduced in comparison to a liquid matrix (commercial milk) (Rinaldi et al., 2014). Although this was attributed to different processing parameters, particularly the severity of the heat treatment applied, which had more impact on milk protein digestion than the matrix structure per se. These studies have helped establish that the matrix structure is also a factor to consider when evaluating the nutritional properties and possible health effects of a food material.

1.5.3 Food structure impact on lipid digestibility

1.5.3.1 Lipid digestion

Fat digestion involves enzymatic hydrolysis (lipolysis) of triglycerides by the enzyme lipase into free fatty acids and glycerol, which can then be absorbed and used by the body. The main steps involved in lipid digestion are outlined in Figure 1.13. Fat digestion begins in the stomach with the aid of lingual lipase and gastric lipase. However, the bulk of lipid digestion occurs in the small intestine due to the action of pancreatic lipase (Porter et al., 2007). When “chyme” enters the duodenum, the hormonal responses trigger the release of bile, which is produced in the liver and stored in the gallbladder. Bile aids in the digestion of lipids, primarily triglycerides, through emulsification. Emulsification involves the breakdown of fat globules in the duodenum into tiny droplets, which provides a larger surface area on which the enzyme pancreatic lipase can act to digest the fats into fatty acids and glycerol. These molecules can pass through the plasma membrane of the cell, entering the epithelial cells of the intestinal lining. The bile salts surround long-chain fatty acids and monoglycerides, forming tiny spheres called micelles (Figure 1.13). The micelles move into the brush border
of the small intestine absorptive cells where the long-chain fatty acids and monoglycerides diffuse out of the micelles into the absorptive cells, leaving the micelles behind in the “chyme” (Fave et al., 2004). The long-chain fatty acids and monoglycerides recombine in the absorptive cells to form triglycerides, which aggregate into globules, and are then coated with proteins. These large spheres are called chylomicrons. Chylomicrons contain triglycerides, cholesterol, and other lipids and contain proteins on their surface. Their surface is also composed of the hydrophilic phosphate "heads" of phospholipids. Together, they enable the chylomicron to move in an aqueous environment without exposing the lipids to water. Chylomicrons leave the absorptive cells via exocytosis, entering the lymphatic vessels (Lairon, 2009). From there, they enter the blood in the subclavian vein. More detailed information about lipid digestion and absorption can be found in the following review articles (Pafumi et al., 2002; Fave et al., 2004; Bauer et al., 2005; Porter et al., 2007).
Figure 1.13 Overview of the steps involved in the digestion and absorption of dietary lipids (Nelson and Cox, 2013).

1. Bile salts emulsify dietary fats in the small intestine, forming mixed micelles.
2. Intestinal lipases degrade triacylglycerols.
3. Fatty acids and other breakdown products are taken up by the intestinal mucosa and converted into triacylglycerols.
4. Triacylglycerols are incorporated, with cholesterol and apoproteins, into chylomicrons.
5. Chylomicrons move through the lymphatic system and bloodstream to tissues.
6. Lipoprotein lipase, activated by apoC-II in the capillary, releases fatty acids and glycerol.
7. Fatty acids enter cells.
8. Fatty acids are oxidised as fuel or reesterified for storage.
There is growing interest in understanding and controlling the digestibility of lipids within the human GIT (McClements et al., 2008; Singh et al., 2009; McClements and Li, 2010) because of the link between over-consumption of certain food lipids (e.g. saturated fats, trans-fatty acids and cholesterol) and increased susceptibility to a range of diseases including cardiovascular disease and obesity (Gesta et al., 2007). The impact of the properties of emulsions (e.g. molecular characteristics of lipid molecules, lipid type, droplet size or interfacial properties) on lipid bioavailability has been studied in detail in simplified model emulsion systems (McClements et al., 2008; Singh et al., 2009; Michalski et al., 2013; Mao and Miao, 2015). However, the lipids in many, if not most, foods are normally present as oil droplets dispersed in a compositionally and structurally complex matrix. Until recently, the role of the structure of the food matrix as a variable in lipid digestion and absorption has generally not been considered. The focus of this discussion will therefore be on how manipulating food matrix structure and composition can influence the rate of lipid digestion and hence the bioavailability of fatty acids (FA).

The rate and extent of lipid digestion within the GIT may depend on how fast the body can breakdown the matrix materials surrounding them since the lipids may have to be exposed to the aqueous phase of digestive products before they can be digested by soluble lipases (Chen et al., 2006; Bornhorst and Singh, 2014). Kong and Singh (2009) have studied the disintegration profiles of a variety of solid foods, and proposed that their disintegration in the stomach is determined by the forces of cohesion that hold the food matrix together and the forces applied on food in the stomach. In addition, the disintegration rate was also governed by water absorption, textural softening and surface erosion during digestion (Kong and Singh, 2009). Also, the initial texture (or physical form) of the food and its rate of softening during digestion have been proposed as good indicators to predict their behaviour during in vitro gastric digestion (Bornhorst et al., 2015). Wooster et al. (2014) demonstrated that yoghurt and
concentrated starch dispersions effectively protected oil droplets against coalescence during *in vitro* gastric digestion. However, these networks did not slow down lipolysis compared to a reference caseinate/monoglyceride (CasMag) stabilised emulsion during *in vitro* intestinal digestion. Infact, Wooster *et al.* (2014) reported that the rate of lipid digestion was lower for the CasMag emulsion and an emulsion-filled gelatine gel mainly because of coalescence reducing fat surface area of oil droplets in these emulsion and filled gel systems during gastric digestion. Therefore, the “natural” foods gave improved digestibility compared to the emulsion and filled gel model systems. Lamothe *et al.* (2012) investigated the kinetics of matrix degradation and free fatty acid (FFA) release for different cheeses (mild Cheddar, aged Cheddar, light Cheddar and Mozzarella) during simulated gastro-intestinal digestion. These authors found that Mozzarella was the most rapidly digested and underwent complete matrix disintegration within 180 min. This was attributed to a more porous protein matrix and lower firmness and cohesiveness in Mozzarella than in the other cheeses. The extent of matrix disintegration of mild Cheddar was significantly lower than that of other cheeses because of its elastic, cohesive and firm structure. This also led to a lower extent of lipid hydrolysis, suggesting that the more physically intact and less proteolysed matrix of the mild Cheddar acted as a protective barrier, limiting the access of enzymes to their substrate. Guo *et al.* (2014) and Guo *et al.* (2016) designed a series of whey protein emulsion gels with different structures and hardness values. These authors reported that a harder gel slowed down the disintegration of the gel matrix and consequently the oil release, which is in contrast to the observations of Wooster *et al.* (2014) who found no effect of hardness on digestion. Guo *et al.* (2014) and Guo *et al.* (2016) observed coalescence of the oil droplets only for the soft gel and this softer gel structure was completely broken down after 4 hours of digestion. These examples show the possible but as yet unresolved, link between food microstructure and lipolysis and hint at the potential of formulating food matrices with different breakdown rates or permeabilities as a potential strategy towards controlling lipid bioavailability. Such an
approach may also provide a means of controlling or targeting the release of lipid-based components to specific sites within the digestive tract (Chen et al., 2006; Parada and Aguilera, 2007).

There is scope to further build upon the knowledge gained in these studies regarding the impact of different food microstructures on lipid digestion. Because the structures of emulsion gels can be easily and precisely manipulated by changing protein/oil ratios, oil droplet sizes and salt content, the emulsion gel model represents an ideal food material for investigating the mechanistic relationships between food structure and food digestion (especially the digestion of emulsified lipid in semi-solid or solid foods with varying structures) and are being used increasingly for this purpose (Dickinson, 2012). Casein-based emulsion gels are reflective matrices of semi-solid dense protein systems containing dispersed oil droplets e.g. processed and analogue cheese. These systems are well characterised in terms of their composition and functional properties (Guinee et al., 2004; Noronha et al., 2008a; El-Bakry et al., 2010a). Another beneficial attribute of these casein-based matrices is the amenability of their formulations to be easily altered, resulting in food materials with customised nutritional, compositional and functional attributes. This may provide an opportunity to design food materials with modulated digestion behaviour that trigger different physiological responses.

1.5.3.2 Influence of calcium on lipid digestion

Besides the structural organisation of the matrix surrounding lipids, minerals present in the matrix can also impact on lipid digestion and metabolism (Michalski et al., 2013). In particular, several in vitro digestion studies have highlighted the important role that calcium plays in determining the rate and extent of lipid digestion (Zangenberg et al., 2001a, b; Hu et al., 2010). Calcium ions (Ca\(^{2+}\)) can potentially impact the lipid digestion process due to a variety of different physico-chemical mechanisms (McClements and Li, 2010). For example, lipase digestion of emulsified lipids can be inhibited by the accumulation of long chain
saturated FA at the droplet surfaces, since this restricts the access of the lipase to the triacylglycerols (Fave et al., 2004). Calcium is known to precipitate these long chain saturated FA, thereby removing them from the lipid droplet surface and allowing the lipase to access the emulsified lipid substrate (Patton and Carey, 1979; Patton et al., 1985). Consequently, Ca\(^{2+}\) ions are able to increase the rate and extent of lipolysis by this mechanism (Zangenberg et al., 2001a, b; Armand et al., 1996; Hwang et al., 2009). Furthermore, calcium has been shown to play an important role in the activity of pancreatic lipase (the main enzyme involved in the digestion of triacylglycerides), acting as a co-factor required for activity (Whayne and Felts, 1971a, b; Kimura et al., 1982). Conversely, several studies have shown that the long chain saturated FAs released during the hydrolysis of triacylglycerides are able to form insoluble soaps with Ca\(^{2+}\) in the small intestine, which can actually reduce the bioavailability of these fatty acid digestion products (Scholz-Ahrens and Schrezenmeir, 2006; Karupaiah and Sundram, 2007; Lorenzen et al., 2007). Although this may seem counter-intuitive, as increased lipolysis might be expected to increase the absorption of FAs, the formation of these insoluble soaps means that the products of increased lipolysis are not available for absorption. It has been demonstrated however that this effect is dependent on the fatty acid composition of the lipid phase (Bonnaire et al., 2008; McClements et al., 2008). MacGregor et al. (1997) reported that the rate of FFA production was independent of calcium concentration for short chain triacylglycerides because the FFAs generated were water-dispersible and easily moved away from the droplet surfaces. This suggests that this effect is only present in the case of medium and long chain FAs.

While it has been shown that calcium can influence the rate or extent of lipid bioavailability in protein-stabilised model oil-in-water emulsion systems, this has not been demonstrated to a great extent in complex multi-component food matrices, which may have different structures and contain other food components that may impact on this effect. Therefore, further research on actual foods is required. A recent study by Ayala-Bribiesca et al. (2016) examined the
effect of calcium enrichment on Cheddar cheese structure and subsequently assessed the impact of that structure on lipid bioaccessibility. Higher calcium levels led to faster lipolysis during the first half of digestion, possibly by enhancing lipase activity. However, a protective effect of the food matrix on lipolysis was observed for the very-high calcium cheese (i.e. the hardest and most cohesive matrix) towards the end of digestion, where less FFAs were detected even in the presence of higher calcium levels, when compared to other cheeses (control and high calcium). These results suggest that lipolysis depends on the calcium content of the food material and also the matrix structure modulating the access of enzymes to their substrates.

It is difficult to alter the calcium concentration of natural cheese and still make an acceptable product. Casein-based food matrices e.g. processed and analogue cheese may prove a more convenient matrix to allow precise exploration of the effect of calcium concentration on lipid digestion. These matrices are concentrated protein gel systems containing lipid, in which the calcium level can be easily tailored and which provide a means of controlling casein concentration, casein type (Sołowiej et al., 2014) and processing parameters (Noronha et al., 2008a). Therefore, further studies using these types of matrices could provide a better understanding of the calcium-matrix interaction affecting lipid bioaccessibility.

1.6 Concluding Remarks

Casein micelles are the primary building blocks of many dairy products; however, controversy about their structure continues. In particular, a more detailed understanding of the effects of calcium concentration and the role of different forms of calcium on their structure and functionality in food systems is required. The chelation of calcium in semi-solid, casein-based food systems e.g. processed and analogue cheese occurs via the action of calcium chelating salts. The existing knowledge of how calcium chelators facilitate structure formation and initiate functionality of the casein present in such matrices is limited, and as
yet, not clearly understood. A very limited number of studies have examined the interactions between calcium chelators, calcium and casein using model systems and the resulting effects on casein structure and functionality have been reported. However, the effects of these interactions have not been investigated in concentrated casein-based systems such as, processed and analogue cheese. Furthermore, the effects of calcium on the structure, functionality (texture, meltability) and subsequent digestibility of such concentrated casein-based food systems have not been clarified. Thus, the present literature review has highlighted a number of knowledge gaps that are addressed in the research objectives of this thesis. These objectives are outlined in the following section.
1.7 Research Objectives

The overall aim of this thesis was to obtain a better understanding of the modulating effect of calcium on the physico-chemical properties of casein matrices with a view to formulating novel casein-based food structures with unique functionalities. More specifically the objectives of this research were:

- To investigate the effects of calcium chelators on calcium distribution and protein solubility in model CaCl\(_2\) solutions and rennet casein dispersions.
- To understand the dynamics of calcium-ion exchange processes as affected by calcium chelators during manufacture of semi-solid casein-based food matrices.
- To examine the effects of altering the calcium levels of casein-based food matrices on their structure formation and functional properties in the presence and absence of calcium chelators.
- To determine if altering the type of cheese flavour and fat content of casein-based food matrices containing no calcium chelators affects their sensory acceptance.
- To establish the effect of calcium concentration on the structure of casein-based food matrices and to assess the impact of that structure on matrix breakdown and lipid digestion using an *in vitro* digestion model.

The research is divided into 5 experimental chapters and the explicit objectives are stated in the introduction to each chapters.
1.8 References


Chapter 2

Effects of Calcium Chelators on Calcium Distribution and Protein Solubility in Rennet Casein Dispersions

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Chapter 2: Effects of calcium chelators on calcium distribution and protein solubility in rennet casein dispersions.

2.1 Introduction

Chelating agents (e.g. citric acid and its derivatives, various phosphates, and salts of EDTA) are known to form complexes with monovalent or polyvalent metal cations e.g. Na\(^+\) or Ca\(^{2+}\). Calcium (Ca) and calcium ions (Ca\(^{2+}\)), can be present in foods in the form of an equilibrium between ionic, soluble non-ionic (i.e. chelates and complexes), and colloidal species e.g. in dairy systems (DeMan, 2013). The Ca\(^{2+}\) ions are of particular importance, these contribute to the internal stability of casein micelles as they form linkages between protein molecules, either as colloidal calcium phosphate (CCP) or directly bound to caseins (Schmidt, 1982; Holt, 1992; Horne, 1998; Tsioulpas et al., 2007). The addition of calcium chelating agents presumably results in the complexation of Ca present in the soluble phase and, then if sufficient chelating agent is added, the Ca associated with the casein is removed. This process can alter the distribution of Ca\(^{2+}\) between the soluble and colloidal phases (Udabage et al., 2000), which can effect the stability (Gaucheron, 2005) and structural integrity of casein micelles (de Kort et al., 2011) and consequently the functionality (e.g. water binding capacity, emulsifying properties, viscosity and solubility) of casein in dairy systems.

Rennet casein is prepared by the enzymatic precipitation of casein from pasteurised skim milk. The high colloidal Ca content retained in the resulting para-casein curd after this process is largely responsible for the lack of solubility of rennet casein in water (Ennis et al., 2000). The ability of rennet casein to disperse in water is governed by the amount of colloidal Ca (Ca phosphate) that is available to cross-link the amorphous para-casein matrix (Fox et al., 2000), and findings of model studies involving dilute suspensions of para-casein, have shown that hydration increases as the level of colloidal Ca decreases (Sood et al., 1979). The
application of calcium chelating salts (CCS), such as disodium phosphate (DSP) and trisodium citrate (TSC) is thought to result in the solubilisation of colloidal Ca (Ca phosphate) from casein micelles (de Kort et al., 2011) through formation of chelates with Ca\(^{2+}\). Ca chelation involves the exchange of the Ca\(^{2+}\) in the \textit{para}-casein network of rennet casein, for the monovalent cations (e.g. Na\(^+\)) of the CCS. This results in the partial hydration of the insoluble \textit{para}-casein and its conversion to a water soluble sodium \textit{para}-caseinate dispersion. In certain applications e.g. processed and analogue cheese, the CCS function is not only to chelate Ca, but also to increase pH which further aids in protein dispersion and hydration (El-Bakry et al., 2011a) by increasing the net negative charge on the caseins. All of the above CCS-induced changes favour a more open reactive \textit{para}-caseinate conformation with enhanced water binding and emulsifying ability (Carić and Kaláb, 1985; Fox et al., 2000). CCS therefore modify the functional properties of rennet casein and, together with the aid of heat and shear, facilitate its use in food applications. The extent and nature of hydration of the protein are critical factors in determining the functional performance of rennet casein during manufacture of processed and analogue cheese and in influencing the end-product functionality (e.g. texture-related and melting properties) of such products.

Some manipulations (e.g. addition of CCS, heat treatment, pH), that one performs during processed and analogue cheese manufacture can influence the degree of casein demineralisation and hydration during processing. While CCS-rennet casein interactions have been studied in dilute systems (Ennis et al., 1998; Ennis and Mulvihill, 1999; Ennis et al., 2000; O’Sullivan and Mulvihill, 2001), to our knowledge the interactive effects of the type and concentration of CCS, temperature and pH have not been investigated in detail in concentrated rennet casein systems. Thus, in this study, relationships between all of these parameters are examined in relation to their influence on the functional performance of rennet casein during processed and analogue cheese manufacture. The aim of the study was to
investigate the effects of adding selected CCS, individually or in mixtures, to rennet casein (RC) dispersions. The effects of varying the proportions of different types of CCS used in the mixture, and the total concentration of CCS applied were investigated under conditions (i.e. pH and temperature) simulating industrial processing of processed and analogue cheese. Preliminary studies were carried out using CaCl₂ solutions to better elucidate CCS-Ca interactions in a simplified system and to establish if this system could then be used to predict or model CCS behaviour in a more complex Ca containing dispersion. Effects of CCS on Ca-ion activity ($A_{Ca^{++}}$) and Ca distribution in both CaCl₂ and RC systems were determined, additionally, the viscosity and protein solubility of RC dispersions with added CCS were studied.

2.2 Materials and Methods

2.2.1 Materials

Rennet casein powder (Kerrynor™ R190) was purchased from Kerry Ingredients Ltd (Listowel, Co. Kerry, Ireland). CCS: disodium phosphate (DSP) (Albright and Wilson Ltd. Cheshire, England), dipotassium phosphate (DPP), trisodium citrate (TSC), and tripotassium citrate (TPC) (Jungbunzlauer GmbH., Pernhofen, Austria) were anhydrous and of food grade quality. Chemical reagents: calcium chloride (CaCl₂) anhydrous, calcium reference standard solution for atomic absorption spectroscopy (AAS) (1000 mg / L), potassium chloride (KCl), lanthanum trichloride heptahydrate (LaCl₃.7H₂O), and nitric acid (HNO₃) were purchased from Sigma Aldrich (Ireland) and were of analytical grade. De-ionized water was prepared in a Milli-Q water purification system.

2.2.2 Experimental design

The first part of the study investigated the effects of different types of CCS (i.e. DSP, DPP, TSC and TPC) and their concentrations (0-100 mmol L⁻¹) on the $A_{Ca^{++}}$ in 50 mmol L⁻¹ CaCl₂
solutions. This calcium concentration was selected, because the casein micelles in concentrated dairy systems have comparable calcium concentrations (de Kort et al., 2009). Samples were prepared at different pHs (8.0 or 6.7) and temperatures (50 °C and 70 °C), to reflect the manufacturing conditions for processed and analogue cheese.

In the second part of the study, the effects of CCS (DSP and TSC) on Ca distribution in both CaCl$_2$ solutions (50 mmol L$^{-1}$) and RC dispersions (15g/100g) were investigated. The CCS used in the manufacture of processed and analogue cheese are mainly sodium salts (Carić and Kaláb, 1985; Cavalier-Salou and Cheftel, 1991), most commonly phosphates and citrates. These CCS show desirable performance characteristics when used in processed and analogue cheese manufacture, therefore only sodium based CCS were used for this part of the study. The CCS at concentrations of 10 and 30 mmol L$^{-1}$ and at DSP:TSC ratios of 1:0, 2:1, 1:1, 1:2 and 0:1 were added to both systems and the pH adjusted; the effect of temperature (22 °C, 50 °C and 70 °C) was only investigated in the case of RC dispersions. Subsequently the Ca and protein contents of the dispersed phase obtained after centrifugation were determined. Concentrations of 10 and 30 mmol L$^{-1}$ CCS were selected, because the largest decrease in $A_{\text{Ca}^{2+}}$ was measured between 0 and 40 mmol L$^{-1}$. Similar concentrations of CCS were used by Udabage et al. (2000) when investigating mineral and casein equilibria in milk protein systems.

**2.2.3 Sample preparation**

Aqueous stock solutions of CCS (100 mmol L$^{-1}$) were prepared and an aliquot added (with stirring) to CaCl$_2$ solutions or RC dispersions to give the final desired concentration of CCS. The pH of solutions was adjusted using 1 mol L$^{-1}$ NaOH or 1 mol L$^{-1}$ HCl. All pH measurements were carried out on a pH meter (EL20, Mettler Toledo, Schwerzenbach, Switzerland) calibrated with standard solutions at pH 4.0 and pH 7.0; measurements were performed at 25.0 ± 0.1 °C. All pH adjusted samples were stirred (IKA RCT basic, IKA®
Werke, GmbH & Co. KG, Germany) for one hour; afterwards, the pH was checked, readjusted if necessary and de-ionized water added to achieve the desired concentrations. RC dispersions were heated to 50 °C or 70 °C using a heated magnetic laboratory stirrer (IKA RCT basic, IKA® Werke, GmbH & Co. KG, Germany). After heating, RC dispersions were allowed to cool to room temperature. The dispersed and insoluble phases were separated by centrifugation (Hettich Rotofix 32 A, Andreas Hettich GmbH & Co. KG, Germany) at 3000 g for 10 minutes (IDF, 2002). All samples were prepared and analysed in triplicate.

2.2.4 Calcium analysis

2.2.4.1 Calcium-ion activity ($A_{Ca}^{++}$)

The $A_{Ca}^{++}$ in samples was measured with a calcium-ion selective electrode (ISE 25 Ca; Radiometer Analytical, Mendes, France) and a reference electrode (“Red Rod” REF 251; Radiometer Analytical, Mendes, France) fitted to a pH meter (EL20, Mettler Toledo AG, Schwerzenbach, Switzerland). Calibration was performed at ambient temperature with standard solutions containing 0.5, 5.0 or 50 mmol L$^{-1}$ CaCl$_2$ and 80 mmol L$^{-1}$ KCl. Addition of this monovalent background electrolyte was necessary, as it keeps the calcium-ion activity coefficient ($\gamma_{Ca}^{++}$) effectively constant in the calibration solutions (de Kort et al., 2009). Preliminary experiments were carried out using 5 CaCl$_2$ standards, selected from the range 0.001 to 100 mmol L$^{-1}$ to confirm a linear response up to 100 mmol L$^{-1}$. An $\gamma_{Ca}^{++}$ of 0.29 was calculated for the calibration solutions using the formula of Davies (1962) below:

\[
\log(\gamma_{Ca}^{++}) = -0.5z^2 \left(\frac{\sqrt{I}}{1 + \sqrt{I}} - 0.2I\right)
\]

In which $z$ is the charge (valence) on the ion and $I$ refers to the ionic strength of the solution in mol / L. The calculated $\gamma_{Ca}^{++}$ value obtained was comparable to that reported by de Kort et al. (2009). The $A_{Ca}^{++}$ was determined by multiplying the experimental $[Ca^{2+}]$ by the activity coefficient of 0.29. The $[Ca^{2+}]$ was calculated from the regression equation, derived from the
calibration curve. All experiments were carried out under continuous stirring with a laboratory stirrer (IKA RCT basic, IKA® Werke, GmbH & Co. KG, Germany) at ambient temperature due to the temperature limitations of the Ca-ISE (i.e. 50°C).

2.2.4.2 Total calcium

A flame atomic absorption spectroscopy method (IDF, 2007) was used to determine total and dispersed Ca contents.

2.2.4.2.1 Sample preparation

Aliquots of ~3 g of the dispersed phase obtained upon centrifugation were weighed into silica crucibles and placed inside a programmable muffle furnace (B180, Nabertherm, GmbH, Germany). The furnace temperature was increased by 50 °C / hr⁻¹ up to 550 °C and maintained at this temperature for 6 hours. The ashed sample was subsequently dissolved in 1 ml of 25 % HNO₃ (v/v) solution and transferred quantitatively into a 250 ml volumetric flask, diluted to volume with water and mixed. The sample was appropriately diluted, and an aliquot of 10 % LaCl₃·7H₂O (v/v) was added to the final analytical solutions. Addition of lanthanum trichloride solution was necessary to inhibit phosphate interference and the ionisation of elements in the atomic absorption spectrometer flame. To avoid contamination from laboratory glassware, supplies and environmental particulate matter (dust) all glassware was soaked in 20 % HNO₃ (v/v) solution for 24 hours and rinsed three times with deionized water and dried prior to use. All samples were prepared and analysed in triplicate.

2.2.4.2.2 Flame atomic absorption spectrometric measurement

Samples were analysed using flame atomic absorption spectroscopy and results expressed as dispersed Ca (mmol L⁻¹). The atomic absorption spectrometer (SpectrAA, 55B, AAS, Varian), was fitted with a Ca hollow cathode lamp (Activion, Halstead, Essex, England) using a wavelength of 422.7 nm and an air-acetylene oxidizing flame for analysis. The instrument was calibrated with reference Ca standards (1.0, 2.0, 3.0, 4.0 and 5.0 mg / L) prepared from
Ca reference solution (1000 mg / L) prior to analysis. An aliquot of 10 % LaCl$_3$.7H$_2$O (v/v) was also added to calibration solutions. The difference between dispersed Ca and $A_{\text{Ca}^{++}}$ was termed dispersed “chelated” Ca.

2.2.4.3 Insoluble calcium

The amount of insoluble Ca (i.e. the Ca associated with the pellet obtained after centrifugation) was calculated as the difference between total Ca and the dispersed Ca content.

2.2.5 Protein solubility

The protein content of the dispersed phase was determined in triplicate for each sample by Macro-Kjeldahl protein analysis (IDF, 1993) and the % soluble protein expressed as follows:

$$\% \text{ Soluble Protein} = \frac{\text{Protein content of supernatant}}{\text{Protein content of initial dispersion}} \times 100 \quad (2)$$

2.2.6 Viscosity measurements

The apparent viscosity (mPa·s) of RC dispersions prepared as described in Section 2.2.3 was determined using a Physica MCR 301 rheometer (Anton Paar Germany GmbH, Ostfildern, Germany) equipped with a cup (CC27-SS) and vane (ST22-4V-40) geometry. All measurements were performed using shear rates from 1 – 100 s$^{-1}$ at 22 °C, and final results were reported at 25 s$^{-1}$. Samples were pre-sheared at 100 s$^{-1}$ for 30 seconds prior to measurement. All samples were prepared and analysed in triplicate.

2.2.7 Statistical analysis

SPSS version 20 (IBM Inc. Chicago, IL, USA) was used to determine a one-way analysis of variance (ANOVA) and means were considered significantly different at $P < 0.05$. 
2.3 Results and Discussion

2.3.1 Changes in the $A_{Ca^{2+}}$ of CaCl$_2$ solutions with added CCS

The effectiveness of CCS in binding Ca$^{2+}$ is reported to depend on valency, type of ionic species forming the CCS, pH, ionic strength, temperature etc. (Lucey et al., 2011). In the present study, addition of DSP and TSC both resulted in a decrease in $A_{Ca^{2+}}$ of CaCl$_2$ solutions (Table 2.1). This occurred in a concentration-dependent fashion with the $A_{Ca^{2+}}$ approaching zero at higher CCS concentrations for both orthophosphate (i.e. DSP) and citrate (i.e. TSC) chelating salt types. However, DSP was a more effective Ca$^{2+}$ binder than TSC, with lower $A_{Ca^{2+}}$ measured across all concentrations (20-100 mmol L$^{-1}$) for CaCl$_2$-DSP solutions (Table 2.1). It has been previously reported in the literature that phosphates have better Ca$^{2+}$ binding ability than citrates (Fox et al., 1996; Guinee et al., 2004) and the general ranking of the Ca$^{2+}$ binding ability of these CCS is in the following order: polyphosphates > pyrophosphates > orthophosphates > sodium aluminium phosphate = citrates (Nakajima et al., 1975; Lee et al., 1986; Cavalier-Salou and Cheftel, 1991; Carić and Kaláb, 1993). A study completed by de Kort et al. (2009) using similar concentrations of DSP resulted in a comparable reduction in $A_{Ca^{2+}}$ in a 50 mmol L$^{-1}$ CaCl$_2$ solution. To our knowledge, no data is available on the reduction in $A_{Ca^{2+}}$ by TSC in CaCl$_2$ solutions under the conditions used in the present study. Our results suggest that substituting the sodium cation of the CCS with potassium (i.e. DPP and TPC) did not have a significant effect ($P > 0.05$) on the ability of the CCS to decrease the $A_{Ca^{2+}}$ in CaCl$_2$ solutions (results not shown). The Ca$^{2+}$ binding ability of CCS is also reported to be strongly influenced by pH (de Kort et al., 2009). Table 2.1 shows significantly ($P < 0.05$) lower values for $A_{Ca^{2+}}$ in CaCl$_2$ solutions with added DSP at pH 8.0 compared to pH 6.7 (at 20 and 40 mmol L$^{-1}$) and these values were also lower for TSC solutions at pH 8.0 (at 20 mmol L$^{-1}$). While our results showed no significant difference ($P > 0.05$) in the Ca$^{2+}$ binding ability of TSC at different pH’s (8.0 or 6.7), consistent with the view of de Kort et al. (2009), DSP was a slightly more effective Ca$^{2+}$ binder at pH 8.0 than at pH
6.7. The increased ion-exchange function at higher pH values is attributed to more complete
dissociation of the sodium phosphate molecules resulting in the formation of a higher valency
anion (van Wazer, 1971), which subsequently enhances the Ca\(^{2+}\) binding efficiency. In the
study completed by de Kort et al. (2009) the calcium-binding capacity of phosphates was
investigated at pH 8.0 as maximal calcium-phosphate interactions were expected at this pH. In
the present study we compared the \(A_{Ca^{+}+}\) at pH 8.0 and 6.7 as the latter is the typical pH
encountered during the manufacture of analogue cheese prepared using CCS (El-Bakry et al.,
2011b). Because of its greater relevance to industrial applications further studies were
conducted at pH 6.7. In the present study temperature (22 °C, 50 °C or 70 °C) was found to
have no substantial effect (\(P > 0.05\)) on the ability of CCS in binding Ca\(^{2+}\) even at appreciable
\(A_{Ca^{+}+}\) (e.g. 20 mmol L\(^{-1}\) CCS addition). These findings are consistent with those described by
de Kort et al. (2009), as similar \(A_{Ca^{+}+}\) were measured before and after sterilization of CaCl\(_2\)-
CCS solutions.
Table 2.1 Changes in $A_{\text{Ca}^{++}}$ of CaCl$_2$ solutions (50 mmol L$^{-1}$) with pH (measured at 20 °C) and temperature (measured at pH 6.7) as a function of calcium chelating salt concentration (0-100 mmol L$^{-1}$).

<table>
<thead>
<tr>
<th></th>
<th>DSP 0 mmol L$^{-1}$</th>
<th>20 mmol L$^{-1}$</th>
<th>40 mmol L$^{-1}$</th>
<th>60 mmol L$^{-1}$</th>
<th>80 mmol L$^{-1}$</th>
<th>100 mmol L$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 8.0</td>
<td>14.90 ±0.05$^a$</td>
<td>4.98 ±0.07$^a$</td>
<td>0.01 ±0.01$^a$</td>
<td>0.01 ±0.01$^a$</td>
<td>0.01 ±0.01$^a$</td>
<td>0.00 ±0.00$^a$</td>
</tr>
<tr>
<td>pH 6.7</td>
<td>15.32 ±0.06$^a$</td>
<td>6.20 ±0.08$^b$</td>
<td>0.18 ±0.02$^b$</td>
<td>0.05 ±0.02$^a$</td>
<td>0.03 ±0.01$^a$</td>
<td>0.03 ±0.01$^a$</td>
</tr>
<tr>
<td>50 °C</td>
<td>14.94 ±0.03$^a$</td>
<td>5.95 ±0.09$^a$</td>
<td>0.01 ±0.00$^a$</td>
<td>0.00 ±0.00$^a$</td>
<td>0.00 ±0.00$^a$</td>
<td>0.00 ±0.00$^a$</td>
</tr>
<tr>
<td>70 °C</td>
<td>15.11±0.02$^a$</td>
<td>5.99 ±0.06$^a$</td>
<td>0.01 ±0.00$^a$</td>
<td>0.01 ±0.00$^a$</td>
<td>0.00 ±0.00$^a$</td>
<td>0.00 ±0.00$^a$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>TSC 0 mmol L$^{-1}$</th>
<th>20 mmol L$^{-1}$</th>
<th>40 mmol L$^{-1}$</th>
<th>60 mmol L$^{-1}$</th>
<th>80 mmol L$^{-1}$</th>
<th>100 mmol L$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 8.0</td>
<td>15.90±0.04$^a$</td>
<td>6.20 ±0.03$^a$</td>
<td>1.70 ±0.04$^a$</td>
<td>0.52 ±0.02$^a$</td>
<td>0.23 ±0.00$^a$</td>
<td>0.14 ±0.02$^a$</td>
</tr>
<tr>
<td>pH 6.7</td>
<td>15.30±0.06$^a$</td>
<td>6.73 ±0.05$^a$</td>
<td>1.66 ±0.04$^a$</td>
<td>0.52 ±0.01$^a$</td>
<td>0.24 ±0.03$^a$</td>
<td>0.14 ±0.02$^a$</td>
</tr>
<tr>
<td>50 °C</td>
<td>14.90±0.02$^a$</td>
<td>6.74 ±0.01$^a$</td>
<td>1.53 ±0.05$^a$</td>
<td>0.48 ±0.02$^a$</td>
<td>0.21 ±0.02$^a$</td>
<td>0.07 ±0.01$^a$</td>
</tr>
<tr>
<td>70 °C</td>
<td>14.70±0.04$^a$</td>
<td>6.70 ±0.06$^a$</td>
<td>1.34 ±0.02$^a$</td>
<td>0.50 ±0.07$^a$</td>
<td>0.24 ±0.04$^a$</td>
<td>0.03 ±0.00$^a$</td>
</tr>
</tbody>
</table>

Numbers represent mean ± standard deviation; n=3

$^a$-$^b$ Means in the same column with different subscript letters differ significantly at P < 0.05

2.3.2 Changes in Ca distribution in CaCl$_2$ solutions with added CCS

The Ca distribution profiles in Figure 2.1 show the effects of the addition of DSP or TSC to 50 mmol L$^{-1}$ CaCl$_2$ solutions at pH 6.7. Compared to starting CaCl$_2$ solutions (containing no CCS), addition of 10 mmol L$^{-1}$ DSP reduced the $A_{\text{Ca}^{++}}$ to ~24 % and rendered 25 % of the Ca insoluble, with the remaining (~50 %) present as dispersed “chelated” Ca. Increasing DSP concentration from 10 to 30 mmol L$^{-1}$ resulted in precipitation of ~85 % of total Ca, the $A_{\text{Ca}^{++}}$
was undetectable and a small proportion (≤ 13 %) was present as dispersed “chelated” Ca. Similar results have been previously reported in CaCl₂ solutions (O’Sullivan and Mulvihill, 2001; de Kort et al., 2009); where, as the concentration of DSP in the solution increased the solubility of Ca decreased. The addition of TSC resulted in a similar decrease in $A_{Ca^{++}}$, however, in this case > 95 % of Ca remained in the dispersed phase at the 30 mmol L⁻¹ level of addition (Figure 2.1). Addition of mixtures of the CCS (DSP:TSC = 2:1, 1:1 or 1:2) to CaCl₂ solutions reduced the $A_{Ca^{++}}$ to 15 % and 5 % at concentrations of 10 and 30 mmol L⁻¹ respectively (Figure 2.1), the decrease in the $A_{Ca^{++}}$ was not significantly different (P > 0.05) for the mixed CCS systems. However, CCS mixtures with a greater proportion of TSC resulted in higher levels of dispersed “chelated” Ca (at 30 mmol L⁻¹ addition). It is considered that CCS function by reducing the chemical activity of metal cations (e.g. Ca²⁺), and are thought to form complexes with these via their unshared electron pair(s) (Lindsay, 1996, Martell and Motekaitis, 2002). In the present study, the calcium distribution profiles (Figure 2.1) suggest that the type of complex formed depends on the type of CCS used. Both DSP and TSC bind Ca²⁺ ions, however, DSP forms insoluble Ca phosphate complexes (e.g. CaHPO₄ or Ca₃(PO₄)₂) that precipitate (Guo et al., 2003), while the Ca citrate complexes formed by TSC remain as soluble complexes (Vujicic et al., 1968; Mizuno and Lucey, 2005). It has been previously reported in the literature that DSP can react with calcium in a ratio of 1:1 or 3:2 to form CaHPO₄ or Ca₃(PO₄)₂ complexes (Pyne, 1934; Upreti et al., 2006; de Kort et al., 2009). The study completed by de Kort et al. (2009) confirms that Ca₃(PO₄)₂ complexes are dominant in 50 mmol L⁻¹ CaCl₂ solution at pH 8.0.
Figure 2.1 Effect of the addition of different concentrations (10 mmol L\textsuperscript{-1} and 30 mmol L\textsuperscript{-1}) of DSP:TSC ratios (1:0, 2:1, 1:1, 1:2 and 0:1) on the distribution of Ca (% of total Ca) between the soluble (“chelated” $\mbox{(\square)} + A_{\text{Ca}^{++}}$ (\blacksquare)) and insoluble (\blacksquare) phases in a 50 mmol L\textsuperscript{-1} CaCl\textsubscript{2} solution at pH 6.7. Standard error bar values represent the data ± standard error of the mean and represent the mean of three replicates.

2.3.3 Appearance of RC dispersions with added CCS

Following dispersion of RC in CCS solutions at different DSP:TSC ratios (1:0, 2:1, 1:1, 1:2 and 0:1), visual observation suggested that in the presence of DSP alone (1:0), visible, dispersed RC particles were swollen in a largely transparent aqueous phase of low viscosity (2.5 mPa\cdot s). When TSC was included in the mix (2:1), a more viscous (1230 mPa\cdot s), opaque dispersion resulted but some swollen rennet casein particles were still evident. When equal concentrations of DSP and TSC (1:1) were used, a lower viscosity (870 mPa\cdot s), opaque liquid containing clearly visible discrete white particles resulted. Dispersions of RC in CCS solutions with higher TSC concentrations (1:2 and 0:1) had a fluid pourable consistency with an opaque appearance and were free of inhomogeneous particles.
2.3.4 Changes in Ca distribution in RC dispersions with added CCS

The addition of DSP to RC dispersions appeared to have had little effect on the distribution of Ca between the dispersed and insoluble phase (Figure 2.2) compared to starting dispersions (with no addition of CCS), with high levels of insoluble Ca present in both. In contrast, addition of TSC resulted in a large increase in dispersed Ca (Figure 2.2) with > 80 % of the total Ca present as dispersed “chelated” Ca. There was no significant effect (P > 0.05) of increasing the total concentration of CCS (from 10 to 30 mmol L\(^{-1}\)) on the distribution of Ca between the dispersed and insoluble phase. In contrast to the behaviour of individual CCS, in the mixed CCS systems (DSP:TSC = 2:1, 1:1 or 1:2) the level of dispersed Ca increased when the total concentration of CCS was increased. High proportions of DSP in the CCS mixtures resulted in lower levels of dispersed “chelated” Ca, whereas A\(_{\text{Ca}}^{++}\) remained low (≤ 5 %) and was not affected (P > 0.05) by the type or concentration of the CCS mixture applied. The type of CCS used in RC dispersions therefore greatly influenced the extent of Ca solubilisation. There were high levels of dispersed “chelated” Ca in dispersions with TSC or CCS mixtures containing a high proportion of TSC. Thus, it appears that TSC chelated Ca from indigenous Ca phosphate and formed soluble Ca citrate complexes, thereby decreasing the insoluble Ca content. In contrast, addition of DSP either on its own or as part of CCS mixtures, resulted in low levels of dispersed Ca. Similar findings were also reported by O'Sullivan and Mulvihill (2001) for RC dispersions with added DSP. These results suggest that DSP may bind Ca by forming an insoluble Ca phosphate complex which may be trapped within the protein matrix. It has been reported that the degree of Ca solubilisation can vary with the calcium chelating abilities of CCS (Ennis and Mulvihill, 1999). The results of the present study indicate that DSP is relatively poor at solubilising Ca and was certainly weaker in this regard than TSC. Therefore, it may be that addition of DSP allows only partial disruption of the Ca-mediated cross-links in the RC, however, this level of disruption was still sufficient to allow the formation of a hydrated matrix. Hence, in RC dispersions containing DSP, Ca-mediated
cross-links may play a greater role in the formation of the matrix than is the case in those dispersions containing TSC. Further studies comparing the mechanical properties of the hydrated matrices formed may help better elucidate the role of Ca in structure formation. Similar to the observations in CaCl$_2$ solutions, temperature had no observable effect on the Ca distribution in the DSP or TSC containing RC dispersions in the present study (data not shown).

![Graph showing the effect of DSP:TSC ratios on Ca distribution](image)

**Figure 2.2** Effect of the addition of different concentrations (10 mmol L$^{-1}$ and 30 mmol L$^{-1}$) of DSP:TSC ratios (0:0, 1:0, 2:1, 1:1, 1:2 and 0:1) on the distribution of Ca (expressed as % of total Ca) between the soluble (“chelated” ) and insoluble phases in rennet casein dispersions (15 g / 100 g) at pH 6.7. Standard error bar values represent the data ± standard error of the mean and represent the mean of three replicates.

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2.3.5 Soluble protein in RC dispersions with added CCS

The protein content (expressed as % of total protein) in the dispersed phase obtained after centrifugation of aqueous mixtures of RC with added CCS are shown in Table 2.2. In the starting dispersions containing no added CCS, the amount of soluble protein corresponded to 2.23 % of total protein. Addition of 10 mmol L\(^{-1}\) DSP increased the protein content of the dispersed phase slightly from 2.23 to 2.45 % of total protein. Increasing the DSP concentration to 30 mmol L\(^{-1}\) resulted in only a marginal rise in soluble protein to 3.13 %. Addition of TSC resulted in a greater level of protein solubilisation, increasing soluble protein to 5.37 % and 11.62 % of total protein for 10 and 30 mmol L\(^{-1}\) TSC respectively. In a manner similar to that observed for the individual CCS, in the mixed CCS systems (DSP:TSC = 2:1, 1:1 or 1:2) the amount of protein in the dispersed phase was overall relatively low (2.9 - 7.28 % of total protein). Higher proportions of DSP in the CCS mixtures resulted in lower levels of soluble protein. Increasing the total concentration of the CCS mixture (from 10 to 30 mmol L\(^{-1}\)) resulted in a significant (P < 0.05) but not substantial increase in soluble protein levels. Temperature (22 °C, 50 °C or 70 °C) had no significant effect (P > 0.05) on the soluble protein content of RC dispersions (results not shown). While there is an absence of literature on the solubility of RC as influenced by temperature, one study reported that RC dispersed with the aid of CCS and heat (80 °C for 60 min) resulted in considerably higher protein solubility values (Mounsey and O’Riordan, 2008) than those achieved in the present study. However, the higher pH (7.2), temperature conditions and holding time, and higher ratio of CCS:protein used in that study, may explain the discrepancies in the solubility values obtained. In agreement with the results of the present study, Konstance and Strange (1991) reported that the solubility of RC dispersions in the presence of various CCS types were not temperature dependent. Our results suggest that DSP and TSC have different effects on Ca solubility. However, although there were significantly higher levels of soluble protein in RC dispersions with added TSC compared to those with DSP added at similar concentrations, neither salt
produced high levels of soluble protein. Correspondingly, higher levels of casein hydration have been reported when TSC rather than DSP was used as the sole CCS in processed and analogue cheese manufacture (Cavalier-Salou and Chefel, 1991; Dimitreli and Thomareis, 2009). Similar findings have also been reported for dilute casein solutions at 50 °C, where it has been found that DSP did not facilitate hydration as well as TSC (Mizuno and Lucey, 2005). In the present study, addition of TSC resulted in the formation of dispersed “chelated” Ca and consequently more of the Ca-mediated cross-links were disrupted, perhaps resulting in greater exposure of charged phosphoserine residues leading to increased charge repulsion between caseins. Several studies have suggested that chelation of Ca by CCS increases the hydration of the protein and thus the extent of solubilisation (Berger et al., 1998; Ennis et al., 1998; Fox et al., 2000). Furthermore, some studies associate this presumed increase in protein solubility with the subsequent emulsification of fat and its incorporation into processed and analogue cheese matrices (Dimitreli and Thomareis 2009; El-Bakry et al., 2011a). There is some evidence to support this as some studies have shown that processed and analogue cheese prepared with CCS known to increase soluble protein levels were highly emulsified (El-Bakry et al., 2011a). While the present study suggests that the level of protein solubilised in RC dispersions by CCS was low, it may be the case that only a low level of soluble protein is required to emulsify the fat in a cheese application given the high emulsifying capacity of casein (Mulvihill and Murphy 1991; Dalgleish, 1997; Sanchez and Rodriguez Patino, 2005). Although there is an absence of literature on the solubility and emulsifying properties of RC specifically, one study reported that size distribution curves for emulsions stabilised with 2 % w/v RC showed a normal distribution, corresponding to small well emulsified fat globules (Mounsey, 2000). These results were comparable to those obtained for sodium caseinate stabilised emulsions (Shokker and Dalgleish, 2000). The same author (Mounsey, 2000) reported reasonable emulsion stability (ES) values (78-65 %) for emulsions stabilised with RC with decreasing casein concentration from 2-1 %.
**Table 2.2 Soluble protein (expressed as % of total protein) in rennet casein dispersions (15 g / 100 g) at pH 6.7.**

<table>
<thead>
<tr>
<th>DSP:TSC</th>
<th>0 mmol L(^{-1}) (Control)</th>
<th>10 mmol L(^{-1})</th>
<th>30 mmol L(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:0</td>
<td>2.23 ±0.39 (^{a,x})</td>
<td>2.45 ±0.19 (^{a,by})</td>
<td>3.13 ±0.39 (^{b,y})</td>
</tr>
<tr>
<td>2:1</td>
<td>2.23 ±0.39 (^{a,x})</td>
<td>2.90 ±0.40 (^{a,x})</td>
<td>5.14 ±0.51 (^{b,y})</td>
</tr>
<tr>
<td>1:1</td>
<td>2.23 ±0.39 (^{a,x})</td>
<td>3.35 ±0.57 (^{a,y})</td>
<td>6.30 ±0.40 (^{b,z})</td>
</tr>
<tr>
<td>1:2</td>
<td>2.23 ±0.39 (^{a,x})</td>
<td>3.90 ±0.20 (^{b,x})</td>
<td>7.26 ±0.39 (^{c,y})</td>
</tr>
<tr>
<td>0:1</td>
<td>2.23 ±0.39 (^{a,x})</td>
<td>5.37 ±0.66 (^{b,y})</td>
<td>11.62 ±0.70 (^{c,z})</td>
</tr>
</tbody>
</table>

Numbers represent mean ± standard deviation; \(n=3\)

\(^{a-c}\) Means in the same column with different subscript letters differ significantly at \(P < 0.05\)

\(^{x-z}\) Means in the same row with different subscript letters differ significantly at \(P < 0.05\)

### 2.4 Conclusion

The CaCl\(_2\) model system used in this study proved a useful predictor of CCS performance in RC dispersions. While DSP and TSC have very different effects on Ca distribution in both these simplified systems, neither salt produces high levels of soluble protein, thus the role of CCS in casein-based matrix formation may be more complex than previously thought. If, as the results of the present study suggest, CCS do not function by solubilising protein, or in the case of DSP, Ca, their contribution to hydration and matrix formation in casein-based matrices may warrant further investigation. The CCS examined in the present study are commonly used in processed and analogue cheese manufacture, therefore despite the fact that the RC system used was relatively concentrated an even more concentrated model protein dispersion may be required to better simulate CCS behaviour in this industrial application.
2.5 References


Chapter 3

Monitoring the Progression of Calcium and Protein Solubilisation as Affected by Calcium Chelators During Small-Scale Manufacture of Casein-Based Food Matrices

Accepted as:
Chapter 3: Monitoring the progression of calcium and protein solubilisation as affected by calcium chelators during small-scale manufacture of casein-based food matrices.

3.1 Introduction

In milk systems calcium is partitioned between colloidal and aqueous phases. Approximately 70% of calcium is present as undissolved complexes in casein micelles, named colloidal calcium phosphate (CCP) (Holt, 1997); the remainder exists as free Ca$^{2+}$ ions or soluble complexes with citrate and HPO$_4^{2-}$ in the aqueous phase (Gao, 2010). Calcium ion equilibria are known to play an important role in the structure and stability of casein micelles (Walstra, 1990; Horne, 1998) and alterations to these equilibria have effects on the physicochemical properties of casein micelles (de Kort et al., 2011). Upon addition of calcium chelating salts (CCS) such as disodium phosphate (DSP) and trisodium citrate (TSC) to casein micelles, solubilisation of CCP and decreased activity or concentration of ionic calcium is observed (Udabage et al., 2000, 2001; Choi et al., 2007; de Kort et al., 2009). The calcium-binding anion of the CCS competes with the phosphoseryl residues and CCP in the casein micelle for Ca$^{2+}$ ions (de Kort et al., 2011). This reduces the extent of calcium-mediated protein cross-linking in casein micelles or the aggregated para-casein network of rennet casein during processed and analogue cheese manufacture (Ennis and Mulvihill, 1999) and consequently enhances the functionality (i.e. water-binding capacity, solubility, viscosity, emulsifying capacity) of the casein (O'Sullivan and Mulvihill, 2001; de Kort et al., 2011; Chapter 2, McIntyre et al., 2016).

Although CCS perturb casein-mineral equilibria affecting Ca$^{2+}$ activity, CCP concentration, and the proportion of casein in the micelle (Udabage et al., 2000), the relative contribution of each of these factors to the formation of a hydrated and functional processed or analogue
cheese matrix is unclear. Previously, the impact of CCS on calcium equilibria has been studied in detail but only in simplified aqueous systems (de Kort et al., 2009; Chapter 2, McIntyre et al., 2016) or semi-concentrated protein dispersions (Ennis et al., 1998; Ennis and Mulvihill, 1999; O’Sullivan and Mulvihill, 2001; Chapter 2, McIntyre et al., 2016). These studies addressed model systems because of the complexity of working with concentrated casein-based matrices containing lipid, however, this can make extrapolation to real food matrices such as processed and analogue cheese difficult. Therefore, despite the fact that some of the protein dispersions previously used were relatively concentrated (Ennis et al., 1998; Ennis and Mulvihill, 1999; Chapter 2, McIntyre et al., 2016), an even more concentrated model system is required to better simulate CCS behaviour during manufacture of the aforementioned products. Recently, a range of processed cheese products have been successfully manufactured on a small-scale using a Thermomix blender cooker (Černíková et al., 2008; Lee et al., 2015; Salek et al., 2015). This mixing system facilitates the manufacture of small batch sizes and allows for rapid sample collection to monitor the changes in calcium equilibria and protein solubility during the hydration, viscosity building and structure development stages of manufacture, enabling a realistic insight into the development of a casein-based food matrix.

The aim of this study was to enhance our knowledge of how ionic species e.g. calcium, partition in concentrated semi-solid casein-based food matrices by developing a small-scale manufacturing protocol for these systems. An additional aim was to validate previous investigations of the effects of CCS on calcium distribution and protein solubility in dilute model systems (Chapter 2) and to determine if such systems are representative of the true realities of processed and analogue cheese manufacture. The final objective of this work was to investigate the suitability of the Thermomix as a small-scale mixer for product manufacture relative to pilot scale manufacture.
3.2 Materials and Methods

3.2.1 Materials

Rennet casein powder (Kerrynor™ R190) with a protein content of 80 % was supplied by Kerry Ingredients Ltd. (Listowel, Co. Kerry, Ireland). Rapeseed oil was sourced from Boyne Valley Foods (Drogheda, Co. Louth, Ireland). Novelose (HI-maize 260) resistant starch was obtained from Univar (Ireland) Ltd. (Rathcoole, Co. Dublin, Ireland). The following food grade ingredients were used: anhydrous disodium phosphate (DSP) (Albright and Wilson Ltd., Cheshire, England), trisodium citrate (TSC), citric acid (Jungbunzlauer GmbH., Pernhofen, Austria), sodium chloride (Salt Union, Cheshire, England) and sorbic acid (Hoechst Ireland Ltd., Dublin, Ireland). Calcium reference standard solution for atomic absorption spectroscopy (AAS) (1000 mg / L), lanthanum trichloride heptahydrate (LaCl₃·7H₂O) and nitric acid (HNO₃) were purchased from Sigma Aldrich (Dublin, Ireland) and were analytical grade. Deionized water was prepared in a Milli-Q water purification system.

3.2.2 Manufacture of casein-based food matrices

3.2.2.1 Pilot scale

A Blentech cooker (Model CC-010, Blentech Corporation, California, U.S.A.), was used to manufacture pilot scale batches (4 kg) of low-fat casein-based food matrices containing resistant starch (Novelose). Processing conditions were similar to those described by El-Bakry et al. (2010a) when manufacturing analogue cheese. An operating speed of 100 rpm was used to manufacture casein matrices with a moisture content of 58 %. The final moisture content was determined by considering the amount of water added in the formulation combined with the moisture content of rennet casein and also allowing for the amount of steam condensed during manufacture. The casein-based food matrices were manufactured according to the following formulation, which is expressed on a dry matter basis (% w/w): 53.09 % rennet
casein, 31.19% novelose, 9.52% rapeseed oil, 2.00% TSC, 0.92% DSP, 1.69% NaCl, 1.14% citric acid and 0.19% sorbic acid. Water and rapeseed oil were added to the mixing chamber and agitated for 1 min at 50°C; the minor ingredients (DSP, TSC, NaCl and sorbic acid) were added and mixed for 1 min. The rennet casein was then added under constant agitation, and the contents blended for 1 min at 50°C. The temperature was increased to 80°C using direct steam injection, and mixing was continued until a homogeneous mass was formed and all free water and oil was absorbed. Novelose was then added and the blend mixed for 2 min. Finally citric acid was added and mixing continued for a further 1 min. The hot mass was discharged from the cooker, filled into a rectangular box lined with a polypropylene bag and placed in a freezer at -18°C and after 1 hour transferred to a refrigerator at 4°C for 24 hours before being vacuum packed (Model C10H, Webomatic® Vacuum Packaging Systems, Maschinenfabrik GmbH, Bochum, Germany). The casein-based food matrices were manufactured in triplicate.

3.2.2.2 Small-scale
Casein-based food matrices were manufactured on a small scale (500 g) using a 2L capacity Vorwerk Thermomix TM 31 blender cooker (Vorwerk & Co. Thermomix; GmbH, Wuppertal, Germany). The Thermomix has a four-blade chopper rotor at the base of the cooker. The cooker is electrically heated at the base and the heating scale ranges from 37 to 100°C. The casein matrices were processed using a method as similar as possible to that used in the Blentech pilot scale cooker. The variable mixing speed was controlled using the speed selector. The temperature of the mixing bowl was regulated by the temperature controls and monitors which were adjusted accordingly to give temperatures of 50°C or 80°C. Ingredients were added to the mixing bowl at 50°C in the same sequence and mixed for the same times as described in Section 3.2.2.1. The increase in mixture temperature from 50°C to 80°C was achieved by adjusting the Thermomix temperature controls, and verifying the temperature
using a hand-held thermometer (HANNA instruments, H19041C, Bedfordshire, UK). This increase in mixture temperature was complete in ~2.25 min. Mixing was continued until a homogeneous mass was formed, after which Novelose was added and the blend mixed for 2 min. Finally citric acid was added and mixing continued for 1 min. Small scale casein-based food matrices were manufactured in triplicate.

### 3.2.3 Compositional analysis

Moisture content was determined gravimetrically after drying the sample in a laboratory oven at 101 °C to constant weight (IDF, 1958); ash content was also determined gravimetrically after the complete incineration of the sample in a muffle furnace (B180, Nabertherm, GmbH, Germany) at 550 °C / 6h (IDF, 2007); the calcium content of the ash residue was assessed using a flame atomic absorption spectroscopy method (IDF, 2007). Fat content was evaluated using the Gerber method (National Standards Authority of Ireland, 1955), and total nitrogen was measured by the Kjeldahl method with a conversion factor of 6.38 for crude protein (IDF, 1993). The potentiometric method (Fox, 1963) was used to evaluate sodium chloride (NaCl) content. Values of pH were determined at ambient temperature by inserting a glass tip electrode of a calibrated pH-meter (EL20, Mettler Toledo, Schwerzenbach, Switzerland) directly into the casein matrix at three randomly chosen locations. All analyses were completed in triplicate for each sample batch.

### 3.2.4 Functional testing

#### 3.2.4.1 Texture profile analysis (TPA)

The texture profile of cylindrical samples (25 mm diameter, 20 mm height) were analysed using an Instron Universal Testing machine (Model 5544, Instron Corp., Canton, Mass., USA) fitted with a 1000 N load cell. Cylindrical samples were cut using a cork borer, wrapped in cling film to prevent dehydration, and allowed to equilibrate to 22 °C for 30 min prior to analysis. Afterwards, the cling film was removed and samples were compressed by 80
% of initial height using a 35 mm diameter plate at a crosshead speed of 50 mm min\(^{-1}\). The uniaxial compression test was performed in two successive cycles, and the textural parameters, hardness and cohesiveness were calculated using Blue Hill 2 software (Instron Corp., Canton, Mass., USA). Five samples were tested for each manufactured batch.

### 3.2.4.2 Dynamic rheology

Changes in the viscoelasticity on heating of cylindrical sample discs from 22 to 90 °C were measured by low amplitude strain oscillation in a controlled stress rheometer (Physica MCR 301, Anton Paar Germany GmbH, Ostfildern, Germany) fitted with a 25 mm parallel plate with a 2.2 mm gap using the method of Mounsey and O’Riordan (1999). Cylindrical sample discs (25 mm diameter, 2.4 mm thick) were cut using a meat slicer (Chef’s Choice International, Model 662, Bristol, UK) and cork borer and allowed to equilibrate to 22 °C for 15 min. Afterwards, samples were placed on the lower plate and compressed by 0.2 mm to prevent slippage, with ~3 min allowed for stress relaxation prior to analysis. A thin layer of mineral oil was applied around the sample to avoid dehydration during testing. Strain sweep experiments (0.005% strain) were undertaken to establish the linear viscoelastic region and temperature sweeps were performed within this region. The temperature of samples was increased at 2 °C min\(^{-1}\) using a peltier heating element and samples were oscillated at a frequency of 1 Hz. The rheological parameters; elastic modulus (\(G'\)), viscous modulus (\(G''\)), loss tangent (\(\tan \delta\)) and cross-over temperature (i.e. where \(G'\)=\(G''\)), were monitored. Five samples were tested for each manufactured batch.

### 3.2.4.3 Heat-induced flowability

Heat-induced flowability was measured as the increase in length of cylindrical samples (25 mm diameter, 20 mm height, 10 ± 0.05 g weight) on heating in an enclosed Pyrex glass tube (250 mm length, 30 mm diameter) at 180 °C for 10 min, as outlined by Mounsey and O’Riordan (1999). The tubes were positioned horizontally inside a pre-heated conventional
oven (Neff B45M54N3GB, Munich, Germany). Following heating, tubes were removed from the oven and, after 1 min at room temperature, the horizontal distance flowed (length) from a reference line was measured in mm and used as an indication of flowability. Five samples were tested for each manufactured batch.

3.2.5 Microstructure

3.2.5.1 Environmental scanning electron microscopy (ESEM)

ESEM was performed using a FEI Quanta 3D FEG DualBeam microscope (FEI Ltd, Hillsboro, Oregon, USA). The detection system used was a Gaseous Secondary Electron Detector. Samples (5 mm cubes) were obtained from the centre of the block (cut surface) and mounted on a Peltier cooling stage set at 5 °C. The samples were examined at a working distance of 5.8-6.5 mm and an accelerating voltage of 20 kV, using the wet mode at a water vapour pressure of 7.25 Torr to ensure that the samples remained fully hydrated. Three samples were analysed for each manufactured batch.

3.2.6 Study of calcium distribution and protein solubility during manufacture

Small-scale casein-based food matrices (58 % Moisture) were manufactured with and without CCS using the protocol described previously (Section 3.2.2.2). The manufacturing protocol was stopped at selected time intervals (1.5, 5.0, 9.0, 11.0 and 13.0 min) and the entire contents of the mixing bowl, including the matrix formed plus any free liquid still not completely incorporated, were collected inside centrifuge bottles (800 g) and centrifuged (Sorvall Legend™ T centrifuge, Thermo Scientific) at 3000 g for 10 minutes (IDF, 2002). After centrifugation, the supernatant layer was carefully removed, leaving a firm pellet. The two phases were weighed and the Ca (i.e. total Ca and Ca-ion activity ($A_{Ca^{++}}$)) and protein contents of the supernatant (dispersed phase) analysed. All analyses were performed in triplicate for each time point during manufacture.
3.2.7 Calcium analysis

3.2.7.1 Calcium-ion activity ($A_{\text{Ca}^{++}}$)

The $A_{\text{Ca}^{++}}$ in samples was measured with a calcium-ion selective electrode (ISE 25 Ca; Radiometer Analytical, Mendes, France) and a reference electrode (“Red Rod” REF 251; Radiometer Analytical, Mendes, France) fitted to a pH meter (EL20, Mettler Toledo AG, Schwerzenbach, Switzerland). Calibration was performed at ambient temperature with standard solutions containing 0.5, 5.0, or 50 mmol L$^{-1}$ CaCl$_2$ and 80 mmol L$^{-1}$ KCl. Addition of this monovalent background electrolyte was necessary, as it keeps the calcium-ion activity co-efficient ($y_{\text{Ca}^{++}}$) effectively constant in the calibration solutions (de Kort et al., 2009). Preliminary experiments were carried out using 5 CaCl$_2$ standards, selected from the range 0.001 to 100 mmol L$^{-1}$ to confirm a linear response up to 100 mmol L$^{-1}$. An $y_{\text{Ca}^{++}}$ of 0.29 was calculated for the calibration solutions using the formula of Davies (1962) below:

$$\log(y_{\text{Ca}^{++}}) = -0.5z^2 \left( \frac{\sqrt{I}}{1 + \sqrt{I}} - 0.2I \right)$$  \hspace{1cm} (1)

In which $z$ is the charge (valence) on the ion and I refers to the ionic strength of the solution in mol / L. The calculated $y_{\text{Ca}^{++}}$ value obtained was comparable to that reported by de Kort et al. (2009). The $A_{\text{Ca}^{++}}$ was determined by multiplying the experimental [Ca$^{2+}$] by the activity coefficient of 0.29. The [Ca$^{2+}$] was calculated from the regression equation, derived from the calibration curve. All experiments were carried out under continuous stirring with a laboratory stirrer (IKA RCT basic, IKA® Werke, GmbH & Co. KG, Germany) at ambient temperature due to the temperature limitations of the Ca-ISE (i.e. 50°C).

3.2.7.2 Total soluble calcium

A flame atomic absorption spectroscopy method (IDF, 2007) was used to determine total and dispersed Ca contents.
3.2.7.2.1 Sample preparation

Aliquots of ~ 3 g of the dispersed phase obtained upon centrifugation were weighed into silica crucibles and placed inside a programmable muffle furnace (B180, Nabertherm, GmbH, Germany). The furnace temperature was increased by 50 °C/hr up to 550 °C and maintained at this temperature for 6 hours. The ashed sample was subsequently dissolved in 1 ml of 25 % HNO₃ (v/v) solution and transferred quantitatively into a 250 ml volumetric flask, diluted to volume with water and mixed. The sample was appropriately diluted, and an aliquot of 10 % LaCl₃·7H₂O (v/v) was added to the final analytical solutions. Addition of lanthanum trichloride solution was necessary to inhibit phosphate interference and the ionization of elements in the atomic absorption spectrometer flame. To avoid contamination from laboratory glassware, supplies and environmental particulate matter (dust) all glassware was soaked in 20 % HNO₃ (v/v) solution for 24 hours and rinsed three times with deionized water and dried prior to use.

3.2.7.2.2 Flame atomic absorption spectrometric measurement

Samples were analysed using flame atomic absorption spectroscopy and results expressed as dispersed calcium (mmol L⁻¹). The atomic absorption spectrometer (SpectrAA, 55B, AAS, Varian), was fitted with a calcium hollow cathode lamp (Activion, Halstead, Essex, England) using a wavelength of 422.7 nm and an air-acetylene oxidizing flame for analysis. The instrument was calibrated with reference calcium standards (1.0, 2.0, 3.0, 4.0 and 5.0 mg / L) prepared from calcium reference solution (1000 mg / L) prior to analysis. An aliquot of 10 % LaCl₃·7H₂O (v/v) was also added to calibration solutions. All samples were prepared and analysed in triplicate.

3.2.8 Protein content

The protein content of the supernatant (dispersed phase) was analysed in triplicate for each sample by the Kjeldahl method with a conversion factor of 6.38 for crude protein (IDF, 1993).
3.2.9 Statistical analysis

All experiments were carried out in triplicate and the mean and standard deviation of replicates were reported. The t-test was applied to test the significance of differences between Blentech and Thermomix casein matrices at a level of $P < 0.05$. For the calcium distribution and protein solubility data, a one-way analysis of variance (ANOVA) was used to determine statistical differences. All statistical analysis were completed using SPSS (version 20) statistical software (IBM Inc. Chicago, IL, USA).

3.3 Results and Discussion

3.3.1 Small-scale manufacture of casein-based food matrices using a Thermomix

In order to establish a method for manufacture of casein-based food matrices in the Thermomix, it was first necessary to standardise the batch size and mixing speed. The criteria used for the standardisation procedures were based largely on the macroscopic observations made during processing and results obtained from compositional analysis. Sample size was an important factor for adequate mixing of the ingredients. The sample sizes used to manufacture casein matrices varied from 250 to 900 g. The aim of this study was to have a relatively small batch size to enable whole sample collection (i.e. matrix formed and free liquid) during manufacture and subsequent separation of the dispersed and insoluble phases by centrifugation. At the smaller sample sizes i.e. $< 400$ g, the ingredients were below the base of the blade in the Thermomix and were not mixing sufficiently. Increasing the sample size improved mixing and a batch size of 500 g was subsequently found to be the smallest batch size to give the best end-product homogeneity, with no visible free water or oil present. Using speed setting 1 (100-200 rev/min) and speeds 3-7 (400-3000 rev/min) on the Thermomix, it did not prove possible to form a homogeneous product due to insufficient or excessive mixing, respectively. A minimum speed of 250-
300 rev/min which corresponds to speed setting 2 on the Thermomix was required to form a cohesive mass and provided adequate mixing of ingredients. Moisture contents of the casein-based food matrices were also varied (60, 58, 50 and 45 %), longer mixing times were required to incorporate higher levels of water into the system, however sufficient moisture uptake and fat emulsification for formation of a homogeneous matrix was achieved in all cases. Results of compositional analysis showed very good repeatability with close approximation to target composition. At reduced moisture levels (e.g. 50 and 45 %) the casein matrices formed were quite hard due to their high solids content and this led to difficulties during their manufacture in the Thermomix. The issues encountered included excessive strain on the Thermomix motor and prolonged mixing times required to form a homogeneous product. Using the newly developed protocol, casein-based food matrices could be reproducibly manufactured on a small scale (500 g) with a range of different formulations.

3.3.2 Comparison of Thermomix and Blentech casein-based food matrices

3.3.2.1 Composition, pH and processing time

The chemical composition and pH of casein matrices (target moisture content: 58 %) manufactured in the Thermomix were not significantly different (P > 0.05) to those of equivalent moisture content made in the Blentech (Table 3.1). However, the manufacture time was ~ 7 min (> 100 %) longer for casein matrices prepared in the Thermomix (Table 3.1). The difference in the action, speed and geometry of the two mixing systems may explain the difference in the manufacture time. The mixing action of the Blentech gives a kneading and stretching of the entire protein mass in the mixer by twin-screw single ribbon augers, which continuously mixes free water throughout the protein mass facilitating more rapid hydration and leads to less shearing of the matrix (Noronha et al., 2008a). The four-blade chopper rotor of the Thermomix operates differently giving a centrally localised mixing zone with a
shearing/cutting action which may require a longer time to allow all of the product to pass through the mixing zone. A similar rationale was offered by Noronha et al. (2008a) to explain differences in processing times of analogue cheese manufactured using Blentech and Stephan mixers.
Table 3.1 Processing time, chemical composition and pH of casein-based food matrices made in the *Thermomix* (batch size 500 g) or the *Blentech* (batch size 4 kg) mixer.

<table>
<thead>
<tr>
<th>Mixer</th>
<th>Processing time (min)</th>
<th>Moisture (%)</th>
<th>Protein (%)</th>
<th>Fat (%)</th>
<th>Salt (%)</th>
<th>Ash (%)</th>
<th>Total Ca (mg/100g)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blentech</td>
<td>6.60&lt;sup&gt;a&lt;/sup&gt; ± 0.70</td>
<td>57.68&lt;sup&gt;a&lt;/sup&gt; ± 0.68</td>
<td>22.93&lt;sup&gt;a&lt;/sup&gt; ± 0.13</td>
<td>4.10&lt;sup&gt;a&lt;/sup&gt; ± 0.24</td>
<td>1.32&lt;sup&gt;a&lt;/sup&gt; ± 0.02</td>
<td>4.01&lt;sup&gt;a&lt;/sup&gt; ± 0.01</td>
<td>652.17&lt;sup&gt;a&lt;/sup&gt; ± 0.36</td>
<td>6.10&lt;sup&gt;a&lt;/sup&gt; ± 0.01</td>
</tr>
<tr>
<td>Thermomix</td>
<td>13.55&lt;sup&gt;b&lt;/sup&gt; ± 0.57</td>
<td>57.90&lt;sup&gt;a&lt;/sup&gt; ± 0.40</td>
<td>22.90&lt;sup&gt;a&lt;/sup&gt; ± 0.20</td>
<td>3.90&lt;sup&gt;a&lt;/sup&gt; ± 0.30</td>
<td>1.30&lt;sup&gt;a&lt;/sup&gt; ± 0.01</td>
<td>3.94&lt;sup&gt;a&lt;/sup&gt; ± 0.05</td>
<td>648.89&lt;sup&gt;a&lt;/sup&gt; ± 0.20</td>
<td>6.15&lt;sup&gt;a&lt;/sup&gt; ± 0.03</td>
</tr>
</tbody>
</table>

Values represent the mean of triplicates. For each column, means with the same letter do not differ significantly at P < 0.05.
3.3.2.2 Texture profile analysis (TPA) and heat-induced flowability

The mean values for TPA-hardness of casein matrices produced with the Thermomix were significantly higher (P < 0.05) and the heat-induced flowability values (Table 3.2) lower than for those matrices manufactured in the Blentech. This indicates that matrices made in the Blentech were softer in texture and more flowable or meltable than those prepared in the Thermomix. Processing parameters such as cook temperature, cook time, and the amount of shear applied during manufacture play a major role in the resulting functional properties of processed and analogue cheese (Rayan et al., 1980; Berger et al., 1998; Glenn et al., 2003). Therefore variations in these parameters could explain the observed differences in functional properties between casein matrices produced by both mixers. It has been reported that analogue cheese functionality is affected by both the mixing action and mixing speed applied during manufacture (Noronha et al., 2008a). Prolonged mixing time was found to reduce the fat globule size and as a consequence a firmer, less meltable cheese was formed. (Noronha et al., 2008a; El-Bakry et al., 2010a). This may explain why in the present study the matrices which required prolonged processing times in the Thermomix were firmer in texture and less meltable. The increased processing times could have also resulted in reduced flowability due to increased protein-protein interactions occuring during prolonged mixing, which is consistent with the findings of other studies (Lee et al., 2003, Noronha et al., 2008b; El-Bakry et al., 2010a).
Table 3.2 TPA-hardness, cohesiveness, cross-over temperature values (Tc) and heat-induced flowability of casein-based food matrices made in the Thermomix (batch size: 500 g) or the Blentech (batch size: 4 kg) mixer.

<table>
<thead>
<tr>
<th></th>
<th>Hardness (N)</th>
<th>Cohesiveness (-)</th>
<th>Tc (°C)</th>
<th>Melt (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blentech</td>
<td>234.07 ± 15.11</td>
<td>0.41 ± 0.08</td>
<td>38.27 ± 0.85</td>
<td>123.2 ± 4.75</td>
</tr>
<tr>
<td>Thermomix</td>
<td>260.39 ± 7.29</td>
<td>0.35 ± 0.05</td>
<td>44.62 ± 0.50</td>
<td>105.1 ± 3.12</td>
</tr>
</tbody>
</table>

Values represent the mean of triplicates. For each column, means with the same letter do not differ significantly at P < 0.05.

3.3.2.3 Microstructure

In the ESEM images the starch particles are clearly visible as discrete spherical particles distributed throughout a continuous protein matrix (Figure 3.1). These starch particles appeared relatively intact and un-swollen with prominent boundaries indicating little or no interaction with the protein matrix. This was observed for casein matrices prepared both in the Blentech (Figure 3.1 a) and Thermomix (Figure 3.1 b) mixers. The ESEM micrographs were similar to those shown by Noronha et al. (2008c) for analogue cheese of similar formulation containing resistant starch. The fat globules were spherical in shape and well distributed throughout the protein matrix for the matrices prepared in the Blentech (Figure 3.1 a). However, the fat globules were difficult to identify in the micrographs obtained from those prepared in the Thermomix (Figure 3.1 b). This may be because these matrices were subjected to greater mixing energy and longer processing times, which may have resulted in reduced fat globule size (Noronha et al., 2008a) and the fat being more finely distributed which may have caused it to become more embedded in the protein matrix. In addition the low fat content
(4 %) of the matrices may also have contributed to difficulties identifying fat globules in the ESEM micrographs.

Figure 3.1 Environmental scanning electron micrographs of casein-based food matrices manufactured in the Blentech (a) or Thermomix (b) mixer. P: protein matrix; F: fat globule; S: starch particle.

3.3.2.4 Dynamic rheology

The effect of temperature (22-95 °C) on \( G' \) was broadly similar for both casein matrices irrespective of cooker type (Figure 3.2 a). However, the matrices produced in the Thermomix had higher \( G' \) values at 22 °C, thus exhibiting more solid-like behaviour, which is consistent with their higher hardness values (Table 3.2). For both matrices, the \( G' \) values decreased as a function of temperature increase, and in the temperature range of 22-50 °C, decreased sharply. This decrease in \( G' \) values reflects a softening of the matrix with increasing temperature that has been reported by many (Mounsey and O’Riordan, 1999; Noronha et al., 2008a; El-Bakry et al., 2010a). The crossover temperature (\( T_c \)), when \( \tan \delta = 1 \) (\( G' = G'' \)), is an indication of melting temperature (Mounsey and O’Riordan, 1999) and there was a significant difference (\( P < 0.05 \)) between these values for casein matrices prepared in both mixers. Those manufactured in the Thermomix had lower flowability and higher \( T_c \) values than matrices prepared in the Blentech (Table 3.2). This may suggest that the higher shearing action occurring in the Thermomix results in greater casein-casein interactions giving a harder matrix.
(Table 3.2) which in turn requires greater energy (higher temperature) for melting and thus exhibits greater resistance to flow.

Figure 3.2 Effect of temperature on $G'$ (a) or tan delta (b) of casein-based food matrices manufactured in different mixers: Blentech (■) or Thermomix (□).
3.3.3 Study of calcium distribution and protein solubility during manufacture of casein-based food matrices

3.3.3.1 Quantification of centrifugal supernatants

Casein-based food matrices were prepared with and without CCS in the Thermomix using the manufacturing protocol described previously (Section 3.2.2.2). Manufacture was interrupted at various times throughout processing and the centrifugal supernatants were obtained as outlined in Section 3.2.6. For matrices manufactured with CCS, there was a significant decrease (P < 0.05) in supernatant volume with increasing processing time (Table 3.3). This reflects water uptake into the casein network during the heating and shearing process due to weakening of protein interactions. Quantification of the supernatant showed that ~130 g, which corresponds to ~45 % of the total added liquid, was initially separable from the matrix after the first 5 min of mixing (Table 3.3). A similar supernatant volume was reported by El-Bakry *et al.* (2011) after 5.5 min of processing analogue cheese containing CCS. Following a further 4 min of processing, a substantial decrease (~26 %) in the volume of separable liquid was recorded, indicating that the casein had absorbed more water and less free liquid was evident during processing. Extending the mixing time to 11 min, resulted in a similar supernatant volume to that recovered at 9 min. From 13 min onwards, a homogeneous mass formed typical of the final matrix, with no free liquid visually present. Upon centrifugation of the matrix formed, only ~14 % of the total added liquid was recovered, presumably as a result of the centrifugation squeezing some of the water from the matrix.

The quantity of supernatant recovered in the CCS-free matrices was significantly higher (P < 0.05) at equivalent processing times (Table 3.3). While there was also a decrease in supernatant volume measured for CCS-free matrices as processing time progressed, this was much more gradual and occurred to a lower extent than in matrices prepared with CCS. For the CCS-free matrices the oil and aqueous phases could not be combined in a homogeneous mix,
even on extending the processing time (> 30 min). Therefore, it was not possible to form a matrix without the addition of CCS. It may be that, due to extensive calcium-mediated cross-links, the casein remained insufficiently hydrated to give emulsification of the oil (Noronha et al., 2008a; El-Bakry et al., 2010b; Guinee and O’Kennedy, 2012). Poorly hydrated casein has been previously reported to be a less active emulsifier and cannot adequately stabilise the oil phase in processed and analogue cheese applications (Ennis and Mulvihill, 1999; Dimitreli and Thomareis, 2008). Visual observation during manufacture showed the partially hydrated, but still particulate, casein, in an excess of free water and oil. Quantification of the supernatant showed that ~109 g, which corresponds to ~40 % of the total added liquid, was still separable from the matrix after 13 min of mixing and this level remained unchanged even after 30 min of processing (data not shown).
Table 3.3 Weights of the dispersed and insoluble phases obtained after centrifugation and the soluble protein (%), total soluble calcium concentration and calcium-ion activity $A_{Ca^{++}}$ (mmol L$^{-1}$) in centrifugal supernatants of casein-based food matrices prepared with or without calcium chelating salts at different processing times.

<table>
<thead>
<tr>
<th>Processing Time (min)</th>
<th>Calcium chelating salts</th>
<th>Pellet weight (g)</th>
<th>Supernatant weight (g)</th>
<th>Protein content (%)</th>
<th>Total soluble calcium content (mmol L$^{-1}$)</th>
<th>$A_{Ca^{++}}$ (mmol L$^{-1}$)</th>
</tr>
</thead>
<tbody>
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</tr>
<tr>
<td>1.5</td>
<td>+</td>
<td>-</td>
<td>293.3$^{a}$$\pm$1.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
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<td>-</td>
<td>297.1$^{a}$$\pm$1.4</td>
<td>-</td>
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<tr>
<td>5.0</td>
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<td>280.0$^{a}$$\pm$6.3</td>
<td>131.8$^{b}$$\pm$7.4</td>
<td>0.33$^{a}$$\pm$0.05</td>
<td>16.64$^{a}$$\pm$2.0</td>
<td>0.26$^{a}$$\pm$0.02</td>
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<tr>
<td></td>
<td>-</td>
<td>227.6$^{b}$$\pm$6.3</td>
<td>180.3$^{c}$$\pm$2.0</td>
<td>0.10$^{b}$$\pm$0.20</td>
<td>9.23$^{b}$$\pm$5.5</td>
<td>0.45$^{b}$$\pm$0.02</td>
</tr>
<tr>
<td>9.0</td>
<td>+</td>
<td>403.4$^{c}$$\pm$3.2</td>
<td>54.8$^{d}$$\pm$2.1</td>
<td>0.53$^{c}$$\pm$0.09</td>
<td>17.08$^{ac}$$\pm$1.4</td>
<td>0.15$^{c}$$\pm$0.03</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>351.9$^{d}$$\pm$3.2</td>
<td>119.0$^{e}$$\pm$1.2</td>
<td>0.10$^{bd}$$\pm$0.20</td>
<td>9.47$^{bd}$$\pm$4.4</td>
<td>0.45$^{bd}$$\pm$0.13</td>
</tr>
<tr>
<td>11.0</td>
<td>+</td>
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<td>51.7$^{f}$$\pm$3.6</td>
<td>1.1$^{ce}$$\pm$0.34</td>
<td>52.64$^{ce}$$\pm$3.0</td>
<td>0.13$^{ce}$$\pm$0.10</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>350.6$^{df}$$\pm$6.0</td>
<td>115.2$^{eg}$$\pm$3.8</td>
<td>0.14$^{bd}$$\pm$0.10</td>
<td>9.87$^{bf}$$\pm$2.2</td>
<td>0.45$^{bf}$$\pm$0.10</td>
</tr>
<tr>
<td>13.0</td>
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<td>422.1$^{ef}$$\pm$5.2</td>
<td>40.9$^{h}$$\pm$2.4</td>
<td>0.98$^{ef}$$\pm$0.11</td>
<td>65.40$^{ef}$$\pm$2.7</td>
<td>0.09$^{ef}$$\pm$0.07</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>362.3$^{h}$$\pm$4.4</td>
<td>109.5$^{i}$$\pm$7.3</td>
<td>0.15$^{h}$$\pm$0.16</td>
<td>8.40$^{h}$$\pm$5.0</td>
<td>0.45$^{hb}$$\pm$0.08</td>
</tr>
</tbody>
</table>

Values represent the mean of triplicates. For each column, means with the same letter do not differ significantly at P < 0.05.

Casein-based food matrices were prepared with (+) or without (−) calcium chelating salts.

* soluble calcium = calcium recovered from the centrifugal supernatant.
3.3.3.2 Analysis of calcium and protein contents of centrifugal supernatants

For casein matrices prepared with CCS there was a significant (P < 0.05) increase in the levels of Ca and decrease in \( A_{Ca}^{++} \) in centrifugal supernatants with increasing processing time (Table 3.3). The levels of soluble Ca equated to \(~6\%\) of the total Ca after \( \leq 9 \) min of processing and significantly increased (P < 0.05) during the latter stages of manufacture (Table 3.3). The protein content of these centrifugal supernatants ranged from \(~0.3\%\) at 5 min up to \(~1\%\) at processing times \( \geq 9 \) min. Therefore, the soluble protein levels up to 11 min of mixing were relatively low (Table 3.3), only \(~3\%\) of total added protein. The centrifugal supernatants obtained during processing of casein matrices which contained no CCS had significantly lower (P < 0.05) Ca levels (Table 3.3), which only equated to \(~2-3\%\) of the total Ca and this level, along with the \( A_{Ca}^{++} \) remained essentially constant throughout the course of manufacture. Hence, the proportion of total calcium recovered in the supernatant was \(~8\) times higher in matrices prepared with CCS than in the CCS-free matrices. In addition, these centrifugal supernatants had even lower protein contents over the first 11 min of mixing compared to those obtained from matrices containing CCS, with only \(~0.4\%\) of the total added protein present in the supernatant liquid. This level also remained practically unchanged throughout the course of manufacture (Table 3.3). Therefore, the proportion of total protein recovered in the supernatant was almost \(~7\) times higher in matrices prepared with CCS than in the CCS-free matrices.

These results suggest that for casein matrices containing CCS, only partial disruption of the calcium-mediated cross-links in rennet casein occurs during the early stages of processing. However, as processing time increases, more of the calcium-mediated cross-links are disrupted and this allows the protein moieties to hydrate and disperse. By the end of manufacture the concentration of Ca in the supernatant was \(~23\%\) of the total Ca. In the study completed in Chapter 2 using dilute rennet casein dispersions (12 % w/w protein) with
similar concentrations (i.e. 30 mM) and ratios of CCS (i.e. 2:1), higher levels of soluble Ca (~88 % of the total Ca) were reported than those obtained in the present study. However, the lower protein content and thus less concentrated system (12 % vs 22 % w/w protein used in the present study) of the rennet casein dispersions, combined with the difference in the mixing conditions used to prepare the dispersions may explain the discrepancies in the solubility values obtained. The CCS mediated demineralisation of casein leads to degradation of intra- and inter-casein calcium cross-links which exposes charged phosphoserine residues thus resulting in an increase in negative charge, which increases the charge repulsion between the casein particles increasing their solubility (Ennis et al., 1998). The greater conformational freedom and increased mobility gained by the proteins results in greater interpenetration of protein chains and allows network formation to proceed to such an extent that the integrity of individual rennet casein particles is lost (O’Sullivan and Mulvihill, 2001). The dispersed and in particular the hydrated protein then contributes to the emulsification of the fat and stability of the emulsion by stabilising free water (Fox et al., 2000) and the dispersion appears macroscopically homogeneous during processing.

In contrast, elimination of CCS prevented successful matrix formation and stabilisation, owing to insufficient Ca removal from, and solubilisation of the casein. The significantly (P < 0.05) lower levels of Ca present in the supernatant liquid obtained from matrices manufactured without CCS, may suggest that only partial disruption of the calcium-mediated cross-links occurred. Although some casein dispersion can be caused solely by the mechanical and thermal energies applied during manufacture (Glenn et al., 2003), these results suggest that in the absence of CCS, a point is reached in the cooking process where the caseins are as dispersed as they can be under the specific conditions (i.e. pH, temperature etc) of manufacture and this level is not sufficient to allow formation of a hydrated matrix. Insufficient hydration of the protein component leads to unsatisfactory incorporation of the oil
phase, and largely explains why the oil and aqueous phases could not be combined in a homogeneous mix. In the absence of CCS, the amount of soluble protein in the centrifugal supernatants was lower than in those obtained from matrices containing CCS (Table 3.3); consequently, there may not have been enough soluble protein present to emulsify the fat. Even though the higher levels of solubilised protein obtained in the presence of CCS might still be considered relatively low, these levels were far higher compared to those present in CCS-free centrifugal supernatants, by \( \sim 7 \) times at processing times \( \geq 11 \) min and consequently the matrices prepared with CCS did come together with the fat well emulsified. The low levels of solubilised protein observed in the present study are consistent with those reported in previous studies in this laboratory (Chapter 2) for rennet casein dispersions (12% w/w protein) with added CCS; in which it was suggested that only low levels of soluble protein may be required to emulsify the fat in an analogue cheese application, given the high emulsifying capacity of casein. If this is the case, although the level of protein solubilised by CCS is low, it may still be sufficient for the formation of a physicochemically stable matrix containing emulsified fat.

### 3.4 Conclusion

The results of this study demonstrate that the Thermomix can be used as a micro-scale manufacturing system for product manufacture which is representative of that made in a traditional pilot scale cooker; this may have consequent cost savings for those engaged in processed and analogue cheese formulation research. More importantly, the results of this study help clarify the role of CCS in modulating hydration and matrix formation during manufacture of such matrices. Monitoring the progression of calcium solubilisation at different processing times during manufacture has not been previously investigated in concentrated systems. The results obtained in the present study strongly suggest that the role of CCS is mainly to deplete colloidal calcium by partial solubilisation, which reduces
calcium-mediated cross-linking sufficiently to allow adequate protein solubilisation to occur for fat emulsification. In the absence of CCS this is simply not possible. Thus, a similar attenuation of calcium mediated cross-links might possibly be achieved by simply modifying the total calcium concentration of these matrices. If, as the results suggest, only ~23 % of colloidal calcium needs to be solubilised to form a hydrated matrix, this may be a useful predictor of the level by which to reduce the total calcium concentration by.

3.5 References


Chapter 4

Manipulating Calcium Level Provides a New Approach for the Manufacture of Casein-Based Food Structures with Different Functionalities

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4.1 Introduction

Rennet casein is a dairy ingredient used in the manufacture of processed and analogue cheese. It comprises a dehydrated, particulate protein network of calcium phosphate para-casein, the physico-chemical properties of which are greatly influenced by molecular interactions between calcium and casein (Chapter 2 and 3, McIntyre et al., 2016). Rennet casein has a limited hydration capacity and solubility in water due to extensive calcium-mediated intermolecular cross-linking among caseins (Ennis et al., 1998). In industrial applications, so-called ‘calcium chelating salts’ (CCS) e.g. sodium phosphates and citrates are commonly used to chelate calcium and disrupt the calcium phosphate-linked para-casein network present in rennet casein. During this process, a calcium-sodium ion exchange occurs which involves the displacement of Ca\(^{2+}\) directly bound to casein by Na\(^+\), causing a decrease in concentration of Ca\(^{2+}\), solubilisation of colloidal calcium (calcium phosphate) from casein micelles (Chapter 2 and 3, McIntyre et al., 2016) and formation of a water-dispersible sodium para-caseinate. This exchange reaction facilitates hydration and enhances the water binding and emulsifying ability of rennet casein during processed and analogue cheese manufacture (Ennis and Mulvihill, 1999). However, the CCS contribute to a relatively high sodium content in the end-product and lead to what is considered an ‘unclean’ label from a consumer perspective.

In recent years there has been an increase in consumer demand for food products formulated with clean label ingredients (Brockman and Beeren, 2011; Lähteenmäki, 2015). This has translated into the clean label trend currently driving product development within the food industry and consequently, formulating products with simple labels using recognisable ingredients is a growing commercial necessity (Boothroyd, 2014). In addition, the global high
prevalence of hypertension and cardiovascular disease has raised concerns regarding the sodium content of the foods which we consume (Dötsch et al., 2009). CCS are food additives which are also sources of sodium in processed and analogue cheese formulations (Johnson et al., 2009). Reducing or eliminating CCS from these products presents a significant challenge, due to their ability to influence several critical events e.g. Ca^{2+} binding, pH adjustment, casein dispersion, fat emulsification, and structure formation during manufacture. Previous research efforts have been focussed on substitution of sodium-based CCS with their potassium equivalents (Metzger and Kapoor, 2007; El-Bakry et al., 2011a), various salt replacers (Schäffer et al., 1999; Schäffer et al., 2001; Černíková et al., 2010), and traditional emulsifiers (Lucey, 2008; Paulus, 2008), often having a negative impact on the functionality or sensory acceptance of the final cheese product. Other approaches have included reducing the level of CCS and altering the ratio of CCS used, both of which had a major influence on cheese manufacture and subsequent functional properties (El-Bakry et al., 2010a; 2011b). In addition, neither approach could yield a CCS reduction of more than 20%.

Since CCS function by reducing or attenuating calcium mediated cross-linking in rennet casein as demonstrated in Chapter 2 and 3, an alternative approach to modifying the level of cross-linking might be to simply formulate matrices with reduced total calcium levels. Thus the objective of this study was to build upon the work described in Chapter 3 and investigate the effect of altering the calcium levels (1080, 775, 673, 569, 514, 462, 410, 358 and 37 mg.100 g^{-1}) of casein-based food matrices by substituting high calcium rennet casein with a lower calcium alternative i.e. acid casein. The structure formation, texture, rheology and flow behaviour of these CCS-free matrices were evaluated and compared to those of a high calcium content (1080 mg.100 g^{-1}) prepared using rennet casein and CCS.
4.2 Materials and Methods

4.2.1 Materials

Rennet casein powder (Kerrynor™ R190) with a protein content of 80 % was supplied by Kerry Ingredients Ltd (Listowel, Co. Kerry, Ireland). Acid casein powder (87 % protein) was provided by Glanbia Ingredients Ltd (Ballyragget, Co. Kilkenny, Ireland). The calcium content of the rennet and acid casein powder as determined by a flame atomic absorption spectroscopy method (IDF, 2007), were 2700 mg.100 g⁻¹ and 100 mg.100 g⁻¹, respectively. Rapeseed oil was obtained from Boyne Valley Foods (Drogheda, Co. Louth, Ireland). The following food grade ingredients were used: disodium phosphate (DSP) anhydrous (Albright and Wilson Ltd., Cheshire, England), trisodium citrate (TSC), citric acid (Jungbunzlauer GmbH, Pernhofen, Austria), sodium chloride (Salt Union, Cheshire, England) and sorbic acid (Hoechst Ireland Ltd., Dublin, Ireland). Chemical reagents: calcium reference standard (1000 mg L⁻¹), lanthanum trichloride heptahydrate (LaCl₃.7H₂O) and nitric acid (HNO₃) were purchased from Sigma Aldrich (Dublin, Ireland) and were analytical grade. Deionized water was prepared in a Milli-Q water purification system.

4.2.2 Manufacture of casein-based food structures

Casein matrices were manufactured in a Thermomix blender cooker (Thermomix TM 31; Vorwerk & Co., GmbH, Wuppertal, Germany) according to the following formulation (% w/w): 58.0 % moisture, 33.0 % total protein, 4.0 % rapeseed oil, 1.0 % NaCl, 0.08 % sorbic acid. The final moisture content of the matrices was determined by considering the amount of water added in the formulation combined with the moisture content of the rennet and/or acid casein powder. Standard high calcium (1080 mg.100 g⁻¹) matrices prepared using CCS were manufactured using rennet casein only as the protein source and contained 1.35 % TSC and 0.61 % DSP; citric acid (0.50 %) was also included in the formulation. An unsuccessful attempt was also made to manufacture a high calcium (1080 mg.100 g⁻¹) matrix using rennet
casein without added CCS. The CCS-free matrices containing various levels of calcium: 775, 673, 569, 514, 462, 410, 358 and 37 mg.100 g⁻¹ were manufactured using mixture ratios of rennet and acid casein of 70:30, 60:40, 50:50, 45:55, 40:60, 35:65, 30:70 and 0:100 w/w, respectively. The casein-based food matrices were grouped depending on their calcium concentration into those containing high (≥ 775 mg.100 g⁻¹), intermediate (673-358 mg.100 g⁻¹) or low (≤ 37 mg.100 g⁻¹) calcium levels. Batches (500 g) of each casein matrix were manufactured by agitating (speed 3, ~250 rpm) the rapeseed oil, water and minor ingredients (i.e. sorbic acid and sodium chloride) in the cooker at 50 °C for 2 min. The rennet and acid casein powders were added in sufficient quantities to give the desired calcium levels between 775-37 mg.100 g⁻¹, the temperature was increased to 80 °C (heating rate ~13.3 °C / min) and maintained with continuous agitation until a homogeneous mass was formed. Following processing, the hot molten matrices were filled into plastic containers and placed in a freezer at -18 °C and after 1 h transferred to a refrigerator at 4 °C for 24 h before being vacuum packed (Model C10H, Webomatic® Vacuum Packaging Systems, Maschinenfabrik GmbH, Bochum, Germany). For the high calcium matrices prepared with CCS; the CCS were added at the same time point as other minor ingredients and, for pH adjustment, food grade citric acid was added toward the end of manufacture. All casein matrices were manufactured in triplicate at each calcium level.

4.2.3 Compositional analysis

Moisture content was determined gravimetrically after drying the sample in a laboratory oven at 101 °C to constant weight (IDF, 1958); ash content was also determined gravimetrically after the complete incineration of the sample in a muffle furnace (B180, Nabertherm, GmbH, Germany) at 550 °C for 6 h (IDF, 2007); the calcium content of the ash residue was assessed using a flame atomic absorption spectroscopy method (IDF, 2007). Fat content was determined using the Gerber method (National Standards Authority of Ireland, 1955), and
total nitrogen was measured by the Kjeldahl method with a conversion factor of 6.38 for crude protein (IDF, 1993). The potentiometric method (Fox, 1963) was used to evaluate sodium chloride (NaCl) content. Values of pH were determined at ambient temperature by inserting a glass tip electrode of a calibrated pH-meter (EL20, Mettler Toledo, Schwerzenbach, Switzerland) directly into the matrix at three randomly chosen locations. All analyses were completed in triplicate.

4.2.4 Functional testing

4.2.4.1 Texture profile analysis (TPA)

TPA was performed on cylindrical samples (25 mm diameter, 20 mm height) using an Instron Universal Testing Machine (Model 5544, Instron Corp., Canton, Mass., USA) fitted with a 1000 N (standard high calcium matrices) or 500 N (CCS-free matrices) load cell. Cylindrical samples were cut using a cork borer, wrapped in cling film to prevent dehydration, and allowed to equilibrate to 22 °C for 30 min prior to analysis. Afterwards, the cling film was removed and samples were compressed by 80 % of initial height using a 35 mm diameter plate at a crosshead speed of 50 mm min⁻¹. The uniaxial compression test was performed in two successive cycles, and the textural parameters, hardness and cohesiveness were calculated. Three batches of five samples were tested for each of the calcium levels studied.

4.2.4.2 Dynamic rheology

Changes in the viscoelasticity of cylindrical sample discs on heating from 22 to 90 °C were measured by low amplitude strain oscillation in a controlled stress rheometer (Physica MCR 301, Anton Paar Germany GmbH, Ostfildern, Germany) fitted with a 25 mm parallel plate with a 2.2 mm gap using the method of Mounsey and O’Riordan (1999). Cylindrical sample discs (25 mm diameter, 2.4 mm thick) were cut using a meat slicer (Chef’s Choice International, Model 662, Bristol, UK) and cork borer and allowed to equilibrate to 22 °C for 15 min. Afterwards, samples were placed on the lower plate and compressed by 0.2 mm to
prevent slippage, with ~3 min allowed for stress relaxation prior to analysis. A thin layer of mineral oil was applied around the sample to avoid dehydration during testing. All measurements were undertaken at an applied shear stress of 100 Pa and a strain of below 1 %, which had been previously established to be within the linear viscoelastic range of analogue cheese (Noronha et al., 2008a). The temperature of samples was increased at 2 °C min⁻¹ using a Peltier heating element and samples were oscillated at a frequency of 1 Hz. The rheological parameters: elastic modulus (G’), viscous modulus (G’”), loss tangent (tan δ) and cross-over temperature (i.e. where G’=G’”), were monitored. Three batches of five samples were tested for each of the calcium levels studied.

4.2.4.3 Heat-induced flowability
Heat-induced flowability was measured as the increase in length of cylindrical samples (25 mm diameter, 20 mm height, 10 ± 0.05 g weight) on heating in an enclosed Pyrex glass tube (250 mm length, 30 mm diameter) at 180 °C for 10 min, as outlined by Mounsey and O’Riordan (1999). The tubes were positioned horizontally inside a pre-heated conventional oven (Neff B45M54N3GB, Munich, Germany). Following heating, tubes were removed from the oven and, after 1 min at room temperature, the horizontal distance flowed (length) from a reference line was measured in mm and used as an indication of flowability. Three batches of three samples were tested for each of the calcium levels studied.

4.2.5 Statistical analysis
All experiments were repeated three times. The data were presented as means ± standard deviations. One-way analysis of variance (ANOVA) was used to determine statistical differences. Where significant differences were displayed (P < 0.05), a Tukey pairwise test was completed to determine the significance. All statistical analysis were completed using SPSS (version 20) statistical software (IBM Inc. Chicago, IL, USA).
4.3 Results and Discussion

4.3.1 Matrix formation

The high calcium matrices produced with CCS and those prepared without CCS at various calcium concentrations were manufactured as outlined in Section 4.2.2. Figure 4.1 shows images of a selection of the products formed during manufacture. The standard high calcium (1080 mg.100 g⁻¹) matrices, produced with CCS formed a smooth homogeneous fluid mass during mixing (Figure 4.1 a) similar to those reported by Noronha et al. (2008a) and El-Bakry et al. (2010b). Similar structures were formed without CCS for matrices manufactured at intermediate calcium levels i.e. 673-358 mg.100 g⁻¹ (Figure 4.1 d and e). This may be because at these calcium levels an optimal balance is achieved between protein-protein and protein-water interactions i.e. the former are reduced sufficiently to allow the latter to occur leading to formation of an adequately hydrated casein matrix. The mixing time required to form a homogeneous mass for matrices at intermediate calcium levels was shorter than for standard high calcium matrices produced with CCS, leading to a ~3 fold decrease in processing times (Table 4.1). Enhanced protein-water interactions may have resulted in greater levels of plasticisation requiring less mixing energy, and which in turn may have allowed for fat incorporation and the formation of a homogeneous matrix to occur more quickly. Figure 4.2 shows images of the cohesive semi-solid blocks formed upon cooling of the matrices containing intermediate calcium levels (Figure 4.2 d and e), which closely resembled those of high calcium content prepared with rennet casein and CCS (Figure 4.2 a).

The CCS-free matrices with high (1080 and 775 mg.100 g⁻¹) or low (37 mg.100 g⁻¹) calcium levels did not form stable structures during manufacture (Figure 4.1 b, c and f). For the highest calcium (1080 mg.100 g⁻¹) matrices containing no CCS, the oil and aqueous phases could not be combined in a homogeneous mix (Figure 4.1 b), even on extending the processing time (Table 4.1). It may be that, due to extensive calcium-mediated cross-links, the
casein remained insufficiently hydrated to give emulsification of the oil (Noronha et al., 2008a; El-Bakry et al., 2010a; Guinee and O’Kennedy, 2012). Poorly hydrated casein has been previously reported to be a less active emulsifier and cannot adequately stabilize the oil phase in processed and analogue cheese (Ennis and Mulvihill, 1999; Dimitreli and Thomareis, 2008). Matrices with calcium levels of either 775 (Figure 4.1 c) or 37 (Figure 4.1 f) mg.100 g⁻¹, similarly appeared to exhibit lower levels of casein hydration and prolonged mixing times were required to emulsify the fat adequately. The 775 mg.100 g⁻¹ matrix lost all solid-like structure at 80 °C and formed a low viscosity milky liquid containing swollen particulates (Figure 4.1 c) and upon cooling exhibited extensive oiling-off and moisture exudation (Figure 4.2 c). For the matrices prepared at low calcium levels (37 mg.100 g⁻¹), the structure formed had a ‘short’ uneven texture, suggesting that there was not enough calcium present to form a cohesive matrix (Figure 4.2 f). Due to the non-uniform nature of the high (1080 and 775 mg.100 g⁻¹) and low (37 mg.100 g⁻¹) calcium matrices prepared without CCS (Figure 4.2 b, c and f), which made reliable sampling impossible, no further characterisation of these products was performed.
Figure 4.1 Photographs of matrix development during manufacture of high calcium (1080 mg.100 g\(^{-1}\)) casein matrices prepared with chelating salts (a); chelating salt-free, high calcium (1080 mg.100 g\(^{-1}\)) matrices (b) and chelating salt-free, matrices containing calcium levels of 775 (c) 673 (d) 358 (e) or 37 mg.100 g\(^{-1}\) (f).
Figure 4.2 Photographs of final matrix structure formed upon cooling of high calcium (1080 mg.100 g⁻¹) casein matrices prepared with chelating salts (a); chelating salt-free, high calcium (1080 mg.100 g⁻¹) matrices (b) and chelating salt-free, matrices containing calcium levels of 775 (c) 673 (d) 358 (e) or 37 mg.100 g⁻¹ (f).
4.3.2 Composition

Table 4.1 shows the composition of high calcium matrices manufactured with CCS and those prepared without CCS at various calcium concentrations. Moisture, fat, protein and NaCl contents of all matrices were similar (P > 0.05). This was expected as all matrices were formulated with uniform composition except for the mineral contents and pH values. The high calcium matrices manufactured with CCS had the highest ash content (4.1 %), while, for the CCS-free matrices as the calcium content was lowered (from 1080 to 37 mg.100 g⁻¹), the ash content of the matrices also decreased. This was caused by the addition of higher levels of acid casein required to lower the calcium content of these matrices. Acid casein has a low mineral content due to the solubilisation of colloidal calcium (calcium phosphate) during isoelectric precipitation (Savello et al., 1989). The final pH value of high calcium matrices prepared with CCS was ~ 6.2 (Table 4.1), as citric acid was added to standardise the pH of these matrices. This is in agreement with values previously reported for analogue cheese manufactured with the same CCS under similar conditions (El-Bakry et al., 2010b). For the CCS-free matrices (not adjusted with citric acid) final pH values progressively decreased with decreasing calcium content from 6.4 to 4.8 for the highest and lowest calcium matrices, respectively. This pH decrease reflects the increasing proportion of low pH acid casein included to reduce the calcium content of the formulations. The target pH value for processed and analogue cheese matrices is typically between 5.6 - 6.0 (Lucey et al., 2011) and 5.9 - 6.1 (Noronha et al., 2008b), respectively. The majority of CCS-free matrices (673-37 mg.100 g⁻¹) did not require pH adjustment as their pH was already below these target values. For the CCS-free matrix with calcium levels of 775 mg.100 g⁻¹, when citric acid was added to the formulation to reduce the pH a very tacky, sticky matrix formed which made reliable sampling impossible; hence these matrices were also formulated without citric acid.
4.3.3 Functional properties

4.3.3.1 Texture profile analysis (TPA)

The standard high calcium matrices prepared with CCS had the highest hardness (~ 697 N) and cohesiveness (0.55) values (Table 4.2). These are consistent with values previously reported for high protein (~35 %) analogue cheese manufactured using CCS (Arimi et al., 2011). All CCS-free matrices were significantly (P < 0.05) softer, with hardness values less than half those of the high calcium matrices containing CCS (Table 4.2). El-Bakry et al. (2010a) had previously reported that at decreased CCS levels (ranging from 0-40 %) hardness of analogue cheese increased. However, it may be the case that at greater levels of CCS reduction, as in the present work, there is a lower level of structural formation of the casein network resulting in decreased hardness and a softer matrix. In the present study, there is also much less calcium (> 40 % less) available for cross-linking during matrix formation. The lower moisture content (50 %), higher pH (6.0) and higher calcium content of the analogue cheese may have contributed to the increased hardness values reported in the study by El-Bakry et al. (2010a).

No trend was observed in hardness among the CCS-free matrices, with calcium concentration (673-410 mg.100 g\(^{-1}\)) having no significant (P > 0.05) effect. It has been reported for natural cheese that hardness decreases with decreasing calcium content (Biswas et al., 2015). Reductions in calcium concentration of processed and analogue cheese might also have been expected to result in softening of the casein matrix through reduced calcium-mediated protein-protein interactions. In the present study, however, it may be that any decrease in hardness which occurred due to reducing calcium levels could have been wholly or partly offset by the lowering of electrostatic repulsion between protein strands caused by the decrease in pH (Lucey et al., 2003) associated with decreasing the calcium level (Table 4.1). The two CCS-free matrices with the lowest calcium levels (410 and 358 mg.100 g\(^{-1}\)) exhibited
the highest hardness values of the CCS-free samples. The lower pH of these matrices may have allowed a greater level of hydrophobic protein-protein interaction, resulting in the slight increase in cheese hardness observed. Similar influences of pH on processed and analogue cheese texture have previously been reported (Lu et al., 2008; Noronha et al., 2008b).
Table 4.1 Composition, pH and processing time of high calcium casein matrices manufactured with chelating salts and those prepared without chelating salts at various calcium concentrations.

<table>
<thead>
<tr>
<th>Composition</th>
<th>Nominal Calcium Content (mg 100 g⁻¹)</th>
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<tbody>
<tr>
<td></td>
<td>1080*</td>
</tr>
<tr>
<td>Moisture (%)</td>
<td>57.87 ±0.22</td>
</tr>
<tr>
<td>Fat (%)</td>
<td>4.40 ±0.30</td>
</tr>
<tr>
<td>Protein (%)</td>
<td>33.49 ±0.71</td>
</tr>
<tr>
<td>Ash (%)</td>
<td>4.14 ±0.03</td>
</tr>
<tr>
<td>NaCl (%)</td>
<td>1.04 ±0.02</td>
</tr>
<tr>
<td>Calcium (mg.100 g⁻¹)</td>
<td>1074.62 ±0.14</td>
</tr>
<tr>
<td>pH</td>
<td>6.16 ±0.02</td>
</tr>
<tr>
<td>Processing time (min)</td>
<td>13.50 ±0.42</td>
</tr>
</tbody>
</table>

*High calcium matrices prepared with chelating salts

Numbers represent mean ± standard deviation; n=3

Means in the same row with different subscript letters differ significantly at P < 0.05
Table 4.2 Texture profile analysis and cross-over temperatures ($T_c$) of high calcium casein matrices prepared with chelating salts and those prepared without chelating salts at various calcium concentrations.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Nominal Calcium Content (mg 100 g$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1080*</td>
</tr>
<tr>
<td>Hardness (N)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$697.25^a$ ±15.03</td>
</tr>
<tr>
<td>Cohesiveness(−)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$0.55^a$ ±0.05</td>
</tr>
<tr>
<td>$T_c$ (°C)</td>
<td>$67.56^a$ ±0.17</td>
</tr>
</tbody>
</table>

n.d. = not determined

*High calcium matrices prepared with chelating salts

Numbers represent mean ± standard deviation; $n=15$

Means in the same row with different subscript letters differ significantly at $P < 0.05$
4.3.3.2 Heat-induced flowability

The standard high calcium matrices containing CCS were characterised by good flow properties, surface sheen and exhibited the greatest flow on heating (Figure 4.3). In comparison, all CCS-free matrices (Figure 4.3) had significantly (P < 0.05) lower flow values. El-Bakry et al. (2010a) reported that at decreased CCS levels (ranging from 0-40 %) a systematic and substantial decrease in the flowability of analogue cheese was observed. Arimi et al. (2011) also reported low flow values for analogue cheese manufactured with reduced levels of CCS. In those studies, the decrease in flowability was attributed to reduced protein-water interactions and a higher level of ‘inflexible’ calcium-mediated cross-linking between caseins due to low CCS levels. In the present study, as the calcium content of CCS-free matrices was reduced from 673 to 358 mg.100 g⁻¹, a progressive decrease (P < 0.05) in flow was observed. Enhanced flow on heating might have been expected at lower calcium levels due to lower levels of cross-linking. However, in the present work flow actually decreased because of increased hydrophobic interactions facilitated by the lower pH (Noronha et al., 2008b; Guinee, 2011) of the reduced calcium matrices. Some moisture exudation observed during heating of the lower calcium, CCS-free matrices further supports the idea that the decrease in pH would decrease the net negative charge on the protein and have a significant impact on charge-related matrix properties such as a decrease in water binding capacity (Teo et al., 1996).
Figure 4.3 Flowability of high calcium casein matrices manufactured with chelating salts* and those prepared without chelating salts at various calcium concentrations.

4.3.3.3 Dynamic rheology

The high calcium matrices produced with CCS had the highest G' values at 25 °C. For CCS-free matrices, the G' values at 25 °C were all lower (Figure 4.4 a) and followed broadly similar trends to hardness values i.e. decreased as calcium concentration decreased from 673-462 mg.100 g⁻¹ and increased for matrices at 410 and 358 mg.100 g⁻¹. The G' values decreased as a function of temperature increase (from 25-45 °C) for casein matrices prepared with and without CCS at all calcium levels. This decrease in G' values reflects a softening of the casein matrix with increasing temperature that has been reported by many (Mounsey and O’Riordan, 1999; Noronha et al., 2008a; El-Bakry et al., 2010b). The tan δ values increased with increasing temperature up to 65 °C for all casein matrices. For CCS-free matrices tan δ values were generally lower compared to high calcium matrices prepared with CCS. The main effects of calcium concentration were observed at temperatures above 50 °C, where the values of tan δ were generally higher for matrices prepared with higher levels of calcium. The cross-
over temperature ($T_c$), when $\tan \delta = 1$, which is an index of melting point (Mounsey and O’Riordan, 1999), was highest ($49.2 \, ^\circ C$) for standard high calcium, CCS matrices. For the CCS-free matrices, $T_c$ values did not significantly differ ($P > 0.05$) at calcium levels between 673-462 mg.100 g$^{-1}$ and at lower calcium levels (410 and 358 mg.100 g$^{-1}$) the matrices did not exhibit a $T_c$ (i.e. $\tan \delta$ remained $< 1$). Although the calcium reduction led to substantially decreased $T_c$ compared to standard high calcium CCS matrices, this did not result in an increase in flowability. The failure of the lower calcium (410 and 358 mg.100 g$^{-1}$), CCS-free matrices to melt, may have contributed to their lower flow values (Figure. 4.3).
Figure 4.4 Changes in the viscoelastic characteristics $G'$ (a) and $\tan \delta$ (b) of casein matrices heated from 25 to 90 °C: high calcium matrices manufactured with chelating salts 1080 (■) and chelating salt-free matrices with calcium levels of 673 (●), 569 (△), 514 (□), 462 (▲), 410 (○) or 358 (◦) mg.100 g$^{-1}$.  


4.4 Conclusion

In this study casein-based food matrices containing lipid were manufactured with a range of calcium concentrations by blending acid and rennet casein. The results show that by manipulating total calcium level, it is possible to form hydrated casein structures to meet a range of functional and compositional requirements without the use of CCS. Fine tuning of the precise calcium level also facilitates the formulation of casein structures suitable for different end-use applications. Structures with higher calcium levels have flow values (106 mm) comparable to cheese analogues prepared with CCS (120 mm) (El-Bakry et al., 2010b). Therefore, similar to cheese analogues, these matrices have potential application as a Mozzarella cheese substitute in frozen pizzas. Casein-based food structures containing intermediate calcium levels may be useful in deep-fried type applications such as breaded sticks, burgers with cheese inserts or cheese products where flow resistance on deep-frying is desired. In addition, the decreased processing times required for manufacture at reduced calcium levels may have important commercial implications from a time and energy cost saving perspective. Finally, the elimination of CCS provides the potential to reduce the sodium content of processed cheese products by up to 60 % and would also present opportunities for end-products with cleaner labels which is highly desirable from a consumer perspective.
4.5 References


Chapter 5

Maximising Sensory Acceptability of Casein-Based Food Matrices Containing Different Enzyme Modified Cheese (EMC) Flavourings and Fat Levels
Chapter 5: Maximising sensory acceptability of casein-based food matrices containing different enzyme modified cheese (EMC) flavourings and fat levels.

5.1 Introduction

Traditionally, casein-based food matrices such as processed and analogue cheese, are manufactured by blending selected ingredients (e.g. natural cheese and/or milk proteins, water, fat, calcium chelating salts (CCS)) together and heating to produce a stable molten oil-in-water emulsion which sets on cooling. The CCS are used to hydrate the protein which subsequently acts as an emulsifier for the oil phase dispersed in the protein/aqueous phase of the cheese system (Ennis et al., 1998). The post-manufacture functionalities, such as texture and melting properties are also affected by the CCS used during manufacture (El-Bakry et al., 2010, 2011). Recent studies in this laboratory (Chapter 4, McIntyre et al., 2016) have demonstrated that manipulating calcium level (using different mixture ratios of rennet and acid casein) provides a new approach for the manufacture of casein-based food matrices, which does not require the use of sodium-based additives e.g. CCS. The resulting matrices have an array of different functionalities (Chapter 4), however, their sensory properties have not been investigated.

A negative property associated with both processed and analogue cheese is their flavour, which often cannot approach the flavour of natural cheese (Bachmann, 2001; Guinee et al., 2004). These types of casein-based food matrices are often reported to be bland and lacking in flavour (Noronha et al., 2008a). This is a particular concern for the newly developed matrices described previously in Chapter 4 due to their high protein (33 %) and low-fat (4 %) contents. Enzyme modified cheeses (EMCs) are produced enzymatically from dairy substrates and are
designed to provide a concentrated source of cheese flavour to processed and analogue cheese products (Kilcawley et al., 2006; Noronha et al., 2008a,b). Therefore, the incorporation of EMCs into the newly developed matrices (Chapter 4) might also be a useful means by which to improve their flavour. The objective of this study was to manufacture flavoured casein-based food matrices with different types of EMCs and to determine which is preferred by sensory panellists. The other objective of this study was to investigate how the sensory acceptability of cheese flavoured casein-based food matrices is influenced by fat level.

5.2 Materials and Methods

5.2.1 Materials

Rennet casein powder (Kerrynor™ R190) with a protein content of 80 % was supplied by Kerry Ingredients Ltd (Listowel, Co. Kerry, Ireland). Acid casein powder (87 % protein) was provided by Glanbia Ingredients Ltd (Ballyragget, Co. Kilkenny, Ireland). The calcium content of the rennet and acid casein powders as determined by a flame atomic absorption spectroscopy method (IDF, 2007), were 2700 mg.100 g⁻¹ and 100 mg.100 g⁻¹, respectively. Rapeseed oil was obtained from Boyne Valley Foods (Drogheda, Co. Louth, Ireland). Anhydrous milk fat was provided by Kerry Ingredients Ltd (Listowel, Co. Kerry, Ireland). The following food grade ingredients were used: citric acid (Jungbunzlauer GmbH, Pernhofen, Austria), sodium chloride (Salt Union, Cheshire, England) and sorbic acid (Hoechst Ireland Ltd., Dublin, Ireland). Three commercial Enzyme Modified Cheese (EMC) flavourings (Emmental, Epinpoise and Cheddar) and yeast extract were received as gifts from Kerry Ingredients Ltd (Listowel, Co. Kerry, Ireland). All EMC products were in paste form and were stored at 4 °C prior to use.
5.2.2 Manufacture of flavoured casein-based food matrices with different types of Enzyme Modified Cheeses (EMCs)

Casein matrices were manufactured in a Thermomix blender cooker (Thermomix TM 31; Vorwerk & Co., GmbH, Wuppertal, Germany) as described previously (Chapter 3 and 4) according to the following formulation: 58.0 % moisture, 33.0 % total protein, 4.0 % rapeseed oil, 1.0 % NaCl and 0.08 % sorbic acid. Casein matrices were manufactured with low levels of calcium (357 mg.100 g⁻¹) without CCS using blends of rennet and acid casein. Batches (500 g) of the casein matrices were manufactured by agitating (speed 3, ~250 rpm) the rapeseed oil, water and minor ingredients (i.e. sorbic acid and sodium chloride) in the cooker at 50 °C for 2 min. The rennet and acid casein powders were then added at a ratio (RC:AC w/w) of 30:70 to give the desired calcium level of 357 mg.100 g⁻¹. The temperature was increased to 80 °C and maintained with continuous agitation until a homogeneous mass was formed. The EMC (Emmental, Epipose or Cheddar) paste (5 % w/w) and yeast extract (0.5 % w/w) (Noronha et al., 2008a,b) were mixed into the molten mass along with citric acid at the end of the manufacturing process for 2 final minutes of mixing at 80 °C. Following processing, the hot molten casein matrices were filled into plastic containers and placed in a freezer at -18 °C and after 1 hour transferred to a refrigerator at 4 °C for 24 hours before being vacuum packed (Model C10H, Webomatic® Vacuum Packaging Systems, Maschinenfabrik GmbH, Bochum, Germany). The casein matrices were manufactured in triplicate with each type of EMC flavouring.

5.2.3 Manufacture of flavoured casein-based food matrices with different fat levels

In addition to the casein-based matrices manufactured in Section 5.2.2, similar matrices were formulated to contain full (34 %), reduced (24 %) or half (17 %) fat levels (Fenelon et al., 2000). These matrices were prepared using anhydrous milk fat and their target composition is
outlined in Table 5.1. Casein matrices were manufactured without CCS with different levels of calcium using blends of rennet and acid casein. The rennet and acid casein powders were mixed at a ratio (RC:AC w/w) of 70:30 to give the desired calcium levels of 583, 684 or 760 mg.100 g\(^{-1}\) in the full, reduced or half fat flavoured matrices respectively.

Table 5.1 Target composition for calcium chelating salt-free flavoured casein-based food matrices formulated with different fat levels.

<table>
<thead>
<tr>
<th></th>
<th>Full Fat</th>
<th>Reduced Fat</th>
<th>Half Fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture (%)</td>
<td></td>
<td>40</td>
<td>45</td>
</tr>
<tr>
<td>Protein (%)</td>
<td></td>
<td>24</td>
<td>29</td>
</tr>
<tr>
<td>Fat (%)</td>
<td></td>
<td>34</td>
<td>24</td>
</tr>
<tr>
<td>NaCl (%)</td>
<td></td>
<td>1.9</td>
<td>1.9</td>
</tr>
<tr>
<td>Calcium (mg.100 g(^{-1}))</td>
<td>583</td>
<td>684</td>
<td>760</td>
</tr>
</tbody>
</table>

Based on the results of the ranked preference test performed on the casein matrices manufactured using different types of EMCs (Section 5.2.2), the most preferred EMC flavouring was reported to be Emmental. For this reason the matrices manufactured with different fat levels were prepared using the Emmental EMC flavouring only. In addition, the level of EMC was reduced to 3.5 % w/w based on the feedback received from the analysis of products tested in Section 5.2.2. The level of addition of yeast extract (0.5 % w/w) was not adjusted. The flavoured casein matrices containing different levels of fat were manufactured using the same protocol as described in Section 5.2.2 and were manufactured in triplicate at each fat level.
5.2.4 Sensory analysis of flavoured casein-based food matrices

5.2.4.1 General

A sensory evaluation of the flavoured casein matrices prepared in Sections 5.2.2 and 5.2.3 was conducted by an untrained 30-member panel on two separate occasions. Panel members were selected from post graduate students and staff of the UCD Institute of Food and Health. All sessions were carried out in a sensory analysis suite equipped with individual testing booths and controlled lighting to avoid visual bias. Products (10 ± 0.05 g cubes) were placed into white disposable plastic containers coded with 3-digit random numbers, equilibrated to room temperature (22 °C) and presented to panellists. Order of presentation was balanced to account for first order and carryover effects (MacFie et al., 1989).

5.2.4.2 Ranked preference sensory analysis

In preference ranking, panellists order products according to their preferences from best to worst. In this study, panellists were asked to rank the product based on preference for flavour and mouthfeel, using the method of Meilgaard et al. (1991). Regarding the casein matrices prepared in Section 5.2.2, panellists (n =30) were presented with 3 samples flavoured with 3 different EMC flavourings: Emmental, Epoisse or Cheddar. In the case of the flavoured casein matrices prepared in Section 5.2.3, panellists (n =30) were presented with 3 samples all flavoured with Emmental EMC but containing 3 different levels of fat: full, reduced or half fat. In both scenarios the assessors were instructed to evaluate samples based on overall flavour and mouthfeel, using a score from 1 (most preferred sample) to 3 (least preferred sample) (see appendix 1). Panellists were provided with still mineral water to cleanse the palate between samples.

5.2.4.3 Acceptability testing

The hedonic test was used to evaluate the acceptability of the Emmental flavoured samples containing either full, reduced or half fat levels. Panellists first ranked these products in their
order of preference (as described in Section 5.2.4.2) and then evaluated them using a 9-point hedonic scale ranging from “1 = dislike extremely” to “9 = like extremely” (see appendix 2). Each panellist evaluated each sample for overall liking, flavour and texture. Panelists were also asked to report any descriptors that they felt characterised the sensory properties of the samples. Panellists were provided with still mineral water to cleanse the palate between samples.

**5.2.5 Statistical analysis**

Data from the ranking preference test was evaluated for their statistical significance (P < 0.05) using Friedman’s non-parametric test and the multiple comparison procedure to determine which products differed from each other (Meilgaard *et al*., 1991). The hedonic test data were analysed using one-way analysis of variance (ANOVA) and paired t-tests to determine significant differences (P < 0.05) between samples. All statistical analysis were completed using SPSS (version 20) statistical software (IBM Inc. Chicago, IL, USA).

**5.3 Results and Discussion**

**5.3.1 Influence of EMC type on the sensory preference of casein-based food matrices**

The ranking sum scores for the flavoured casein matrices manufactured with different EMCs revealed that panellists had a preference for those flavoured with Emmental (43) over Cheddar (62) and Eppoise (51), however this was only significant (P < 0.05) in the case of Cheddar. There were no differences (P > 0.05) in preference between Cheddar or Eppoise flavoured matrices, both were considered ‘strong’ and ‘cheesy’ flavours by panellists.
5.3.2 Influence of fat level on the sensory acceptance of casein-based food matrices

The ranking sum scores for the Emmental flavoured casein matrices showed that panellists had a significant (P < 0.05) preference for full fat matrices (35) describing them as ‘smoother’ and ‘creamier’ in the mouth. There were no differences (P > 0.05) in preference between flavoured matrices containing half (51) or reduced (53) fat levels, these were both termed ‘grainy’ by panellists. The full fat matrices received the highest rating for all 3 attributes (i.e. flavour, texture and overall liking) tested on the hedonic scale (Table 5.2). These ratings were significantly higher (P < 0.05) compared to those received for the half or reduced fat flavoured matrices for all 3 attributes. There was no significant differences (P > 0.05) between the rating scores obtained for the half or reduced fat flavoured matrices for any of the attributes studied (Table 5.2).

Table 5.2 Mean values for the scores given by panellists for texture, flavour and overall liking for calcium chelating salt-free flavoured casein-based food matrices containing different fat levels.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Texture</th>
<th>Flavour</th>
<th>Overall liking</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full fat</td>
<td>6.37\textsuperscript{a}</td>
<td>6.16\textsuperscript{a}</td>
<td>6.96\textsuperscript{a}</td>
</tr>
<tr>
<td>Half fat</td>
<td>4.75\textsuperscript{b}</td>
<td>4.54\textsuperscript{b}</td>
<td>4.58\textsuperscript{b}</td>
</tr>
<tr>
<td>Reduced fat</td>
<td>4.83\textsuperscript{b}</td>
<td>4.66\textsuperscript{b}</td>
<td>4.54\textsuperscript{b}</td>
</tr>
</tbody>
</table>

Means with the same letter, in the same column, do not differ significantly (P > 0.05)

Muir et al. (1997) compared the sensory characteristics of full, reduced and low fat processed cheese spreads, the authors reported that as the fat content of these products was decreased, there was a decrease in the ‘creamy’ attribute and an increase in the ‘acid’ and ‘bitter’
attributes of the processed cheese spreads. Moreover, with the decrease in the fat content there was an increase in the ‘graininess’ and ‘stickiness’ of the products, similar to that reported in the present study. Noronha et al. (2008a) reported that analogue cheese prepared with higher fat levels (13 %) modulated a greater retention of fat-based flavour compounds and improved their release during consumption compared to those containing lower fat levels (2 %). In addition, the same authors in a similar study (Noronha et al., 2008b) reported that a reduction in the pH of the analogue cheese base increased perceived cheese flavour intensity. Consequently, analogue cheese containing higher fat levels (13 %) and which had a lower pH (5.5) were rated higher by sensory panellists (Noronha et al., 2008a,b). Many studies on natural cheese have also reported that as fat content was decreased, sensory acceptance also decreases (Fenelon et al., 2000; Drake, 2008; Johnson et al., 2009; Miri and Habibi Najafi, 2011). Fat is responsible for the richness and mouthfeel of cheese, and lowering its content generally has a negative impact on sensory attributes (Drake and Swanson, 1995). It has also been reported that another reason why natural cheese samples with varying fat content are perceived differently is that they behave differently in the mouth; both with respect to flavour release and structure breakdown (Wendin et al., 2000).

5.4 Conclusion

The results show that, of the three types examined, Emmental was the most preferred type of EMC used to flavour casein-based food matrices with low fat contents (4 %). An important finding of this study was that even though sensory panellists preferred full fat flavoured matrices and rated these higher in terms of texture, flavour and overall liking; there were no significant differences (P > 0.05) detected between those containing half or reduced fat levels for any of these attributes. This suggests that greater levels of fat reduction may not impact negatively on the sensory properties of casein-based food matrices, which may have important consequences for those concerned with reducing the fat contents of such matrices. Further
studies comparing the sensory properties (including textural attributes) of matrices flavoured with a broader range of EMCs at different levels of addition, are required to optimise the sensory acceptance of these matrices.

5.5 References


Chapter 6

Altering the Level of Calcium Changes the Physical Properties and Digestibility of Casein-Based Emulsion Gels

Published as:

Chapter 6: Altering the level of calcium changes the physical properties and digestibility of casein-based emulsion gels.

6.1 Introduction

Driven by concerns regarding obesity and other diet and health related issues, there is an ever increasing demand to develop foods with enhanced nutritional properties (Norton et al., 2015). To develop these innovative food products, it is necessary to understand the behaviour of food as it is processed within the human digestive system, from its initial physical breakdown, to the transformation and absorption of its constituent nutrient molecules (Bornhorst et al., 2016). In particular, the deliberate design of food structures that impact on lipid digestion has received increasing attention because of the link between over-consumption of certain food lipids (e.g. saturated fats, trans-fatty acids, and cholesterol) and increased susceptibility to a range of diseases e.g. obesity and metabolic syndrome (McClements et al., 2009). From this attention has come the recent recognition that the matrix in which lipids are presented in the diet can influence the rate of lipid digestion and hence the bioavailability of fatty acids (FA) (Lamothe et al., 2012; Wooster et al., 2014; Ayala-Bribiesca et al., 2016; Guo et al., 2016). Consequently, there is now heightened interest in understanding how food matrix structure / properties can be manipulated so that lipid digestion under physiological conditions is impacted. Such knowledge could facilitate the design of novel foods with specific lipid digestion profiles that provide e.g. regulation of lipid digestion, enhanced satiation, protective encapsulation or targeted delivery; while still retaining the desirable taste and mouthfeel characteristics of lipids.

Besides the structural organisation of the matrix surrounding lipids, minerals present in the matrix can also impact on lipid digestion and metabolism (Michalski et al., 2013). Long-chain saturated FA released during the hydrolysis of triacylglycerides (TAG) are able to form
insoluble soaps with dietary divalent cations (mainly calcium but also magnesium), that are excreted in the faeces (Bendsen et al., 2008). Calcium enhances lipolysis during digestion by this mechanism but this in turn may reduce lipid bioaccessibility (Lorenzen et al., 2007; Lopez and Gaucheron, 2008). However, the impact of calcium ions (Ca$^{2+}$) on lipid digestion and absorption can be attenuated by the presence of other food components within a food matrix, such as calcium chelating agents (Hu et al., 2010). Controlling the molecular characteristics of lipid molecules, altering lipid type, droplet size or interfacial properties are some other approaches that have also been reported to influence the rate or extent of lipid bioavailability in protein stabilized oil-in-water emulsions (McClements et al., 2009; Singh et al., 2009). However, our understanding of how complex food matrices (e.g. semi-solid or solid foods) with different compositions, physical properties and structures are affected by such variables remains unclear.

Composite high protein emulsion gels e.g. processed and analogue cheese are semi-solid proteinaceous food matrices containing dispersed oil droplets. These matrices can be formulated by blending selected ingredients (e.g. water, protein, fat, calcium chelating salts (CCS)) together and heating to produce a stable molten oil-in-water emulsion which sets on cooling and acquires texture characteristics similar to those of cheese. Although the rate and extent of lipid digestion have been previously investigated in natural cheese (Lamothe et al., 2012; Ayala-Bribiesca et al., 2016), these have not been evaluated in processed or analogue cheese. A unique attribute of the latter food matrix is the ease with which the product may be altered to yield matrices with customised nutritional, compositional and functional attributes e.g. high vs low moisture, low-fat, high melt vs low melt, firm texture etc. as demonstrated in Chapters 4 and 5. In addition, these matrices are traditionally prepared using CCS. Hence, if calcium plays a crucial role in the lipid digestion process, then the rate of lipid digestion may be influenced by such food components that can bind calcium (Hu et al., 2010).
The objective of this study was to investigate the effect of calcium concentration on the structure of casein-based emulsion gels (prepared with and without CCS) and to assess the impact of that structure on matrix breakdown and lipid digestion using an in vitro digestion model. The physical characteristics and in vitro digestibility of casein-based emulsion gels containing different lipid phases (i.e. milk fat or rapeseed oil) were also compared.

6.2 Materials and Methods

6.2.1 Materials

Rennet casein powder (Kerrynor™ R190) was supplied by Kerry Ingredients Ltd (Listowel, Co. Kerry, Ireland). Acid casein powder was provided by Glanbia Ingredients Ltd (Ballyragget, Co. Kilkenny, Ireland). The calcium contents of the rennet and acid casein powder were determined by flame atomic absorption spectroscopy (IDF, 2007) and were 2700 mg 100 g⁻¹ and 100 mg 100g⁻¹, respectively. Anhydrous milk fat was provided by Kerry Ingredients Ltd (Listowel, Co. Kerry, Ireland). Rapeseed oil was obtained from Boyne Valley Foods (Drogheda, Co. Louth, Ireland). The following food grade ingredients were used to manufacture the casein-based emulsion gels: disodium phosphate (DSP) anhydrous (Albright and Wilson Ltd., Cheshire, England), trisodium citrate (TSC), citric acid (Jungbunzlauer GmbH., Pernhofen, Austria), sodium chloride (Salt Union, Cheshire, England) and sorbic acid ( Hoechst Ireland Ltd., Dublin, Ireland). Chemical reagents: calcium reference standard (1000 mg L⁻¹), lanthanum trichloride heptahydrate (LaCl₃.7H₂O) and nitric acid (HNO₃) were purchased from Sigma Aldrich (Dublin, Ireland) and were analytical grade. All reagents and extracts for in vitro digestion were also obtained from Sigma-Aldrich (Dublin, Ireland). Deionized water was prepared in a Milli-Q water purification system.
6.2.2 Manufacture of casein-based emulsion gels

Casein-based emulsion gels were manufactured in a Thermomix blender cooker (Thermomix TM 31; Vorwerk & Co., GmbH, Wuppertal, Germany) as described previously in Chapter 4 (McIntyre et al., 2016). In this study the rennet casein-CCS emulsion gels with high moisture content (58 %) were termed “standard” emulsion gels. Standard matrices (723 mg Ca 100g⁻¹) were manufactured using rennet casein as the only protein source (23 % protein) and contained CCS (1.35 % TSC, 0.61 % DSP) and citric acid (0.50 %). CCS-free matrices (48 % moisture and 34 % protein) containing high (774 mg 100g⁻¹) or low (357 mg 100g⁻¹) levels of calcium were manufactured using blends of rennet and acid casein to manipulate the calcium content. All emulsion gels were manufactured with milk fat or rapeseed oil and contained 13 % fat. Batches (500 g) of each emulsion gel were manufactured by agitating (speed 3, ~250 rpm) the fat (i.e. milk fat or rapeseed oil), water and minor ingredients (i.e. sorbic acid and sodium chloride) in the cooker at 50 °C for 2 min. The rennet and acid casein powders were mixed at ratios (RC:AC; w/w) of 70:30 and 30:70 to give the calcium levels of 774 and 357 mg 100g⁻¹, respectively. The temperature was increased to 80 °C and maintained with continuous agitation until a homogeneous mass was formed. Following processing, the hot molten emulsion gels were filled into plastic containers, placed in a freezer at -18 °C and after 1 hour transferred to a refrigerator at 4 °C for 24 hours before being vacuum packed (Model C10H, Webomatic® Vacuum Packaging Systems, Maschinenfabrik GmbH, Bochum, Germany). For the standard emulsion gels prepared with CCS, the CCS were added at the same time point as the other minor ingredients and, for pH adjustment, food grade citric acid was added toward the end of manufacture. All casein matrices were manufactured in triplicate at each calcium level, for each lipid type.
6.2.3 Compositional analysis

Moisture content was determined gravimetrically after drying the sample in a laboratory oven at 101 °C to constant weight (IDF, 1958); ash content was also determined gravimetrically after the complete incineration of the sample in a muffle furnace (B180, Nabertherm, GmbH, Germany) at 550 °C / 6h (IDF, 2007); the calcium content of the ash residue was assessed using a flame atomic absorption spectroscopy method (IDF, 2007). Fat content was determined using the Gerber method (National Standards Authority of Ireland, 1955) and total nitrogen was measured by the Kjeldahl method with a conversion factor of 6.38 for crude protein (IDF, 1993). Values of pH were determined at ambient temperature by inserting a glass tip electrode of a calibrated pH-meter (EL20, Mettler Toledo, Schwerzenbach, Switzerland) directly into the matrix at three randomly chosen locations. All analyses were completed in triplicate.

6.2.4 Texture profile analysis (TPA)

TPA was performed on cylindrical samples (25 mm diameter, 20 mm height) using an Instron Universal Testing Machine (Model 5544, Instron Corp., Canton, Mass., USA) fitted with a 1000 N load cell. Cylindrical samples were cut using a cork borer, wrapped in cling film to prevent dehydration, and allowed to equilibrate to 22 °C for 30 minutes prior to analysis. Afterwards, the cling film was removed and samples were compressed by 80 % of initial height using a 35 mm diameter plate at a crosshead speed of 50 mm / min⁻¹. The uniaxial compression test was performed in two successive cycles, and the textural parameters, hardness and cohesiveness were calculated. Three batches of five samples were tested for each emulsion gel studied.

6.2.5 Microstructure

Environmental scanning electron microscopy (ESEM) was performed using a FEI Quanta 3D FEG DualBeam microscope (FEI Ltd, Hillsboro, Oregon, USA). The detection system used
was a Gaseous Secondary Electron Detector. Samples (5 mm cubes) were obtained from the centre of the block (cut surface) and mounted on a peltier cooling stage set at 5 °C. The samples were examined at a working distance of 5.8-6.5 mm and an accelerating voltage of 20 kV, using the wet mode at a water vapour pressure of 7.25 Torr to ensure that the samples remained fully hydrated. The size of lipid droplets were estimated from ESEM images using ImageJ software. Three samples were analysed for each manufactured batch.

**6.2.6 Nuclear Magnetic Resonance (NMR)**

All NMR experiments were performed using an Oxford Instruments Maran Ultra Spectrometer (Oxford Instruments, Tubney Woods, Oxfordshire, UK) equipped with an 18 mm variable temperature probe with a resonance frequency of 23 MHz. Grated samples (~5 g) were placed inside NMR sample tubes (18 mm diameter) and tempered in a water bath for 2 hours at 25 °C prior to analysis. Samples were measured at 25 ± 0.5 °C. T$_2$ relaxation times were obtained using the Carr, Purcell, Meiboom and Gill (CPMG) pulse sequence with a relaxation delay of 5000 ms and a 90-180° pulse gap of 0.15 ms. The 90° pulse sequence was 7.4 µs and 180° pulse was 14.8 µs in lenght. A total of 8192 echoes were aquired for each of the 32 scans. T$_2$ distributions were calculated as a continuous distribution of exponentials using Origin Pro software. Five samples were analysed for each manufactured batch.

**6.2.7 In vitro digestion**

The digestion process was simulated using the *in vitro* approach developed by Versantvoort *et al.* (2005). The constituents and concentrations of the various synthetic juices used in this *in vitro* digestion model are described in appendix 3. Emulsion gels were cut into pieces weighting 20 ± 0.5 g. The samples were prepared using an electric mincer (Kenwood chef-major; multifoood grinder, AT950 attachment, Kenwood Ltd, UK) to simulate oral breakdown (Minekus *et al.*, 2014). The approximate size of the minced product was ~3.5 mm cubes. The minced sample (4.5 g) was transferred to a 250 ml plastic container with 2.5 g of
glass beads, mixed with 6 ml of simulated saliva (pH 6.8 ± 0.02), and incubated without agitation for 5 min at 37 °C. Afterwards, 12 ml of simulated gastric juice (pH 1.3 ± 0.02) was added and the mixture incubated for 2 hours at 37 °C in a shaking water bath (160 movements per min) to mimic gastrointestinal tract conditions (Parrot et al., 2003). Following the gastric phase, 12 ml of simulated duodenal juice (pH 8.1 ± 0.02), 6 ml of bile juice (pH 8.2 ± 0.02) and 2 ml of bicarbonate solution (1 M) were added to each container, and the mixtures were agitated for a further 3 hours at 37 °C. At specific times during digestion (5, 60, 120, 150, 180 and 300 min), containers were removed from agitation for immediate analyses. All emulsion gels showed similar pH profiles during the different stages of digestion. However, the standard emulsion gels containing CCS had a slightly higher pH, presumably due to the buffering capacity of DSP and TSC. Upon addition of the gastric and intestinal digestive juices, pH adjustment was required to compensate for any buffering effect of the emulsion gels and to maintain the desired pH of 2.0-3.0 and pH 6.5-7.0, to simulate the digestive processes in the stomach and small intestine, respectively (Versantvoort et al., 2005).

6.2.8 Matrix degradation

Emulsion gel degradation was determined from the proportion of undigested solids that were finely dispersed in the digestion juices as a result of the in vitro digestion process described in Section 6.2.7. The entire contents of each digestion container were filtered through a metallic sieve (1.5 mm x 1.5 mm mesh), as previously described (Lamothe et al., 2012; Ayala-Bribiesca et al., 2016). The undigested particles were rinsed twice with 5 ml of appropriate digestive juice at 37 °C to remove any digested material. Blotting paper was placed around the metallic sieve for 10 min to drain residual digestive juice. The particles were then transferred to a pre-weighed aluminium dish and dried in an oven overnight and the matrix degradation index (MDI) was calculated using the following equation:
\[ MDI (\%) = \frac{CS_o-CS_t}{CS_o} \times 100 \]  

(1)

Where \( CS_o \) is the mass of solids originally present in the digestion container as determined gravimetrically after oven drying (IDF, 1958) and \( CS_t \) is the mass of solids remaining at digestion time \( t \). Analyses were completed in triplicate for each time point during the digestion process.

6.2.9 Free fatty acid measurement

Fatty acid release during digestion was measured using a free fatty acid (FFA) colorimetric assay kit (Roche Diagnostics, Indianapolis, IN, USA). The drained liquid from the MDI samples was diluted 100-fold with a solution containing Triton-X-100 (5.6 %) and ethanol (6 %) in water to solubilise the FFA and stop lipase activity (Lamothe et al., 2012; Ayala-Bribiesca et al., 2016). The assay was carried out according to the instructions provided by the manufacturer. The red fatty acid-dye complex was determined at 546 nm. Oleic acid (0-0.8 mM) was used as the calibration standard. The FFAs were expressed as the percentage of total fatty acids that could theoretically be released after complete digestion, assuming the maximum release of 2 fatty acids per TAG molecule and an average molecular weight of 247 g mol\(^{-1}\) for milk fatty acids (Jones and Kubow, 2006) and 308 g mol\(^{-1}\) for rapeseed oil fatty acids (Ratnayake and Daun, 2009). Analyses were completed in triplicate for each time point during the digestion process.

6.2.10 Statistical analysis

All analyses were repeated three times and results were presented as mean values and standard deviations. Data were analysed for statistical differences by a one-way analysis of variance (ANOVA) and Tukey’s pairwise comparisons were used to determine significant differences between the various treatments. A three-way ANOVA was conducted to determine the effects of calcium level, lipid source and digestion time on MDI (\%) and
lipolysis (%) for the *in vitro* digestion experiments. All statistical analyses were carried out using SPSS version 20 statistical software (IBM Inc. Chicago, IL, USA) and statistical differences were considered significant at P < 0.05.

### 6.3 Results and Discussion

#### 6.3.1 Composition

The composition of standard casein-based emulsion gels manufactured with CCS and those prepared without CCS at high and low calcium concentrations are shown in Table 6.1. The moisture was higher (P < 0.05) and protein content lower (P < 0.05) for standard emulsion gels compared to those containing no CCS. Preliminary experiments showed that the maximum level of fat inclusion possible for the matrices formulated without CCS was 13 % and this was only achievable at the lower moisture level of 48 %. However, a standard emulsion gel with 13 % fat was only possible to manufacture in our mixing system at 58 % moisture.

There were differences (P < 0.05) in pH between all of the emulsion gels. This was a result of the inclusion of different proportions of rennet and acid casein in the formulations to manipulate calcium level (Chapter 4, McIntyre *et al.*, 2016). The final pH value of the standard emulsion gels prepared with CCS was 6.1 (Table 6.1), as citric acid was added to standardise the pH of these matrices. The target pH value for processed and analogue cheese is typically between 5.6-6.0 (Lucey *et al.*, 2011) and 5.9-6.1 (Noronha *et al.*, 2011), respectively. The CCS-free matrices did not require pH adjustment as their pH was already between or below these target values. The chemical composition and pH of the casein matrices manufactured from milk fat were not significantly different (P > 0.05) from those of equivalent composition made from rapeseed oil.
Table 6.1 Composition of the standard casein-based emulsion gels prepared with chelating salts and the chelating salt-free high and low calcium emulsion gels manufactured with either milk fat or rapeseed oil.

<table>
<thead>
<tr>
<th>Matrix calcium level</th>
<th>Calcium chelating salts</th>
<th>Fat source</th>
<th>Moisture (%)</th>
<th>Protein (%)</th>
<th>Fat (%)</th>
<th>Total Ca (mg/100g)</th>
<th>pH (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>+</td>
<td>Milk Fat</td>
<td>57.94 ± 0.59&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>23.33 ± 0.66&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>12.85 ± 0.22&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>715.44 ± 0.13&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>6.12 ± 0.04&lt;sup&gt;ax&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rapeseed oil</td>
<td>57.71 ± 0.61&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>23.28 ± 0.60&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>12.80 ± 0.57&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>712.15 ± 0.08&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>6.13 ± 0.01&lt;sup&gt;ax&lt;/sup&gt;</td>
</tr>
<tr>
<td>High</td>
<td>-</td>
<td>Milk Fat</td>
<td>48.64 ± 0.70&lt;sup&gt;bx&lt;/sup&gt;</td>
<td>34.14 ± 0.24&lt;sup&gt;bx&lt;/sup&gt;</td>
<td>12.67 ± 0.61&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>767.80 ± 0.15&lt;sup&gt;bx&lt;/sup&gt;</td>
<td>5.96 ± 0.07&lt;sup&gt;bx&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rapeseed oil</td>
<td>48.45 ± 0.34&lt;sup&gt;bx&lt;/sup&gt;</td>
<td>34.89 ± 0.43&lt;sup&gt;bx&lt;/sup&gt;</td>
<td>12.92 ± 0.66&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>770.51 ± 0.06&lt;sup&gt;bx&lt;/sup&gt;</td>
<td>5.98 ± 0.03&lt;sup&gt;bx&lt;/sup&gt;</td>
</tr>
<tr>
<td>Low</td>
<td>-</td>
<td>Milk Fat</td>
<td>48.09 ± 0.60&lt;sup&gt;bx&lt;/sup&gt;</td>
<td>34.74 ± 0.65&lt;sup&gt;bx&lt;/sup&gt;</td>
<td>12.42 ± 0.49&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>353.43 ± 0.25&lt;sup&gt;cx&lt;/sup&gt;</td>
<td>5.50 ± 0.01&lt;sup&gt;cx&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rapeseed oil</td>
<td>48.04 ± 0.51&lt;sup&gt;bx&lt;/sup&gt;</td>
<td>34.52 ± 0.65&lt;sup&gt;bx&lt;/sup&gt;</td>
<td>13.00 ± 0.49&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>351.64 ± 0.10&lt;sup&gt;cx&lt;/sup&gt;</td>
<td>5.49 ± 0.06&lt;sup&gt;cx&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values represent the means of triplicates.

For each calcium level (a, b, c) or fat source (x, y), means with different superscript letters differ significantly at P < 0.05.

Casein-based emulsion gels were prepared with (+) or without (-) calcium chelating salts.
6.3.2 Texture

The results for texture properties of the emulsion gels are presented in Table 6.2. The CCS-free emulsion gels with the lowest calcium concentration had the highest hardness values (P < 0.05) of all matrices (Table 6.2); whereas, those with high calcium levels showed the lowest degree (P < 0.05) of cohesiveness. The standard emulsion gels prepared with CCS were the softest but most (P < 0.05) cohesive matrices. This was most likely caused by their higher moisture and lower protein content and also the inclusion of CCS in their formulation. The low calcium (CCS-free) emulsion gels had almost double the hardness values of the other matrices (Table 6.2). Regarding the CCS-free matrices, it might be expected that at the lower calcium concentrations, there would be a lower level of structure formation due to less calcium being available for cross-linking the casein network, resulting in decreased hardness and a softer matrix. However, in the present study the higher hardness values obtained for the low calcium matrices, may have been caused by their lower pH (Table 6.1) and the prolonged processing time required for their manufacture (Table 6.2). It has been reported for processed and analogue cheese that the extensive shearing associated with prolonged processing/cook times or increased shearing speed can result in the formation of a harder matrix (Shirashoji et al., 2006; Noronha et al., 2008a; El-Bakry et al., 2010) In addition, the lower pH of these matrices may have resulted in reduction in electrostatic repulsion between protein strands also contributing to increased hardness (Noronha et al., 2008a). These results are in agreement with the high hardness values previously reported in Chapter 4 for low calcium, casein-based matrices manufactured without CCS (McIntyre et al., 2016).

There was no difference (P > 0.05) in hardness values for the standard and high calcium (CCS-free) emulsion gels manufactured from milk fat; however, the cohesiveness of the standard matrices was significantly higher (P < 0.05). All emulsion gels manufactured from milk fat were harder (P < 0.05) compared to those of equivalent composition prepared from
rapeseed oil. This may be attributed to the solid state of the fat at room temperature, with milk fat having a much higher melting point (+35 to +40 °C) compared to that of rapeseed oil (-10 to +2 °C) (Michalski et al., 2013).
Table 6.2 Manufacture time and texture of the standard casein-based emulsion gels prepared with chelating salts and the chelating salt-free high and low calcium emulsion gels manufactured with either milk fat or rapeseed oil.

<table>
<thead>
<tr>
<th>Matrix calcium level</th>
<th>Calcium chelating salts</th>
<th>Fat source</th>
<th>Manufacture time (min)</th>
<th>Hardness (N)</th>
<th>Cohesiveness (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>+</td>
<td>Milk Fat</td>
<td>13.38 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>359.82 ± 20.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.38 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rapeseed oil</td>
<td>14.27 ± 0.27&lt;sup&gt;b&lt;/sup&gt;</td>
<td>277.68 ± 18.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.40 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>High</td>
<td>-</td>
<td>Milk Fat</td>
<td>07.04 ± 0.06&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>361.79 ± 12.75&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>0.17 ± 0.03&lt;sup&gt;hx&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rapeseed oil</td>
<td>07.10 ± 0.11&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>322.26 ± 15.14&lt;sup&gt;cy&lt;/sup&gt;</td>
<td>0.17 ± 0.02&lt;sup&gt;hx&lt;/sup&gt;</td>
</tr>
<tr>
<td>Low</td>
<td>-</td>
<td>Milk Fat</td>
<td>24.01 ± 0.02&lt;sup&gt;cx&lt;/sup&gt;</td>
<td>649.33 ± 12.36&lt;sup&gt;dx&lt;/sup&gt;</td>
<td>0.29 ± 0.03&lt;sup&gt;cx&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rapeseed oil</td>
<td>24.04 ± 0.04&lt;sup&gt;cx&lt;/sup&gt;</td>
<td>585.03 ± 18.53&lt;sup&gt;ey&lt;/sup&gt;</td>
<td>0.33 ± 0.03&lt;sup&gt;cx&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values represent the means of triplicates.

For each calcium level (a, b, c) or fat source (x, y), means with different superscript letters differ significantly at P < 0.05.

Casein-based emulsion gels were prepared with (+) or without (-) calcium chelating salts.
6.3.3 Microstructure of casein-based emulsion gels prior to digestion

The ESEM images show that the standard emulsion gels manufactured with CCS presented a compact protein matrix with individual or small aggregates of well emulsified fat globules embedded (Figure 6.1 A1 and A2). These fat globules were smooth surfaced, mostly spherical in shape and appeared to be slightly larger in size (14.7 - 29.5µm) for standard emulsion gels made from rapeseed oil (Figure 6.1 A2) compared to those containing milk fat (< 5µm) (Figure 6.1 A1). It has been previously reported that the melting point of fat affects the emulsification and resulting fat globule size (Singh et al., 2009). Hence, the differences in melting points between rapeseed oil (-10 to +2 °C) and milk fat (+35 to +40 °C) may explain the dissimilarities in the ESEM micrographs of emulsion gels containing different fat sources (Michalski et al., 2013).

For the CCS-free emulsion gels with high levels of calcium, ESEM images revealed these matrices to be quite porous and inhomogeneous in nature, with large areas apparently devoid of fat globules (Figure 6.1 B1 and B2). In contrast, the images of the low calcium emulsion gels showed fat globules, which were not uniformly distributed, throughout the protein matrix. There appeared to be a larger number of smaller fat globules (8.6 - 16.0µm) (especially for those containing milk fat) (Figure 6.1 C1) within a homogeneous, somewhat mottled protein matrix, more similar to that observed in standard emulsion gels. The fat globules appeared much larger in size (13.6 - 39.6µm) in low calcium emulsion gels prepared from rapeseed oil (Figure 6.1 C2) compared to those containing milk fat (Figure 6.1 C1), similar to the differences observed for the standard matrices (Figure 6.1 A1 and A2).
Figure 6.1 Environmental scanning electron micrographs (ESEM) of standard (A), high calcium, CCS-free (B), and low calcium, CCS-free (C) casein-based emulsion gels manufactured from milk fat (1) or rapeseed oil (2) (magnification 500 X).
6.3.4 Nuclear magnetic resonance (NMR)

Moisture mobility within the emulsion gels was monitored using NMR T$_2$ relaxometry (Figure 6.2). Three relaxation states were observed in all emulsion gels these were denoted T$_{2-1}$, T$_{2-2}$ and T$_{2-3}$, and were broadly similar to those reported for processed and analogue cheese (Stafford, 2011; Chen and Liu, 2012). The component with the shortest relaxation time, T$_{2-1}$ (1-2 ms), corresponds to protons in a less mobile fraction of water i.e. water that is very tightly bound (Noronha et al., 2008b) and increased in the order; high calcium (CCS-free) < low calcium (CCS-free) < standard emulsion gels. The T$_{2-2}$ relaxation times ranged from 32 ms for the standard emulsion gels to 20 ms and 8 ms for the low and high calcium gels (CCS-free), respectively. T$_{2-2}$ relaxation times (~12-28 ms) are representative of water that is bound to protein (Budiman et al., 2000; Noronha et al., 2008b). This may suggest that the water in the emulsion gels interacts with protein differently and consequently the gels exhibit different hydration behaviour depending on whether they contain CCS and/or on their total calcium concentration. The width of the high calcium (CCS-free) emulsion gel T$_{2-2}$ peak may indicate a less uniform distribution of water in the very porous inhomogeneous matrix observed in ESEM images (Figure 6.1 B1). By comparison, the standard and low calcium (CCS-free) emulsion gels both had narrow T$_{2-2}$ peaks, perhaps suggesting more uniform water distribution contributing to the homogeneous matrices presented in Figure 6.1 A and C. NMR relaxation times for T$_{2-1}$ and T$_{2-2}$ were greatest for standard emulsion gels, it might be expected that these matrices would contain more tightly bound or less mobile water due to the presence of CCS; however, these emulsion gels contained 10 % more moisture than CCS-free gels (Table 6.1).

The shoulder peak of the second component (Figure 6.2), T$_{2-2}$ (~58 ms), was ascribed to protons from the fat phase of the emulsion gels. To verify this, a high calcium (CCS-free) emulsion gel containing 0 % fat was analysed and was not found to have this component (data...
not shown). The presence of the shoulder peak only in the CCS-free matrices may be because the fat is poorly emulsified and not uniformly distributed as seen in the ESEM images of these emulsion gels (Figure 6.1 B and C). It may be the case that because the fat is more finely emulsified in the standard emulsion gels containing CCS (Figure 6.1 A1 and A2) the shoulder peak coincides with $T_{2,2}$ to a greater extent and so is not observed, and this would also account for the higher peak observed at $T_{2,2}$ for these matrices. $T_{2,2}$ relaxation times of between 50 and 130 ms have previously been associated with the fat phase of analogue cheese containing CCS (Budiman et al., 2000; Noronha et al., 2008b). A longer relaxation time, $T_{2,3}$ (~140-160 ms) corresponded to a more mobile water fraction within samples, correlating with water that is moderately bound. $T_{2,3}$ times were very similar ($P > 0.05$) for all emulsion gels.

Figure 6.2 Distribution of spin-spin relaxation time of standard casein-based emulsion gels prepared with chelating salts (—) and the chelating salt-free high (---) and low (…) calcium emulsion gels manufactured with milk fat.
6.3.5 Breakdown of casein-based emulsion gels during *in vitro* digestion

The physical breakdown (MDI) of emulsion gels during *in vitro* digestion is shown in Figure 6.3. All emulsion gels showed different (P < 0.05) physical rates of breakdown during the gastric phase. MDI values were higher (P < 0.05) for high calcium emulsion gels (CCS-free), than for those obtained for matrices with low calcium levels containing no CCS. The standard emulsion gels had the lowest (P > 0.05) MDI values in the gastric environment. These trends were similar for matrices made from both milk fat (Figure 6.3 a) and rapeseed oil (Figure 6.3 b). After 30 min of the intestinal phase, the physical breakdown of all emulsion gels accelerated substantially. This increase was most likely due to the action of *trypsin* and *chymotrypsin* (contained in the *pancreatin* extract), which rapidly completed the hydrolysis of the hydrated and exposed protein matrix (Ayala-Bribiesca *et al*., 2016), combined with the increase in pH (6.0-6.5). Upon completion of *in vitro* digestion (i.e. after 300 min), all emulsion gels had disintegrated to particle sizes of < 1.5 mm, with MDI values of 100% obtained.

The higher MDI values obtained for the high calcium emulsion gels (CCS-free) during the gastric phase may be due to their low cohesiveness values (Table 6.2) and therefore higher brittleness (Lamothe *et al*., 2012), which probably contributed greatly to the enhanced rate at which they disintegrated in the gastric environment. A greater degree of brittleness of high calcium (CCS-free) emulsion gels could be visually observed during sample preparation. Ayala-Bribiesca *et al*. (2016) showed that high calcium cheese had the highest MDI values during digestion of Cheddar cheeses with different calcium levels. In that study, and similar studies concerning natural cheese, higher MDI values after the oral phase were attributed to brittle texture (Lamothe *et al*., 2012; Ayala-Bribiesca *et al*. 2016). In addition, the high calcium emulsion gels (CCS-free) had the shortest $T_{2,1}$ and $T_{2,2}$ relaxation times, suggesting they had the least water mobility of all the gels. It has been reported for analogue cheese that
matrices in which the water is more tightly bound tend to be more brittle, whereas those with higher water mobility have a softer texture (Noronha et al., 2011). This correlates with the texture and NMR results observed in the present study. The CCS-free matrices with low levels of calcium were significantly (P < 0.05) harder and more cohesive, which may have caused limited accessibility of digestive enzymes to the matrix, resulting in reduced physical breakdown. This may suggest that the extent of breakdown depends on the physical properties of the matrix modulating the access of enzymes to their substrate.

Although the standard matrices had comparable calcium concentrations and broadly similar hardness values to the high calcium gels (CCS-free), particularly those made from milkfat, their physical breakdown was considerably (P < 0.05) lower during the gastric phase. This resistance to gastric conditions may be due to their significantly (P < 0.05) higher cohesiveness, which could have slowed the rate at which the standard emulsion gels disintegrated. Their higher moisture to protein ratio may also have contributed to their resistance to physical breakdown. A study by Marshall (1990) showed that moisture in cheese matrices acted as a plasticiser, making the matrix more elastic and less likely to fracture. The inclusion of CCS and their contribution to structure formation may also play a critical role in how the standard matrices disintegrate during digestion. Resistance to gastric degradation has consequences for the kinetics of nutrient release and may also be relevant for the design of functional dairy foods (Lamothe et al., 2012). With only ~27% degradation at the end of the gastric phase, the standard emulsion gels might be a useful matrix for the transport and protection of bioactive compounds sensitive to the gastric environment. In addition, the differences observed in MDI values in the gastric phase suggests that the structure of these and similar types of emulsion gels could be tailored to delay the rate of gastric emptying; which could be a useful approach to develop functional foods with enhanced satiation (Cardoso-Junior et al., 2007; Kong and Singh, 2008).
Figure 6.3 Kinetics of matrix degradation during *in-vitro* digestion of a standard high calcium casein-based emulsion gel prepared with chelating salts (■) and chelating salt-free emulsion gels, containing high (▲) or low (●) calcium levels manufactured with milk fat (a) or rapeseed oil (b) in a simulated gastro-intestinal environment. Standard error bar values represent the data ± standard error of the mean and represent the mean of three replicates.
6.3.6 Release of free fatty acids (FFAs) from casein-based emulsion gels during in *vitro* digestion

Before the addition of the simulated intestinal fluids, the FFAs detected in the digestates were lower than 0.01 %. No lipases were used in the oral or gastric phase, so lipolysis did not occur until the intestinal fluids were added. At the beginning of the intestinal phase i.e. after 120 min, lipolysis increased for all emulsion gels (Figure 6.4). However, no significant difference (P > 0.05) was observed between the emulsion gels manufactured from milk fat (Figure 6.4 a). Conversely, for those manufactured from rapeseed oil, high calcium gels (CCS-free) showed a higher rate (P < 0.05) and extent of FFAs release (at 150 and 180 min) compared to the other emulsion gels (Figure 6.4 b). There were no differences (P > 0.05) in lipolysis between the low calcium (CCS-free) and standard matrices prepared with CCS.

The Ca$^{2+}$ can increase the rate of lipid digestion by binding and precipitating the long chain fatty acids produced during the lipolysis of emulsified TAGs (Armand *et al*., 1992; Zangenberg *et al*., 2001; Hwang *et al*., 2009). This may explain why the CCS-free, high calcium matrices had greater FFAs release compared to the low calcium emulsion gels (CCS-free). These differences are most likely attributable to calcium concentration, as both matrices had similar (P > 0.05) MDI values at 150 and 180 min and there were no differences (P > 0.05) in their composition otherwise (Table 6.1). It might be expected that as the standard matrices had comparable calcium concentrations to the high calcium matrices (CCS-free), that their lipolysis progression would be higher or enhanced. However, while calcium has been shown to increase the rate of lipid digestion, the addition of CCS has the opposite effect (Hu *et al*., 2010). This decrease is due to the ability of CCS to bind some of the Ca$^{2+}$, and thereby prevent them from either activating the lipase and/or precipitating the long chain fatty acids at the droplet surface (Hu *et al*., 2010).
It has been reported that the effects of calcium concentration on the rate of lipid digestion is dependent on the fatty acid composition of the lipid phase (Bonnaire et al., 2008; McClements et al., 2009). MacGregor et al. (1997) reported that the rate of FFA production was independent of calcium concentration for short chain TAGs because the FFAs generated were water-dispersible and easily moved away from the droplet surfaces. The fatty acid composition of milk fat and rapeseed oil vary greatly with milk fat containing a much larger proportion of shorter chain fatty acids (Michalski et al., 2013). This may explain why in the present study, we observed no effect of calcium concentration on the FFAs release of emulsion gels manufactured from milk fat.

An additional observation was the higher rates of lipolysis evident in the standard and low calcium (CCS free) matrices manufactured using milk fat (Figure 6.4 a) compared to those prepared from rapeseed oil (Figure 6.4 b) at equivalent calcium concentrations. This effect may have been caused by the more rapid breakdown of these matrices during gastric digestion (Figure 6.3 a) which may have facilitated greater access of the enzyme lipase to the substrate. In addition, the micrographs of the standard and low calcium (CCS free) matrices containing milk fat (Figure 6.1 A & C) show smaller lipid droplet sizes compared to those manufactured using rapeseed oil which presumably resulted in increased fat surface area and may have caused enhanced lipolysis. However, further investigations where lipid droplet size is more tightly controlled are required to determine the impact of this parameter on the rate and extent of lipolysis.
Figure 6.4 Evolution of lipolysis (NEFA, non-esterified fatty acids) during the intestinal phase of the in vitro digestion of a standard high calcium casein-based emulsion gel prepared with chelating salts (■) and chelating salt-free casein-based emulsion gels containing high (▲) or low (●) calcium levels manufactured with milk fat (a) or rapeseed oil (b) in a simulated gastro-intestinal environment. Standard error bar values represent the data ± standard error of the mean and represent the mean of three replicates.
6.4 Conclusion

The results of this study show that modifying the composition (i.e. calcium concentration, moisture to protein ratio, inclusion of CCS) and consequently the physical characteristics (e.g. texture, microstructure and water mobility) of casein-based emulsion gels affects their behavior during in vitro digestion. These results are a clear example that food matrix microstructures can be modified to alter resistance to gastric degradation which may have consequences for the kinetics of nutrient release, encapsulation of nutrients or bioactive ingredients, and satiety, all of which are relevant for the design of functional dairy foods. The results of the present study revealed that the effect of calcium concentration on enhancing lipolysis was dependent on fat type. However, further work on casein-based emulsion gels with similar composition and structure may be required to better elucidate the effect of calcium on lipid bioaccessibility.
6.5 References


Chapter 7
General Discussion
7.0 General Discussion

For several decades, the scientific community has been actively seeking to describe and understand the complexity of casein micelles in terms of their composition, structure and functional properties. In spite of this intense research, it is still necessary to improve our knowledge on the structure, organisation and techno-functionality of these complex association colloids (Broyard and Gaucheron, 2015). Among the most important factors to be resolved are the interactions between calcium and protein within these particles, and how this can be related to the properties of casein-based food systems. The lack of understanding of this relationship may limit the use of such matrices as, for example, delivery vehicles for bioactives and in the development of ‘structurally tailored’ foods which may be beneficial to human health. Consequently, knowledge of the critical role of calcium and its various forms in influencing the aggregation, self-assembly behaviour and functionality of casein micelles is required in order to facilitate the development of casein-based matrices for such target applications.

7.1 Key Findings

7.1.1 Chapter 2

The research presented in this thesis investigates the modulating effect of calcium on the physico-chemical properties of rennet casein which is largely composed of micellar casein and was completed with a view to developing novel casein-based food structures with unique functionalities. Initially, the research completed in Chapter 2 established the impact of individual and mixtures of calcium chelating salts (CCS) on calcium distribution in model systems comprising of CaCl₂ solutions or dilute rennet casein dispersions. The type of calcium chelator applied induced very different changes to calcium distribution in both model systems studied. In the case of trisodium citrate (TSC) addition, high levels of dispersed “chelated” calcium were present; conversely, disodium phosphate (DSP) addition resulted in
the formation of greater levels of insoluble calcium, while the $A_{Ca^{++}}$ decreased with increasing concentration of both CCS types. Quite surprisingly and in contrast to previous reports in the literature, calcium chelator addition did not result in high levels of soluble protein in the model rennet casein dispersions used. This information is useful in understanding the role of calcium in casein solubility and its impact on functional performance. In addition, these results suggest that the calcium-mediated cross-links in rennet casein, which limit its solubility and hydration capacity are disrupted by CCS in different ways. The chelation of $Ca^{2+}$ ions by TSC results in the formation of soluble complexes in the aqueous phase. However, the mechanism by which DSP facilitates disruption of calcium-mediated cross-links is less clearly understood, as it is not simply by increasing calcium solubility, but rather by forming some type of insoluble calcium phosphate complex. This may also be linked / associated with casein but presumably not in a cross-linking way. Although the results presented in Chapter 2 give a useful indication of the different behaviours of TSC and DSP in calcium-casein systems, investigations into their behaviour in more concentrated and industrial relevant applications e.g. processed and analogue cheese manufacture were required.

7.1.2 Chapter 3

Therefore, the focus of the research undertaken in Chapter 3 was to monitor the progression of calcium and protein solubilisation as affected by calcium chelators during small-scale manufacture of concentrated casein-based food matrices e.g. processed and analogue cheese. To achieve this, a small-scale manufacturing protocol was developed and the resulting matrices were confirmed to be reflective of those manufactured in a pilot-scale system. The progression of calcium and protein solubilisation was monitored throughout the manufacturing period of these dense protein systems. Such investigations had not been reported previously in the literature and provided a realistic insight into the role of CCS in the development of a casein-based food matrix. Thus, Chapter 3 advances the current knowledge,
by demonstrating that the role of CCS is mainly to deplete colloidal calcium by partial solubilisation. In turn this reduces calcium-mediated cross-linking in the para-casein network of rennet casein sufficiently to allow adequate protein solubilisation to occur for fat emulsification, thereby contributing to the formation of a homogeneous casein-based food matrix. However, similar to the results obtained using the model rennet casein dispersions in Chapter 2, the levels of protein solubilised by calcium chelators during manufacture of these concentrated casein matrices were relatively low. This demonstrates that while CCS facilitate the hydration of the protein present in the system, this is not solubilised to a great extent. The results obtained in Chapter 3 suggest that a similar attenuation of calcium-mediated cross-links could possibly be achieved by simply modifying the total calcium concentration of these matrices and provided an indication of the levels by which to reduce the total calcium concentration (by \(\sim 23\%\)) in order to form hydrated and functional casein-based food matrices. Therefore, it was hypothesised that this might be an alternative approach to modifying calcium-casein interactions and thus develop casein-based food matrices with novel functionalities.

### 7.1.3 Chapter 4

To achieve this, in Chapter 4 casein-based food matrices were manufactured with a range of calcium concentrations by substituting high calcium rennet casein with a low calcium acid casein. The target calcium concentrations were chosen based on the findings of Chapter 3 and the results obtained demonstrate that this was a novel and successful approach to facilitate the formation of hydrated and functional casein-based food matrices without the use of calcium chelators. This had not been reported previously in the literature and thus one of the principal objectives set out in this thesis was accomplished. Eliminating CCS affords the possibility of reducing the sodium content of processed and analogue cheese products by up to 60\% which is a considerable advance on previous attempts reported in the literature and is also of substantial nutritional significance. Another advantage of this research was that precise
control of calcium concentration facilitated the formulation of casein-based food matrices with tailored functionality and composition – e.g. high melt vs low melt; high moisture vs low moisture; hard vs soft texture etc. The results generated demonstrate that homogeneous matrices were successfully formed at intermediate calcium levels (673-358 mg.100 g\(^{-1}\)) without CCS. Another possible way this might be achieved would be to adjust the pH of rennet casein-based matrices by adding citric acid directly to obtain a final pH similar to the values obtained for those CCS-free matrices formed at intermediate calcium levels. This may be an interesting avenue to explore for further research.

7.1.4 Chapter 5
The results in Chapter 3 and 4 provide insights into the role of CCS in the formation of hydrated and functional casein-based food systems with the outcome of the development of CCS-free matrices. However, these studies were completed using low-fat and unflavoured matrices, which are not representative of the processed and analogue cheese-type products available commercially. Therefore, Chapter 5 fulfil the role of developing flavoured matrices containing the different fat levels (full, reduced and half fat) available in commercial products. While the findings indicate the most preferred type of enzyme modified cheese (EMC) used to flavour the newly developed matrices was Emmental, more importantly, the results demonstrate that it is possible to produce full, reduced and half fat casein-based food matrices without CCS. Although the full fat flavoured matrices were most preferred by sensory panellists and were rated higher in terms of their texture, flavour and overall liking; there were no significant differences detected between those containing half or reduced fat levels for any of these attributes. Therefore, the results obtained gave an indication of the extent to which the fat content of these CCS-free matrices could be reduced without impacting negatively on their sensory acceptability. Further investigations are required to compare the sensory properties (including textural attributes) of the casein-based food
matrices flavoured with a broader range of EMCs at different levels of addition to optimise the sensory acceptance of these novel matrices.

7.1.5 Chapter 6
The results generated in Chapters 3-5 led to the development of a novel approach to modifying calcium-casein interactions in concentrated casein systems without the need for calcium chelators. In Chapter 6 the impact of manipulating the total calcium concentration on the microstructure and the digestibility of these matrices was examined. More specifically, the impact of the composition (i.e. calcium concentration, moisture to protein ratio, inclusion of calcium chelators) and consequently the physical characteristics (e.g. texture, microstructure and water mobility) of the CCS-free casein matrices on their digestibility was determined using an in-vitro digestion model. The study showed that matrix degradation in the gastric environment was related to the physical characteristics of the casein-based food structure. The high calcium CCS-free systems, which presented a more porous protein matrix with lower levels of firmness and cohesiveness, were the most rapidly digested during simulated gastric digestion. In contrast, for those casein matrices which had a more cohesive structure, more compact protein matrix and more uniform water distribution (i.e. the standard and the low calcium, CCS-free formulations), the extent of matrix disintegration in the gastric environment was significantly lower. These results are a clear example that food matrix microstructure can be modified to alter resistance to gastric degradation and may also suggest that they can be designed in such a way that their modulated digestion behaviour triggers different physiological responses. However, only the high calcium, CCS-free matrices showed a higher extent of free fatty acid release, which was attributed to the ability of Ca$^{2+}$ to increase the rate of lipid hydrolysis. This effect was found to be dependent on the type of lipid phase present in the matrix. Therefore, further studies are required to better elucidate the effect of calcium on the bioaccessibility of different lipid types.
7.2 Recommendations / Future Work

The studies presented in this thesis have provided new insights into the interactions between calcium chelators and calcium and their influence on the behaviour of micellar casein and on the physico-chemical properties of concentrated casein-based food systems e.g. processed and analogue cheese. Furthermore, the research generated has facilitated a greater understanding of the modulating effect of calcium on the physico-chemical properties of casein which were subsequently manipulated to develop novel casein-based semi-solid food systems with different structures, functional properties (i.e. textural attributes and melting characteristics) and modulated digestion behaviour. Such matrices may have a number of potential applications, for example, the functional food industry has seen major advances in the development of new delivery systems for nutraceuticals and bioactive compounds (Singh, 2016). Previously, many food materials have been exploited to create delivery systems to incorporate and protect these compounds within a food matrix. In particular, the use of biopolymers for the delivery of functional food ingredients and pharmaceuticals has increased dramatically. In this context, milk proteins have received growing attention (Livney, 2010; Elzoghby et al., 2011; Tavares et al., 2014; Ranadheera et al., 2016) due to their superior functional qualities and nutritional properties. The knowledge generated as part of this thesis advances our understanding of the modulating effect of calcium on the physico-chemical properties of casein and how this can be manipulated to influence the structure and functional properties of casein-based food matrices formed. Food structure is of critical importance for the delivery of bioactives; some bioactives are acid labile, for example, and need to be protected from the stomach acids and released in the neutral pH of the small intestine. The results presented in Chapter 6 certainly suggest that some of the casein-based food matrices developed have appropriate structure and exhibit sufficient gastric resistance to achieve this. Therefore, there is further scope to investigate if these matrices can be used as delivery systems for the entrapment of sensitive bioactives for functional food development.
Manipulating food structure / texture has recently been shown to be an efficient way to influence oral processing characteristics (e.g. bite size and oral residence duration) and thus food intake (Bolhuis et al., 2014; Campbell et al., 2016). Investigating the effect of food structure / texture on energy intake is relevant from a nutritional point of view, because semi-solid foods account for the majority (~80 %) of our daily energy intake (Bolhuis et al., 2014). Although, existing studies are rather limited and very little research has been completed in this field to date, the evidence available suggests that subtle changes in food texture can impact on the satiating effect of foods and may lead to decreased energy intake (Bolhuis et al., 2014; Campbell et al., 2016). Some studies have reported weaker satiating capacities of liquid foods compared to semi-solid foods (De Wijk et al., 2008; De Graaf, 2011; Viskaal-van Dongen et al., 2011). This effect has been attributed to the minimal sensory exposure of liquid foods in the oral cavity due to the fast rate of consumption (De Graaf and Kok, 2010; De Graaf, 2011). However, further studies are required to investigate the impact of different semi-solid food textures on eating rate and energy intake. In Chapters 4-6 of this thesis casein-based food structures with different textures were generated by manipulating key components within the food matrix i.e. calcium, fat and protein. Thus, it may be interesting to look at the satiating effects of the different food structures developed and to link this effect to differences in food oral processing characteristics.

Recently the link between food structure and the sensory perception they evoke has also been the subject of much research (Foegeding et al., 2015). The pattern by which food structure breaks down during oral processing, generating texture, flavour and taste, determines the acceptability of a food. However, breakdown patterns and textural perception associated with desirable food qualities are not well understood. With regard to the results of the sensory study presented in Chapter 5, further research investigating the influence of matrix texture and formulation on flavour release using a technique such as temporal dominance of sensations.
(TDS) may be a useful means of determining how the structural changes associated with the
different matrices may affect oral sensory properties.

7.3 Conclusion

In summary, the research completed in the Chapters of this thesis present a comprehensive
and cohesive set of studies on the impact of CCS on the behaviour of micellar rennet casein
systems. Beginning with a very simplified CaCl$_2$ system and building through model casein
dispersions in Chapter 2 and applying those to more realistic and industrial relevent
concentrated sample matrices in Chapters 3, 4 and 5 has provided an alternative approach to
modifying calcium-casein interactions with the outcome of the development of novel CCS-
free matrices. Finally, in Chapter 6 the impact of the implications of such modifications on
the digestibility of the matrices formed was examined. The research generated has facilitated
the development of novel casein-based semi-solid food matrices which have different
structures, functionality and digestion behaviour and thus gives scope for lots of future work
in this area.

7.4 References

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scientific and technological challenge. Dairy Science and Technology, 95, 831-862.

Campbell, C. L., Wagoner, T. B. and Foegeding, E. A. (2016). Designing foods for satiety:
The roles of food structure and oral processing in satiation and satiety. Food


Appendices
Appendices

Appendix 1:

Sensory Analysis Cheese Analogue – Ranking Test

Assessor’s No: _____ Assessor’s Name_____________________ Date____/____/______
Time____

Characteristic Studied: Preference between cheese analogue varieties

Instructions:

1. Receive the sample tray and note each sample code below according to its position
   on the tray.

2. Taste the samples from left to right, chewing for 30 seconds and note your
   preference in terms of flavour and mouthfeel of the sample.

   Wait at least 30 seconds between samples and clean the palate with water and
   cracker provided.

3. Write ‘1’ in the box of the sample which you most prefer.

   Write ‘2’ for the next and 3 for the least preferred.

4. If two samples appear the same, make a ‘best guess’ as to their rank order.

Code  _______ _______ _______

Rank
☐ ☐ ☐

Any additional comments:
________________________________________________________________________________
________________________________________________________________________________
Appendix 2:

Sensory Analysis Cheese Analogue – Acceptability Test

Assessor’s No: _____ Assessor’s Name ___________________ Date ___/___/______
Time ______

**Instructions:** Please rinse your mouth with the water provided before starting the test. You are given three coded cheese analogue samples; please taste the samples from left to right. Assess the first sample using the 1-9 scale below.

Sample number: __________

<table>
<thead>
<tr>
<th>TEXTURE</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLAVOUR</td>
<td></td>
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<tr>
<td>OVERALL LIKING</td>
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</tbody>
</table>

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Please rinse your mouth with the water provided and assess the second sample using the 1-9 scale below.

Sample number: __________

<table>
<thead>
<tr>
<th>TEXTURE</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLAVOUR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OVERALL LIKING</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

***************
Please rinse your mouth with the water provided and assess the third sample using the 1-9 scale below.

Sample number: __________

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEXTURE</td>
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<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>FLAVOUR</td>
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</tr>
<tr>
<td>OVERALL LIKING</td>
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</tr>
</tbody>
</table>

Any additional comments:
__________________________________________________________________________________
__________________________________________________________________________________
__________________________________________________________________________________

You have reached the end of the test
Thank You
### Appendix 3: Constituents and concentrations of the various synthetic juices of the in vitro digestion model representing fed conditions (Versantvoort et al., 2005).

<table>
<thead>
<tr>
<th></th>
<th>Saliva</th>
<th>Gastric juice</th>
<th>Duodenal juice</th>
<th>Bile juice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inorganic solution</td>
<td>10 ml KCl (89.6 g/l)</td>
<td>15.7 ml NaCl (175.3 g/l)</td>
<td>40 ml NaCl (175.3 g/l)</td>
<td>30 ml NaCl (175.3 g/l)</td>
</tr>
<tr>
<td></td>
<td>10 ml KSCN (20g/l)</td>
<td>3.0 ml NaH$_2$PO$_4$ (88.8 g/l)</td>
<td>40 ml NaHCO$_3$ (84.7 g/l)</td>
<td>68.3 ml NaHCO$_3$ (84.7 g/l)</td>
</tr>
<tr>
<td></td>
<td>10 ml NaH$_2$PO$_4$ (88.8 g/l)</td>
<td>9.2 ml KCl (89.6 g/l)</td>
<td>10 ml KH$_2$PO$_4$ (8 g/l)</td>
<td>4.2 ml KCl (89.6 g/l)</td>
</tr>
<tr>
<td></td>
<td>10 ml NaSO$_4$ (57 g/l)</td>
<td>18 ml CaCl$_2$.H$_2$O (22.2 g/l)</td>
<td>6.3 ml KCl (89.6 g/l)</td>
<td>150 µl HCl (37% g/g)</td>
</tr>
<tr>
<td></td>
<td>1.7 ml NaCl (175.3 g/l)</td>
<td>10 ml NH$_4$Cl (30.6 g/l)</td>
<td>10 ml MgCl$_2$ (5 g/l)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20 ml NaHCO$_3$ (84.7 g/l)</td>
<td>6.5 ml HCl (37% g/g)</td>
<td>180 µl HCl (37% g/g)</td>
<td></td>
</tr>
<tr>
<td>Organic solution</td>
<td>8 ml urea (25 g/l)</td>
<td>10 ml glucose (65 g/l)</td>
<td>4 ml urea (25 g/l)</td>
<td>10 ml urea (25 g/l)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 ml glucuronic acid (2 g/l)</td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>3.4 ml urea (25 g/l)</td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10ml glucoseamine hydrochloride (33 g/l)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Add to mixture of</td>
<td>290 mg α-amylase</td>
<td>1 g BSA</td>
<td>9 ml CaCl$_2$.H$_2$O (22.2 g/l)</td>
<td>10 ml CaCl$_2$.H$_2$O (22.2 g/l)</td>
</tr>
<tr>
<td>organic + inorganic</td>
<td>15 mg uric acid</td>
<td>2.5 g pepsin</td>
<td>1 g BSA</td>
<td>1.8 g BSA</td>
</tr>
<tr>
<td>solution</td>
<td>25 mg mucin</td>
<td>3 g mucin</td>
<td>9 g pancreatin</td>
<td>30 g bile</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.5 g lipase</td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>6.8 ± 0.2</td>
<td>1.30 ± 0.02</td>
<td>8.1 ± 0.2</td>
<td>8.2 ± 0.2</td>
</tr>
</tbody>
</table>