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Role of calcium equilibrium in modulating the textural and functional properties of brine-salted cheese

Thesis presented by

Lisa Norma McAuliffe, B.Sc. (Hons.) *(NUI)*

for the degree of

Doctor of Philosophy

in

Food Science and Technology

May, 2017

Table of Contents

Declaration **I**

Acknowledgements **II**

Summary **IV**

Chapter 1 **1**

A general review on the influence of calcium on the textural and rheological properties of cheese

Chapter 2 **16**

Literature review: Encapsulation technologies and their application in the food industry

Objectives **117**

Chapter 3 **119**

Effect of cooking temperature and addition of calcium chelating salts on the calcium equilibrium, textural and rheological properties of Gouda-type cheese during ripening

Chapter 4 **170**

Manufacture and incorporation of liposome-entrapped calcium chelating salts into model miniature Gouda-type cheese and subsequent effect on starter viability, pH and moisture content

Chapter 5 **219**

Calcium solubilisation, texture and functionality in a model Gouda-type cheese manufactured with liposome-entrapped calcium chelating salts

Chapter 6 **268**

Chymosin-mediated proteolysis, calcium solubilisation and texture development during the ripening of Gouda-type cheese

Chapter 7 **316**

Characterisation of a Swiss-Cheddar hybrid cheese during ripening

Chapter 8 **368**

Conclusions and recommendations

Appendix

McAuliffe, L. N., Kilcawley, K. N., Sheehan, J. J. & McSweeney, P. L. H. (2016). Manufacture and incorporation of liposome-entrapped ethylenediaminetetraacetic acid into model miniature Gouda-type cheese and subsequent effect on starter viability, pH, and moisture content. *Journal of Food Science*, **81**, C2708-2712.

Declaration

I hereby declare that the work submitted is entirely my own and has not been submitted to any other university or higher education institute, or for any other academic award in this university.

Date: _____

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Lisa McAuliffe

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Summary

The role of calcium (Ca) in modulating the textural and rheological properties of brine-salted Gouda-type cheese during ripening was investigated, with particular emphasis on the alteration of Ca between the soluble (SOL) and insoluble (INSOL) phases. Several studies, mostly pertaining to dry-salted Cheddar and Mozzarella variants, have described the dynamic equilibrium which exists between INSOL INSOL Ca phosphate in milk/cheese (CCP) and the SOL Ca in the aqueous phase of dairy products, and its influence on cheese texture and rheology. This thesis aims to explore the association between the changes in CCP concentration and the textural, functional and rheological properties of brine-salted continental-type cheese during ripening.

Direct addition of EDTA to the curds/whey mixture during manufacture resulted in decreased pH and increased moisture content. Increasing EDTA addition levels and reducing the cook temperature of Gouda-type cheese resulted in a significant reduction in the levels of total Ca, INSOL Ca and pH, as well as an increase in moisture content. Reduction of INSOL Ca content of cheese increased Gouda-type cheese meltability to a greater extent than increasing the moisture content, while the textural properties (hardness, chewiness and gumminess) were more influenced by moisture content rather than the level of INSOL Ca.

In an effort to overcome the unfavorable effects of direct addition of EDTA to cheese, a novel method was developed in which EDTA was entrapped within liposomes and added to cheese-milk during manufacture of miniature Gouda-type cheese. Liposome-entrapped EDTA did not affect the rennet gelation or fermentation of milk, cheese moisture content, or pH. Thus, the potential for incorporation of EDTA entrapped in liposomes into brine-salted cheese was demonstrated and later explored for modifying cheese texture and functionality. Addition of liposome-entrapped EDTA into Gouda-type cheese caused no differences in composition; however, a reduction in the level of INSOL Ca was evident. Cheese supplemented with liposome-entrapped EDTA exhibited reduced hardness, chewiness and gumminess values, as well as, increased melting properties, due to the altered distribution of Ca between the INSOL and SOL forms.

It was determined that chymosin-mediated proteolysis of α_{s1}-casein at Phe_{23}-Phe_{24} was not a prerequisite for softening of Gouda-type cheese texture during the early stages of ripening. Instead, the reduction in the level of INSOL Ca during early ripening was more associated with lower hardness values of Gouda-type cheese as ripening progressed.

A Swiss-Cheddar-hybrid cheese was manufactured and characterised fully during ripening. The pH increased progressively during ripening, while the concentration of INSOL Ca decreased as ripening progressed up to 112 d, as expected, and then appeared to increase slightly for the remainder of ripening (224 d), indicating the possible reformation of INSOL Ca. These factors were attributed to the limited changes to the textural, rheological and functional properties of Swiss-Cheddar cheese observed during ripening.

In conclusion, the work undertaken in this thesis has expanded knowledge of the role of physicochemical and biochemical process, and their interactions, with particular emphasis on calcium equilibrium, in the development of textural and rheological properties of brine-salted continental-type cheese during ripening.

Chapter 1

A general review on the influence of calcium on the textural and rheological properties of cheese

1.1 Colloidal calcium phosphate

The salts associated with casein micelles in milk are collectively referred to as colloidal calcium phosphate (CCP) (Holt, 1985). Despite CCP being one of the minor constituents of the casein micelle by weight (Fox and McSweeney, 1998) it is in fact one of its major structural components (Horne, 1998; Lucey and Horne, 2009) (Figure 1.1). The main role of CCP is to act as a cross linking agent within the casein micelles, helping to maintain their integrity.

1.2 Forms of calcium in cheese

Calcium (Ca) in cheese exists in two forms, insoluble Ca (INSOL; Ca associated with the CCP; INSOL Ca) and soluble Ca (SOL; Ca present in the aqueous serum phase within the cheese). During cheese manufacture, most of the serum Ca is lost in the whey during drainage and INSOL Ca is partially dissolved as the draining pH is decreased (Lucey and Fox, 1993a). For a considerable time, it was believed that the CCP in milk was completely solubilised during cheesemaking and that Ca in the cheese was only present in the serum phase (Pyne and McGann, 1960; Choi *et al*., 2007). Lucey and Fox (1993a) suggested that the total Ca concentration in cheese is not the best indicator or predictor of cheese texture. Instead they proposed that it is the amount of INSOL Ca (amount of Ca still associated with the casein matrix of the cheese), that plays a major role in controlling cheese structure, texture and functionality. Ca associated with the casein particle (CCP) is believed to be a principal structural constituent in cheese (Lucey and Fox, 1993; Horne, 1998), and any decrease in the amount of INSOL Ca would be expected to modify cheese texture (Lucey *et al*., 2003, 2005).

1.3 Changes in calcium equilibrium of cheese during ripening

As in milk, a dynamic equilibrium exists between INSOL Ca bound to the CCP within the casein micelle and SOL Ca present in the aqueous serum phase of cheese. It is believed that Ca is solubilized from the residual CCP in the *para*-casein matrix during ripening to become part of the aqueous phase of cheese in order to attain a so-called 'pseudoequilibrium' of Ca phosphate between the SOL and INSOL phases of cheese (Hassan *et al*., 2004; Lucey *et al*., 2005; O'Mahony *et al.*, 2006), commonly termed 'Ca-equilibrium' of cheese. In early ripening of cheese, starter bacteria metabolise residual lactose leading to an increase in lactic acid (Cogan and Hill, 1993). In an effort to resist changes in pH caused by increasing lactic acid production, CCP experiences partial solubilisation, resulting in the release of phosphate ions which can react with hydrogen ions, resulting in buffering (Figure 1.2). Hassan *et al*. (2004) and O'Mahony *et al*. (2006) both demonstrated in Cheddar cheese that there is a significant reduction in the INSOL Ca content of the cheese during ripening, with the proportion of INSOL Ca decreasing from ~70 to 56% during the first 3 months of maturation. It has been observed that the major decrease in INSOL Ca of Cheddar and Colby-type cheeses occurred during the first month of ripening and thereafter few changes were evident (Hassan *et al*., 2004; Lucey *et al*., 2005; Lee *et al*., 2005, 2011).

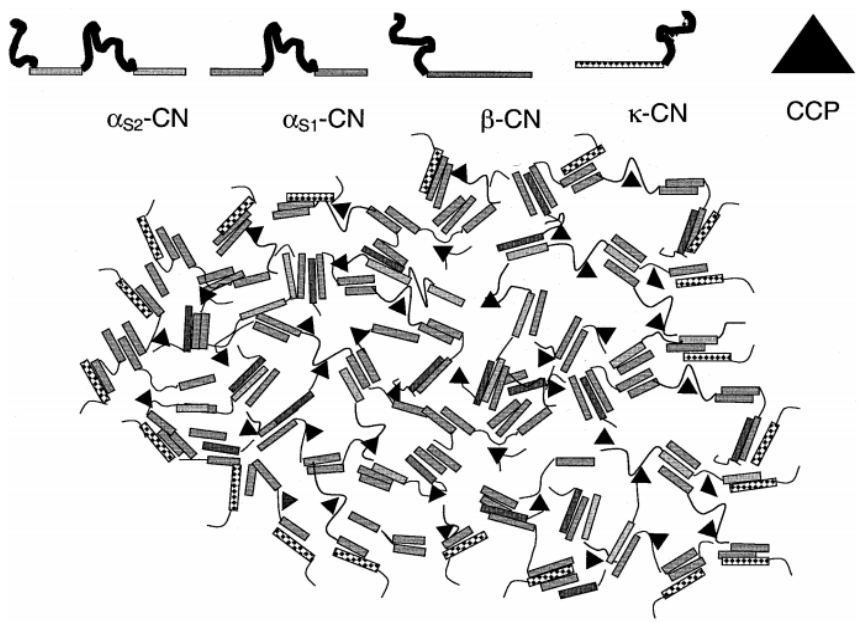


Figure 1.1 Schematic representation of the 'dual-binding model' of the casein micelle (Horne, 1998) revised by Lucey and Horne (2009), showing the role of colloidal calcium phosphate (CCP) in micelle integrity.

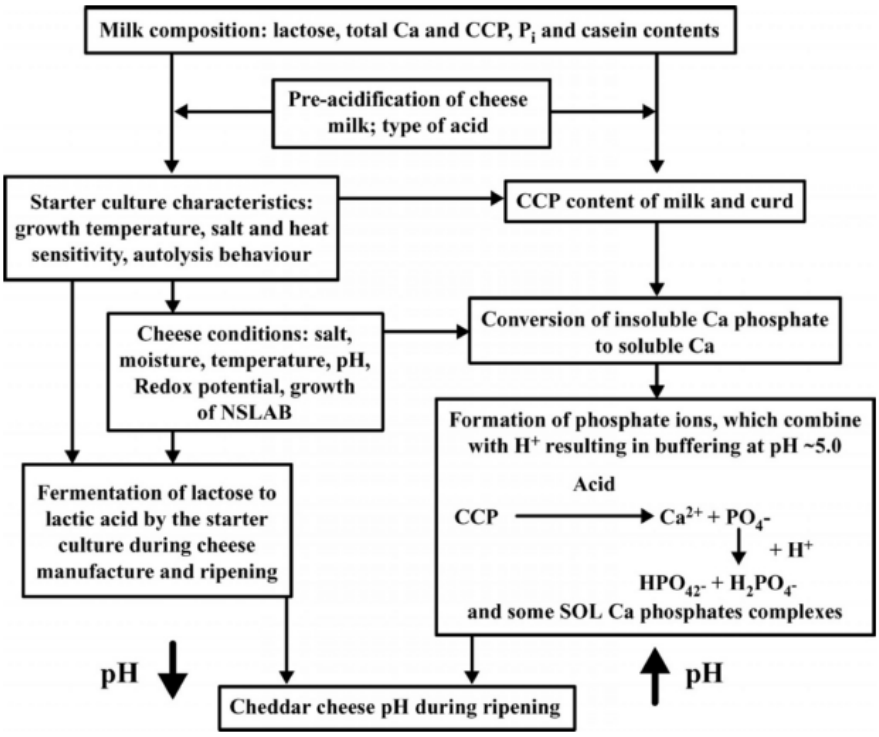


Figure 1.2 Schematic representations of the possible relationships between metabolism of lactose, solubilisation of colloidal calcium phosphate (CCP) and changes in pH during ripening of Cheddar cheese (source from Hassan *et al*., 2004).

1.4 Methods to determine calcium equilibrium in cheese

Accurate quantification of the distribution of total Ca between the INSOL and SOL forms in cheese during ripening is possible by using either the "cheese juice" method (Morris *et al*., 1988; Hassan *et al*., 2004; Lee *et al*., 2005, 2010) or the acid-base titration method (Lucey *et al*., 1993b, 2005; Hassan *et al*., 2004; O'Mahony *et al*., 2005; Lee *et al*., 2011).

The first approach involves the determination of Ca concentration in the aqueous phase of the cheese ("cheese juice") extracted from grated cheese by applying hydraulic pressure. The extracted 'juice' is assumed to be compositionally equal to the serum phase of cheese and thus contains the SOL Ca at the same concentration as the serum phase of the cheese. The INSOL Ca content of the cheese can be estimated by subtracting the Ca concentrationof the juice from the total Ca concentration of the cheese.

The second approach is the acid-base titration method which correlates the buffering capacity of cheese to the amount of Ca associated with the casein micelles/particles. In this method, the buffering capacity of the cheese, as well as the milk it was made from are determined. The buffering peaks (Figure 1.3) observed in milk between ~pH 5.8 to 4.1 and in cheese from ~pH 5.1 to 4.0 are an index of their CCP content (Lucey *et al*., 1993a, b; Hassan *et al*., 2004). The buffering capacities and Ca contents of the milk and cheese are used to calculate the INSOL Ca content. Hassan *et al*. (2004) reported no statistical difference between these two methods for accuracy in their determination of % INSOL Ca in cheese (Figure 1.4).

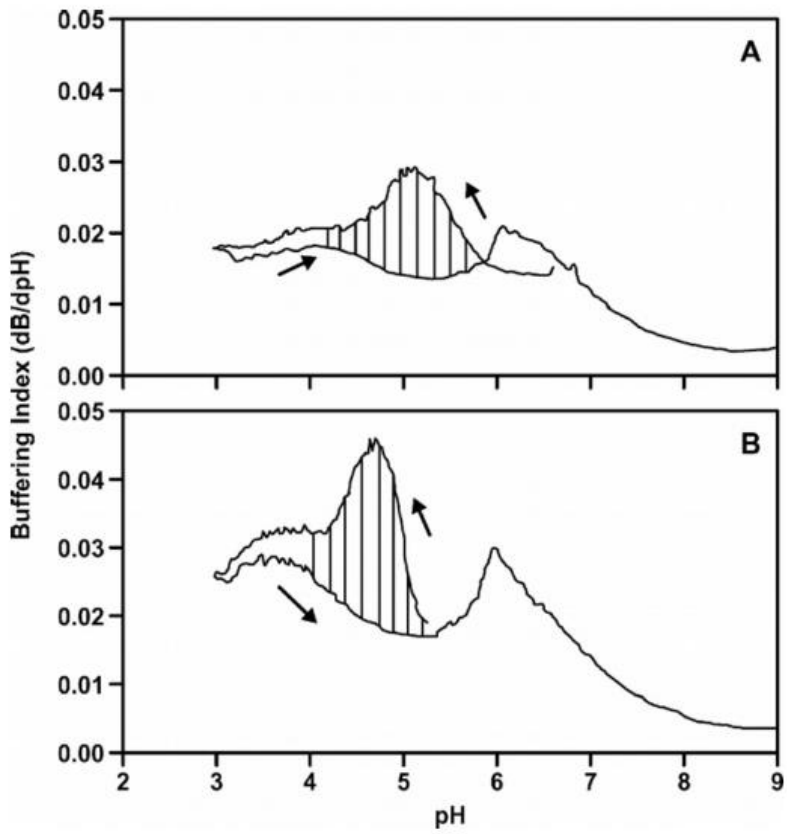


Figure 1.3 Buffering curves of milk (**a**) and Cheddar cheese (**b**) titrated from initial pH to pH 3.0 with 0.5 N HCl and then back-titrated to pH 9.0 with 0.5 N NaOH. Hatched area represents the buffering due to colloidal calcium phosphate. Arrows indicate the direction of the titration (Hassan *et al*., 2004).

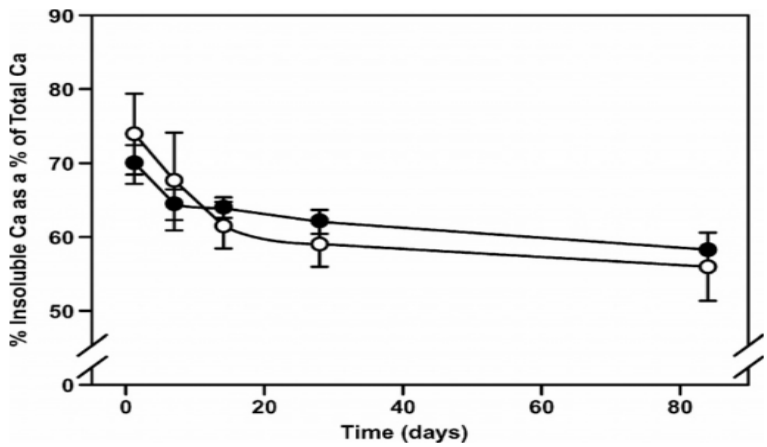


Figure 1.4 Changes in the % insoluble Ca content (expressed as a % of total cheese Ca) as a function of ripening time in Cheddar cheese determined by acid-base titration (○) and cheese juice (●) methods (Hassan *et al*., 2004).

1.5 Manipulation of calcium equilibrium in cheese

Alteration of cheese manufacturing variables such as changing pH values during cheesemaking, altering the lactose content of cheese milk before manufacture and different curd washing methods have all been found to alter the Ca equilibrium of cheese. Lee *et al*. (2005, 2010) reported that altering acid development during manufacture of Cheddar or Colby cheese, through modification of pH at renneting and drainage and varying lactose levels, resulted in cheeses with very low pH values (<4.9), which contained lower total Ca and INSOL Ca than cheeses with a higher pH. Lee *et al*. (2011) demonstrated that different curd washing methods significantly reduced the INSOL Ca content of Colby cheese particularly during the first month of ripening.

Addition of calcium chelating agents such as trisodium citrate (TSC) and ethelynediaminetetraacetic acid (EDTA) to milk and/or curd/whey mixture during cheese manufacture has been found to cause a reduction in the availability of INSOL Ca, resulting in cheese with softer textural, rheological and melting properties. Mizuno and Lucey (2005) and Brickley *et al*. (2009) supplemented non-fat *pasta filata* and Cheddar cheeses, respectively, with TSC at the dry-salting step of cheese manufacture, which resulted in a lower initial INSOL Ca level, as well as a decrease in INSOL Ca level during the first month of ripening. These authors attributed the decrease in INSOL Ca to the calcium-sequestering ability of TSC causing solubilisation of a proportion of CCP in the cheeses. Direct acidified cheeses with reduced INSOL Ca levels were produced by Choi *et al*. (2008) by lowering the pH of the cheese milk and by addition of EDTA to cheese milk.

Pastorino *et al*. (2003) decreased the pH of Cheddar cheese after manufacture by high pressure injecting cheese blocks one, three or five times with 20% (wt/wt) glucono-δ-lactone solution at 10 d of ripening, causing a decrease in INSOL Ca content. O'Mahony *et al*. (2006) immersed Cheddar cheese slices in synthetic Cheddar cheese aqueous phase (SCCAP) solutions of varying calcium concentrations. Authors observed an increase in the CCP concentration of cheese when the calcium concentration of the SCCAP solution exceeded that of the cheese serum. The opposite effect was observed with SCCAP solutions containing lower calcium concentrations than the cheese serum, where solubilisation of CCP in cheese occurred. These observations occurred due to calcium equilibration between the cheese and solutions in which it was immersed.

1.6 Influence of calcium on cheese texture, rheology and functionality

Ripening of cheese involves a series of biochemical, physicochemical and microbiological changes which collectively are responsible for the conversion of the rubbery, bland young product into a mature cheese with characteristic flavour texture and aroma (Fox *et al*., 1990; Fox and McSweeney, 1998; Lucey *et al*., 2003). The most critical factors governing the textural and rheological properties of cheese include the total Ca content, proteolysis and pH (Lucey and Fox, 1993; Watkinson *et al*., 2001; Guinee *et al*., 2002; Lucey *et al*., 2003; Sheehan and Guinee, 2004). Proteolysis was long considered to be the fundamental process responsible for the textural changes in cheese during ripening (Lucey and Fox, 1993); however, in recent years it has been demonstrated that calcium content plays a more critical role in modulating the textural, rheological and functional properties of cheese; in particular, the changes in INSOL Ca have been found to

be more closely related to the initial changes in texture during the first month of ripening (Lucey *et al*., 2003; Lucey *et al*., 2005; O'Mahony *et al*., 2005).

O'Mahony *et al*. (2005) observed significant softening of Cheddar cheese during early ripening (first 21 d) and correlated (r = 0.92; P<0.001) this with the decrease in INSOL calcium content in cheeses within the first month of ripening. In agreement with these results, Lucey *et al*. (2005) found that the increasing melt during early ripening of Cheddar cheese was more highly correlated with the reduced concentration of insoluble calcium than with extent of proteolysis.

Ong *et al*. (2013) and Brickley *et al*. (2009) both observed an increase in texture profile analysis (TPA) hardness values of Cheddar cheese supplemented with $CaCl_2$ added to the cheese milk and cheese during salting, respectively. These authors attributed this increase in hardness to increased CCP content in the calcium supplemented cheese. A decrease in TPA hardness values of Cheddar cheese supplemented with TSC at the salting stage of cheesemaking was demonstrated by Mizuno and Lucey (2005) and Brickley *et al*. (2009), due to reduction of CCP crosslinks *via* calcium sequestration by TSC forming soluble calcium citrate complexes.

A reduction in the INSOL Ca content of cheese has been found to increase LT values in cheese heated at pH values > 5.0 (Pastorino *et al*., 2003; Lee *et al*., 2005, 2010; Choi *et al*., 2008). O'Mahony *et al*. (2006) found a significant decrease in loss tangent (LT) and increase in G′ values at 70 ˚C for Cheddar cheese with increasing CCP concentration, while the opposite effect was observed with decreasing CCP concentration, indicating that CCP concentration had a

strong influence on the small deformation rheological properties of Cheddar cheese during ripening.

1.7 Conclusions

It has been shown that the INSOL Ca content of cheese is a critical factor affecting cheese texture and functionality, particularly during the early stages of ripening (first month). Several studies have identified that the shift in Ca equilibrium in dry-salted cheese, i.e., the shift from INSOL to SOL form of Ca, can be controlled or altered by the cheese manufacturing variables, such as acid development during crucial stages (pH at renneting and whey drainage) of cheese making, lactose content of cheese milk, curd washing and curd washing methods, as well as addition of $CaCl_2$ or Ca chelating agents such as TSC or EDTA to the cheese milk or to the cheese during manufacture. Currently, most research on Ca equilibrium has focused on Cheddar-type and Mozzarella-type cheeses; therefore it would be beneficial to extend these studies to a wider range of cheese varieties that are currently used for, or have potential as, food ingredients, in particular brine-salted cheese systems.

References

Brickley, C. A., Lucey, J. A. & McSweeney, P. L. H. (2009). Effect of the addition of trisodium citrate and calcium chloride during salting on the rheological and textural properties of Cheddar-style cheese during ripening. *International Journal of Dairy Technology*, *62*, 527-534.

Choi, J., Horne, D. S. & Lucey, J. A. (2007). Effect of insoluble calcium concentration on rennet coagulation properties of milk. *Journal of Dairy Science*, *90*, 2612–2623.

Choi, J., Horne, D. S., Johnson, M. E. & Lucey, J. A. (2008). Effects of the concentration of insoluble calcium phosphate associated with casein micelles on the functionality of directly acidified cheese. *Journal of Dairy Science*, *91*, 513-522.

Choi, J., Horne, D. S., Johnson, M. E. & Lucey, J. A. (2008). Effects of the concentration of insoluble calcium phosphate associated with casein micelles on the functionality of directly acidified cheese. *Journal of Dairy Science*, *91*, 513– 522.

Fox, P. F., Lucey, J. A. & Cogan, T. M. (1990). Glycolysis and related reactions during cheese manufacture and ripening. *Critical Reviews in Food Science and Nutrition*, *29*, 237-253.

Fox, P. F. & McSweeney, P. L. H. (1998). *Dairy Chemistry and Biochemistry*. Blackie Academic & Professional, London, UK.

Guinee, T. P., Feeney, E. P., Auty, M. A. E. & Fox, P. F. (2002). Effect of pH and calcium concentration on some textural and functional properties of Mozzarella cheese. *Journal of Dairy Science, 85,* 1655-1669.

Hassan, A., Johnson, M. E. & Lucey, J. A. (2004). Changes in the proportions of soluble and insoluble calcium during the ripening of Cheddar cheese. *Journal of Dairy Science*, 87, 854-862.

Holt, C. (1985). The milk salts: their secretion, concentrations and physical chemistry. In, *Developments in Dairy Chemistry: 3. Lactose and Minor Constituents* (Fox, P. F., Ed.) (pp. 143–181). Elsevier Applied Science Publishers, London, UK.

Horne, D. S. (1998). Casein interactions: Casting light on the black boxes, the structure in dairy products. *International Dairy Journal*, *8*, 171–177.

Lee, M. R., Johnson, M. E. & Lucey, J. A. (2005). Impact of modifications in acid development on the insoluble calcium content and rheological properties of Cheddar cheese. *Journal of Dairy Science, 88,* 3798-3809.

Lee, M. R., Johnson, M. E., Govindasamy-Lucey, S., Jaeggi, J. J. & Lucey, J. A. (2010). Insoluble calcium content and rheological properties of Colby cheese during ripening. *Journal of Dairy Science*, *93*, 1844–1853.

Lee, M. R., Johnson, M. E., Govindasamy-Lucey, S., Jaeggi, J. J. & Lucey, J. A. (2011). Effect of different curd-washing methods on the insoluble Ca content and rheological properties of Colby cheese during ripening. *Journal of Dairy Science*, *94*, 2692-2700.

Lucey, J. A. & Fox, P. F. (1993a). Importance of calcium and phosphate in cheese manufacture: A review. *Journal of Dairy Science, 76*, 1714–1724.

Lucey, J. A., Gorry, C. & Fox, P. F. (1993b). Changes in the acid-base buffering curves during the ripening of Emmental cheese. *Milchwissenschaft, 48*, 183– 186.

Lucey, J. A., Johnson, M. E. & Horne, D. S. (2003). Invited review: Perspectives on the basis of the rheology and texture properties of cheese. *Journal of Dairy Science, 86*, 2725–2743.

Lucey, J. A., Mishra, R., Hassan, A. & Johnson, M. E. (2005). Rheological and calcium equilibrium changes during the ripening of Cheddar cheese. *International Dairy Journal, 15*, 645–653.

Lucey, J. A. & Horne, D. S. (2009). Milk salts: technological significance. In, *Advanced Dairy Chemistry*: *Volume. 3. Lactose, Water, Salts and Minor Constituents* (3rd Edition) (pp. 351–389) (McSweeney, P. L. H., & Fox, P. F. Eds.). Springer, New York, New York, USA.

Mizuno, R. & Lucey, J. A. (2005). Effects of two types of emulsifying salts on the functionality of nonfat *pasta filata* cheese. *Journal of Dairy Science, 88*, 3411-3425.

Morris, H. A., Holt, C., Brooker, B. E., Banks, J. M. & Manson, W. (1988). Inorganic constituents of cheese: analysis of juice from a one-month-old Cheddar cheese and the use of light and electron microscopy to characterize the crystalline phases. *Journal of Dairy Research, 55*, 255–268.

O'Mahony, J. A., Lucey, J. A. & McSweeney, P. L. H. (2005). Chymosin-mediated proteolysis, calcium solubilization, and texture development during the ripening of Cheddar cheese. *Journal of Dairy Science*, *88*, 3101-3114.

O'Mahony, J. A., McSweeney, P. L. H. & Lucey, J. A. (2006). A model system for studying the effects of colloidal calcium phosphate concentration on the rheological properties of Cheddar cheese. *Journal of Dairy Science*, *89*, 892-904.

Ong, L., Dagastine, R. R., Kentish, S. E. & Gras, S. L. (2013). The effect of calcium chloride addition on the microstructure and composition of Cheddar cheese. *International Dairy Journal*, *33*, 135–141.

Pastorino, A. J., Hansen, C. L. & McMahon, D. J. (2003). Effect of pH on the chemical composition and structure-function relationships of Cheddar cheese. *Journal of Dairy Science*, *86*, 2751–2760.

Pyne, G. T. & McGann, T. C. A. (1960). The colloidal phosphate of milk: II. Influence of citrate. *Journal of Dairy Research*, *27*, 9-17.

Sheehan, J. J. & Guinee, T. P. (2004). Effect of pH and calcium level on the biochemical, textural and functional properties of reduced-fat Mozzarella cheese. *International Dairy Journal*, *14*, 161-172.

Watkinson, P., Coker, C., Crawford, R., Dodds, C., Johnston, K., McKenna, A. & White, N. (2001). Effect of cheese pH and ripening time on model cheese textural properties and proteolysis. *International Dairy Journal*, *11*, 455-464.

Chapter 2

Literature review: Encapsulation technologies and their application in the food industry

2.1 Introduction

Encapsulation involves the packing of molecules, either solid, liquid or gas (Poshadri and Aparna, 2010; Ho *et al*., 2014; Tackenberg and Kleinebudde, 2015), referred to as the core or active, within a secondary uniform material, referred to as the matrix or shell, in order to create small capsules (Madene *et al*., 2006; Augustin and Hemar, 2009; Nedovic *et al*., 2011). The core/active may be a crystalline material, a jagged adsorbent particle, an emulsion, a suspension of solids or a suspension of smaller microcapsules (Gibbs *et al*., 1999). The encapsulated core material is isolated and protected from the surrounding environment and is only released in response to a stimulus. Different triggers can be used to prompt the release of the encapsulated ingredient, such as, pH change, mechanical stress, temperature, enzymatic activity, time and osmotic force (Gibbs *et al*., 1999; Gouin, 2004; Augustin and Hemar, 2009; Lakkis, 2016); it is this unique characteristic that enables the controlled release and delivery of core material to a specific target site (Augustin and Hemar, 2009).

2.2 Functionality of encapsulates within a food system

There are several reasons why a food manufacturer would encapsulate an ingredient. The benefits associated with encapsulation of ingredients are not merely associated with the end use of the ingredient but can be seen throughout the storage, manufacture and finally ingestion of the active ingredient, as illustrated in Figure 2.1.

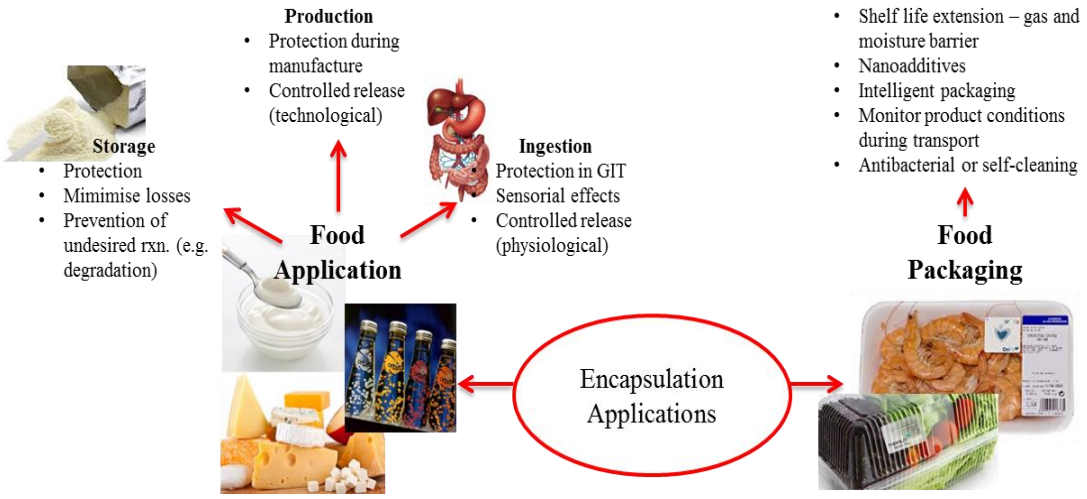


Figure 2.1 Advantages associated with the encapsulation of compounds throughput manufacture, storage and ingestion.

Selected examples of ingredients used in the food industry that benefit from encapsulation are highlighted in Table 2.1. Most recently, the use of encapsulates in food packaging has emerged as a new trend, capable of providing food products with increased bioactivity and health benefits (Sozer and Kokini, 2009; Duran and Marcato, 2013; Ajaykumar, 2014). Different clay–polymer nanomaterials have been developed which limit the permeation of gases and water vapour, therefore providing substantial improvements in barrier properties of food packaging materials (Bharadwaj *et al*., 2002; Silvestre *et al*., 2011). As well as this, active packaging containing metal nanoparticles such as silver, gold and zinc, have been developed which provide food products with increased protection from spoilage caused by microbial effects (Kumar and Münstedt, 2005; Silvestre *et al*., 2011).

Table 2.1 Selected examples of active ingredients used in the food industry that benefit from encapsulation. (Source: adapted from McClements, 2012).

Active	Example	Potential advantages of encapsulation	Reference
Flavours	Citrus oils	Allows incorporation in aqueous medium Facilitates storage and utilization Retards chemical degradation Controls flavour release profile	Madene *et al*., 2006; Brückner *et al*., 2007
Antimicrobials	Essential oils	Improves matrix compatibility Facilitates storage and utilization Retards chemical degradation Masks off-flavours Increases potency	Were *et al*., 2003;da Silva Malheiros *et al*., 2010;Malheiros *et al*., 2016
Antioxidants	Carotenoids	Allows incorporation in aqueous medium Facilitates storage and utilization Retards chemical degradation Increases efficacy	Ersus and Yurdagel, 2007;Nayak and Rastogi, 2010;Tonon *et al*., 2010
Bioactive peptides	Polyphenols	Retards degradation in stomach Reduces bitterness and astringency Controls release profile and bioactivity	Kerdudo *et al*., 2014; Zou *et al*., 2014
Minerals	Iron	Avoids undesirable oxidative reactions Prevents precipitation Enhances bioavailability Reduces off flavours and astringency	Albaldawi *et al*., 2005;Kosaraju *et al*., 2006
Vitamins	Vitamin D	Allows incorporation in aqueous medium Improves ease of utilization Prevents chemical degradation Increases bioavailability	Tesoriere *et al*., 1996; Banville *et al*., 2000; Kosaraju *et al*., 2006
Bioactive lipids	Omega-3 fatty acids	Allows incorporation in aqueous medium Improves ease of utilization Avoids chemical degradation (oxidation) Controls delivery in GI tract Increases bioavailability	Eratte *et al*., 2015; Stratulat *et al*., 2015
Probiotics	Lactic acid bacteria	Avoids degradation in stomach Improves cell viability in product	Annan *et al*., 2008; Valero-Cases and Frutos, 2015;Amakiri and Thantsha, 2016

2.2.1 Storage

One of the most important reasons for encapsulation of active ingredients is to provide improved stability during processing and storage. Prolonged storage of many ingredients, particurarly those with bioactive properties, such as vitamins, minerals, probiotics, omega-3-fatty acids, phytosterols and polyphenols, can result in the loss of nutritional value due to degradation caused by light, heat, oxygen or water (Desai and Jin Park, 2005; de Vos *et al*., 2010; Nedovic *et al*., 2011). Encapsulation of such ingredients can provide them with protection from the destabilisation processes mentioned. The stability of bioactive compounds is a critical parameter for their successful incorporation into various food systems. If bioactive compounds form degradation products such as off-flavours, off-colours, or carcinogenic compounds, the shelf life of the fortified product will be limited. Gandía-Herrero *et al*. (2010) encapsulated the bioactive pigment of *Opuntia* fruits within a maltodextrin matrix using a spray drying procedure. These researchers demonstrated that encapsulation strongly increased the stability of the pigment and that the pigment remained stable for months in the absence of light at different temperatures (-20, 4, and 20˚C). Likewise, Igual *et al*. (2014) and Parthasarathi *et al*. (2013) encapsulated *Solanum quitoense* L. pulp and *Garcinia cowa* Roxb. fruit extract, respectively; increased antioxidant activity of the extracts was reported and the authors attributed this to the increased protection of the bioactive material from degradation due to encapsulation.

Flavours can be among the most valuable ingredients in any food formulation. Even small quantities of an aroma substance can be expensive, and because they are

usually sensitive and volatile, preserving them is often a top concern of food manufacturers. Encapsulation can be employed to treat flavours so as to impart some degree of protection against evaporation, which leads to the loss of valuable aroma compounds during storage (Madene *et al*., 2006). Brückner *et al*. (2007) encapsulated the aromatic compound 3-methylbutyraldehyde in eight different materials using a combined emulsification and spray drying process and found that the resultant dry microcapsules were easy to handle and protected the aromatic compound from evaporation. A critical review on flavour encapsulation and controlled release is provided by Madene *et al*. (2006). In addition to protecting the important volatile flavour compounds in foods, it is also of critical importance to mask unpleasant taste and flavour aroma compounds such as polyphenols in food products, in order to increase consumer acceptability of a product; Fang and Bhandari (2010) provide a good review on how encapsulation can effectively alleviate this deficiency.

2.2.2 Production

Encapsulation can facilitate improved flow properties and easier handling of an ingredient by the conversion of a liquid active agent into a powder (Nedovic *et al*., 2011; Zuidam and Shimoni, 2010). Additional benefits include the reduction of dust generation and a neutralisation of odour, as well as an increase in dispersibility of the active ingredient (Dias *et al*., 2015).

Encapsulation reduces the possibility of evaporation, chemical reactions (flavour-flavour interactions, oxidation or light-induced reaction) and migration of

volatiles during processing (de Roos, 2003; Madene *et al*., 2006; Nedovic *et al*., 2011). Charve and Reineccius (2009) evaluated the potential of selected protein ingredients (sodium caseinate, soy and whey protein isolates (WPI)), gum acacia and modified starch as materials for flavour encapsulation by spray drying. Depending on the matrix and core material used, oxidation was effectively limited (>70% limonene retained using protein materials) and nonenzymatic browning did not occur when gum acacia and modified starch were used during processing.

Biologically active compounds are susceptible to degradation due to chemical reactions affected by heat, light and oxygen (Desai and Jin Park, 2005; de Vos *et al*., 2010). During processing of most food products at least one of these factors will become an issue. Therefore, encapsulation may provide protection to the bioactive ingredient from degradative reactions during manufacture. Ersus and Yurdagel (2007), Nayak and Rastogi (2010) and Tonon *et al*. (2010) obtained anthocyanin extracts from *Daucus carota* L. roots, *Garcinia indica* Choisy fruit pulp and *Euterpe oleracea* Mart. fruit pulp respectively, and encapsulated them within maltodextrins. The authors reported increased stability and antioxidant activity, indicating that encapsulation was efficient in protecting these compounds from degradation. Also, some microbes such as lactobacilli and bifidobacteria have been shown to benefit from a Ca-alginate immobilisation matrix during dehydration and lyophilisation (Kim *et al*., 1996).

Co-encapsulation has been proposed to encourage the synergistic relationship between probiotics and prebiotics in which the prebiotics are consumed by probiotics

as sources of carbon and energy, favoring their colonisation of the intestinal tract over colonisation by pathogenic microorganisms (Homayouni *et al*., 2008), a scenario sometimes referred to as 'synbiotics' (Crowley and O'Mahony, 2016). Okuro *et al*. (2013) produced and evaluated solid lipid microparticles in which *Lactobacillus acidophilus*, a probiotic, was co-encapsulated with a prebiotic, either inulin or polydextrose, using spray chilling technology. The authors demonstrated that all of the microcapsules investigated increased the survival rate of *Lactobacillus acidophilus* exposed to simulated gastric fluids and simulated intestinal fluids compared to that of free probiotic cells. Recently, *Bifidobacterium* species were effectively encapsulated within the prebiotics inulin, oligofructose, and oligofructose-enriched inulin; thermal results demonstrated a higher stability of microcapsules produced with prebiotics than those produced with reconstituted skim milk (RSM) only (Fritzen-Freire *et al*., 2012).

2.2.3 Ingestion

2.2.3.1 Targeted delivery

Once a food product has been consumed, biological components are subjected to rapid intestinal and first-pass metabolism (concentration of the active ingredient is greatly reduced before it reaches the systematic circulation), causing the transformation of its chemical structure and changes in their bioactivities. Therefore, it is advantageous to ensure stability in the gastrointestinal tract (GIT) and to allow a controlled release at the appropriate target site. Encapsulating bioactive ingredients

has been found to increase the uptake and bioavailability of compounds (Nedovic *et al*., 2011). Alginate-coated gelatin microspheres were produced by Annan *et al*. (2008) to encapsulate the probiotic *Bifidobacterium adolescentis* 15703T. The alginate coat prevented pepsin-induced degradation of the gelatin microspheres in simulated gastric juice (pH 2.0, 2 h), resulting in significantly ($P < 0.05$) higher numbers of surviving bifidobacteria due to the buffering effect of intact microspheres. Radiolabelled bioactive formulations containing fish oil, tributryin and resveratrol were produced by Augustin *et al*. (2011) as free components or encapsulated mixtures within an oil-in-water emulsion stabilized by a heated mixture of a milk protein, glucose and a modified resistant starch. These authors reported increased levels of radioactivity from the microencapsulated bioactives in the blood and liver, consistent with an increase in the bioavailability of the agents. Several techniques exist (Gibbs *et al*., 1999; Gouin, 2004; Augustin and Hemar, 2009) which allow for the timely release of active ingredients from the matrix core; however, none of them can be considered as a universally applicable procedure for bioactive food components. This is due to the fact that individual bioactive food components have their own characteristic molecular structure (Augustin and Hemar, 2009). Gunasekaran *et al*. (2007) demonstrated that whey proteins, as hydrogels, exhibit pH-sensitive swelling ability especially at pH above their isoelectric point, and that the release kinetic of the hydrogels paralleled that of their swelling ability. The authors also noted that the release properties can be conveniently altered by coating the beads with sodium alginate.

2.2.3.1 Sensory

The unpleasant bitter taste and astringency of most phenolic compounds limits their application in food. However, the utilization of encapsulated polyphenols, instead of free compounds, can effectively alleviate this problem (Nedovic *et al*., 2011). Liposomal formulations have been found to be useful for masking the unpleasant taste of bitter bioactives. Egg phosphatidyl choline was found to mask the bitterness of chloroquine phosphate at pH 7.2, and a lipoprotein composed of phosphatidic acid and β-lactoglobulin showed a good ability to mask the bitterness of quinine and propranolol (Katsuragi *et al*., 1995). The bitter taste of grapefruit or mandarin juices decreased substantially when 0.3% β-cyclodextrin was added prior to a heat treatment of canned juices. The bitter substances, naringin and limonin form stable inclusion complexes with β-cyclodextrin resulting in the reduction of free bitter substances in solution (Shaw and Wilson, 1983).

2.3 Encapsulation design considerations

Although the scientific rational behind the use of encapsulation technology in food products is strong, several considerations, such as cost, increased complexity and availability of materials must be critically evaluated by the food manufacturer before this technique should be employed in food applications and these are discussed in the following section.

Cost constraints - From a food manufacturer's perspective, it is essential to determine the cost in use and feasibility of scale-up in order to generate commercially viable encapsulation techniques with broad-spectrum product applications. Currently, the maximum acceptable cost of an encapsulation process is rather low, €0.1/kg of a new product (Gouin, 2004; Đorđević *et al*., 2015).

Increased complexity and technical limitations - Encapsulation of a food ingredient brings with it increased complexity of the production process and/or supply chain. Manufacturers must be aware of the necessary changes to processing and storage required, as well as the heightened cost associated with such alterations to the production process (Frost and Sullivan, 2005). For example, a product which normally does not need to be refrigerated may now require storage and transport at low temperatures in order to prevent heat-induced release of the active component. Therefore, it is essential that the benefit associated with the encapsulated active compound will outweigh the heightened complexity and/or cost associated.

Food-grade material - The food industry is severely limited with respect to the compounds that can be used for encapsulation. Many synthetic chemical polymer coatings used in the non-food sectors simply cannot be used as food ingredients. Materials used for encapsulation (active core component and encapsulate material) must be food-grade, biodegradable and granted GRAS (generally regarded as safe) status in order to be considered for use in encapsulation formulas intended for addition into food products (Pegg and Shahidid, 2007; Augustin and Sanguansri,

2012). A list of permitted carrier materials for delivery of food components is given in Table 2.2.

Stability - An important requirement is that the encapsulation system protects the active component from chemical degradation and evaporation in order to keep the active component fully functional throughout storage, processing and ingestion. Food technologists therefore must develop encapsulation systems that are capable of effective and efficient delivery of bioactive food components through the hazardous events that occur during passage through the GIT but also the deleterious circumstances during storage in the product that serves as vehicle for the bioactive components.

Loading capacity and retention - It is desirable that a delivery system is capable of encapsulating a large amount of encapsulated component per unit mass of carrier material, and should efficiently retain the encapsulated component until it needs to be delivered (McClements, 2012).

Triggers and release mechanisms - Any kind of trigger can be used to prompt the release of an encapsulated ingredient, such as pH change, mechanical stress, temperature, enzymatic activity and osmotic force (Gouin, 2004). Therefore, the delivery system must be designed so that it releases the active component at the correct target site at the specific time; during storage (e.g., release of an antimicrobial or antioxidant) or within the human body (e.g., release in the mouth, stomach, small intestine, large intestine or colon) (McClements, 2012).

Table 2.2 Selection of encapsulating materials for food applications. (Source: adapted from Augustin and Sanguansri, 2012).

Material	Examples
Proteins	Egg albumen, caseinates, gelatin, gluten, peptides, soy protein, pea proteins, whey proteins, zein, haemoglobin
Simple sugars	Fructose, galactose, glucose, maltose, sucrose
Carbohydrates/gums	Chitosan, corn syrup solids, cyclodextrin, dried glucose syrup, maltodextrins, starches and modified starch derivatives, agar, alginates, carrageenan, gum arabic, pectin, gellan gum, agar
Lipids	Edible fats and oils, fractionated fats, hardened fats, beeswax, paraffin
Emulsifiers	Mono- and di-glycerides, lecithin, liposomes, food-grade surfactants
Cellulose	Acetylcellulose, carboxymethyl cellulose, cellulose acetate butyrate, cellulose acetate phthalate, ethyl cellulose, methyl cellulose

Particle size and density - It is desirable that the addition of bioactive ingredients should not affect the sensory properties, colour or flavour of food products and the food industry usually aims to prevent this. As particle size affects texture, the addition of large particles is undesirable in most cases, explaining why micro-encapsulation becomes desirable in the food sector (Champagne and Fustier, 2007).

Compatibility within food matrix - Several encapsulation methods have been investigated but none of them can be considered as universally applicable for bioactive food components. This is due to the fact that individual bioactive food components have their own characteristic molecular structure (molecular weight, polarity and solubility) (Augustin and Hemar, 2009; de Vos *et al*., 2010). As a result,

it is essential that different encapsulation approaches are applied in order to meet the specific physicochemical and molecular requirements of a specific bioactive component (Kailasapathy, 2002; Augustin and Hemar, 2009; de Vos *et al*., 2010). However, compatibility with the active component is not the only requirement an encapsulation procedure has to meet. Many food components may interfere with the bioactivity of the added active food component and therefore, it is essential that the encapsulating material has specific characteristics to withstand influences from the outside environment (Augustin and Hemar, 2009; de Vos *et al*., 2010).

2.4 Structure of encapsulates

Depending on the physicochemical properties of the core, the wall composition, and the encapsulation technique employed, different capsule structures can be obtained (Figure 2.2): matrix type, containing several core particles embedded in a continuous matrix of wall material; core/shell (reservoir) type, consisting of a simple sphere surrounded by a coating of uniform thickness; multi-wall type, made up of multi-walled capsules; multi-core type, consisting of several distinct cores within the same capsule and phospholipid bilayer type, entrapping water-soluble, lipid-soluble and amphiphilic material, simultaneously (Gibbs *et al*., 1999; Desai and Jin Park, 2005; Madene *et al*., 2006; Augustin and Hemar, 2009; Fang and Bhandari, 2010; Zuidam and Shimoni, 2010; Đorđević *et al*., 2015).

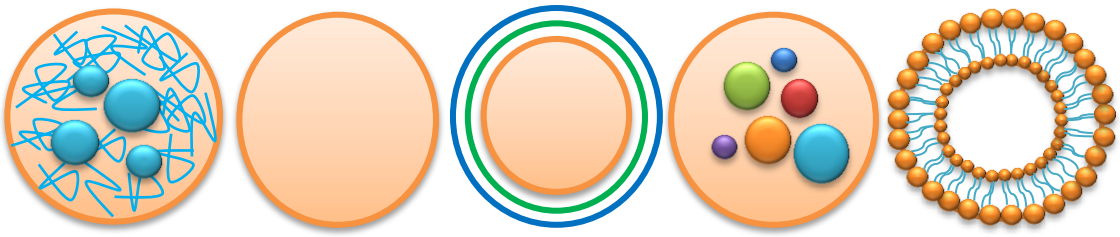


Figure 2.2 Schematic illustrations of different types of encapsulate structures. (a) matrix type; (b) core/shell (reservoir) type; (c) multi-wall type; (d) multi-core type and (e) phospholipid bilayer.

The size and shape of encapsulates depend on two factors; the materials (core and matrix) and methods (processing technique) used to prepare them. The different types of encapsulates are produced from a wide range of wall materials. The most commonly used matrix ingredients include biopolymers (carbohydrates and proteins), low molecular weight surfactants, co-polymers (two different types of monomers joined in the same polymer chain) and fats (McClements *et al*., 2007; Augustin and Sanguansri, 2008; Augustin and Hemar, 2009), while the food ingredients which have been encapsulated on or within the matrix material are described in Table 2.1.

A large number of very different processes exist to produce encapsulated ingredients. The techniques employed most commonly in the food industry include spray drying, spray cooling, spray chilling, freeze drying, fluid bed coating, extrusion, coacervation, liposome entrapment, molecular inclusion and co-

crystallization (Gibbs *et al*., 1999; Madene *et al*., 2006; Augustin and Hemar, 2009; Nedovic *et al*., 2011; Đorđević *et al*., 2015).

2.5 Encapsulation versus entrapment

In recent years encapsulation and entrapment/immobilization have been mentioned interchangeably. This, however, is not completely correct as they were originally introduced as two separate concepts for different types of technologies. Therefore, it is important to distinguish between encapsulation and entrapment of food ingredients. Encapsulation refers to a technology in which the encapsulated component (i.e., solid particles, droplets of liquids, or gas cells) is completely enveloped and covered by a matrix without any protrusion of the active components. In contrast, entrapment refers to the trapping of encapsulates within or throughout a matrix (e.g., gel, crystal) but not necessarily enveloped by the matrix. A small percentage of the entrapped ingredients will normally be exposed at the particle surface, whereas this would not be the case for an encapsulated product. Although this distinction is rarely being made in the food literature (Kailasapathy, 2002; McClements *et al*., 2009), it is necessary to make this separation in order to identify correctly the right encapsulation system for a specific product. For example, in the case of probiotics, immobilized cells hold the risk of leakage of the product which may result for example in undesired growth of probiotics in dairy products which consequently may interfere with the shelf life and the efficacy of the product. Also, immobilization will not always be sufficient to protect cells from hazardous substances in the direct microenvironment.

2.6 Material composition

Wall materials used in encapsulation as film-coating or matrix-forming components can be selected from a range of natural or synthetic polymers (Table 2.2), depending on the functional ingredient to be coated and the characteristics desired in the final encapsulated particle (Lakkis, 2016). The first essential requirement of a matrix material is that it is food grade and has GRAS status. The material must also be biodegradable and capable of forming a barrier between the internal core phase and its surroundings. A summary of the main encapsulating materials used in the food industry to entrap functional components intended for food applications is shown in Table 2.3, and the benefits and disadvantages of each material depending on the food application is highlighted.

Table 2.3 Encapsulating materials widely used in food applications. (Source adapted from Fang and Bhandari, 2012).

Category	Coating material	Method	Application	Properties	Advantages	Disadvantages
Carbohydrate	Starch, maltodextrin, chitosan, corn syrup solids, dextran, modified starch, cyclodextrins, modified cyclodextrins, sucrose, chitin, cellulose	Spray-drying, freeze-drying, extrusion, coacervation, edible films, inclusion complexation	Aroma compounds, fat replacers, emulsion stabilisers, lipids, pigments, nutrients	Low viscosity at high solids, good solubility, wide spectrum of MW, good emulsion stability	Low cost, diverse, bland flavour, produces strong films, controlled flavour release, prevents oxidation, light-induced reactions, evaporation loss	Poor emulsifier and low retention of volatile compounds, unsuitable for oil-based ingredients, not considered natural, off-flavour, poor protection to oxidisabe flavourings
Protein	Gluten, casein, whey proteins, polypeptone, soy protein, gelatin, albumin, peptides	Emulsion, spray-drying, solvent evaporation technique	Volatile compounds, oily substances, tallow and vegetable oils	Excellent solubility, viscosity, emulsification and film-forming properties	Good oxidation barrier, stabilise emulsions, better controlled release ability	Expensive, poor solubility in cold water and potential to react with carbonyls (polypeptone, soy protein and gelatin)
Lipid	Wax, paraffin, beeswax, diacylglyerols, acetoacrylglycerols, lecithins, liposomes	Emulsion, liposomes, film formation, spray-drying, extrusion	Antimicrobials, enzymes, water-soluble ingredients	Capsules formed at low temperatures	Good barrier properties, low water vapor permeability or diffusion	Use of organic solvents during manufacture, lack of stability in presence of oils or hydrophobic proteins
Gum	Gum acacia, agar, sodium alginate, agar, carrageenan	Spray-drying, syringe method (gel beads)	Volatile compounds, flavour oils, viscous high fat ingredients, powdered aroma concentrates	Tasteless, good solubility and emulsification properties, gelation low viscosity	Good retention of volatile compounds, increases elasticity, prevents oxidation	Expensive, availability fluctuates

2.7 Controlled release profiles

As previously mentioned, one of the major benefits of encapsulating an active food component is the ability to release the core material at a specific concentration-time profile at the site of action (McClements, 2012). The types of controlled-release profiles which exist include the following:

- *Burst release* - rapid release of most of the encapsulated component over a short time.
- *Sustained release* - prolonged release of the encapsulated component at a relatively constant rate.
- *Triggered release* - release of the encapsulated component in response to a trigger.
- *Targeted release* - release of the encapsulated component at a specific location in the GIT (e.g., mouth, stomach, small intestine, colon).

2.8 Release triggers

Controlled-release systems can be designed to respond to one or a combination (see below list) of stimuli that can trigger the release of the entrapped active component, therefore meeting the desired release target site and/or rate (Gibbs *et al*., 1999; Gouin, 2004; Augustin and Hemar, 2009; Lakkis, 2016).

- *Shear* - physical fracture, chewing and grinding represent physical means of release of core component during oral consumption.
- *pH* - ideal for the release of active ingredients from enteric-coated particles.
- *Moisture* - capable of releasing entrapped actives from hydrophilic matrices.
- *Temperature* - employed to release active compounds from fat/wax matrices, gelatin and other meltable polymers.
- *Enzyme action* - shown to release actives from enteric-coated particles caused by the disintegration of wall material due to amylase, protease and lipase activity.

2.9 Release mechanisms

Active core material may be released from the matrix surrounding it by several different physicochemical mechanisms (McClements, 2012), as outlined below.

- *Diffusion* - Active core component moves through and out of the matrix by diffusion. Release rate of active depends on the size, shape, structure and composition of the particle, the concentration gradient between the core and the surrounding medium and the translation diffusion coefficient through the various components in the matrix.
- *Fragmentation* - Active component is released due to physical disruption of the matrix material. Rate of release depends on the fracture properties of the particle. The active component may still diffuse out of the fragments, but

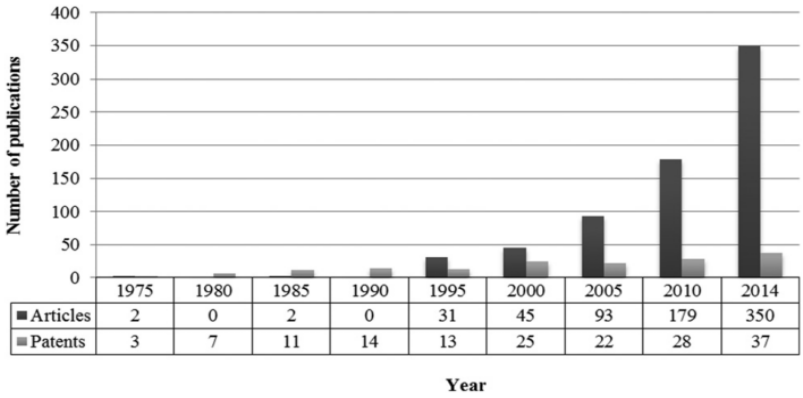
release will be quicker due to the increased surface area and decreased diffusion path.

- *Erosion* - Active ingredient is released from the matrix by erosion of the outer layer of the matrix. Release rate depends on the rate at which erosion occurs, which depends on the composition and structure of the matrix, as well as the magnitude and duration of the factor responsible for erosion (e.g., shear force, acid strength, enzyme type and concentration).
- *Swelling* - Active is released from the matrix material when it absorbs solvent and swells. Active release rate depends on the swelling rate, and the time taken for the compounds to diffuse through the swollen matrix.

2.10 Encapsulation technologies

Encapsulation was originally developed by the pharmaceutical industry, whose goal was to control and/or modify the release of drug substances. While the pharmaceutical sector still represents the major field using encapsulation (68% of the sector), the food industry has recently emerged as a sector finding great use for encapsulation technology, and currently accounts for 13% of the sector using encapsulation technologies (Martins, 2014; Dias *et al*., 2015). Dias *et al*. (2015) reported that the number of scientific reports and patents regarding encapsulation for food purposes (Figure 2.3a) is indicative of the growing interest in this technique regarding the incorporation of bioactive extracts and compounds.

A



	1975	1980	1985	1990	1995	2000	2005	2010	2014
Articles	2	0	2	0	31	45	93	179	350
Patents	3	7	11	14	13	25	22	28	37

B

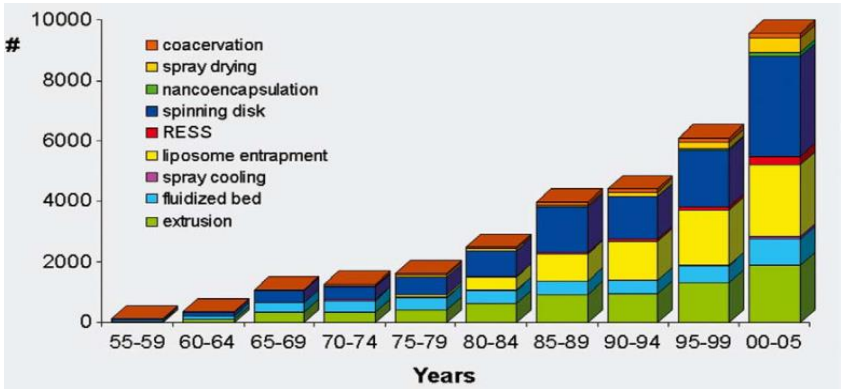


Figure 2.3 (**a**) Number of research articles and reviews, and patents published in the period from 1970 to 2014 regarding microencapsulation for food purposes (Source: Dias *et al*., 2015) and (**b**) trends in microencapsulation technologies in the period 1955 to 2005 (Source: Gouin, 2004).

Gouin (2004) also reported the growing interest of food technologists in encapsulation, demonstrated by the exponential increase in the number of publications (non-scientific and scientific articles and patents) over the years since 1955, as shown in Figure 2.3b. Liposome entrapment and spinning disk, as well as

coacervation to a lesser extent, have experienced the most rapid growth in interest from researchers and technologists.

In theory, encapsulation methods can be broken down into a three-step approach (Table 2.4); *incorporation* of active material within a microcapsule core and/or matrix, *dispersion* of the core as a droplet or by mixing of the core particles, and finally, the *stabilisation/solidification* of the droplet or application of a coating material around the particle.

Numerous techniques exist for the manufacture of encapsulated or entrapped ingredients for use in food applications. The most commonly employed methods will be discussed in detail throughout the next section. These include mechanical methods such as spray drying, spray cooling/chilling, fluid bed coating and extrusion, and chemical methods such as emulsification, coacervation and liposome entrapment techniques. This, however, is not an exhaustive list; other techniques such as, freeze drying, molecular inclusion complexation and co-crystalisation, are used to encapsulate food ingredients; however their application is not as great as the ones discussed in detail in this review.

Table 2.4 A simple three step approach to microencapsulation technologies.

1. Incorporation		*Liquid core*			*Solid core*	
2. Dispersion		*Droplet extrusion*	*Spraying*	*Emulsion*	*Air suspension*	*Liquid suspension*
3. Stabilisation	*Solidification*	Prilling	Spray cooling		Hot melt spray coating	
	Evaporation (drying)		Spray drying	Solvent evaporation	Spray coating	
	Gelation	Hydrogels	Spray chilling	Internal gelation		
	Polymerisation			Interfacial polymerization		
	Precipitation coacervation			Coacervation		Polymer coating
	Molecular interaction	Interfacial coacervation		Liposomes	Dry powder coating	Ionic coating

2.10.1 Spray drying

Spray drying accounts for the production of the majority of commercial entrapped materials in food products (~ 90%) (Nedovic *et al*., 2013; Đorđević *et al*., 2015**)** and is one of the oldest and best known encapsulation/entrapment techniques (Iwami *et al*., 1988; Moreau and Rosenberg, 1996; Gibbs *et al*., 1999; Pegg and Shahidi, 2007; Zuidam and Shimoni, 2010; Fang and Bhandari, 2012; Dias *et al*., 2015). Spray drying is an entrapment/immobilisation technology rather than an encapsulation technique and has application in the entrapment of flavours, volatiles, bioactive materials, vitamins, minerals, enzymes, unsaturated oils and probiotics (Madene *et al*., 2006; Augustin and Hemar 2009; de Vos *et al*., 2010; Fang and Bhandari, 2010). Spray drying is a flexible and continuous process, making it very cost effective and consequently, the most economical amongst several common

entrapment methods. It can be easily industrialized in terms of equipment and materials, which have a low cost compared with other available techniques (Dias *et al*., 2015).

The basic mechanism of entrapment by spray drying involves the *homogenisation* of core material for entrapment with the wall material, followed by *atomisation* of the mixture through a high pressure nozzle or centrifugal wheel (rotary atomiser), *dehydration* of the atomised material using heated air, separation by a cyclone or bag-filter from the drying air at the outlet at a lower temperature and finally collection in powder form (Gharsallaoui *et al*., 2007; de Vos *et al*., 2010; Fang and Bhandari, 2010; Zuidam and Shimoni, 2010; Fang and Bhandari, 2012). Spray dried entrapped particles generally form a matrix structure with a typical spherical shape, and the particle size may vary from very fine (10-50 μm) to large if the spray drying process is integrated with an agglomeration process (2-3 mm) (Gharsallaoui *et al*., 2007; Fang and Bhandari, 2012).

Homogenisation - The first step is the creation of a fine and stable emulsion consisting of the core material, which is usually hydrophobic, within the wall solution, with which it is immiscible. The resultant dispersion is then heated and homogenised, with or without the addition of an emulsifier depending on the emulsifying properties of the coating materials (i.e., the degree of interfacial activity of a material) (Gharsallaoui *et al*., 2007). Prior to dehydration, it is essential that the emulsion formed is stable for a defined period of time; the oil droplets should be small and viscosity must be low in order to prevent air inclusion in the particle

(Drusch *et al*., 2007). Particle size distribution and emulsion viscosity significantly affect entrapment efficiency by spray-drying. Rosenberg *et al*. (1990) demonstrated that high viscosity negatively interferes with atomisation, leading to the creation of elongated, large droplets that adversely affect the capsule drying rate.

Atomisation - The core-wall emulsion is fed into the spray dryer where it is atomised through either a pressure nozzle or rotary atomiser (Figure 2.4) and this can be situated either at the top or the bottom of the spray dryer chamber as illustrated in Figure 2.5c. Atomised droplet size depends on several factors including the surface tension and viscosity of the liquid, nozzle pressure and spray velocity (Gharsallaoui *et al*., 2007; Zuidam and Shimoni, 2010; Fang and Bhandari, 2012). Additionally, the droplet size determines the drying time and particle size (Zuidam and Shimoni, 2010; Fang and Bhandari, 2012).

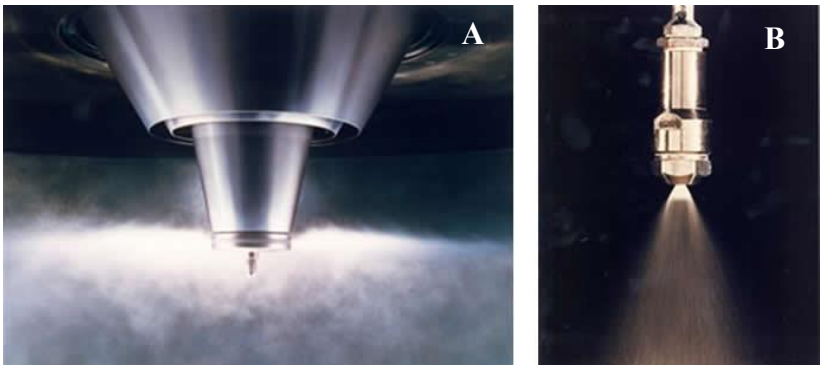


Figure 2.4 (**a**) Rotary nozzle and (**b**) direct pressure nozzle used in spray drying technology. (Images from: Anonymous, (2011), accessed 22.09.16).

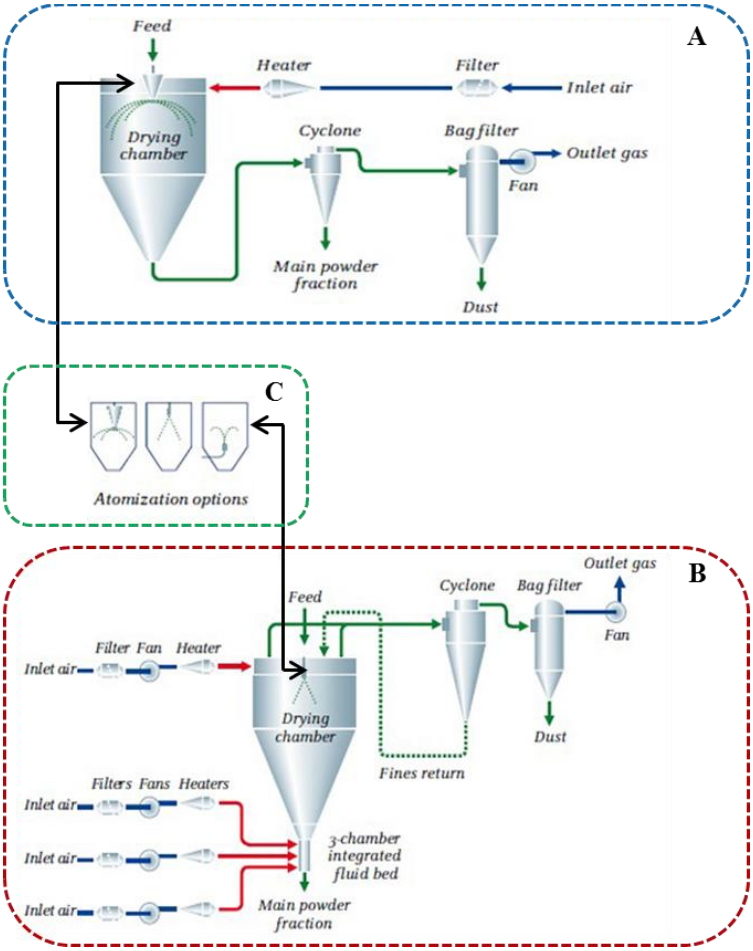


Figure 2.5 Schematic diagram of (**a**) an open mode design, (**b**) a multi stage design spray drying process and (**c**) the atomisation options available for each method. (Source adapted from Anonymous, (2012), accessed 22.09.16)

Dehydration - Depending on the placement of the feed inlet atomiser containing the core-wall emulsion to the hot air distributor, co-current or counter-current drying of particles can occur (Figure 2.6). In co-current process the liquid is sprayed in the same direction as the flow of hot air. The inlet temperature is typically 150-220˚C (Gharsallaoui *et al*., 2007; Zuidam and Shimoni, 2010 Fang and Bhandari, 2012; Đorđević *et al*., 2015), resulting in instantaneous evaporation; this allows for minimum exposure of dry powder to moderate temperatures (generally 50–80 ˚C), which limits the risk of thermal degradation (Gharsallaoui *et al*., 2007; Fang and Bhandari, 2012). Conversely, during counter-current drying, the liquid emulsion is sprayed in the opposite direction to the flow of hot air, causing the dry product to be exposed to high temperatures which limits its applications to less thermo-sensitive products. However, the main advantage of the counter-current process is that it is considered more economical in terms of energy consumption (Gharsallaoui *et al*., 2007). Typically, droplets are dehydrated into spheres with a diameter from 10 to 300 μm in the heated chamber of the spray dryer (Marison *et al*., 2004; Madene *et al*., 2006; Fang and Bhandari, 2010; Đorđević *et al*., 2015).

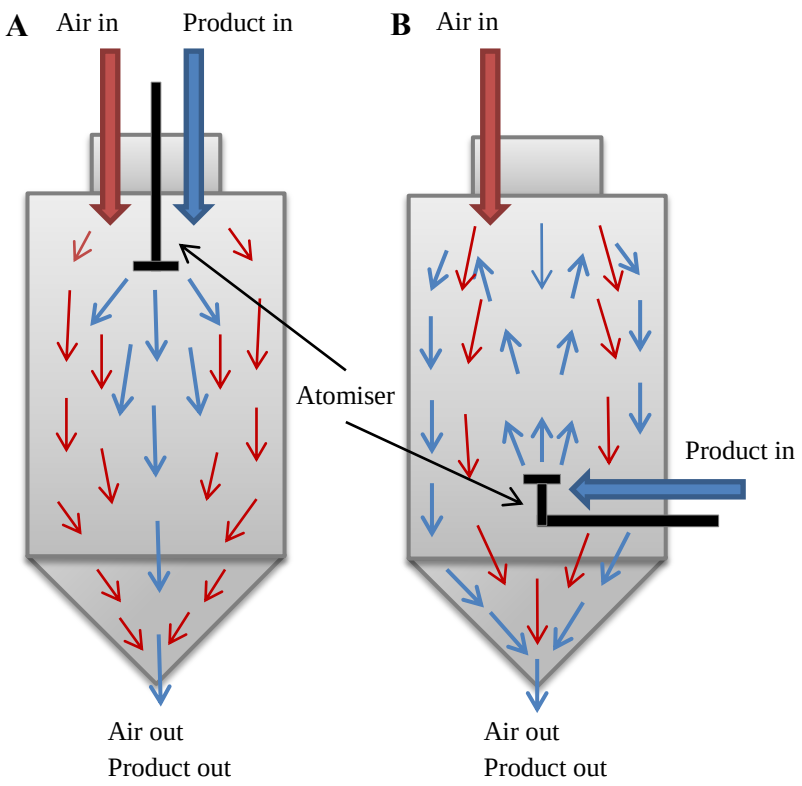


Figure 2.6 Schematic of (**a**) co-current and (**b**) counter-current air flow systems in spray drying.

Factors that affect the entrapment efficiency (%EE) of spray dried particles within the drying chamber include the feed concentration, temperature and flow rate, as well as the air inlet/outlet temperatures. Feed concentration and temperature are capable of altering the viscosity of the feed solution, its fluidity and its capacity to be sprayed homogeneously (Fang and Bhandari, 2012). Increasing the feed temperature results in a concomitant decrease in droplet size; however, high temperatures (> 220 ˚C) can also cause volatilisation or degradation of some heat-sensitive ingredients (Gharsallaoui *et al*., 2007; Fang and Bhandari, 2012). Atomizer feed rate is adjusted

to ensure that each sprayed droplet reaches the desired drying level before it comes in contact with the drying air (Fang and Bhandari, 2012). The difference between inlet and outlet air temperatures is directly proportional to the microcapsule drying rate and the final water content (Zbicinski *et al*., 2002), which should be set to a certain level that can safely be used without damaging the product or creating operating hazards (Fogler and Kleinschmidt, 1938). The low air inlet temperature will result in a low evaporation rate, which causes the formation of microcapsules with high density membranes, high water contents, poor fluidity, and ease of agglomeration. However, too high an air inlet temperature will cause excessive evaporation, and membrane cracks may occur and lead to subsequent premature release and degradation or loss of encapsulated cores. Therefore, it is paramount that the outlet air temperature is controlled in order to control the properties of powdered encapsulates (Gharsallaoui *et al*., 2007; Fang and Bhandari, 2012).

2.10.1.1 Advantages, disadvantages and food applications of spray drying

The advantages and disadvantages associated with the use of spray drying for the entrapment of active ingredients within matrices is summarised in Table 2.5. Spray drying can be employed to entrap water-soluble, oil-soluble, and even solid core materials, which means that a wide range of core materials are compatible with this technique (Fang and Bhandari, 2012). Spray drying also converts liquid into powder (Gouin, 2004 Nedovic *et al*., 2011; Zuidam and Shimoni, 2010) facilitating improved flow properties and easier handling of the entrapped active ingredient.

Table 2.5 Advantages and disadvantages of using spray drying for microencapsulation.

Advantages	**Disadvantages**
Low operating cost	Not suitable for heat sensitive materials
Rapid, large scale, continuous process	Produces non-uniform particles
High stability of finished product	Risk of oxidation and off-flavours
Good volatile retention	Water-based dispersion only
Good oxygen barrier properties	Bioactive exposure
High solids at low viscosity	Limitation in choice of wall material (reduced viscosity at relatively high concentrations)
Small droplet size	Further processing generally required (e.g. agglomeration)
Rapid capsule solubility	

Matsuno and Adachi (1993) stated that encapsulation of lipids can retard their auto-oxidation, enhance stability, control release of lipid-soluble flavour compounds, mask bitter taste of lipid-soluble substances, and protect dissolved substances against enzyme hydrolysis. Jimenez *et al*. (2004) reported increased oxidation stability of conjugated linoleic acid when encapsulated with a whey protein concentrate wall using spray drying. Shaw *et al*. (2007) also showed an increase in the oxidation stability of omega-3 fatty acids encapsulated in lecithin-chitosan multilayer wall by spray drying.

A major limitation of spray drying technology for entrapment is the limited number of shell materials available (Gouin, 2004; Fang and Bhandari, 2010). Due to the fact that almost all spray drying processes in the food industry are carried out from aqueous feed formulations, the wall material must have good solubility in water

(Gouin, 2004; Fang and Bhandari, 2012). Therefore, its application is limited to entrapment of water-based dispersions (de Vos *et al*., 2010).

Spray drying is an entrapment/immobilisation technology rather than an encapsulation technique which implies that some bioactive components may be exposed, and therefore at high risk of degradation (Gouin, 2004; de Vos *et al*., 2010; Dias *et al*., 2015). This becomes especially problematic when considering this method for entrapment and protection of probiotics, where the bacteria may leak into the continious phase of the product when some hydration occurs. Another limitation is the high temperature that is required during the entrapment process that is not compatible with the survival of all types of probiotic bacteria. O'Riordan *et al*. (2001) demonstrated that bifidobacteria exhibited high sensitivity to elevated inlet temperatures during drying. Another major disadvantage of spray drying is the production of particles of non-uniform size and shape, as well as particles with a tendency to aggregate (Đorđević *et al*., 2015). This can result in particles with projections or concavities on the outer surface caused by rapid evaporation of drops of liquid during the drying process in the atomizer (Rocha *et al*., 2012; Yang *et al*., 2012; Đorđević *et al*., 2015). Particles containing pores on the surface can also be formed as a consequence of pressure within the capsules (Favaro-Trindade *et al*., 2010; Yang *et al*., 2012), which may allow the active core material to be released in an untimely manner. As well as this, the inclusion of air within capsules can cause off-flavour development. Spray drying technology typically produces fine microcapsule powders with small particle size which may need further processing

such as agglomeration to improve their handling properties (Fang and Bhandari, 2012).

2.10.2 Spray cooling/chilling

Spray cooling/chilling is the least expensive entrapment technology and is routinely used for the entrapment of a number of organic and inorganic salts as well as various water soluble ingredients such as enzymes, flavours, protein hydrolysates and other functional ingredients such as probiotics to improve heat stability, delay release in wet environments, and/or convert liquid hydrophilic ingredients into free flowing powders (Gouin, 2004; Madene *et al*., 2006; Champagne and Fustier, 2007; Pegg and Shahidi, 2007; Augustin and Hemar, 2009; de Vos *et al*., 2010; Đorđević *et al*., 2015). A variety of atomization methods are available, including pressure nozzles, vibrating nozzles, and spinning disc atomizers. Microspheres are the most common entrapment morphology prepared with this technique, with an active ingredient dispersed homogeneously throughout the entrapment matrix (Oxley, 2012). Common matrix materials for spray cooling/chilling include fats, waxes, lipids, and gelling hydrocolloids.

Spray cooling/chilling processes are similar to that of spray drying in that both involve dispersing the core material into a liquefied shell material and spraying through heated nozzles to a controlled temperature (Pegg and Shahidi, 2007; Oxley, 2012). The major factor that distinguishes spray cooling/chilling from spray drying is that there is no evaporation of water (spray drying); instead the carrier/active mixture droplets are cooled or gelled into particles (Madene *et al*., 2006; Zuidam and

Shimoni, 2010; Oxley, 2012). Further differentiating factors are summarized in Table 2.6. The products of spray chilling/cooling resemble fine spherical beads of large particle size, are water-soluble but capable of releasing their contents at or near the melting point (MP) of the wall material (Pegg and Shahidi, 2007). Spray chilling differs from spray cooling only in the melting point (MP) of the carrier material employed (Đorđević *et al*., 2015), MP of 32–42 ˚C (hydrogenated vegetable oils) and MP of 45–122 ˚C (vegetable oils), respectively (de Vos *et al*., 2010; Zuidam and Shimoni, 2010; Đorđević *et al*., 2015).

Particles produced by spray cooling/chilling are generally matrix type (Figure 2.2) where the active ingredient is dissolved or dispersed throughout the volume of the particle and not just in the centre (Augustin and Hemar, 2009; de Vos *et al*., 2010; Oxley, 2012; Okuro *et al*., 2013b; Đorđević *et al*., 2015). Therefore, this technology is considered an immobilisation technique rather than a true encapsulation method (de Vos *et al*., 2010). Similar to spray drying, the particle size is dependent on both formulation and operating parameters. Formulation parameters include the size and concentration of the active ingredient as well as the viscosity of the feed (Okuro *et al*., 2013b). The operating parameters consist of the configuration of the atomisation device and working conditions (i.e., disc configuration, disc rotational speed, air nozzle pressure, and temperature) (Zuidam and Shimoni, 2010; Okuro *et al*., 2013b). An additional coating may be applied to microparticles obtained by spray cooling/chilling, using spray coating technology, to ensure a complete coverage of the particle and to eliminate undesirable interactions during embedding and storage (Lakkis, 2007).

Table 2.6 Differences between spray chilling and spray drying techniques. (Source: Okuro *et al*., 2013b).

	Spray drying	Spray chilling
Energy flow	energy applied in the droplet forcing evaporation of the medium	energy removed from the droplet forcing the solidification of the medium
Equipment	feeding tubes without heating	heated feeding tubes (to avoid solidification)
Flow in the chamber	hot air	cold air or liquid N_2
Particle size	5–150 μm	20–200 μm
Dissolution mechanism	dissolution	diffusion, heating, lipases and bile salts (GI tract)
Particle morphology	geometric particle with porous and irregular surface due to solvent evaporation	dense, spherical and smooth surface (no effects of solvent evaporation)
Carrier	water-soluble polymers	waxes, fatty acids, water-soluble polymers and water-insoluble monomers
Food ingredients	vitamins, flavours, starter cultures, carotenoids, oils, fats, enzymes and acidulants	iron sulphate, vitamins, minerals, acidulants, enzymes and probiotics
Process steps	dispersing or dissolving the active compound in aqueous solution coating, atomisation, dehydration	dispersing or dissolving the active compound in the molten lipid mixture, atomisation, cooling
Load/%	5–50	10–20

2.10.2.1 Advantages, disadvantages and food applications of spray chilling/cooling

Spray chilling and spray cooling technologies have been described as environmentally friendly methods that are rapid, safe, reproducible, easy to scale-up and are continuous (Gouin, 2004; Madene *et al*., 2006; Champagne and Fustier, 2007; Pegg and Shahidi, 2007; Augustin and Hemar, 2009; de Vos *et al*., 2010; Đorđević *et al*., 2015). Encapsulates obtained by spray cooling/chilling have the ability to provide delayed release of actives at temperatures around the melting point of the coating material (Gouin, 2004; Barbosa-Canovas *et al*., 2005) and by enzymatic degradation (de Lara Pedroso *et al*., 2012; Okuro *et al*., 2013b). The release of active ingredients from particles can occur via erosion and leaching of the matrix.

Spray chilled/cooled products have applications in bakery products, dry soup mixes, and foods containing high levels of fat (Barbosa-Canovas *et al*., 2005). However, solid lipid particles prepared by spray chilling/cooling are insoluble in water due to the lipid carrier, which can limit their applications in food (Đorđević *et al*., 2015). Only limited delayed release of a water-soluble active core in high moisture foods can be achieved as some of the core molecules are at the surface of the particle (Gouin, 2004; Augustin and Hemar, 2009), and therefore, this kind of entrapment is not recommended for application in foods with high water contents. As well as this, spray chilling/cooling technologies provide little or no barrier against flavour loss by diffusion if the flavour is soluble in fat. A possible solution to this issue is to use as the carrier a fat or wax that is immiscible with the flavour compounds, but it is difficult to find such materials that are not expensive but are food grade (Đorđević *et al*., 2015).

A major application where spray cooling/chilling has great advantages over other methods of encapsulation/immobilisation is in the entrapment of probiotics. Firstly, this technique does not require the use of organic solvents or high temperatures which are detrimental to the survival of probiotic strains. Additionally, the probiotics can be released directly in a controlled manner in the intestine, as a result of lipid breakdown caused by the action of lipases present in the intestinal lumen. Okuro *et al*. (2013a) produced solid lipid micro-particles in which *Lactobacillus acidophilus* was co-encapsulated with a prebiotic, either inulin or polydextrose, using spray chilling technology. The authors reported an increased survival rate of *Lactobacillus acidophilus* exposed in all microcapsules manufactured

compared to that of free probiotic cells. Additionally, de Lara Pedroso *et al*. (2012) entrapped *Bifidobacterium lactis* and *Lactobacillus acidophilus* by spray chilling using interesterified palm fat and palm kernel oil, and found that the solid lipid micro-particles provided protection for both probiotics against the simulated gastric and intestinal fluids.

2.10.3 Fluidised Bed Coating

Fluidised bed coating is a powerful tool for the production of microparticles aimed to enhance the functionality and bioavailability of food products (Meiners, 2012; Đorđević *et al*., 2015). Fluidised bed coating has been used in encapsulation/immobilisation of vitamins, acidulants, flavours, minerals and pigments intended for food applications (Madene *et al*., 2006; Zuidam and Shimoni, 2010; Meiners, 2012; Đorđević *et al*., 2015). This technique is based on the additional coating of powder particles containing the active ingredient, either by batch process or a continuous setup (Zuidam and Shimoni, 2010; Đorđević *et al*., 2015). This technique is considered a relatively low-cost technology for entrapment in comparison to other drying methods (Chua and Chou, 2003; Meiners, 2012).

Fluidised bed coating can be described as a three step process; (1) the particles to be coated are suspended by an air stream, at a predefined temperature within the coating chamber, (2) the coating material is sprayed through the atomising nozzle onto the particles, (3) followed by a succession of wetting and drying stages

(Augustin and Hemar, 2009; Zuidam and Shimoni, 2010; Đorđević *et al*., 2015). The particles intended for coating should be dense, spherical with a narrow particle size distribution and good flow properties (Pegg and Shahidi, 2007; Zuidam and Shimoni, 2010; Đorđević *et al*., 2015). Depending upon the specific application, the airflow may be heated or cooled within the coating chamber (Pegg and Shahidi, 2007). The shell/wall material may be a concentrated solution or dispersion, a hot melt or an emulsion (Augustin and Hemar, 2009). In the case of hot melts, the coating material is hardened by solidification in cool air, while solvent-based coating material is hardened by evaporation of the solvent in hot air (Pegg and Shahidi, 2007).

2.10.3.1 Fluidised bed configurations

Like spray drying, several different fluidised bed configurations exist; these include top spray, bottom spray (Würster system) and tangential spray fluidised bed coating system (Figure 2.7; Meiners, 2012). The major differentiating feature of each system is, as the name suggests, the placement of the atomising nozzle delivering the coating material.

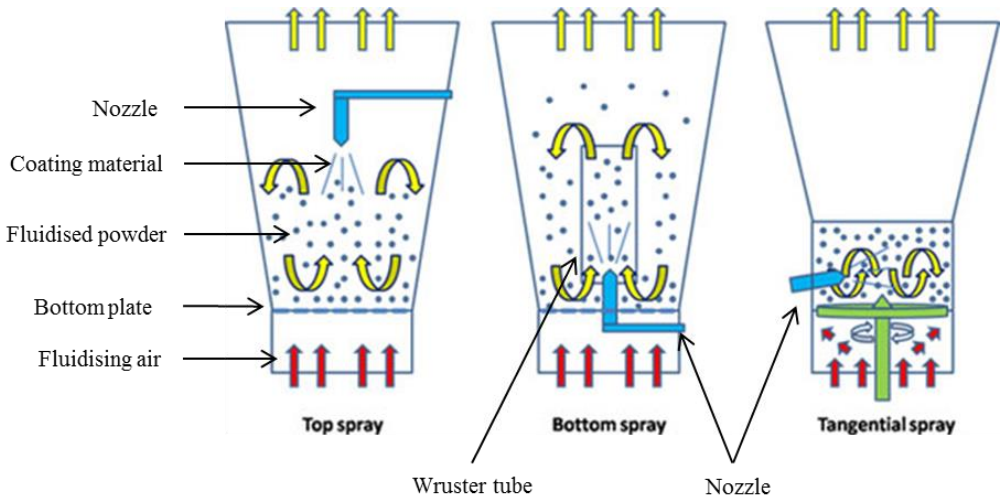


Figure 2.7 Schematic diagram of a top, bottom and tangential spray fluidised bed. (Source adapted from Anonymous, (2016), accessed on 23.09.16).

2.10.3.2 Advantages, disadvantages and food applications of fluidised bed coating

Fluidised bed coating has been used for the entrapment of vitamins (B and C), ferrous salts and numerous minerals. Encapsulates of sorbic and lactic acid, potassium sorbate, and calcium propionate produced by fluid bed coating have wide applications in bakery products (Dewettinck and Huyghebaert, 1999; Đorđević *et al*., 2015). Also, some acids, pigments, and flavours have been manufactured using fluidised bed coating for applications in the meat industry (Dezarn, 1995; Dewettinck and Huyghebaert, 1999; Đorđević *et al*., 2015). Additionally, Schell and Beermann (2014) used fluidized bed coating to entrap *Lactobacillus reuteri* within sweet whey and shellac. The authors reported an increase in acid resistance of *Lactobacillus reuteri* which enabled improved survival upon transit through the GIT.

Fluidised bed coating is an advantageous technique as it enables the utilisation of a wider range of wall coating materials (proteins, fats, carbohydrates, gums and emulsifiers) compared to the traditional spray drying method (Meiners, 2012). Coating materials utilised in food applications range from aqueous solutions of hydrocolloids such as gums and proteins to melted fats and waxes (Gouin, 2004; Augustin and Hemar, 2009; de Vos *et al*., 2010; Đorđević *et al*., 2015). As a result of the vast array of wall materials that can be applied to particles, it is therefore possible to develop particles with very different controlled release properties (Augustin and Hemar, 2009; de Vos *et al*., 2010). Also, fluidised bed coating can be applied to provide spray dried powders or a sensitive core product (oils or flavours) with a second coating, increasing protection of the active ingredient from degradation (Augustin and Hemar, 2009; de Vos *et al*., 2010).

However, a major disadvantage associated with the fluidised bed technique is the uncontrolled agglomeration of particles due to coalescence of the wet-coated material through the formation and solidification of liquid bridges between particles (Figure 2.8; Saleh *et al*., 2003). Agglomeration occurs when the temperature of the particle surface is above the glass transition temperature of the coating substance (Đorđević *et al*., 2015). Previous studies have highlighted the main variables that should be controlled in order to diminish agglomeration, these include the inlet air temperature, atomization pressure and the coating solution concentration (Jiménez *et al*., 2006; Hede *et al*., 2007; Prata *et al*., 2012; Đorđević *et al*., 2015).

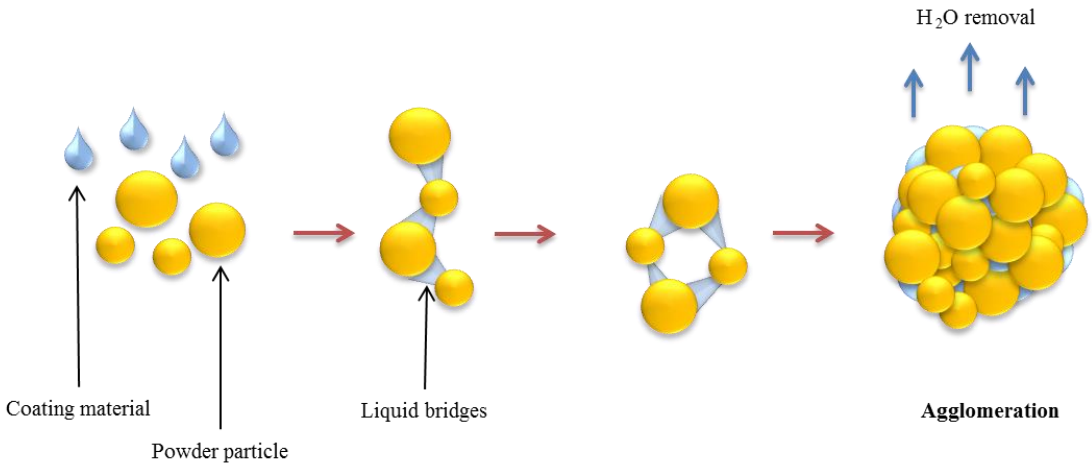


Figure 2.8 Schematic representation of the mechanism of agglomeration in microparticles manufacture using fluidised bed technology.

2.10.4 Extrusion techniques

Extrusion technologies have been applied for the encapsulation of proteins, enzymes, bacteria, yeast cells, volatiles and flavours (Gouin, 2004; de Vos *et al*., 2010; Dias *et al*., 2015; Đorđević *et al*., 2015). This technology has been described as less hazardous than techniques which require heat or solvents, but also a more laborious process of encapsulation (de Vos *et al*., 2010). Extrusion methods typically consist of dropping droplets of an aqueous solution consisting of a polymer (most often 0.6-4.0 wt. % sodium alginate) and an active component into a gelling bath (usually 0.05-1.50 M calcium chloride solution) in order to form encapsulated microspheres (Zuidam and Shimoni, 2010; Nedovic *et al*., 2011).

The dripping tool employed to create the beads can be simply a pipette, syringe, vibrating nozzle, spraying nozzle, jet cutter, atomizing disk, coaxial air-flow, or electric field (Figure 2.9). Particles with a diameter between 0.2 and 5 mm can be formed depending on the dripping tool and the viscoelasticity of the alginate solution (Zuidam and Shimoni, 2010). The smaller the inner diameter of the nozzle or openings, the smaller the resultant capsules (de Vos *et al*., 2010). The extrusion technique has been used in conjunction with a concentric nozzle (co-extrusion), to form core-shell type microspheres consisting of a hydrophobic core and a hydrophilic or hydrophobic shell (Zuidam and Shimoni, 2010).

Prüsse *et al*. (2008) evaluated different extrusion methods based on their ability to process fluids of varying viscosities. The authors reported suitability of all technologies to produce microspheres (800 μm in diameter) from low-viscosity alginate solutions (up to 2% w/w), while high viscousity alginate solutions (>3% w/w sodium alginate) could not be manufactured using the vibration technique. Microspheres were produced with a narrow size distribution range using electrostatic, jet-cutter, and coaxial air-flow technologies. The microsphere production rates of the coaxial air-flow and the electrostatic technology are very low, therefore, are limited to small/lab-scale applications. Vibration technology exhibited a 50% higher production rate than the aforementioned technologies, thus, are suitable for lab-scale as well as larger scale applications, assuming that multi-nozzle devices (Kailasapathy, 2002) are used for larger scales. The jet-cutter was found to be the best technology for large-scale/industrial applications although it is not limited to such scales and can also be used for small/lab-scale applications.

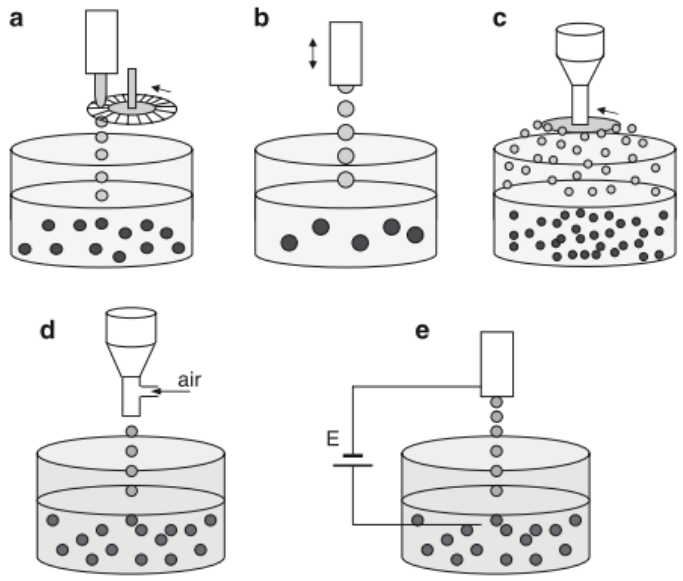


Figure 2.9 Set-ups of three different ways of making microspheres. Aqueous solution of, e.g., sodium alginate and active are atomized by jet-cutter (**a**), pipette or vibrating nozzle (**b**), atomizing disk (**c**), coaxial air-flow (**d**), or electrostatic potential (**e**). The droplets fall into a batch of 0.05-1.5 M calcium chloride, resulting in instantaneous formation of calcium alginate microspheres (Source: Zuidam and Shimoni, 2010).

2.10.4.1 Advantages, disadvantages and food applications of extrusion techniques

Extrusion technology provides true encapsulation in that the core material is completely surrounded by the wall material (de Vos *et al*., 2010) and have been used in the encapsulation of proteins, enzymes, bacteria, yeast cells, volatiles and flavours for food applications (Gouin, 2004; de Vos *et al*., 2010; Dias *et al*., 2015; Đorđević *et al*., 2015).

The major advantage of this process is the very long shelf-life imparted to normally oxidation-prone volatiles and flavour compounds, due to the extremely slow diffusion through the hydrophilic glassy carbohydrate matrix, thus providing an almost impermeable barrier against oxygen (Gouin, 2004). Extrusion technologies possess many advantages for encapsulation of microbes, namely, it is a relatively gentle technology which does not involve the use of severe temperature or harmful solvents and it can be carried out under both aerobic and anaerobic conditions (de Vos *et al*., 2010; Đorđević *et al*., 2015). Encapsulation of active ingredients by extrusion can allow for controlled release of core material. Deladino *et al*. (2008) encapsulated Yerba mate lyophilized extracts within calcium alginate and calcium alginate–chitosan systems and found that the release rate of antioxidant could be controlled depending on the presence or absence of the chitosan coating layer.

A drawback associated with this process is that large particles are formed (typically 500-1000 μm), which may limit the use of extruded core materials in applications where mouthfeel is critical (Gouin, 2004). Conversely, the formation of large particles may be beneficial in some food applications where visible flavour pieces are desirable (Pegg and Shahidi, 2007). To date, the shell materials available for encapsulation of core materials by extrusion are limited; with most applications using carbohydrates of various dextrose equivalents, starch and a mixture of additives (Gouin, 2004). Diffusion of flavours from extruded carbohydrates has been found to occur in encapsulates containing structural defects such as pores, cracks or thin walls formed during or after processing (Wampler, 1992; Madene *et al*., 2006). Additionally, this technique of encapsulation is difficult to scale-up, with only the jet-

cutter showing the ability to be easily scalable, resulting in a low production rate (Đorđević *et al*., 2015).

2.10.5 Emulsification techniques

Emulsions are, by definition, colloidal systems, consisting of oil, water and surfactant (lecithin, gelatin, sodium caseinate, sorbitans etc.) phases (Đorđević *et al*., 2015). Various emulsion-based systems have been used for the entrapment and delivery of lipophilic food ingredients such as, omega-3 fatty acids, carotenes and tocopherols, with the water phase of the emulsion capable of delivering water-soluble food components (Augustin and Hemar, 2009). Emulsion-based systems can either be oil-in-water emulsions (O/W) or water-in-oil emulsions (W/O), as illustrated in Figure 2.10. Control over the properties of emulsion-based encapsulation systems has been achieved by tailoring the characteristics of the dispersed phase (size, charge, interfacial properties and composition of droplets) (Figure 2.11), as well as the microstructure of the emulsions (McClements *et al*., 2007).

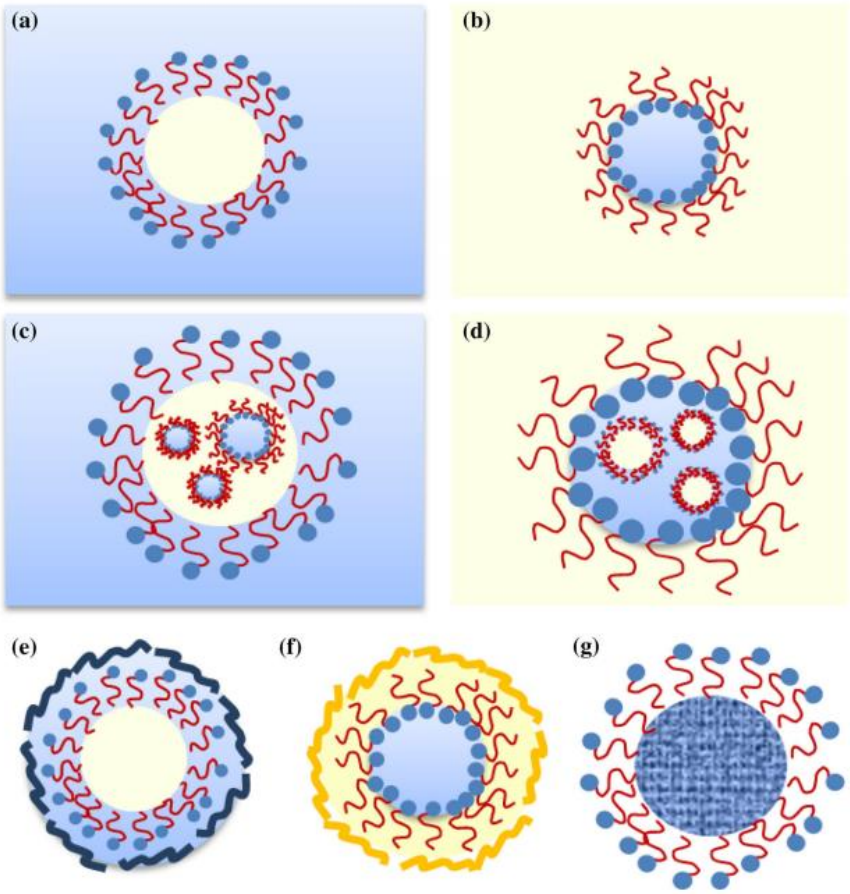


Figure 2.10 Schematic illustrations of different emulsion-based delivery systems. (**a**) oil-in-water emulsion; (**b**) water-in-oil emulsion; (**c**) water-in-oil-in-water double emulsion; (**d**) oil-in-water-in-oil double emulsion; (**e**) multilayer emulsion oil in water; (**f**) multilayer emulsion water in oil; (**g**) solid lipid particle emulsions (SLPE) (Source: Đorđević *et al*., 2015).

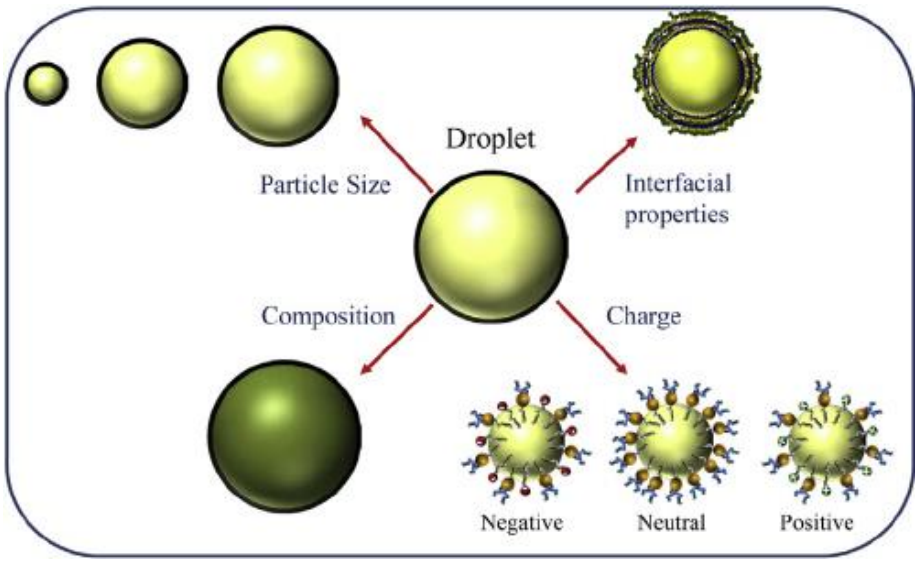


Figure 2.11 Schematic of the ways in which the physicochemical and structural properties of an emulsion, such as size, composition, charge and interfacial properties can be varied in order to design emulsions to have different functional performances (Source: Piorkowski and McClements, 2014).

2.10.5.1 Classification and structure of emulsion-based systems

Emulsion-based systems can be classified according to particle size into one of the following categories; emulsions/macroemulsions, microemulsions, or nanoemulsions (Piorkowski and McClements, 2014; Đorđević *et al*., 2015).

Emulsions/Macroemulsions typically have a particle size > 100 nm, are kinetically but not thermodynamically stable.

Nanoemulsions generally have a particle size < 100 nm, require high mechanical shear for formation and higher concentrations of surfactant for

stabilization compared to microemulsions. Nanoemulsions are metastable dispersions, and therefore have a tendency to break down during storage. However, they are more resistant to oxidation than microemulsions.

Microemulsions have a particle size < 100 nm, are thermodynamically stable, transparent, isotropic dispersions with low viscosity. Microemulsions have a large solubilisation capacity for lipophilic and hydrophilic molecules which is beneficial in protection of the solubilised component from degradation. Microemulsions are stabilised by a set of surfactants, generally in conjunction with a co-surfactant, such as short and medium-chain alcohols, which are required to lower the interfacial tension further (Augustin and Hemar, 2009).

Apart from particle size, emulsion-based systems used to encapsulate food ingredients are also classified based on their method of preparation. Processing methods include low-energy and high-energy procedures. Low-energy processes include the spontaneous emulsification and the phase inversion temperature (PIT) method (Figure 2.12). In spontaneous emulsification, the emulsion is prepared only by mixing the oil, water, surfactant, and co-surfactant at constant temperature. The PIT method includes a transitional inversion induced by a change in temperature, pH/or and salt concentration. In general, low-energy methods are suitable for the encapsulation of thermally sensitive compounds. However, production of microemulsions for food applications by low-energy procedures is problematic due to complexity of these systems (multicomponent systems) and the limited choice of food-grade ingredients (Santana *et al*., 2013). High-energy emulsification processes

involve the use of high-pressure homogenisers, which are capable of obtaining small droplet diameters (between 0.3 and 1 μm) with a very narrow size distribution by controlling energy input, and are used industrially (Đorđević *et al*., 2015). This system allows the use of a variety of emulsifiers and co-emulsifiers (e.g., proteins and GRAS polysaccharides), but the drawbacks are the expensive equipment, low energetic efficiencies, and overall high production costs (Santana *et al*., 2013; Đorđević *et al*., 2015). Emulsions formed using these methods include multi-layered emulsions (Figure 2.10e and 2.10f), double emulsions (Figure 2.10c and 2.10d) and solid lipid particle emulsions (SLPE) (Figure 2.10g).

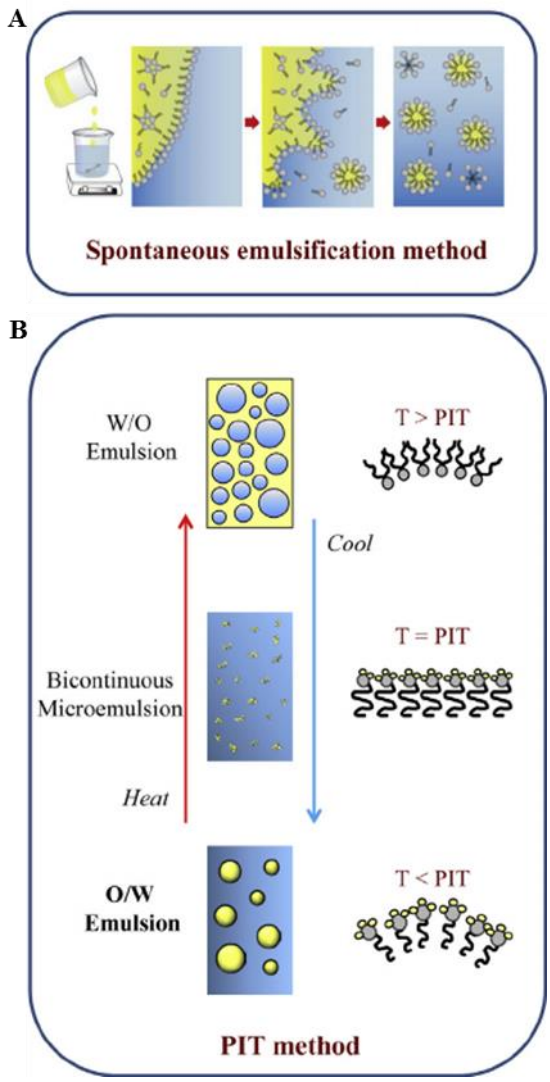


Figure 2.12 Schematic representation of two low-energy approaches used to produced emulsions, (**a**) spontaneous emulsification and (**b**) phase inversion temperature (PIT) methods (Souce adapted from: Piorkowski and McClements, 2014).

2.10.5.2 Advantages, disadvantages and food applications of emulsion systems

Emulsions have been particularly utilized for the entrapment of lipophilic actives that can be further incorporated into aqueous-based foods or beverages (Velikov and Pelan, 2008), as well as minerals, vitamins, amino acids, polyphenolic compounds and probiotics (Jiménez-Colmenero, 2013; Đorđević *et al*., 2015). Numerous advantages of encapsulation of functional ingredients have been reported (McClements *et al*., 2009), including the controlled release of functional ingredients in response to specific environmental triggers, protection of entrapped components from chemical degradations and masking of undesirable sensory attributes.

Double emulsions have the potential to improve the lipid characteristics of foods by reducing fat content or providing healthier fatty acid profiles (Jiménez-Colmenero, 2013; Đorđević *et al*., 2015). Lobato-Calleros *et al*. (2006, 2009) manufactured cottage cheese and stirred yoghurt, respectively, by replacing milk fat with multiple emulsions thus improving their nutritional quality by reducing the fat and saturated fatty acid contents.

Recently, it has been suggested that double and multi-layer emulsions could be used as a strategy to reduce salt in foods (Jiménez-Colmenero, 2013). Koliandris (2011) stated that salt contained in the outer aqueous phase of multiple emulsions will only be perceived upon consumption, and therefore any salt contained in the inner aqueous phase would not contribute to saltiness perception. So, if salt is only present in the outer aqueous phase of the double emulsion, the perception of saltiness in the

product could be greater than is justified by the actual salt content. Given such conditions, salt reductions of up to 80% have been postulated (Norton and Norton, 2010).

The application of emulsions in food formulations has been limited due to the small choice (Đorđević *et al*., 2015), as well as the toxicity of surfactants and co-surfactants available (Augustin and Hemar, 2009). The manufacture of emulsions (micro and nano) from edible oils (e.g., fish oil, corn oil, and soybean oil) is also problematic due to relatively high viscosity and interfacial tension of edible oils, therefore limiting their application in food (Đorđević *et al*., 2015). Another issue associated with delivery of actives using SLPE is a burst release effect caused by the presence of actives in the outer shell. However, by decreasing the surfactant concentration and storage temperature, it is possible to diminish the burst effect (Fathi *et al*., 2012; Đorđević *et al*., 2015).

2.10.6 Coacervation

Coacervation is the second most commonly employed entrapment technique used for food applications, after spray drying (Dias *et al*., 2015). Often described as a modified emulsification technology, the principle is relatively simple; when a solution of active component is mixed with a matrix molecule of opposite charge, a complex is formed (Figure 2.13) (de Vos *et al*., 2010). The microcapsules thus formed are then usually dried, either by spray drying or freeze drying (Champagne *et al*., 1992; Nazzaro *et al*., 2012). This technique is predominantly driven by electrostatic interactions but hydrophobic interactions and hydrogen bonding can also contribute significantly to complex formation (Augustin and Hemar, 2009; de Vos *et al*., 2010). Coacervation is typically used to entrap flavours, vitamins, nutrients, polyphenols, oils and water-soluble actives (Gibbs *et al*., 1999; Gouin, 2004; de Vos *et al*., 2010; Fang and Bhandari, 2010; Augustin and Hemar, 2009; Dias *et al*., 2015). Coacervation is an immobilisation technique rather than a true encapsulation technology.

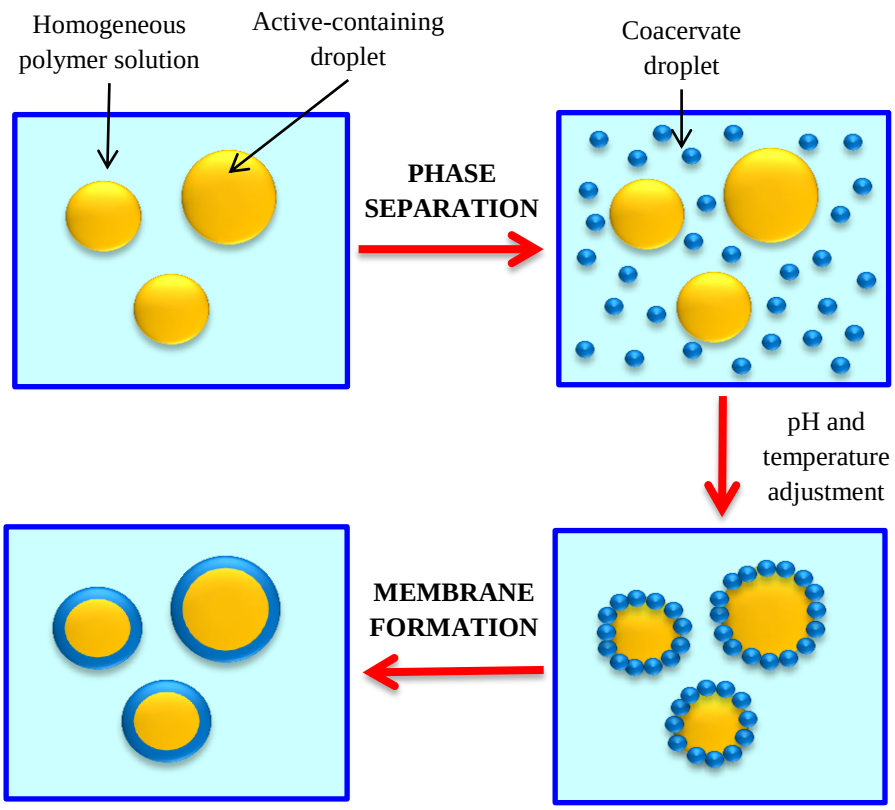


Figure 2.13 Schematic representation of coacervation technology.

2.10.6.1 Complex versus simple coacervation

This technology can be divided into complex or simple coacervation. Simple coacervation involves systems containing only one soluble polymer that is precipitated by changing the pH or temperature. Whereas complex coacervation deals with systems containing two oppositely charged soluble polymers that form a strong polymeric shell or matrix due to ionic interaction at the interface (Pegg and Shahidi, 2007). Chitosan and alginate are the most commonly used wall polymer materials

used in complex coacervation (Baruch and Machluf, 2006; Dias *et al*., 2015). Chitosan has low toxicity, antimicrobial activity, and biocompatibility, but it is mainly "mucoadherence" that allows transmucosal absorption and better release of the active ingredient (Dias *et al*., 2015). The most common wall materials used in simple coacervation include milk proteins (Weinbreck *et al*., 2004a) and pectins with polyglycerol polyricinoleate (PGPR). Other coacervation systems have been studied, which exhibit good properties, these include, gelatin/gum arabic (Dong *et al*., 2011), soybean protein isolate/gum Arabic (Jun-Xia *et al*., 2011) and whey proteins/carrageenan (Weinbreck *et al*., 2004b).

Coacervates may be cross-linked in order to increase their robustness. Augustin and Hemar (2009) stated that glutaraldehyde is an effective cross-linking material; however, legislative issues exist with regards to its use. Therefore, enzymatic cross-linkers, such as transglutaminase (TGase), are more widely accepted for use in the food industry (Đorđević *et al*., 2015). TGase mediates a chemical reaction between glutamine and lysine, thus providing covalent amide bonds that serve to reinforce the matrix. Chen *et al*. (2012) demonstrated that laccase addition generated the cross-linking of beet pectin, resulting in the reinforcement of WPI/beet pectin complex coacervates. However, Weinbreck *et al*. (2004a) demonstrated that cross-linked capsules gave a lower flavour release in Gouda cheese than non-cross-linked capsules, highlighting that the use of cross-linking material must be carefully considered for each individual food application.

2.10.6.2 Factors affecting coacervate stability

Several factors including the biopolymer type (MW, flexibility and charge), pH, ionic strength, pressure, concentration and the ratio of the biopolymers affect the strength of the interactions between the biopolymers and the nature of the complex formed (Figure 2.14) (Gibbs *et al*., 1999; Augustin and Hemar, 2009; Lakkis, 2016). Baruch and Machluf (2006) investigated the influence of different types of chitosan and varying pH conditions on the long-term encapsulation of cells within chitosan-alginate microcapsules manufactured using coacervation. The authors found that the use of high molecular weight (MW) chitosan glutamate and low MW chitosan chloride provided high cell viability levels (> 93%), as well as good mechanical properties. In addition, when using low MW chitosan, better cell viability levels (195%) were obtained at a pH of 6 and a reaction time of 30 min when compared with the alginate poly-L-lysine (PLL) encapsulation system which served as a control.

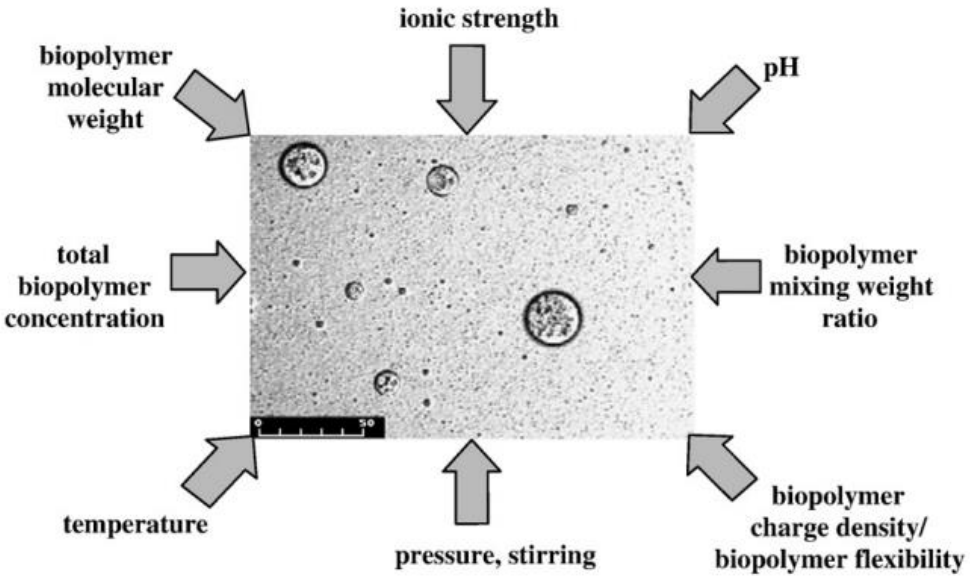


Figure 2.14 Overview of physicochemical parameters influencing complex formation and coacervation in protein/polysaccharide systems. Scale bar is 50 μm (Source: Schmitt and Turgeon, 2011).

2.10.6.3 Advantages, disadvantages and food applications of coacervation

A great advantage associated with using coacervation as a method of entrapment is the very high pay load achievable, up to 99% retention of the active ingredient (Gouin, 2004; Weinbreck *et al*., 2004; Pegg and Shahidi, 2007; Dias *et al*., 2015). Microcapsules produced by coacervation are heat resistant and therefore possess excellent and versatile controlled-release options based on different triggers such as, mechanical stress, biological mechanisms and pH (Gibbs *et al*., 1999; Gouin, 2004; Dias *et al*., 2015; Đorđević *et al*., 2015). Complex coacervates also exhibit

excellent stability upon storage. For example, Dong *et al*. (2011) encapsulated peppermint oil in gelatin/gum Arabic microcapsules and reported that only ~7% of the encapsulated flavour oil was released during 40 d storage in cold water. In addition, complex coacervates are capable of protecting sensitive compounds from oxidation during storage. Comunian *et al*. (2013) encapsulated ascorbic acid within gelatin/gum Arabic microcapsules and found that 80% of the vitamin remained in the microcapsules after 30 d storage at 20 ˚C, while ascorbic acid in the free form was totally degraded under the same conditions.

However, coacervation is an expensive method of entrapment (Fang and Bhandari, 2010) and its application is limited due to the number of approved food-grade entrapment materials available to food manufacturers (Pegg and Shahidi, 2007). Another factor that limits the use of coacervates in entrapment is their sensitivity to pH and ionic strength. However, as previously mentioned, the addition of cross-linking components can increase the robustness of coacervates. Moreover, entrapment of hydrophilic compounds by coacervation has proven difficult, meaning this technique is more appropriate to hydrophobic compounds as core materials (Đorđević *et al*., 2015). In order to successfully entrap hydrophilic compounds it is necessary to make several adjustments to the method (i.e., addition of a double emulsion step at the beginning), which will add time and cost to the procedure (Comunian *et al*., 2013; Đorđević *et al*., 2015).

2.10.7 Liposome entrapment

Liposomes are spherical bilayer vesicles that are formed by the dispersion of polar lipids in aqueous media (Law and King 1985; Risch and Reineccius 1995; Zuidam and Shimoni, 2010; Singh *et al.*, 2012; Đorđević *et al*., 2015). Once formed, a liposome can be unilamellar or multi-lamellar and range in size from 20 nm to several micrometres (Gibbs *et al*., 1999; Walde and Ichikawa, 2001; Fang and Bhandari, 2010; Zuidam and Shimoni, 2010; Maherani *et al*., 2011; Singh *et al*., 2012; Đorđević *et al*., 2015; Mohammadi *et al*., 2015). The main constituents of liposomes are phospholipids, which are amphiphilic molecules containing a water-soluble, hydrophilic head section and a lipid-soluble, hydrophobic tail section. The underlying mechanism for the formation of liposomes is basically the hydrophilic–hydrophobic interactions between phospholipids and water molecules (Gibbs *et al*., 1999; Fang and Bhandari, 2010; Zuidam and Shimoni, 2010; Maherani *et al*., 2011; Đorđević *et al*., 2015; Mohammadi *et al*., 2015). This property of phospholipids gives liposomes unique characteristics, such as self-sealing, in aqueous media and makes them an ideal carrier system (Gibbs *et al*., 1999). Due to the fact that liposomes contain both lipid and aqueous phases, they can be utilised in the entrapment, delivery and release of water-soluble, lipid-soluble and amphiphilic materials, simultaneously (Figure 2.1b) (Gibbs *et al*., 1999; Fang and Bhandari, 2010; Maherani *et al*., 2011). Most commonly liposomes release entrapped material slowly by diffusion out through the phospholipid membrane as the concentration in the external phase decreases. Environmental changes (i.e., pH, temperature) can also be employed

to create appropriate conditions for destabilization of liposomes and release of their contents (Singh *et al*., 2012).

2.10.7.1 Liposome classification

Liposomes are classified based on vesicle size, number of lamella and preparation method. Liposome classifications are shown in Figure 2.15. Liposomes containing only a single bilayer membrane are called unilamellar vesicles (ULV). Unilamellar vesicles can be sub-divided further depending on size; small unilamellar vesicles (SUV, less than 100 nm) and large unilamellar vesicles (LUV, larger than 100 nm) (Risch and Reineccius 1995; Walde and Ichikawa, 2001; Maherani *et al*., 2011). Furthermore, liposomes composed of a number of concentric lipid bilayers are referred to as multilamellar vesicle (MLV) (Risch and Reineccius 1995; Walde and Ichikawa, 2001), while liposomes composed of several non-concentric vesicles entrapped within a single bilayer are known as a multivesicular vesicle (MVV) (Maherani *et al*., 2011).

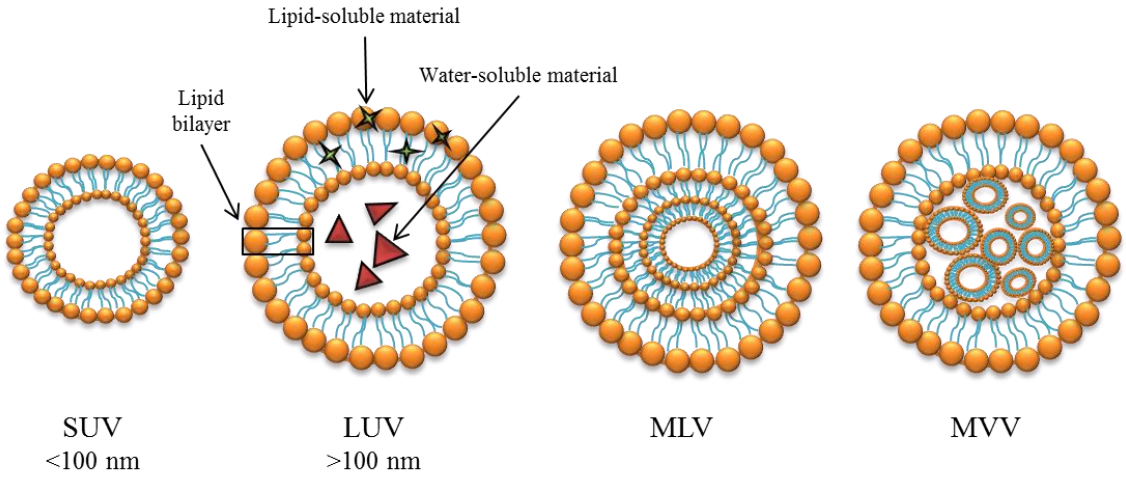


Figure 2.15 Liposome vesicle classification structures; small unilamellar vesicles (SUV), large unilamellar vesicles (LUV), multilamellar vesicle (MLV) and multivesicular vesicle (MVV). Aqueous central and bilayer membrane parts of a liposome with capability of containing water- and lipid-soluble materials.

2.10.7.2 Liposome composition

The main factor influencing liposome bilayer characteristics such as mean size, zeta potential, release mechanism and polydispersity index is the composition of the lipid bilayer (Đorđević *et al*., 2015) and its method of preparation (Singh *et al*., 2012) and these differences can affect a number of liposome characteristics, including stability and the rate of release of entrapped material. The principal ingredients of liposomes are phospholipids, consisting of a hydrophilic head and two hydrophobic tails (Maherani *et al*., 2011; Mohammadi *et al*., 2015). The most common phospholipid used in the liposome bilayer is phosphatidylcholine (PC) as the hydrophilic and hydrophobic portions of the molecule are roughly equal in size

(Singh *et al*., 2012). Phosphatidylglycerol (PG), phosphatidylinositol (PI) and phosphatidyl acid (PA), dipalmitoyl phosphatidylcholine (DPPC) and cholesterol have also been added to modify the properties of different bilayer systems. Due to their charge they are capable of promoting electrostatic repulsion, thus preventing aggregation or fusion (Singh *et al*., 2012; Đorđević *et al*., 2015). Liposome characteristics (entrapment efficiency, toxicity and stability) are closely related to the chemical properties of the phospholipids used for their preparation (Singh *et al*., 2012). It has been demonstrated that saturated phospholipids such as DPPC form rigid and impermeable bilayer while unsaturated PC provide more permeable and less stable bilayers (Đorđević *et al*., 2015). Small vesicles have a tendency to fuse or aggregate, and may develop into micron-sized liposomes during storage. This may be prevented by electrostatic repulsion, by addition of charged lipids in the membrane, or steric stabilisation (Mozafari and Khosravi-Darani 2007; Zuidam and Shimoni, 2010). Cholesterol is the most commonly used molecule added to liposomes to increase stability by reducing the permeability of the liposomal membrane to solutes (Singh *et al*., 2012).

2.10.7.3 Methods of liposome manufacture

Formation of liposomes of a specific size and lamellarity requires energy (Singh *et al.*, 2012). MLV form immediately when bilayer-forming polar lipids are dissolved in aqueous media under mild agitation. In order to produce LUV and SUV, considerable energy inputs are required that are sufficient to disrupt MLV structures and force the generation of monomodal vesicles (New, 1990). Typically, the energy

applied to form the desired liposome is generally either non-mechanical or mechanical energy.

Traditionally, liposome preparation was carried out using conventional, non-mechanical methods such as thin-film hydration method, reversed-phase evaporation, solvent injection method and heating-based method. The major disadvantage of these techniques is that they produce liposomes of a large size (MLV, up to 500 μm or LUV, 100–1000 nm) with a broad size distribution range. In order to overcome this issue and create liposomes of a smaller size (SUV, <100 nm), mechanical techniques such as, extrusion (Turanek, 1994), sonication (Maa and Hsu, 1999), high-pressure homogenisation (McAuliffe *et al*., 2016), or microfluidisation (Thompson and Singh, 2006) need to be employed as an additional preparation step. Apart from a reduction in liposome size (controlled by varying the pressure used and the number of times the dispersion is recirculated through the homogenizing chambers), additional energy can lead to increase in entrapment efficiency (Nongonierma *et al*., 2009). In addition, most conventional, non-mechanical methods usually involve the use of organic solvents or detergents which are not desirable from a food manufacturing perspective as they may leave residues within the food product and therefore are unsuitable for food applications (Singh *et al*., 2012). The major techniques employed in the formation of liposomes are outlined in Table 2.7, including advantages and disadvantages associated with each method.

Table 2.7 Mechanisms, advantages and disadvantages associated with the most commonly used methods for liposome production.

Method	Mechanism	Advantage	Disadvantage
Non-Mechanical			
Reverse-phase evaporation (REV)	Lipids dissolved in organic solvent, aqueous phase added, then the solution sonicated to produce inverted micelles. The organic solvent is removed using a rotary evaporator and a viscous gel forms.	High entrapmentefficiency	Risk of enzyme denaturation Unsuitable for food application due to residual solvent in final product
Injection method			
Ether	Solution of lipids dissolved in diethyl ether or ether/methanol mixture, slowly injected to an aqueous solution of the material to be entrapped at 55-65˚C or under reduced pressure. Subsequent removal of ether under vacuum leads to the formation of liposomes.		Heterogeneous liposome (70-190 nm) Unsuitable for food application due to residual solvent in final product
Ethanol	A lipid solution of ethanol is rapidly injected to a vast excess of buffer. MLVs are immediately formed.		Heterogeneous liposome (30-110 nm) Liposomes are very dilute Inactivation of biologically active macromolecules due to the presence of even low amounts of ethanol
Thin-film hydration	Process involves the mixing of organic solvent with dissolved lipid fraction. Followed by solvent removal to yield a lipid film, which is thoroughly dried and finally rehydrated using aqueous medium.		Unsuitable for food application due to residual solvent in final product
Heating method	Hydration of the phospholipid components in an aqueous solution containing glycerol (3%) and increasing the temperature to 60 ˚C or 120 ˚C, depending on the absence or presence of cholesterol, respectively.	No organic solvent required No degradation of lipid ingredient Further sterilisation not required	Multi-step technique

Table (continued)

Method	**Mechanism**	**Advantage**	**Disadvantage**
Dehydration-rehydration	Initially a suspension of water-containing SUVs are made, and then the active to be entrapped is added followed by lyophilisation of the mixture. Upon rehydration, the vesicles re-form, passively entrapping the active.	Up-scale easily	Produces MLV and SUV
Mechanical			
Sonication method			
Probe	Disruption of MLV liposome suspensions using sonic energy.	Rapid method High energy input Produces SUV (15-50 nm)	Enzyme degradation by ultrasonic energy Sample contamination with titanium Poor reproducibility Unstable liposomes produced, capable of fusion
Bath	Disruption of LMV liposome suspensions using sonic energy.	Simple method Sample isolated – not in contact with equipment Produces SUV (15-50 nm)	Enzyme degradation by ultrasonic energy Low efficiency in manufacturing homogenous vesicles Poor reproducibility Unstable liposomes produced
Extrusion	Involves passing a dispersion of MLVs formed via thin film rehydration through a small orifice or a membrane with a defined pore size.	Easy control over liposome size (filter pore size) High entrapment efficiencies	Only appropriate for small volumes as the process is operating at low flow rates
High-pressure homogenisation	Mainly used in the formation of liposomes. Involves forcing a dispersion of MLVs at high pressure through small holes.	No organic solvent required Easy control over size of liposomes (pressure and number of passes applied)	Material loss Difficult to scale-up

Table (continued)

Method	**Mechanism**	**Advantage**	**Disadvantage**
Microfluidisation	Mainly used in the formation of liposomes. Involves forcing a dispersion of MLVs at very high pressure through small holes.	No organic solvent required Easy control over size of liposomes (pressure and number of passes applied) Rapid, continuous process Stable preparations produced	Possible degradation of active material due to high energy and pressure input Material loss Difficult to scale-up

2.10.7.4 Advantages, disadvantages and applications of liposomes in food

Liposome systems are considered a popular choice for entrapping active food components due to their low toxicity, their capacity to entrap virtually any molecule (hydrophobic and hydrophilic) regardless of its structure, and the ability to obtain the desired physicochemical characteristics by manipulating the size, composition and bilayer fluidity (Singh *et al*., 2012). Most recently, liposomes have been used as delivery systems for several food components including enzymes (Kheadr *et al*., 2000; Lee *et al*., 2000; Kheadr *et a*l., 2002; Nongonierma *et al*., 2013), proteins (da Silva Malheiros *et al*., 2010), vitamins (Tesoriere *et al*., 1996; Banville *et al*., 2000; Kosaraju *et al*., 2006), flavours (Liolios *et al*., 2009), minerals (Albaldawi *et al*., 2005; Kosaraju *et al*., 2006), polyphenols (Kerdudo *et al*., 2014; Zou *et al*., 2014), antioxidants (Mozafari *et al*., 2006; Rashidinejad *et al*., 2014) and antimicrobials (Benech *et al*., 2003; Laridi *et al*., 2003; Were *et al*., 2003; Colas *et al*., 2007; da Silva Malheiros *et al*., 2010).

Entrapment of such components within liposomes has been found to increase the stability of the entrappeded material against environmental changes (temperature, moisture, pH and ionic strength) both during processing and thereafter (Kirby *et al*., 1991; Farhang *et al*., 2012). Another major advantage of their use in food is the ability to control the release rate and of the entrapped material and deliver it to a specific site at the correct time (Schafer *et al*., 1992; Kheadr *et al*., 2000). Liposomes are also non-toxic and therefore, suitable for addition into food (Gibbs *et al*., 1999). Moreover, bioactive agents entrapped into liposomes can be protected from digestion in the stomach, and show significant

levels of absorption in the gastrointestinal tract, leading to the enhancement of bioactivity and bioavailability (Takahashi *et al*., 2008).

To date, the most widely used application of liposomes within food is for the entrapment of enzymes used in cheese manufacture. Enzymes entrapped in liposomes have been successfully used in cheese manufacture to accelerate ripening (Kirby *et al*., 1987; Anjani *et al*., 2007; Nongonierma *et al*., 2013), to increase shelf-life through entrapment of antimicrobial agents such as nisin and lysozyme (Thapon and Brule, 1986; Laridi *et al*., 2003; da Silva Malheiros *et al*., 2012), to fortify cheese with liposome-entrapped vitamins and antioxidants (Kirby *et al*., 1991; Bainville *et al*., 2000; Rashidinejad *et al*., 2014) and to alter textural and rheological properties of cheese (Kheadr *et al*., 2000; Lee *et al*., 2000; McAuliffe *et al*., 2016).

Liposomes also have potential for use in other dairy products. For example, they could be employed to control the release of minerals during heat treatment, thus minimizing unwanted aggregation (Westhaus and Messersmith, 2001). Another application would be the addition of liposome entrapped *β*-galactosidase, to aid in the digestion of lactose in dairy products. The entrapped enzyme will be released in the upper intestine in the presence of bile salts (Rodríguez-Nogales and López, 2006). In addition, during cheesemaking a substantial fraction of the enzyme is lost in the whey stream, increasing product cost through the requirement for a high initial enzyme concentration and limiting downstream whey processing options; entrapment of enzymes may alleviate this problem.

2.10.7.5 Future trends of liposomes entrapment in food

Most literature pertaining to the use of liposomes as encapsulating/entrapping systems has focused on egg and soy as obvious sources of phospholipids. However, in recent years there has been growing interest in employing milk-derived phospholipids as ingredients for liposomes (Thompson and Singh, 2006; Thompson *et al*., 2006). As a result of increasing commercial availability of these dairy phospholipids, the interest in their use in different food applications is also rising. Farhang *et al*. (2012) has demonstrated that, using microfluidisation, it was possible to obtain small, unilamellar milk phospholipids liposomes that were stable for 7 weeks at neutral pH. Amongst the advantages of using milk phospholipids are their unique composition and the reported health benefits associated with their consumption (Spitsberg, 2005; Zheng *et al*., 2014). Milk phospholipids contain PC, phosphatidylethanolamine (PE), sphingomyelin (SM) and phosphatidylserine (PS), while egg and soy phospholipids predominantly contain only PC and a mixture of PC, PE and PI, respectively. The high ratio of PC to SM in milk phospholipids imparts unique properties of the bilayer (Barenholtz and Thompson, 1980). Thompson *et al*. (2006) reported that liposomes prepared with milk phospholipids showed higher phase transition temperatures, lower membrane permeability, a thicker membrane and better stability than those prepared with soy phospholipids. Moreover, SM are increasingly recognized as bioactive molecules (Vesper *et al*., 1999) making formulations containing these milk phospholipids very attractive in neutraceutical and health products.

2.8 Methods used to characterise encapsulates

After manufacture, characterisation of microparticles and microcapsules is required to qualify, quantify and approve the encapsulate capability for particular applications. It is essential that methods of characterisation are precise and rapid. Size and composition are contributing factors affecting the stability, permeability, surface activity and affinity of encapsulates (Gibbs *et al*., 1999).

The most common methods (Figure 2.16) used to determine the *morphology* and *size distribution* of encapsulates are spectroscopic techniques such as dynamic light scattering (DLS), microscopy techniques such as transmission electron microscopy (TEM), scanning electron microscopy (SEM) and atomic force microscopy (AFM) and chromatographic techniques, including gel permeation, size exclusion chromatography (SEC), capillary electrophoresis (CE), hydrodynamic chromatography (HDC) and field-flow fractionation (FFF) (Tiede *et al*., 2008; Maherani *et al*., 2011; Katouzian and Jafari, 2016). DLS provides information on the size of the vesicle but not on its shape. Electron microscopy techniques (TEM and SEM) enable the determination of the shape of the vesicle, as well as observing any fusion or aggregation. AFM is a very high-resolution type of scanning probe microscopy that can create three dimensional micrographs with resolution down to the nanometer, ideal for structural characterization of particles. Gel permeation chromatography compares the elution characteristics, size distribution and homogeneity of vesicles, while SEC separates particles on the basis of size and makes it possible to estimate the molecular mass of a compound.

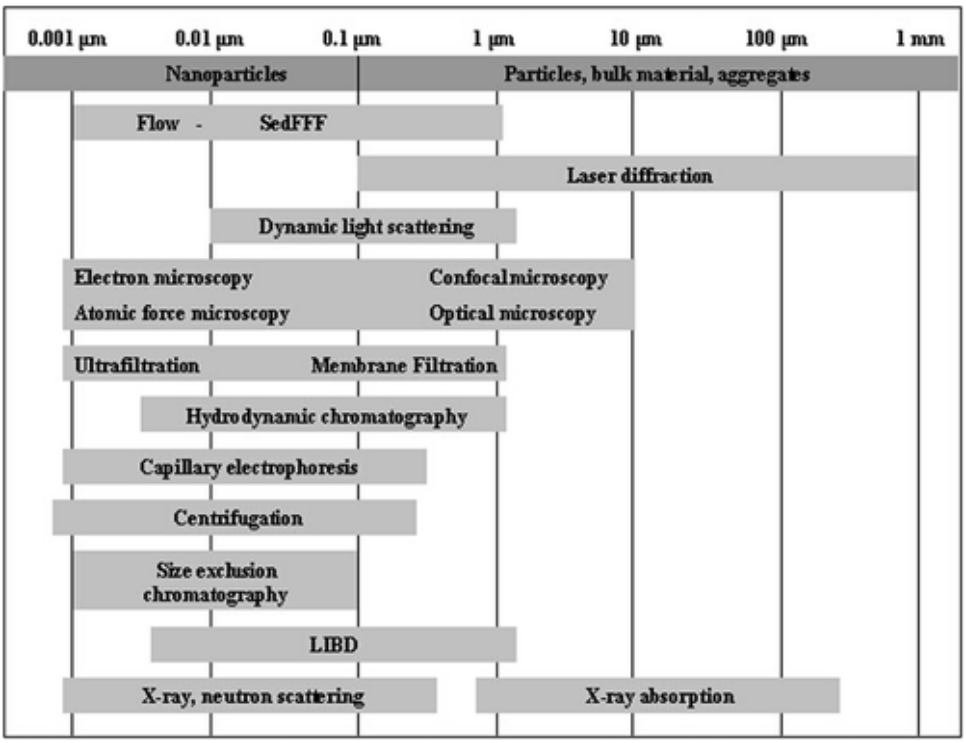


Figure 2.16 Sizing methods and their size ranges for particle measurements (from Tiede *et al*., 2008).

Particle *charge* is a crucial parameter for predicting the stability of colloidal delivery systems. Stability is increased when charged vesicles repel each other and thus overcome the natural tendency to fuse, aggregate and precipitate during storage (Matalanis *et al*., 2011). This can be achieved by increasing inter-particle repulsion, either by electrostatic or steric repulsion (Keller, 2001). Particle charge also influences how they interact with other charged species in the surrounding medium. If a particle has an opposite charge to another ionic ingredient within a food, then it may form an electrostatic complex that may precipitate and sediment (Matalanis *et al*., 2011). As highlighted by Matalanis *et al*. (2011), electrical charge also determines how particles interact with different surfaces of the human digestive system. A cationic encapsulate particle may bind

to the anionic surface of the tongue thereby causing perceived astringency. Conversely, a cationic encapsulate particle could be designed to bind to a specific location within the gastrointestinal tract ("mucoadhesion") to delay its transit through the body and release its bioactive at a particular site. The charge density of particle surfaces and the binding affinity of various ions to lipid vesicles can be determined by measuring a parameter called *zeta potential* (Maherani *et al*., 2011). Zeta potential is a function of the surface charge of the lipid vesicle and is influenced by particle composition (Maherani *et al*., 2011). Zeta potential is not measurable directly, but it can be calculated using theoretical models as determined by electrophoretic mobility (Filion and Phillips, 1997) or dynamic electrophoretic mobility (laser doppler electrophoresis or Zetasizer) (Nongonierma *et al*., 2013; McAuliffe *et al*., 2016). The electrical characteristics of particles can be controlled by selecting one or more ingredients with different charge *versus* pH profiles (Matalanis *et al*., 2011).

Material composition and processing method both influence the *entrapment/encapsulation efficiency (%EE)* of encapsulate formulations. The experimental methods applied to determine the %EE of lipid based encapsulates (i.e., emulsions and liposomes) usually require removal of the free (unencapsulated) active ingredient from encapsulated active ingredient by the following methods: column chromatography (Zhang *et al*., 2004), SEC (Were *et al*., 2004), ultracentrifugation (Were *et al*., 2004; McAuliffe *et al*., 2016), and ultrafiltration (Nii *et al*., 2003), before quantification of the entrapped material. The analytical techniques employed to quantify entrapped material (solid and liquid particles) include spectrometry (Zhang *et al*., 2004; Barbosa *et al*., 2005),

HPLC (McAuliffe *et al*., 2016), UV spectroscopy (El Bahri and Taverdet, 2007) and spectrofluorimetry (Taylor *et al*., 2007). Liposomal encapsulation efficiency using the fluorescent dye calcein based on fluorescence quenching of the untrapped calcein by addition of cobalt cation was demonstrated by Oku *et al.* (1982). This method, in contrast to the common entrapment efficiency evaluation methods, does not require application of any separation technique.

The *release kinetics* of a microcapsule/microparticle must be studied in order to ensure the encapsulate is releasing the entrapped active at the correct time under the correct conditions (pH, temperature, osmotic pressure, etc.). Measuring the release rate of a particle usually involves a two-step process: firstly, the particle is subjected to conditions that induce the release of the active from the particle (i.e., dissolution reactor, dialysis bag). This is followed by analysis of the released component solution by methods such as HPLC and UV spectroscopy (El Bahri and Taverdet, 2007; Xu *et al*., 2016).

2.8.1 Further characterisation methods used for emulsions and liposomes

Encapsulates containing high concentrations of phospholipids (liposomes) have a tendency to oxidise, hydrolyse, aggregate and fuse during long term storage. Maximum *stability* can be ensured by using freshly prepared lipid and solvents to prepare the liposomes or emulsions, avoiding exposure of the formulation to oxygen as much as possible (using inert atmosphere, e.g., nitrogen), limiting excessive temperatures, adding stabilising agents, antioxidants and complexing agents (e.g., EDTA) to avoid charge neutralization by metals and using proper storage conditions (Mozafari *et al*., 2008; Singh *et al*., 2012).

Hydrolysis can be minimized by using pure solvents and removing as much of the water as possible. Holding the temperature above the phase transition temperature and increasing liposome charge helps to avoid fusion. Since neutral liposomes will still aggregate due to van der Waals' forces, addition of 5% phosphatidic acid or phosphatidyl glycerol can reduce this (Gibbs *et al*., 1999). Liposome stability is evaluated using the following methods: SEC (Márquez-Ruiz *et al*., 2000), HPLC (Zuidam *et al*., 2003), evaporative light scattering detectors (ELSD) (Sas *et al*., 1999) and mass spectrometry (MS) analysis in combination with HPLC (Vernooij *et al*., 1998). Lipid peroxidation (LPO) rate is measured and quantified using gas chromatography (individual fatty acids) and HPLC (cholesterol) (Lang and Vigo-Pelfrey, 1993).

The *phase transitions*, *fluidity* and *lamellarity* of phospholipid membranes are important in the manufacture and application of liposomes. The phase behaviour of encapsulates containing phospholipid membranes determines properties such as permeability, fusion, aggregation and protein binding of encapsulates, all of which affect stability (Maherani *et al*., 2011). Consequently, determining the transition temperature is very important. Differential scanning calorimetry (DSC) has been used extensively for the determination of transition temperatures of phospholipids (Saroglou *et al*., 2006). Electron paramagnetic resonance (EPR), nuclear magnetic resonance (NMR) spectroscopy and depolarization of fluorescence methods are generally used to study liposome fluidity (Sułkowski *et al*., 2005; Saroglou *et al*., 2006; Ruozi *et al*., 2007). Lamellarity refers to the number of lipid bilayers surrounding the inner aqueous space of the lipid vesicles and it typically regarded as one of the most

characteristics of lipid vesicles. Liposome lamellarity is commonly determined using direct microscopy techniques but NMR has also been used. Ruozi *et al*. (2007) used NMR and the EPR to determine the lamellarity, the permeability of the bilayer and the influence of particle size on the liposomal transport of bioactive molecules.

2.9 Conclusions

It can be seen that the utilisation of encapsulated/entrapped food ingredients in place of free ingredients can lead to improvements in the stability, functionality and bioavailability of the compounds *in vivo* and *in vitro*. In addition, encapsulation facilitates improved flow properties and easier handling of ingredients by the conversion of a liquid active agent into a powder.

Cost remains a major consideration when developing new encapsulant materials and delivery systems for the food industry. It is essential to determine the cost of both the encapsulant material and the technology employed to create the microparticle in order to generate commercially viable encapsulation techniques with broad-spectrum product applications. In cases where costly materials and processes are the only possible solution to address the requirements of the food ingredient being encapsulated, the benefits offered by the delivery system must either clearly outweigh the cost, or the final product must be able to bear the additional cost.

Several encapsulation/entrapment techniques exist but none of them can be considered as a universally applicable procedure for entrapment of different food components. Therefore, development of novel methods for the manufacture of microparticles and microcapsules should be pursued in order to determine which methods are most efficient in terms of cost and flexibility.

There is a need to develop and introduce new and functional GRAS encapsulant materials due to the limited selection allowed for food applications.

This presents an opportunity to modify food-grade materials using either new emerging processes or standard food processing conditions to develop new food grade materials that are more functional as encapsulant materials. Additionally, the creation of hypoallergenic encapsulant materials and non-protein delivery systems is necessary due to allergenicity issues associated with protein ingredients. Despite non-protein encapsulant materials existing, the majority of them available are chemically modified and therefore they are unsuitable and not appealing to food manufacturers looking for a natural or clean label.

To date, research has shown a vast array of applications for the use of encapsulation technologies in food products. This being said, several more potential applications are yet to be investigated. From a cheese manufacturing perspective, most literature pertaining to the use of encapsulation/entrapment (mostly liposome entrapment) has focused mainly on applications such as acceleration of ripening, increasing shelf-life through encapsulation of antimicrobial agents and fortification of cheese with entrapped vitamins and antioxidants. However, further research needs to be carried out in order to investigate its potential application in modifying the textural, functional and rheological properties of cheese through the encapsulation of calcium chelating agents which have been shown to alter such properties.

References

Albaldawi, A., Brennan, C. S., Alobaidy, K., Alammar, W. & Aljumaily, D. (2005). Effect of flour fortification with haem liposome on bread and bread doughs. *International Journal of Food Science and Technology*, *40*, 825-828.

Amakiri, A. C. & Thantsha, M. S. (2016). Survival of *Bifidobacterium longum* LMG 13197 microencapsulated in Vegetal or Vegetal-inulin matrix in simulated gastrointestinal fluids and yoghurt. *Springer Plus*, *5*, 1343-1355.

Anjani, K., Kailasapathy, K. & Phillips, M. (2007). Microencapsulation of enzymes for potential application in acceleration of cheese ripening. *International Dairy Journal*, *17*, 79-86.

Ajaykumar, T. M. (2014). Nanotechnology: Emerging tool in food sector. *Research Journal of Biotechnology*, *9*, 12-24.

Annan, N. T., Borza, A. D. & Hansen, L. T. (2008). Encapsulation in alginate-coated gelatin microspheres improves survival of the probiotic *Bifidobacterium adolescentis* 15703T during exposure to simulated gastro-intestinal conditions. *Food Research International*, *41*, 184-193.

Anonymous. (2011, December 31). Retrieved from http://kodichina.com/newsshow.asp?nclassname=60&nid=22 on 22.09.16.

Anonymous. (2012). Retrieved from http://www.china-dryer.com/en/html/Library3/27.html on 22.09.16.

Anonymous. (2016). Retrieved from http://www.colorcon.com/products-formulation/process/fluid-bed-coating on 23.09.16.

Augustin, M. A. & Sanguansri, L. (2008). Encapsulation of bioactives. In: J. M. Aguilera & P. J. Lillford (Eds), *Food Materials Science – Principles and Practice* (pp. 577-601). New York, USA: Springer.

Augustin, M. A. & Hemar, Y. (2009). Nano-and micro-structured assemblies for encapsulation of food ingredients. *Chemical Society Reviews*, *38*, 902-912.

Augustin, M. A., Abeywardena, M. Y., Patten, G., Head, R., Lockett, T., De Luca, A. & Sanguansri, L. (2011). Effects of microencapsulation on the gastrointestinal transit and tissue distribution of a bioactive mixture of fish oil, tributyrin and resveratrol. *Journal of Functional Foods*, *3*, 25-37.

Augustin, M. A. & Sanguansri, L. (2012). Challenges in developing delivery systems for food additives, nutraceuticals and dietary supplements. In: Garti, N. & McClements, D. J. (Eds), *Encapsulation Technologies and Delivery Systems for Food Ingredients and Nutraceuticals* (pp. 19-48). Cambridge, UK: Woodhead Publishing Limited.

Banville, C., Vuillemard, J. C. & Lacroix, C. (2000). Comparison of different methods for fortifying Cheddar cheese with vitamin D. *International Dairy Journal*, *10*, 375-382.

Barbosa, M. I. M. J., Borsarelli, C. D. & Mercadante, A. Z. (2005). Light stability of spray-dried bixin encapsulated with different edible polysaccharide preparations. *Food Research International*, *38*, 989-994.

Barbosa-Canovas, G. V., Ortega-Rivas, E., Julijano, P. & Yan, H. (2005). *Food Powders: Physical Properties, Processing, and Functionality.* New York, USA: Kluwer/Plenum.

Barenholz, Y. & Thompson, T. E. (1980). Sphingomyelins in bilayers and biological membranes. *Biochimica et Biophysica Acta (BBA)-Reviews on Biomembranes*, *604*, 129-158.

Baruch, L. & Machluf, M. (2006). Alginate–chitosan complex coacervation for cell encapsulation: Effect on mechanical properties and on long-term viability. *Biopolymers*, *82*, 570-579.

Benech, R. O., Kheadr, E. E., Lacroix, C. & Fliss, I. (2003). Impact of nisin producing culture and liposome-encapsulated nisin on ripening of *Lactobacillus* added Cheddar cheese. *Journal of Dairy Science*, *86*, 1895-1909.

Bharadwaj, R. K., Mehrabi, A. R., Hamilton, C., Trujillo, C., Murga, M., Fan, R., Chavira, A. & Thompson, A. K. (2002). Structure–property relationships in cross-linked polyester–clay nanocomposites. *Polymer*, *43*, 3699-3705.

Brückner, M., Bade, M. & Kunz, B. (2007). Investigations into the stabilization of a volatile aroma compound using a combined emulsification and spray drying process. *European Food Research and Technology*, *226*, 137-146.

Champagne, C. P., Gaudy, C., Poncelet, D. & Neufeld, R. J. (1992). *Lactococcus lactis* release from calcium alginate beads. *Applied and Environmental Microbiology*, *58*, 1429-1434.

Champagne, C. P. & Fustier, P. (2007). Microencapsulation for the improved delivery of bioactive compounds into foods. *Current Opinion in Biotechnology*, *18*, 184-190.

Charve, J. & Reineccius, G. A. (2009). Encapsulation performance of proteins and traditional materials for spray dried flavours. *Journal of Agricultural and Food Chemistry*, *57*, 2486-2492.

Chen, B., Li, H., Ding, Y. & Suo, H. (2012). Formation and microstructural characterization of whey protein isolate/beet pectin coacervations by laccase catalyzed cross-linking. *LWT-Food Science and Technology*, *47*, 31-38.

Chua, K. J. & Chou, S. K. (2003). Low-cost drying methods for developing countries. *Trends in Food Science and Technology*, *14*, 519-528.

Colas, J. C., Shi, W., Rao, V. M., Omri, A., Mozafari, M. R. & Singh, H. (2007). Microscopical investigations of nisin-loaded nanoliposomes prepared by Mozafari method and their bacterial targeting. *Micron*, *38*, 841-847.

Comunian, T. A., Thomazini, M., Alves, A. J. G., de Matos Junior, F. E., de Carvalho Balieiro, J. C. & Favaro-Trindade, C. S. (2013). Microencapsulation of ascorbic acid by complex coacervation: Protection and controlled release. *Food Research International*, *52*, 373-379.

Crowley, S. V. & O'Mahony, J. A. (2016). Drying: effect on nutrients, composition, and health. In: Caballero, B., Finglas, P. M. & Toldrá, F. (Eds). *Encyclopedia of Food and Health*, 1st Ed., pp. 439-444. Elsevier Ltd., Oxford, GB.

da Silva Malheiros, P., Daroit, D. J. & Brandelli, A. (2010). Food applications of liposome-encapsulated antimicrobial peptides. *Trends in Food Science and Technology*, *21*, 284-292.

da Silva Malheiros, P., Daroit, D. J. & Brandelli, A. (2012). Inhibition of *Listeria monocytogenes* in Minas Frescal cheese by free and nanovesicle-encapsulated nisin. *Brazilian Journal of Microbiology*, *43*, 1414-1418.

de Lara Pedroso, D., Thomazini, M., Heinemann, R. J. B. & Favaro-Trindade, C. S. (2012). Protection of *Bifidobacterium lactis* and *Lactobacillus acidophilus* by microencapsulation using spray-chilling. *International Dairy Journal*, *26*, 127-132.

de Roos, K. B. (2003). Effect of texture and microstructure on flavour retention and release. *International Dairy Journal*, *13*, 593-605.

de Vos, P., Faas, M. M., Spasojevic, M. & Sikkema, J. (2010). Encapsulation for preservation of functionality and targeted delivery of bioactive food components. *International Dairy Journal*, *20*, 292-302.

Deladino, L., Anbinder, P. S., Navarro, A. S. & Martino, M. N. (2008). Encapsulation of natural antioxidants extracted from *Ilex paraguariensis*. *Carbohydrate Polymers*, *71*, 126-134.

Desai, K. G. H. & Jin Park, H. (2005). Recent developments in microencapsulation of food ingredients. *Drying Technology*, *23*, 1361-1394.

Dewettinck, K. & Huyghebaert, A. (1999). Fluidized bed coating in food technology. *Trends in Food Science and Technology*, *10*, 163-168.

Dezarn, T. J. (1995). Food ingredient encapsulation. In: Risch, S. J. & Reineccius, G. A. (Eds.), *Encapsulation and Controlled Release of Food Ingredients* (pp. 74–86). Washington DC, USA: American Chemical Society.

Dias, M. I., Ferreira, I. C. & Barreiro, M. F. (2015). Microencapsulation of bioactives for food applications. *Food and Function*, *6*, 1035-1052.

Dong, Z., Ma, Y., Hayat, K., Jia, C., Xia, S. & Zhang, X. (2011). Morphology and release profile of microcapsules encapsulating peppermint oil by complex coacervation. *Journal of Food Engineering*, *104*, 455-460.

Đorđević, V., Balanč, B., Belščak-Cvitanović, A., Lević, S., Trifković, K., Kalušević, A., Kostić, I., Komes, D., Bugarski, B. & Nedović, V. (2015). Trends in encapsulation technologies for delivery of food bioactive compounds. *Food Engineering Reviews*, *7*, 452-490.

Drusch, S. (2007). Sugar beet pectin: A novel emulsifying wall component for microencapsulation of lipophilic food ingredients by spray-drying. *Food Hydrocolloids*, *21*, 1223-1228.

Duran, N. & Marcato, P. D. (2013). Nanobiotechnology perspectives. Role of nanotechnology in the food industry: a review. *International Journal of Food Science and Technology*, *48*, 1127-1134.

El Bahri, Z. & Taverdet, J. L. (2007). Elaboration and characterisation of microparticles loaded by pesticide model. *Powder Technology*, *172*, 30-40.

Eratte, D., McKnight, S., Gengenbach, T. R., Dowling, K., Barrow, C. J. & Adhikari, B. P. (2015). Co-encapsulation and characterisation of omega-3

fatty acids and probiotic bacteria in whey protein isolate–gum Arabic complex coacervates. *Journal of Functional Foods*, *19*, 882-892.

Ersus, S. & Yurdagel, U. (2007). Microencapsulation of anthocyanin pigments of black carrot (*Daucus carota* L.) by spray drier. *Journal of Food Engineering*, *80*, 805-812.

Fang, Z. & Bhandari, B. (2010). Encapsulation of polyphenols - a review. *Trends in Food Science and Technology*, *21*, 510-523.

Fang, Z. & Bhandari, B. (2012). Spray drying, freeze drying and related processes for food ingredient and nutraceutical encapsulation. In: Garti, N. & McClements, D. J. (Eds), *Encapsulation Technologies and Delivery Systems for Food Ingredients and Nutraceuticals* (pp. 73-109). Cambridge, UK: Woodhead Publishing Limited.

Farhang, B., Kakuda, Y. & Corredig, M. (2012). Encapsulation of ascorbic acid in liposomes prepared with milk fat globule membrane-derived phospholipids. *Dairy Science and Technology*, *92*, 353-366.

Fathi, M., Mozafari, M. R. & Mohebbi, M. (2012). Nanoencapsulation of food ingredients using lipid based delivery systems. *Trends in Food Science and Technology*, *23*, 13-27.

Favaro-Trindade, C. S., Santana, A. S., Monterrey-Quintero, E. S., Trindade, M. A. & Netto, F. M. (2010). The use of spray drying technology to reduce bitter taste of casein hydrolysate. *Food Hydrocolloids*, *24*, 336-340.

Filion, M. C. & Phillips, N. C. (1997). Toxicity and immunomodulatory activity of liposomal vectors formulated with cationic lipids toward immune effector cells. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1329*, 345-356.

Fogler, B. B. & Kleinschmidt, R. V. (1938). Spray drying. *Industrial and Engineering Chemistry Research*, *30*, 1372–1384.

Fritzen-Freire, C. B., Prudêncio, E. S., Amboni, R. D., Pinto, S. S., Negrão-Murakami, A. N. & Murakami, F. S. (2012). Microencapsulation of bifidobacteria by spray drying in the presence of prebiotics. *Food Research International*, *45*, 306-312.

Frost & Sullivan (2005). Oppertunities in the microencapsulated food market. London. Retrieved from http://www.frost.com/prod/servlet/report-brochure.pag?id=B716-01-00-00-00 on 19.09.16.

Gandía-Herrero, F., Jiménez-Atiénzar, M., Cabanes, J., García-Carmona, F. & Escribano, J. (2010). Stabilization of the bioactive pigment of *Opuntia* fruits through maltodextrin encapsulation. *Journal of Agricultural and Food Chemistry*, *58*, 10646-10652.

Gharsallaoui, A., Roudaut, G., Chambin, O., Voilley, A. & Saurel, R. (2007). Applications of spray-drying in microencapsulation of food ingredients: An overview. *Food Research International*, *40*, 1107-1121.

Gibbs, B. F., Kermasha, S., Alli, I. & Mulligan, C. N. (1999). Encapsulation in the food industry: a review. *International Journal of Food Sciences and Nutrition*, *50*, 213-224.

Gouin, S. (2004). Microencapsulation: industrial appraisal of existing technologies and trends. *Trends in Food Science and Technology*, *15*, 330-347.

Gunasekaran, S., Ko, S. & Xiao, L. (2007). Use of whey proteins for encapsulation and controlled delivery applications. *Journal of Food Engineering*, *83*, 31-40.

Hede, P. D., Bach, P. & Jensen, A. D. (2007). Small-scale top-spray fluidised bed coating: Granule impact strength, agglomeration tendency and coating layer morphology. *Powder Technology*, *176*, 156-167.

Ho, T. M., Howes, T. & Bhandari, B. R. (2014). Encapsulation of gases in powder solid matrices and their applications: a review. *Powder Technology*, *259*, 87-108.

Homayouni, A., Azizi, A., Ehsani, M. R., Yarmand, M. S. & Razavi, S. H. (2008). Effect of microencapsulation and resistant starch on the probiotic survival and sensory properties of synbiotic ice cream. *Food Chemistry*, *111*, 50-55.

Igual, M., Ramires, S., Mosquera, L. H. & Martínez-Navarrete, N. (2014). Optimization of spray drying conditions for lulo (*Solanum quitoense* L.) pulp. *Powder Technology*, *256*, 233-238.

Iwami, K., Hattori, M., Yasumi, T. & Ibuki, F. (1988). Stability of gliadin-encapsulated unsaturated fatty acids against autoxidation. *Journal of Agricultural and Food Chemistry*, *36*, 160-164.

Jiménez, M., Garcia, H. S. & Beristain, C. I. (2004). Spray-drying microencapsulation and oxidative stability of conjugated linoleic acid. *European Food Research and Technology*, *219*, 588-592.

Jiménez, T., Turchiuli, C. & Dumoulin, E. (2006). Particles agglomeration in a conical fluidized bed in relation with air temperature profiles. *Chemical Engineering Science*, *61*, 5954-5961.

Jiménez-Colmenero, F. (2013). Potential applications of multiple emulsions in the development of healthy and functional foods. *Food Research International*, *52*, 64-74.

Jun-Xia, X., Hai-Yan, Y. & Jian, Y. (2011). Microencapsulation of sweet orange oil by complex coacervation with soybean protein isolate/gum Arabic. *Food Chemistry*, *125*, 1267-1272.

Kailasapathy, K. (2002). Microencapsulation of probiotic bacteria: technology and potential applications. *Current Issues in Intestinal Microbiology*, *3*, 39-48.

Katouzian, I. & Jafari, S. M. (2016). Nano-encapsulation as a promising approach for targeted delivery and controlled release of vitamins. *Trends in Food Science and Technology*, *53*, 34-48.

Katsuragi, Y., Sugiura, Y., Lee, C., Otsuji, K. & Kurihara, K. (1995). Selective inhibition of bitter taste of various drugs by lipoprotein. *Pharmaceutical Research*, *12*, 658-662.

Keller, B. C. (2001). Liposomes in nutrition. *Trends in Food Science and Technology*, *12*, 25-31.

Kerdudo, A., Dingas, A., Fernandez, X. & Faure, C. (2014). Encapsulation of rutin and naringenin in multilamellar vesicles for optimum antioxidant activity. *Food Chemistry*, *159*, 12-19.

Kheadr, E. E., Vuillemard, J. C. & El Deeb, S. A. (2000). Accelerated Cheddar cheese ripening with encapsulated proteinases. *International Journal of Food Science and Technology*, *35*, 483-495.

Kheadr, E. E., Vuillemard, J. C. & El-Deeb, S. A. (2002). Acceleration of Cheddar cheese lipolysis by using liposome-entrapped lipases. *Journal of Food Science*, *67*, 485-492.

Kim, K. I., Yoon Y. H. & Baek Y. J. (1996). Effects of rehydration media and immobilization in Ca-alginate on the survival of *Lactobacillus casei* and *Bifidobacterium bifidum*. *Korean Journal of Dairy Science*, *18*, 193-198.

Kirby, C. J, Brooker, B. E. & Law, B. A. (1987). Accelerated ripening of cheese using liposome-encapsulated enzyme. *International Journal of Food Science and Technology*, *22*, 355-375.

Kirby, C. J., Whittle, C. J., Rigby, N., Coxon, D. T. & Law, B. A. (1991). Stabilization of ascorbic acid by microencapsulation in liposomes. *International Journal of Food Science and Technology*, *26*, 437-449.

Koliandris, A. L. (2011). *Flow behaviour of biopolymer solutions and effect on saltiness perception*. Doctoral dissertation, University of Nottingham.

Kosaraju, S. L., Tran, C. & Lawrence, A. (2006). Liposomal delivery systems for encapsulation of ferrous sulfate: preparation and characterization. *Journal of Liposome Research*, *16*, 347-358.

Kuang, S. S., Oliveira, J. C. & Crean, A. M. (2010). Microencapsulation as a tool for incorporating bioactive ingredients into food. *Critical Reviews in Food Science and Nutrition*, *50*, 951-968.

Kumar, R. & Münstedt, H. (2005). Silver ion release from antimicrobial polyamide/silver composites. *Biomaterials*, *26*, 2081-2088.

Lang, J. K. & Vigo-Pelfrey, C. (1993). Quality control of liposomal lipids with special emphasis on peroxidation of phospholipids and cholesterol. *Chemistry and Physics of Lipids*, *64*, 19-29.

Laridi, R., Kheadr, E. E., Benech, R. O., Vuillemard, J. C., Lacroix, C. & Fliss, I. (2003). Liposome encapsulated nisin Z: optimization, stability and release during milk fermentation. *International Dairy Journal*, *13*, 325-336.

Law, B. A. & King, J. S. (1985). Use of liposomes for proteinase addition to Cheddar cheese. *Journal of Dairy Research*, *52*, 183-188.

Lee, D. H., Jin, B. H., Hwang, Y. I. & Lee, S. C. (2000). Encapsulation of bromelain in liposome. *Journal of Food Science and Nutrition*, *5*, 81–85.

Liolios, C. C., Gortzi, O., Lalas, S., Tsaknis, J. & Chinou, I. (2009). Liposomal incorporation of carvacrol and thymol isolated from the essential oil of *Origanum dictamnus* L. and in vitro antimicrobial activity. *Food Chemistry*, *112*, 77-83

Lakkis, J. M. (2007). Encapsulation and controlled release in bakery applications. In: Lakkis, J. M. (Ed.), *Encapsulation and Controlled Release Technologies in Food Systems* (pp. 116–177). Ames, IA, USA: Blackwell Publishing.

Lobato-Calleros, C., Rodriguez, E., Sandoval-Castilla, O., Vernon-Carter, E. J. & Alvarez-Ramirez, J. (2006). Reduced-fat white fresh cheese-like products obtained from W1/O/W2 multiple emulsions: Viscoelastic and high-resolution image analyses. *Food Research International*, *39*, 678-685.

Lobato-Calleros, C., Recillas-Mota, M. T., Espinosa-Solares, T., Alvarez-Ramirez, J. & Vernon-Carter, E. J. (2009). Microstructural and rheological properties of low-fat stirred yoghurts made with skim milk and multiple emulsions. *Journal of Texture Studies*, *40*, 657-675.

Maa, Y. F. & Hsu, C. C. (1999). Performance of sonication and microfluidisation for liquid–liquid emulsification. *Pharmaceutical Development and Technology*, *4*, 233-240.

Madene, A., Jacquot, M., Scher, J. & Desobry, S. (2006). Flavour encapsulation and controlled release–a review. *International Journal of Food Science and Technology*, *41*, 1-21.

Maherani, B., Arab-Tehrany, E., R Mozafari, M., Gaiani, C. & Linder, M. (2011). Liposomes: a review of manufacturing techniques and targeting strategies. *Current Nanoscience*, *7*, 436-452.

Malheiros, P. S., Cuccovia, I. M. & Franco, B. D. (2016). Inhibition of *Listeria monocytogenes* in vitro and in goat milk by liposomal nanovesicles containing bacteriocins produced by *Lactobacillus sakei* subsp. *sakei* 2a. *Food Control*, *63*, 158-164.

Marison, I., Peters, A. & Heinzen, C. (2004). Liquid core capsules for applications in biotechnology. In: Nedovic, V. & Willaert, R. (Eds),

Fundamentals of Cell Immobilisation Biotechnology (pp. 185-204). Dordrecht, Netherlands: Springer.

Martins, I. M., Barreiro, M. F., Coelho, M. & Rodrigues, A. E. (2014). Microencapsulation of essential oils with biodegradable polymeric carriers for cosmetic applications. *Chemical Engineering Journal*, *245*, 191-200.

Márquez-Ruiz, G., Velasco, J. & Dobarganes, C. (2000). Evaluation of oxidation in dried microencapsulated fish oils by a combination of adsorption and size exclusion chromatography. *European Food Research and Technology*, *211*, 13-18.

Matalanis, A., Jones, O. G. & McClements, D. J. (2011). Structured biopolymer-based delivery systems for encapsulation, protection, and release of lipophilic compounds. *Food Hydrocolloids*, *25*, 1865-1880**.**

Matsuno, R. & Adachi, S. (1993). Lipid encapsulation technology-techniques and applications to food. *Trends in Food Science and Technology*, *4*, 256-261.

McAuliffe, L. N., Kilcawley, K. N., Sheehan, J. J. & McSweeney, P. L. H. (2016). Manufacture and incorporation of liposome-entrapped ethylenediaminetetraacetic acid into model miniature Gouda-type cheese and subsequent effect on starter viability, pH, and moisture content. *Journal of Food Science, 81, C2708-2712.*

McClements, D. J. (2012). Requirements for food ingredient and nutraceutical delivery systems. In: Garti, N. & McClements, D. J. (Eds), *Encapsulation*

Technologies and Delivery Systems for Food Ingredients and Nutraceuticals (pp. 3-18). Cambridge, UK: Woodhead Publishing Limited.

McClements, D. J. & Li, Y. (2010). Review of *in vitro* digestion models for rapid screening of emulsion-based systems. *Food and Function*, *1*, 32-59.

McClements, D. J., Decker, E. A. & Weiss, J. (2007). Emulsion-based delivery systems for lipophilic bioactive components. *Journal of Food Science*, *72*, R109-R124.

McClements, D. J., Decker, E. A., Park, Y. & Weiss, J. (2009). Structural design principles for delivery of bioactive components in nutraceuticals and functional foods. *Critical Reviews in Food Science and Nutrition*, *49*, 577-606.

Meiners, J. A. (2012). Fluid bed microencapsulation and other coating methods for food ingredient and nutraceutical bioactive compounds. In: Garti, N. & McClements, D. J. (Eds), *Encapsulation Technologies and Delivery Systems for Food Ingredients and Nutraceuticals* (pp. 151-176). Cambridge, UK: Woodhead Publishing Limited.

Mohammadi, R., Mahmoudzade, M., Atefi, M., Khosravi-Darani, K. & Mozafari, M. R. (2015). Applications of nanoliposomes in cheese technology. *International Journal of Dairy Technology*, *68*, 11-23.

Moreau, D. L. & Rosenberg, M. (1996). Oxidative stability of anhydrous milkfat microencapsulated in whey proteins. *Journal of Food Science*, *61*, 39-43.

Mozafari, M. R., Flanagan, J., Matia-Merino, L., Awati, A., Omri, A., Suntres, Z. E. & Singh, H. (2006). Recent trends in the lipid-based nanoencapsulation of antioxidants and their role in foods. *Journal of the Science of Food and Agriculture*, *86*, 2038-2045.

Nazzaro, F., Orlando, P., Fratianni, F. & Coppola, R. (2012). Microencapsulation in food science and biotechnology. *Current Opinion in Biotechnology*, *23*, 182-186.

Nayak, C. A. & Rastogi, N. K. (2010). Effect of selected additives on microencapsulation of anthocyanin by spray drying. *Drying Technology*, *28*, 1396-1404.

Nedovic, V., Kalusevic, A., Manojlovic, V., Levic, S. & Bugarski, B. (2011). An overview of encapsulation technologies for food applications. *Procedia Food Science*, *1*, 1806-1815.

Nedovic, V., Kalusevic, A., Manojlovic, V., Petrovic, T. & Bugarski, B. (2013). Encapsulation systems in the food industry. In: Yanniotis, S., Taoukis, P., Stoforos, N. G. & Karathanos, V. T. (Eds), *Advances in Food Process Engineering Research* (pp. 229-253). New York, USA: Springer.

New, R. R. C. (1990). Preparation of liposomes. In: New, R. R. C. (Ed), *Liposomes: A Practical Approach*, *Vol 1*, 1st Ed. (pp. 33-104). New York, USA: Oxford University Press.

Nii, T., Takamura, A., Mohri, K. & Ishii, F. (2003). Factors affecting physicochemical properties of liposomes prepared with hydrogenated

purified egg yolk lecithins by the microencapsulation vesicle method. *Colloids and Surfaces B: Biointerfaces*, *27*, 323-332.

Nongonierma, A. B., Abrlova, M., Fenelon, M. A. & Kilcawley, K. N. (2009). Evaluation of two food grade proliposomes to encapsulate an extract of a commercial enzyme preparation by microfluidisation. *Journal of Agricultural and Food Chemistry*, *57*, 3291-3297.

Nongonierma, A. B., Abrlova, M. & Kilcawley, K. N. (2013). Encapsulation of a lactic acid bacteria cell-free extract in liposomes and use in Cheddar cheese ripening. *Foods*, *2*, 100-119.

Norton, J. E. & Norton, I. T. (2010). Designer colloids - towards healthy everyday foods? *Soft Matter*, *6*, 3735-3742.

O'Riordan, K., Andrews, D., Buckle, K. & Conway, P. (2001). Evaluation of microencapsulation of a *Bifidobacterium* strain with starch as an approach to prolonging viability during storage. *Journal of Applied Microbiology*, *91*, 1059-1066.

Okuro, P. K., Thomazini, M., Balieiro, J. C., Liberal, R. D. & Fávaro-Trindade, C. S. (2013a). Co-encapsulation of *Lactobacillus acidophilus* with inulin or polydextrose in solid lipid microparticles provides protection and improves stability. *Food Research International*, *53*, 96-103.

Okuro, P. K., de Matos Junior, F. E. & Favaro-Trindade, C. S. (2013b). Technological challenges for spray chilling encapsulation of functional food ingredients. *Food Technology and Biotechnology*, *51*, 171-182.

Oxley, J. D. (2012). Spray cooling and spray chilling for food ingredient and nutraceutical encapsulation. In: Garti, N. & McClements, D. J. (Eds), *Encapsulation Technologies and Delivery Systems for Food Ingredients and Nutraceuticals* (pp. 110-130). Cambridge, UK: Woodhead Publishing Limited.

Parthasarathi, S., Ezhilarasi, P. N., Jena, B. S. & Anandharamakrishnan, C. (2013). A comparative study on conventional and microwave-assisted extraction for microencapsulation of *Garcinia* fruit extract. *Food and Bioproducts Processing*, *91*, 103-110.

Piorkowski, D. T. & McClements, D. J. (2014). Beverage emulsions: Recent developments in formulation, production, and applications. *Food Hydrocolloids*, *42*, 5-41.

Poshadri, A. & Aparna, K. (2010). Microencapsulation technology: a review. *Journal of Research ANGRAU*, *38*, 86-102.

Prata, A. S., Maudhuit, A., Boillereaux, L. & Poncelet, D. (2012). Development of a control system to anticipate agglomeration in fluidised bed coating. *Powder Technology*, *224*, 168-174.

Rashidinejad, A., Birch, E. J., Sun-Waterhouse, D. & Everett, D. W. (2014). Delivery of green tea catechin and epigallocatechin gallate in liposomes incorporated into low-fat hard cheese. *Food Chemistry*, *156*, 176-183.

Risch, S. J. & Reineccius, G. (1995). Encapsulation and controlled release of food ingredients. ACS Symposium Series. American Chemical Society.

Rocha, G. A., Fávaro-Trindade, C. S. & Grosso, C. R. F. (2012). Microencapsulation of lycopene by spray drying: characterization, stability and application of microcapsules. *Food and Bioproducts Processing*, *90*, 37-42.

Rodríguez-Nogales, J. M. & López, A. D. (2006). A novel approach to develop *β*-galactosidase entrapped in liposomes in order to prevent an immediate hydrolysis of lactose in milk. *International Dairy Journal*, *16*, 354-360.

Ruozi, B., Tosi, G., Leo, E. & Vandelli, M. A. (2007). Application of atomic force microscopy to characterize liposomes as drug and gene carriers. *Talanta*, *73*, 12-22.

Santana, R. C., Perrechil, F. A. & Cunha, R. L. (2013). High-and low-energy emulsifications for food applications: a focus on process parameters. *Food Engineering Reviews*, *5*, 107-122.

Saleh, K., Steinmetz, D. & Hemati, M. (2003). Experimental study and modeling of fluidized bed coating and agglomeration. *Powder Technology*, *130*, 116-123.

Saroglou, V., Hatziantoniou, S., Smyrniotakis, M., Kyrikou, I., Mavromoustakos, T., Zompra, A., Magafa, V., Cordopatis, P. & Demetzos, C. (2006). Synthesis, liposomal formulation and thermal effects on phospholipid bilayers of leuprolide. *Journal of Peptide Science*, *12*, 43-50.

Sas, B., Peys, E. & Helsen, M. (1999). Efficient method for (lyso) phospholipid class separation by high-performance liquid chromatography using an

evaporative light-scattering detector. *Journal of Chromatography A*, *864*, 179-182.

Schell, D. & Beermann, C. (2014). Fluidized bed microencapsulation of *Lactobacillus reuteri* with sweet whey and shellac for improved acid resistance and in-vitro gastro-intestinal survival. *Food Research International*, *62*, 308-314.

Schmitt, C. & Turgeon, S. L. (2011). Protein/polysaccharide complexes and coacervates in food systems. *Advances in Colloid and Interface Science*, *167*, 63-70.

Shaw, P. E. & Wilson, C. W. (1983). Debittering citrus juices with *β*-cyclodextrin polymer. *Journal of Food Science*, *48*, 646-647.

Shaw, L. A., McClements, D. J. & Decker, E. A. (2007). Spray-dried multilayered emulsions as a delivery method for ω-3 fatty acids into food systems. *Journal of Agricultural and Food Chemistry*, *55*, 3112-3119.

Silvestre, C., Duraccio, D. & Cimmino, S. (2011). Food packaging based on polymer nanomaterials. *Progress in Polymer Science*, *36*, 1766-1782.

Singh, H., Thompson, A., Liu, W. & Corredig, M. (2012). Liposomes as food ingredients and nutraceutical delivery systems, In: Garti, N. & McClements, D J. (Eds), *Encapsulation Technologies and Delivery Systems for Food Ingredients and Nutraceuticals* (pp. 287-318). Cambridge, UK: Woodhead Publishing Limited.

Sozer, N. & Kokini, J. L. (2009). Nanotechnology and its applications in the food sector. *Trends in Biotechnology*, *27*, 82-89.

Spitsberg, V. L. (2005). Invited review: Bovine milk fat globule membrane as a potential nutraceutical. *Journal of Dairy Science*, *88*, 2289-2294.

Stratulat, I., Britten, M., Salmieri, S., Fustier, P., St-Gelais, D., Champagne, C. P. & Lacroix, M. (2015). Enrichment of cheese with vitamin D 3 and vegetable omega-3. *Journal of Functional Foods*, *13*, 300-307.

Sułkowski, W. W., Pentak, D., Nowak, K. & Sułkowska, A. (2005). The influence of temperature, cholesterol content and pH on liposome stability. *Journal of Molecular Structure*, *744*, 737-747.

Tackenberg, M. W. & Kleinebudde, P. (2015). Encapsulation of liquids via extrusion-A review. *Current Pharmaceutical Design*, *21*, 5815-5828.

Takahashi, M., Kitamoto, D., Imura, T., Oku, H., Takara, K. & Wada, K. (2008). Characterization and bioavailability of liposomes containing a ukon extract. *Bioscience, Biotechnology, and Biochemistry*, *72*, 1199-1205.

Taylor, T. M., Gaysinsky, S., Davidson, P. M., Bruce, B. D. & Weiss, J. (2007). Characterization of antimicrobial-bearing liposomes by ζ-potential, vesicle size, and encapsulation efficiency. *Food Biophysics*, *2*, 1-9.

Tesoriere, L., Bongiorno, A., Pintaudi, A. M., D'Anna, R., D'Arpa, D. & Livrea, M. A. (1996). Synergistic interactions between vitamin A and vitamin E against lipid peroxidation in phosphatidylcholine liposomes. *Archives of Biochemistry and Biophysics*, *326*, 57-63.

Tiede, K., Boxall, A. B., Tear, S. P., Lewis, J., David, H. & Hassellöv, M. (2008). Detection and characterization of engineered nanoparticles in food and the environment. *Food Additives and Contaminants*, *25*, 795-821.

Thapon, J. L. & Brule, G. (1986). Effects of pH and ionic strength on lysozyme-casein affinity. *Lait*, *66*, 19-30.

Thompson, A. K. & Singh, H. (2006). Preparation of liposomes from milk fat globule membrane phospholipids using a microfluidizer. *Journal of Dairy Science*, *89*, 410-419.

Thompson, A. K., Haisman, D. & Singh, H. (2006). Physical stability of liposomes prepared from milk fat globule membrane and soya phospholipids. *Journal of Agricultural and Food Chemistry*, *54*, 6390-6397.

Tonon, R. V., Brabet, C. & Hubinger, M. D. (2010). Anthocyanin stability and antioxidant activity of spray-dried açai (*Euterpe oleracea* Mart.) juice produced with different carrier agents. *Food Research International*, *43*, 907-914.

Turanek, J. (1994). Fast-protein liquid chromatography system as a tool for liposome preparation by the extrusion procedure. *Analytical Biochemistry*, *218*, 352-357.

Valero-Cases, E. & Frutos, M. J. (2015). Effect of different types of encapsulation on the survival of *Lactobacillus plantarum* during storage with inulin and in vitro digestion. *LWT-Food Science and Technology*, *64*, 824-828.

Velikov, K. P. & Pelan, E. (2008). Colloidal delivery systems for micronutrients and nutraceuticals. *Soft Matter*, *4*, 1964-1980.

Vernooij, E. A. A. M., Kettenes-van den Bosch, J. J. & Crommelin, D. J. A. (1998). Rapid determination of acyl chain position in egg

phosphatidylcholine by high performance liquid chromatography/electrospray mass spectrometry. *Rapid Communications in Mass Spectrometry*, *12*, 83-86.

Vesper, H., Schmelz, E. M., Nikolova-Karakashian, M. N., Dillehay, D. L., Lynch, D. V. & Merrill, A. H. (1999). Sphingolipids in food and the emerging importance of sphingolipids to nutrition. *Journal of Nutrition*, *129*, 1239-1250.

Walde, P. & Ichikawa, S. (2001). Enzymes inside lipid vesicles: preparation, reactivity and applications. *Biomolecular Engineering*, *18*, 143-177.

Wampler, D. J. (1992). Flavour encapsulation: a method for providing maximum stability for dry flavour systems. *Cereal Foods World*, *37*, 817-820.

Weinbreck, F., Minor, M. & De Kruif, C. G. (2004a). Microencapsulation of oils using whey protein/gum Arabic coacervates. *Journal of Microencapsulation*, *21*, 667-679.

Weinbreck, F., Nieuwenhuijse, H., Robijn, G. W. & de Kruif, C. G. (2004b). Complexation of whey proteins with carrageenan. *Journal of Agricultural and Food Chemistry*, *52*, 3550-3555.

Were, L. M., Bruce, B. D., Davidson, P. M. & Weiss, J. (2003). Size, stability, and entrapment efficiency of phospholipid nanocapsules containing polypeptide antimicrobials. *Journal of Agricultural and Food Chemistry*, *51*, 8073-8079.

Were, L. M., Bruce, B., Davidson, P. M. & Weiss, J. (2004). Encapsulation of nisin and lysozyme in liposomes enhances efficacy against *Listeria monocytogenes*. *Journal of Food Protection*, *67*, 922-927.

Westhaus, E. & Messersmith, P. B. (2001). Triggered release of calcium from lipid vesicles: a bioinspired strategy for rapid gelation of polysaccharide and protein hydrogels. *Biomaterials*, *22*, 453-462.

Xu, Y., Wang, L., Tong, Y., Xiang, S., Guo, X., Li, J., Gao, H. & Wu, X. (2016). Study on the preparation, characterization, and release behavior of carbosulfan/polyurethane microcapsules. *Journal of Applied Polymer Science*. DOI: 10.1002/app.43844.

Yang, S., Mao, X. Y., Li, F. F., Zhang, D., Leng, X. J., Ren, F. Z. & Teng, G. X. (2012). The improving effect of spray-drying encapsulation process on the bitter taste and stability of whey protein hydrolysate. *European Food Research and Technology*, *235*, 91-97.

Zbicinski, I., Delag, A., Strumillo, C. & Adamiec, J. (2002). Advanced experimental analysis of drying kinetics in spray drying. *Chemical Engineering Journal*, *86*, 207-216.

Zhang, X. M., Patel, A. B., de Graaf, R. A. & Behar, K. L. (2004). Determination of liposomal encapsulation efficiency using proton NMR spectroscopy. *Chemistry and Physics of Lipids*, *127*, 113-120.

Zheng, H., Jiménez-Flores, R., Gragson, D. & Everett, D. W. (2014). Phospholipid architecture of the bovine milk fat globule membrane using

giant unilamellar vesicles as a model. *Journal of Agricultural and Food Chemistry*, *62*, 3236-3243.

Zou, L. Q., Liu, W., Liu, W. L., Liang, R. H., Li, T., Liu, C. M., Cao, Y. L., Niu, J. & Liu, Z. (2014). Characterization and bioavailability of tea polyphenol nanoliposome prepared by combining an ethanol injection method with dynamic high-pressure microfluidisation. *Journal of Agricultural and Food Chemistry*, *62*, 934-941.

Zuidam, J. N., De Vruech, R. & Crommelin, D. J. A. (2003). Characterization of liposomes. In: Torchilin, V. P. & Weissig, V. (Eds), *Liposomes: A Practical Approach*, 2nd Ed. (pp.50.53). New York, USA: Oxford University Press.

Zuidam, N. J. & Shimoni, E. (2010). Overview of microencapsulates for use in food products or processes and methods to make them. In: *Encapsulation Technologies for Active Food Ingredients and Food Processing* (pp. 3-29). New York, USA: Springer.

Objectives

Ireland is a major producer of Cheddar cheese; with almost half of Cheddar being manufactured exported to the UK market. European cheese consumers in Italy, France, Germany, Switzerland, Norway, etc. are less likely to consume Cheddar cheese due to their preference for continental-type cheese varieties. As a result, any threat to the UK market may negatively impact Irish Cheddar cheese producers. Therefore, it is necessary for Cheddar cheese manufactures to develop and expand the Cheddar and hybrids, together with continental-type cheese portfolios in a bid to remain competitive within new and existing markets.

As a result, research pertaining to the physicochemical, textural and functional properties of Cheddar-hybrid cheese varieties is essential in order to determine their suitability for manufacture on existing Cheddar plants and their potential applications (composition and functionality). Additionally, research into the feasibility of manufacturing continental-type cheese varieties within established cheese production facilities is also essential for the development of novel products with diverse characteristics and functionalities (texture and rheology) in order to attract new customers/markets.

The major objective of this thesis was to investigate the role of calcium (Ca) in modulating the textural and rheological properties of brine-salted Gouda-type cheese during ripening, with particular emphasis on the alteration of Ca between the soluble (SOL) and insoluble (INSOL) phases. Several studies, mostly pertaining to dry-salted Cheddar and Mozzarella variants, have described the dynamic equilibrium which exists between INSOL Ca phosphate in milk/cheese

(CCP) and the SOL Ca in the aqueous phase of dairy products, and its influence on cheese texture and rheology. This thesis aimed to explore the association between the changes in CCP concentration and the textural, functional and rheological properties of brine-salted continental-type cheese during ripening. In addition, this thesis aimed to investigate a Swiss-Cheddar hybrid-type cheese with the potential to be easily manufactured on most existing Cheddar cheese plants in order to diversify a cheese manufacturer's portfolio, with little need for major investment (i.e., can be produced in an existing plant, no new equipment/staff needed). The major areas investigated included the development of proteolysis, lipolysis and Ca equilibrium in a Swiss-Cheddar-hybrid cheese and their influence on the volatile flavour profile and textural and functional changes throughout ripening.

The work undertaken in this thesis has expanded knowledge of the role of physicochemical and biochemical process, and their interactions, with particular emphasis on Ca equilibrium, in the development of textural and rheological properties of brine-salted continental-type and Cheddar-hybrid-type cheese varieties during ripening. Additionally, the subject matter of this thesis is relevant to the developments in the Irish and global dairy industry and will be of great assistance to the future developments in cheese product design and development.

Chapter 3

Effect of cooking temperature and addition of calcium chelating salts on the calcium equilibrium, textural and rheological properties of Gouda-type cheese during ripening

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Abstract

Altering moisture content through varying cooking temperature (31 and 38 ˚C) and insoluble (INSOL) calcium (Ca) levels by addition of ethylenediamine tetraacetic acid (EDTA) on the textural and rheological properties of Gouda-type cheese during ripening was investigated. Four Gouda-type cheeses were manufactured: a control (cook temperature (CT): 38 ˚C), high moisture control (HMCC) (CT: 31 ˚C), low EDTA (LEDTA) (CT: 38 ˚C, 2.5 g EDTA/L whey removed) and high EDTA cheese (HEDTA) (CT: 38 ˚C, 5 g EDTA/L whey removed). The EDTA was added to the curd/whey mixture during a second wash stage, with an equal volume of water added to the control and HMCC cheese. Moisture content increased and total Ca, INSOL Ca and pH decreased with increasing EDTA addition levels and decreased cooking temperature. Physicochemical changes were greatest for cheese manufactured with the highest concentration of EDTA. Hardness of cheeses decreased throughout ripening - the magnitude of which was greatest for cheese containing the highest moisture content (HMCC). Meltability increased in all cheeses during ripening, with the HEDTA cheese containing the lowest levels of INSOL Ca exhibiting the greatest % increase in melt diameter. The results of this study suggest that increasing moisture content has as great an effect on manipulating the textural properties of Gouda-type cheese as changing the ratio of soluble to INSOL Ca.

3.1 Introduction

The factors responsible for influencing the textural, rheological and melting properties of cheese include composition (moisture, salt, salt in moisture (S/M) ratio, fat and protein) (Chevanan *et al*., 2006), pH during manufacture and ripening (Lawrence *et al*., 1987; Watkinson *et al*., 2001; Pastorino *et al*., 2003; Sheehan and Guinee, 2004; Lee *et al*., 2005; Upreti and Metzger, 2007), total and insoluble (INSOL) calcium (Ca) content (Lucey *et al*., 2005; Mizuno and Lucey, 2005; O'Mahony *et al*., 2006; Chevanan *et al*., 2007; Choi *et al*., 2008; Fröhlich-Wyder *et al*., 2009), proteolysis (O'Mahony *et al*., 2005; Fathollahi *et al*., 2010), curd washing technique (Lee *et al*., 2011; Hou *et al*., 2014) and acidification methods (Joshi *et al*., 2003).

Studying the effects of these factors independently has proven difficult as they are inter-related: as the pH of cheese is reduced during manufacture due to acidification, Ca is lost from the casein (CN) particles (Lucey and Fox, 1993), subsequently resulting in increased proteolysis (Lucey *et al*., 2005). Numerous authors have examined the effect of Ca binding salts such as trisodium citrate (TSC), pH and calcium phosphate addition on Cheddar cheese properties and found that lower Ca content in cheese resulted in decreased hardness and more rapid softening leading to increased meltability during ripening (Lucey and Fox, 1993; Guinee *et al*., 2002; Joshi *et al*., 2003; Brickley *et al*., 2009). Recently however, it has been suggested that total Ca alone is not the most accurate predictor of the textural and functional properties of cheese, but rather the distribution of INSOL and soluble (SOL) Ca in the cheese during ripening.

Evidence is growing that the CN-bound Ca; i.e., INSOL Ca, may play a more significant role in controlling cheese textural, rheological and melting properties due to its direct influence on casein-casein interactions (Lucey and Fox, 1993; Pastorino *et al*., 2003; Lucey *et al*., 2003, 2005; Hassan *et al*., 2004; Lee *et al*., 2005; Wang *et al*., 2011). O'Mahony *et al*. (2006) demonstrated that Cheddar cheese containing reduced total and INSOL Ca levels had greater meltability compared with the same cheese with increased total and INSOL Ca levels.

Addition of Ca chelating salts to cheese milk prior to rennet addition or at the salting stage of Cheddar cheese manufacture causes reduced INSOL Ca levels, resulting in the solubilisation and hydration of caseins, leading to softer textural properties. Mizuno and Lucey (2005) reported that addition of TSC to *pasta filata* cheese during manufacture caused a reduction in INSOL Ca, due to Ca binding, resulting in the solubilisation and hydration of the caseins and ultimately reduced hardness and increased meltability. Choi *et al*. (2008) manufactured directly acidified cheeses with varying levels of INSOL Ca through the addition of EDTA to skim milk prior to acidification. The authors reported higher loss tangent (LT) values (meltability index) in EDTA-treated cheeses and attributed this to the reduction in INSOL Ca content. Brickley *et al*. (2009) observed a reduction in the buffering curves of Cheddar cheeses supplemented with TSC added at salting, which is related to the levels of INSOL Ca present in the cheese. These authors found that cheese supplemented with the highest concentration of TSC had the softest texture and attributed this result to the changes in Ca equilibrium of the cheese (i.e., shift in proportion of INSOL to SOL forms as well as the increased total Ca content with the addition of $CaCl_2$).

To date, work relating to the study of the effects of total Ca concentration, Ca equilibrium or both in influencing the textural and melting properties of cheese has been largely concentrated on dry-salted Cheddar (Creamer *et al*., 1985; Lee *et al*., 2005; O'Mahony *et al*., 2005) and Mozzarella cheeses (Metzger *et al*., 2000; Joshi *et al*., 2003; Sheehan and Guinee, 2004). Consequently, little knowledge exists with regards to the influence of these factors on the textural properties of brine-salted continental cheese varieties such as Gouda. Therefore, the objectives of this study were to investigate the effects of the addition of EDTA to the curd/whey mixture during washing, and of decreased cooking temperature, on the changes to Ca equilibrium, textural and rheological properties of brine-salted Gouda-type cheese during ripening; in a bid to diversify this already well established cheese variety on already existing chees manufacturing facilities.

3.2 Materials and methods

3.2.1 Cheese manufacture

Four 5 kg Gouda-type cheeses were manufactured in triplicate in the food processing facility at University College Cork, Ireland. A control, high moisture control (HMCC), low concentration EDTA (LEDTA) and high concentration EDTA (HEDTA) cheese was manufactured as described in Table 3.1. Raw milk (200 L) was heated to 50 ˚C, separated and standardised to a casein:fat ratio of 0.9:1.0. Standardised milk was pasteurised, separated into 4 vats, each containing 50 L of milk and cooled to 31 ˚C. B11 starter culture (*Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp *lactis*, *Leuconostoc mesenteroides* subsp. *cremoris* and a citrate-positive *Lactococcus lactis*) (Chr. Hansen Ltd., Little Island, Co. Cork, Ireland) was added to milk at a level of 0.03% (w/v) and sodium nitrate (Sigma Aldrich, Dublin, Ireland) at a level of 0.006% (w/v) and allowed 30 min to ripen. Following 30 min ripening, 1 M $CaCl_2$ (Sigma Aldrich, Dublin, Ireland) and chymosin (CHY-MAX® M, Chr. Hansen Ltd., Little Island, Co. Cork, Ireland) at a rate of 0.09% and 0.035% (w/v), respectively, was added to each vat, stirred gently for 2 min and given 50 min to coagulate. Once the coagulum had formed, the curds were cut, allowed to heal for 5 min, before stirring gently for 20 min. After stirring, the curds were washed; one third of the whey was removed (12 L) from all vats followed by the addition of warm water (54 ˚C) until the curds/whey mixture reached 38 ˚C (vat 1, 3 and 4). The same volume of water (31 ˚C) was added to vat 2 (HMCC). Curds were cooked at 38 ˚C (vat 1, 3 and 4) or 31 ˚C (vat 2) for 20 min.

Table 3.1 Cheese manufacture cooking temperatures and levels of addition of EDTA at cooking (g EDTA/L whey removed) for control, high moisture control (HMCC), low concentration EDTA (LEDTA) and high concentration EDTA (HEDTA) Gouda-type cheeses.

Treatment	**Vat**	**EDTA**	**Cook temperature**
	#	(g/L whey removed)	(˚C)
Control	1	0.0	38
HMCC	2	0.0	31
LEDTA	3	2.5	38
HEDTA	4	5.0	38

Following cooking, a second wash stage was implemented, 15 L of whey was removed and replaced with 10 L of water (control and HMCC vats) or EDTA dissolved in 10 L of water (Table 3.1), this was followed by 90 min stirring. Following stirring, the whey was drained and collected, curds were placed in a 5 kg mould lined with cheese cloth, the mould was placed back in the vat with a 30 kg weight placed on top, the whey was reintroduced back into the vat and the curds were allowed to press under the whey for 30 min. After pressing under the whey, the moulds were removed from the vats and placed on a vertical press (1.03 bar) overnight. The cheeses were removed from the press and placed in brine solutions (10 kg H_2O, 20% (w/v) NaCl and 0.05% (w/v) $CaCl_2$) for 35 h. After brining, the cheeses were vacuum packed and ripened at 10 ˚C for 112 d. All Gouda-type cheeses were manufactured in triplicate on three separate manufacturing days.

3.2.2 Chemical analysis

The composition and pH of the control, HMCC, LEDTA and HEDTA Gouda-type cheeses were determined at 14 d post manufacture. The moisture content of the cheeses was determined using an oven-drying method (IDF, 1982). The pH was measured using a calibrated pH meter on cheese slurry made from 10 g cheese and 10 mL of deionised water. Protein content was determined by the macro-Kjeldahl method (N x 6.38; IDF, 1986), fat by the Gerber method (IIRS, 1955) and salt was measured by using a potentiometric end-point determination method (Fox, 1963).

3.2.3 Determination of the proportions of soluble and insoluble calcium

The proportion of total calcium in the insoluble form in Gouda-type cheeses was determined at 7, 28 and 112 d of ripening using the acid-base titration method described by Hassan *et al*. (2004). The total calcium content of the cheeses was determined using atomic absorption spectroscopy as described by O'Mahony *et al*. (2005).

3.2.4 Proteolysis

The pH 4.6 soluble and insoluble fractions of cheese at 7 and 112 d of ripening were prepared in triplicate as described by Kuchroo and Fox (1982). The nitrogen content of the pH 4.6-soluble fraction was determined by the macro-Kjeldahl method (N x 6.38; IDF, 1986).

Urea–polyacrylamide gel electrophoresis (urea-PAGE) was carried out on the pH 4.6-insoluble fractions of cheeses using the procedure described by O'Mahony *et al*. (2005). Samples were run through the stacking gel at 280 V and the separating gel at 300 V. Gels were stained using Coomassie Brilliant Blue G250 (Blakesley and Boezi, 1977) and destained by several washes with distilled water.

Peptide profiles of pH 4.6-soluble fractions were obtained by reverse-phase HPLC using an ultra-performance liquid chromatographer (UPLC) which consisted of a Waters Acquity UPLC H-Class Core System with an Acquity UPLC TUV Detector (dual wavelength) and Acquity Column Heater 30-A. The system was interfaced with Empower 3 software (Waters Corp., Milford, MA, USA). The core system includes an Acquity UPLC H-Class quaternary solvent manager, an H-Class Sample Manager-FTN and a CH-A column heater. The column used was an Acquity UPLC® Peptide BEH C18, 130 Å, 1.7 m, 2.1 x 100 mm column. Elution was monitored at 214 nm and a mobile phase of two solvents, A, 0.1 % (v/v) trifluoroacetic acid (TFA, sequential grade, Sigma-Aldrich, St Louis, MO, USA) in deionized HPLC grade water (Milli-Q system, Millipore, Cork, Ireland) and B, 0.1 % (v/v) TFA in acetonitrile (HPLC grade, Sigma-Aldrich, Steinheim am Albuch, Germany) was used. The pH 4.6-soluble fraction samples were filtered through 0.22 μm cellulose acetate filter (Sartorius GmbH, Gottingen, Germany) and an aliquot (3.3 μL) of the filtrate was injected on the column at an eluent flow rate of 0.32 mL min^{-1}. The elution gradient used is shown in Table 3.2. Data acquisition is from 0 to 20.01 min. Time between

injections was 2 min at 100 % solvent A. A blank of 100% solvent A was used at the start.

3.2.5 Determination of textural and rheological properties of Gouda-type cheese during ripening

3.2.5.1 Texture profile analysis

Texture profile analysis (TPA) was performed using a Texture Analyser TA-XT2i (Stable Micro Systems, Godalming, Surrey, UK) according to the method of Cooke and McSweeney (2013). Hardness was defined according to Bourne (1978) and measured at 7, 14, 28, 56, 84 and 112 d of ripening. Five replicate samples from each cheese were compressed at each ripening time point.

3.2.4.2 Dynamic small amplitude oscillatory rheology

Rheological analysis of cheese samples was performed using a AR-G2 Controlled Stress Rheometer (TA Instruments, Leatherhead, UK) as describe by Cooke and McSweeney (2013), with the following modifications: cheese discs were not glued to the base plate of the Rheometer; storage modulus (G'), loss modulus (G'') and loss tangent (LT) were recorded continuously at a low amplitude shear strain of 0.1 % at a frequency of 1.0 Hz over 20 min during which the temperature was increased from 20 to 80 ˚C.

Table 3.2 Elution gradient for reverse phase ultra-performance liquid chromatography (RP-UPLC) analysis of cheese.

Time	**Flow Rate**	**Solvent A**	**Solvent B**
min	mL min^{-1}	%	%
0.00	0.32	100	0
0.58	0.32	100	0
11.27	0.32	50	50
12.43	0.32	50	50
13.21	0.32	5	95
14.38	0.32	5	95
15.15	0.32	0	100
16.51	0.32	100	0
20.01	0.32	100	0

3.2.5.3 Cheese meltability analysis

Cheese meltability was performed using the Schreiber melt test procedure (232 ˚C) (Altan *et al*., 2005). Cheese samples were prepared and stored according to the procedure described by Cooke and McSweeney (2013). Results were expressed as a % increase in diameter of the cheese discs. Analysis on each cheese sample was performed in triplicate at 7, 14, 28, 56, 86 and 112 d of ripening.

3.2.6 Quantification of EDTA in cheese and whey

The concentration of EDTA present in whey and cheese fractions of Gouda-type cheeses were determined according to the method described by McAuliffe *et al*. (2016). The pH 4.6-soluble fractions were extracted from the cheese using the method described by Kuchroo and Fox (1982). The pH 4.6-soluble extracts and whey samples were diluted 1:100 using de-ionised water (Milli-Q system,

Millipore, Cork, Ireland.) and filtered through 0.45 μM Minisart RC 15 (Sartorius, Goettingen, Germany) before injection onto the column.

3.2.7 Statistical analysis

Analysis of variance (ANOVA) was performed on cheese composition and proteolysis at a significance level of $P \leq 0.05$. A split-plot design was used to evaluate the effect of treatment (control, HMCC, LEDTA and HEDTA), ripening time and their interactions on proteolysis, meltability, hardness, chewiness, gumminess, springiness, G', G'' and LT_{max} values. The ANOVA for the split-plot designs was performed using a general linear model (GLM) procedure. When significant differences were found ($P \leq 0.05$), the treatment means were analysed by Tukey's multiple comparison test. All analyses were performed using MiniTab® 16 (MiniTAB Inc., State College, PA, USA).

3.3 Results and discussion

Preliminary trials involved the addition of EDTA at different levels (7.5, 19, 23, 31, 39 and 47 g EDTA/L whey removed) to the curds and whey mixture of Gouda-type cheese which resulted in a decrease in pH, with the magnitude of this effect increasing with increasing EDTA levels (Figure 3.1). At addition levels greater than 7.5 g EDTA/L whey, curd pieces presented an inability to fuse together and brittleness increased, which was attributed to the low pH of these cheeses. Further preliminary trials highlighted that in addition to decreasing the pH of cheese, EDTA (2.5 and 5 g EDTA/L whey) also caused an increase in the moisture content of the cheese. Therefore, a high moisture cheese (not containing EDTA) was manufactured in order to act as a control cheese for the HEDTA cheese, to enable the distinction of the effects of increased moisture content and INSOL Ca content on the textural and rheological properties of the cheese.

3.3.1 pH Changes during cheese manufacture

The changes in pH during Gouda cheese manufacture for control, HMCC, LEDTA and HEDTA cheeses are shown in Figure 3.2. During cheese manufacture the pH of all Gouda-type cheeses decreased due to acidification caused by the starter culture, as expected. This decrease in pH remained steady and constant for both the control and HMCC cheeses. However, following addition of EDTA to the curds and whey mixture of the LEDTA and HEDTA cheeses, the pH decreased dramatically.

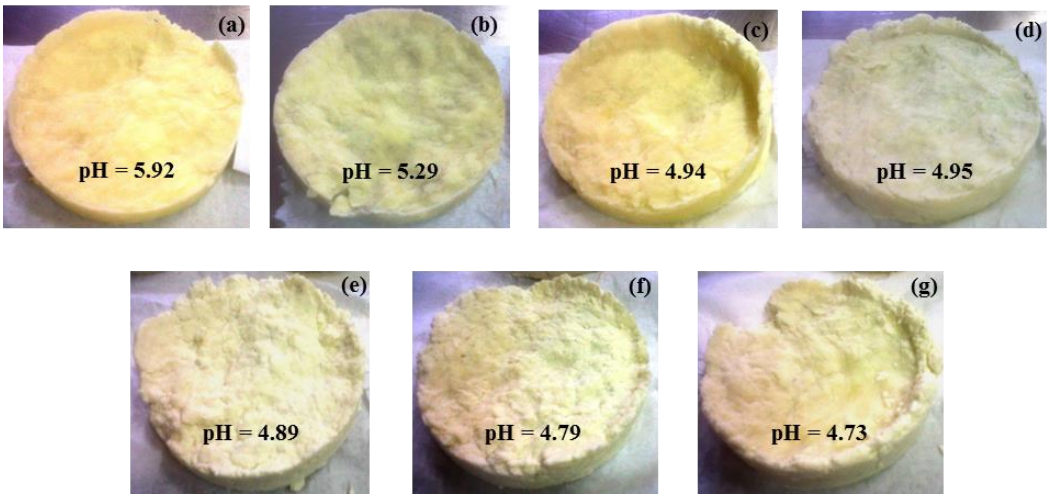

Figure 3.1 Images and pH values of Gouda-type cheeses manufactured in preliminary trials supplemented with (**a**) 0.0 (control), (**b**) 7.5, (**c**) 19, (**d**) 23, (**e**) 31, (**f**) 39 and (**g**) 47 g EDTA/L whey removed.

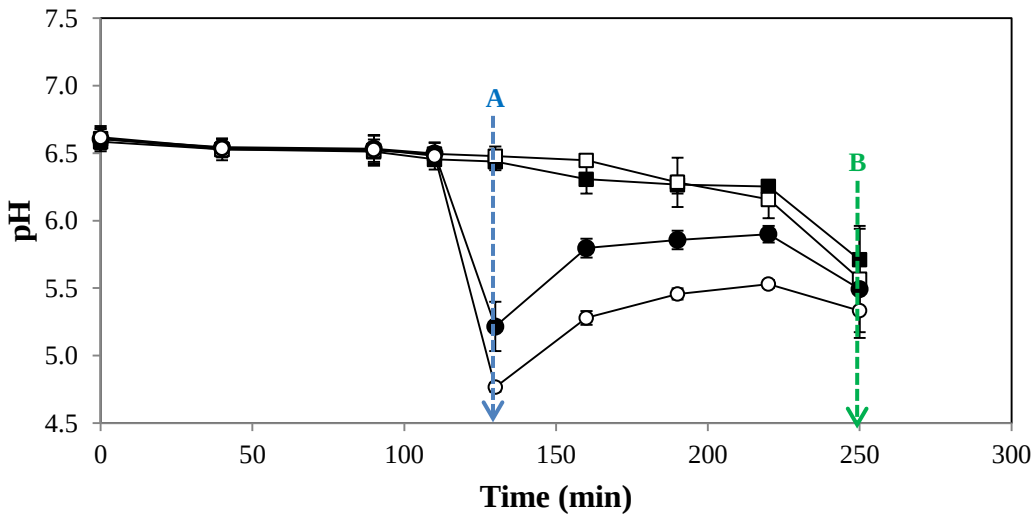

Figure 3.2 Changes in pH during Gouda cheese manufacture for control (■), high moisture control (□), low EDTA (●) and high EDTA (○) cheeses. Arrow A indicates the time of the second wash step (EDTA was added to cheese vats 3 and 4) and arrow B signifies the time of whey drainage completion.

The pH of cheese is influenced by two major factors: the degree of acid production and the level of Ca phosphate in the cheese. There was no significant

difference in the rate of decrease in pH between the cheeses up until EDTA was added. Once EDTA was added to the curd/whey mixture of the LEDTA and HEDTA cheese vats, some Ca present in the cheese was chelated. With less Ca phosphate now available, the curd pH and buffering capacity of the curd is decreased (Lucey and Fox, 1993; O'Mahony *et al*., 2006). The pH values for the LEDTA and HEDTA cheeses increased throughout the cooking and pressing stages of manufacture, possibly due to the Ca phosphate buffering effect becoming apparent by picking up hydrogen ions; however, this effect did not occur to the same extent for the control and HMCC cheeses.

3.3.2 pH and compositional analysis

Composition and pH values for control, HMCC, LEDTA and HEDTA Gouda-type cheeses at 14 d of ripening are shown in Table 3.3. No significant differences in the fat, fat in dry matter (FDM), salt and salt in moisture (S/M) was evident between the control and treatment cheeses (Mizuno and Lucey, 2005). The moisture, moisture non-fat solids (MNF) and protein contents of cheeses showed significant differences between control and treatment cheeses. The moisture and MNFS values increased with increasing concentration of EDTA. The HMCC cheese had the highest moisture and MNFS contents of all cheeses. There was no significant difference in moisture and MNFS contents between the HMCC and HEDTA cheese, indicating that decreasing the cook temperature from 38 ˚C (HEDTA) to 31 ˚C (HMCC) and increasing the EDTA addition levels to cheese, increased the moisture content of Gouda-type cheese to the same extent.

Table 3.3 Composition, pH, levels of pH 4.6-soluble N and EDTA concentrations of control, high moisture control, low EDTA and high EDTA Gouda-type cheese at 14 d of ripening.

Parameter	**Treatment**			
	Control	**HMCC**	**LEDTA**	**HEDTA**
Protein (%)	24.5[a] (0.36)	22.1[c] (0.46)	24.3[a] (0.70)	22.9[b] (0.48)
Fat (%)	20.1[a] (1.58)	18.3[a] (1.10)	19.7[a] (0.60)	18.1[a] (1.02)
Moisture (%)	41.3[c] (1.26)	48.3[a] (1.78)	44.2[b] (0.35)	47.2[a] (1.28)
MNFS[2] (%)	51.7[c] (2.21)	59.1[a] (1.69)	55.1[b] (1.07)	57.6[a] (1.77)
FDM[3] (%)	34.2[a] (3.33)	35.4[a] (2.27)	35.3[a] (2.09)	34.3[a] (2.31)
NaCl (%)	2.28[b] (0.27)	2.60[ab] (0.39)	2.69[ab] (0.54)	2.96[a] (0.56)
S/M[4] (%)	5.53[a] (0.71)	5.36[a] (0.64)	6.08[a] (1.19)	6.27[a] (1.08)
pH (5 d)	5.36[a] (0.06)	5.10[c] (0.07)	5.25[b] (0.16)	5.03[d] (0.02)
pH (14 d)	5.35[a] (0.09)	5.11[c] (0.06)	5.25[b] (0.04)	4.99[d] (0.03)
pH (28 d)	5.47[a] (0.06)	5.14[c] (0.06)	5.31[b] (0.04)	5.07[d] (0.03)
pH (112 d)	5.80[a] (0.04)	5.39[c] (0.09)	5.56[b] (0.05)	5.16[d] (0.04)
pH 4.6-SN%TN (7 d)	5.93[c] (0.56)	8.80[a] (0.74)	6.42[bc] (0.43)	7.32[b] (1.72)
pH 4.6-SN%TN (112 d)	25.5[b] (2.47)	28.8[a] (0.71)	23.4[c] (1.46)	23.0[c] (0.77)
mg EDTA/g cheese	0.00[c] (0.00)	0.00[c] (0.00)	0.88[b] (0.06)	2.06[a] (0.09)
Total Ca (mg/100g cheese)	1226[a] (31.0)	970[c] (28.3)	1036[b] (34.3)	836[d] (43.8)
Insoluble Ca (% of total Ca) 7 d	67.1[a] (3.90)	58.2[a] (4.92)	64.8[a] (4.33)	33.9[b] (4.22)
Insoluble Ca (% of total Ca) 28 d	60.9[a] (8.98)	47.7[ab] (6.36)	50.8[ab] (12.6)	27.2[b] (6.74)
Insoluble Ca (% of total Ca) 112 d	53.8[a] (9.57)	45.7[ab] (9.65)	41.3[ab] (9.11)	24.8[b] (6.13)

[1]Values are the means of triplicate trials with standard deviations in parentheses; means within rows not sharing a common letter differ statistically ($P \leq 0.05$)
[2]Moisture in nonfat solids of the cheese
[3]Fat in dry matter of the cheese
[4]Salt in moisture phase of cheese

It is well established that decreasing cook temperature during cheese manufacture decreases syneresis, leading to increased curd moisture (Sheehan, 2007). It has also been shown that lower draining pH results in lower Ca content with a concomitant increase in moisture content of cheese (Yun *et al*., 1995). A concomitant decrease in protein content of cheese with increasing moisture content was observed, with the control having the highest (24.5%) and the HMCC

cheese having the lowest (22.1%) protein concentration. Similar results were found by Hennelly *et al*. (2005) who reported decreased levels of protein with increasing moisture content for imitation cheese.

The pH of all cheeses increased throughout ripening, as expected. Significant differences in pH values were observed between control and treatment cheeses at all stages of ripening. The HEDTA cheese exhibited the lowest pH values throughout ripening, while the control cheese had the highest. The pH of the HMCC cheese was significantly lower than the control and LEDTA cheese at all ripening times. As a result of lower cook temperature used during the manufacture of HMCC cheese, syneresis was reduced leading to a higher moisture cheese. Cheese containing higher moisture content generally contains increased levels of lactose and lactate which result in a higher lactate to protein ratio and thus a lower buffering capacity and a lower cheese pH (Sheehan, 2007). As well as this, Chevanan and Muthukumarappan (2007) reported lower pH in Cheddar cheese containing low S/M and low Ca and P levels compared to cheeses with high S/M and high Ca and P. At whey drainage, all treatment cheeses exhibited a lower pH compared to the control cheese. It has been well established that a lower pH at whey drainage results in a lower pH of the final cheese due to a reduction in the levels of total Ca (Lawrence *et al*., 1987; Lucey and Fox, 1993; Yun *et al*., 1995; Lee *et al*., 2005). It has also been demonstrated by several authors that a reduction in S/M results in a concomitant decrease in pH (Thomas and Pearce, 1981; Upreti and Metzger, 2007).

The concentration of EDTA present in the pH 4.6-SN fraction of cheese and whey samples was determined using HPLC and the results are shown in Table

3.3 and are presented as mg EDTA/g cheese. No EDTA was detected in the control and HMCC cheese and whey, as expected. EDTA was detected at levels of 0.88 and 2.05 mg/g cheese in the LEDTA and HEDTA cheeses, respectively. Results indicate that ~ 90% of the EDTA originally added to the curds and whey mixture of both the LEDTA and HEDTA cheeses was lost in the whey. However, despite this, a significant concentration (~ 10% of that added to the curds/whey mixture) of EDTA was retained in the curds of cheeses supplemented with EDTA. In a previous study, EDTA (0, 2, 4, or 6 m*M*) was supplemented to skim milk that was subsequently acidified to pH 6.0, resulting in significantly decreased INSOL Ca levels and reduced pH (Choi *et al*., 2008). Conversely, McMahon *et al*. (2005) added 15 g of EDTA into the whey of high pH, directly-acidified, nonfat Mozzarella cheese during cooking, in order to decrease Ca levels in the finished cheese. However, the Ca contents of cheeses treated with EDTA were unaffected by EDTA addition and the structural and functional characteristics of EDTA-treated cheeses were similar to that of the control cheese.

3.3.4 Total and insoluble calcium

The acid-base buffering curves used to quantify the INSOL Ca content of the control and experimental cheeses are shown in Figure 3.3. For all cheeses a buffering peak was observed at ~ pH 4.8 during titration with 0.5 N HCl from the initial curd pH to pH 3.0, caused by solubilisation of CCP in the cheese, which released phosphate ions that can combine with H^+ in solution resulting in buffering (Lucey and Fox, 1993; Lucey *et al*., 1993; Hassan *et al*., 2004; O'Mahony *et al*., 2006). A buffering peak was also observed at pH 6.0 (not shown) during the back titration of cheese samples from pH 3.0-9.0 with 0.5 N

NaOH. This peak at pH 6.0 has been attributed to the precipitation of calcium phosphate with the release of H^+ (Lucey *et al*., 1993; Hassan *et al*., 2004; O'Mahony *et al*., 2006; Shirashoji *et al*., 2006).

The buffering peak at ~ 4.8 and the magnitude of the net titration peak area between the pH limits 4.1 and 5.2 decreased with both increasing concentrations of EDTA and decreased cooking temperature, indicating a reduction in the INSOL Ca content of cheese due to solubilisation of INSOL Ca (Lucey and Fox, 1993; Hassan *et al*., 2004). Choi *et al*. (2008) and Brickley *et al*. (2009) both found a reduction in the buffering curves of directly acidified cheeses manufactured from milk with added EDTA and Cheddar cheese containing TSC added at salting, with the decrease greatest for cheeses containing the highest level of EDTA and TSC. A decrease in the buffering peak was observed by Shirashoji *et al*. (2006) upon increasing ES concentration in processed cheese. Cheeses made with ≥ 2.38 % TSC did not have any peak at pH 4.8, which suggested that INSOL Ca was no longer present due to removal of Ca from CCP by TSC. Additionally, O'Mahony *et al*. (2006) demonstrated a steady increase in the buffering curves of Cheddar cheese slices incubated in synthetic Cheddar cheese aqueous phase solutions (SCCAP) with increasing Ca concentrations.

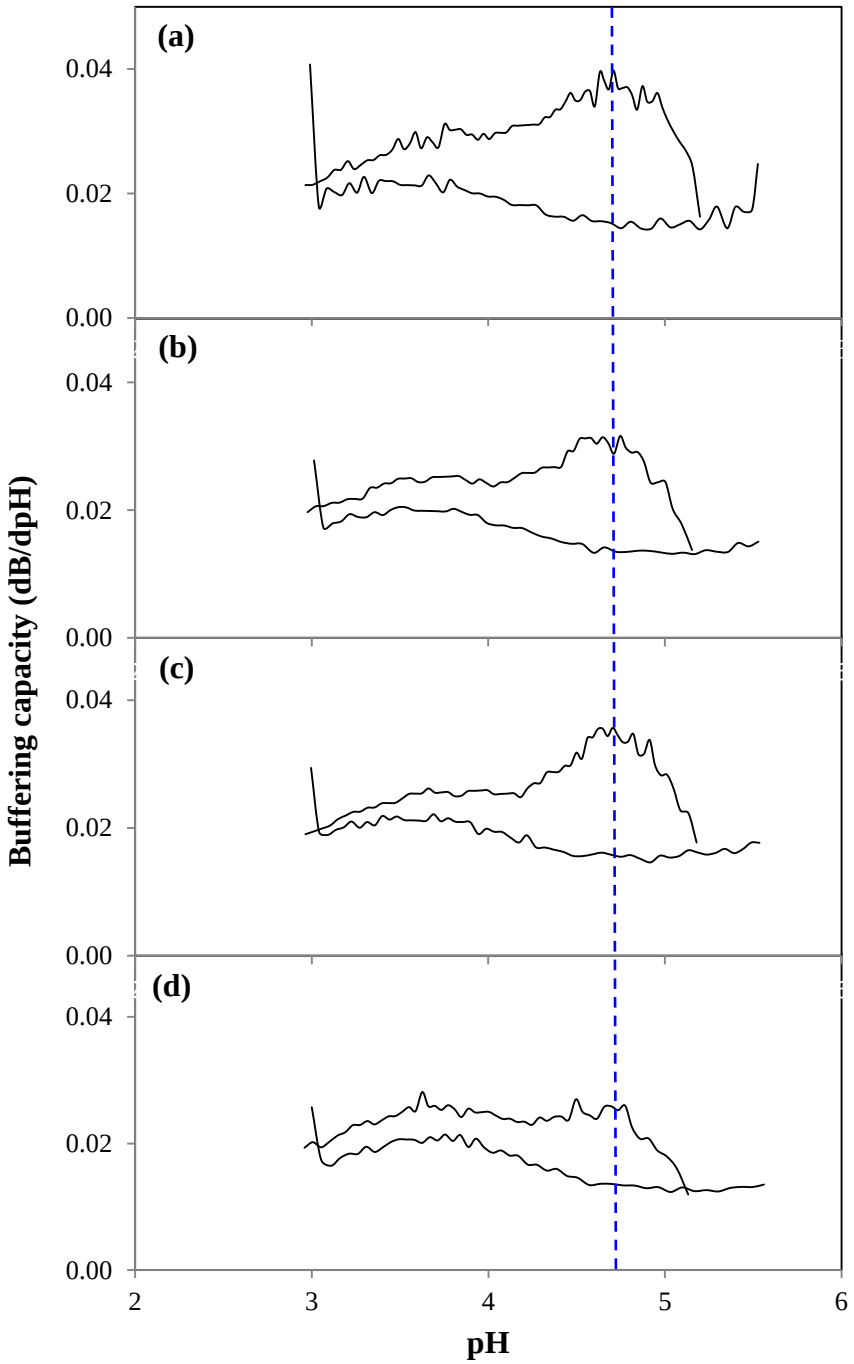

Figure 3.3 Acid-base buffering curves for cheese titrated from initial cheese pH to pH 3.0 with 0.5 N HCl and then back-titrated to pH 6.0 with 0.5 N NaOH, for control (**a**), high moisture (**b**), low EDTA (**c**) and high EDTA (**d**) Gouda-type cheeses. Vertical dashed line indicates buffering peak centered at pH 4.8.

The total and INSOL Ca concentrations of control, HMCC, LEDTA and HEDTA Gouda-type cheese throughout ripening are shown in Table 3.3. The total Ca levels in cheeses treated with EDTA significantly decreased, with the decrease greatest for the HEDTA cheese. This suggests that the EDTA rapidly bound to the Ca, forming a complex, which was subsequently lost in the whey fraction. The HMCC cheese also exhibited significantly reduced total Ca levels compared to the control cheese; however, the extent of this reduction was not as great as that of the HEDTA-treated cheese. The retention of total Ca in cheese has been found to be dramatically affected by processing conditions, particularly pH at whey drainage and cooking temperature (Lucey and Fox, 1993; Yun *et al*., 1995; Lee *et al*., 2005). In cheeses where the pH is low, substantial losses of total Ca occur when the whey is drained (Lawrence *et al*., 1987; Lucey and Fox, 1993; Lucey *et al*., 1993). Cottage cheese has been found to have the lowest Ca concentrations due to the fact that the pH at the time of whey drainage is typically less than 5.0 (Kosikowski and Mistry, 1997; Lee and Lee, 2009). The point of whey removal is shown in Figure 3.2. Results indicate that the HEDTA cheese had the lowest pH at whey drainage and therefore, the reduced total Ca level is most likely attributed to the lower pH value of the HEDTA cheese compared with the control, 5.33 and 5.71, respectively, as well as, the effects of Ca-EDTA complex formation (removed with the whey) and colloidal calcium phosphate (CCP) solubilisation caused by EDTA addition. Mizuno and Lucey (2005) reported lower total Ca concentrations in *pasta filata* cheese manufactured with 5 % TSC compared with control cheese, despite no significant differences in pH between the cheeses. Lucey *et al*. (1993) found that increasing cooking temperature caused an increase in Ca levels and buffering capacity due to reduced solubility of CCP. Therefore,

the reduction in Ca concentration in the HMCC cheese can be attributed to the lower cooking temperature applied during manufacture as well as the low pH at whey drainage.

Levels of INSOL Ca decreased during ripening for all Gouda-type cheeses (Table 3.3), reflecting the slow solubilisation of some of the residual INSOL Ca present in cheese. This phenomenon has been well established in dry-salted cheese varieties such as Cheddar (Hassan *et al*., 2004; Lee *et al*., 2005; Lucey *et al*., 2005; Brickley *et al*., 2009; Wang *et al*., 2011). The INSOL Ca levels (% of the total Ca) of the control, HMCC, LEDTA and HEDTA were 67, 58, 65 and 34%, respectively at 7 d. Increasing the EDTA addition levels significantly decreased the levels of INSOL Ca in Gouda-type cheese. The decrease in INSOL Ca concentration paralleled the decrease in total Ca content of the cheese with increasing EDTA concentration. Similar findings were reported by Mizuno and Lucey (2005), who found that the addition of TSC to *pasta filata* cheese during manufacture caused a reduction in both INSOL Ca and P_i. These authors attributed the decrease in INSOL Ca and P_i to the ability of TSC to bind some of the INSOL Ca remaining in the cheese, resulting in the solubilisation and hydration of the caseins.

Decreasing the cooking temperature of the HMCC caused a considerable reduction in the concentration of INSOL Ca in the cheese when compared with the control cheese. This reduction in INSOL Ca is most likely attributed to increased SOL Ca expulsion in the whey as a result of the lower cooking temperature (Table 3.1) applied to the HMCC cheese, 31 ˚C instead of 38 ˚C (control, LEDTA and HEDTA), during cheese manufacture (Salaün *et al*., 2005).

The INSOL Ca levels in the HMCC and HEDTA-treated cheeses differed significantly (58.2 and 33.9%, respectively), while no significant ($P \leq 0.05$) differences in moisture content of each cheese was apparent (48.3 and 47.2%, respectively). This facilitated the effect of INSOL Ca on the textural properties of cheese to be evaluated independently from the effect of moisture content.

3.3.5 Proteolysis

The urea-PAGE gel electrophoretograms of all Gouda-type cheeses from Trial 2 are shown in Figure 3.4 and are typical of the cheeses from the other trials (results not shown). Results show progressive degradation of both α_{s1}-CN and β-CN throughout ripening for all cheeses, with the extent of α_{s1}-CN breakdown being greater than that of β-CN for all cheeses, except the HEDTA cheese which exhibited a greater breakdown in β-CN than α_{s1}-CN. Degradation resulted in the accumulation of the breakdown product of both α_{s1}-CN (f 24-199) and γ-caseins (β-CN f106-209, f29-201, f108-209). At both 7 and 112 d of ripening the extent of proteolytic breakdown of α_{s1}-CN was slightly greater for the HMCC cheese, most likely attributed to the reduced inactivation of chymosin due to the lower cooking temperature used during manufacture (31 ˚C) (Matheson, 1981; Rampilli *et al*., 1998; Sheehan *et al*., 2007).

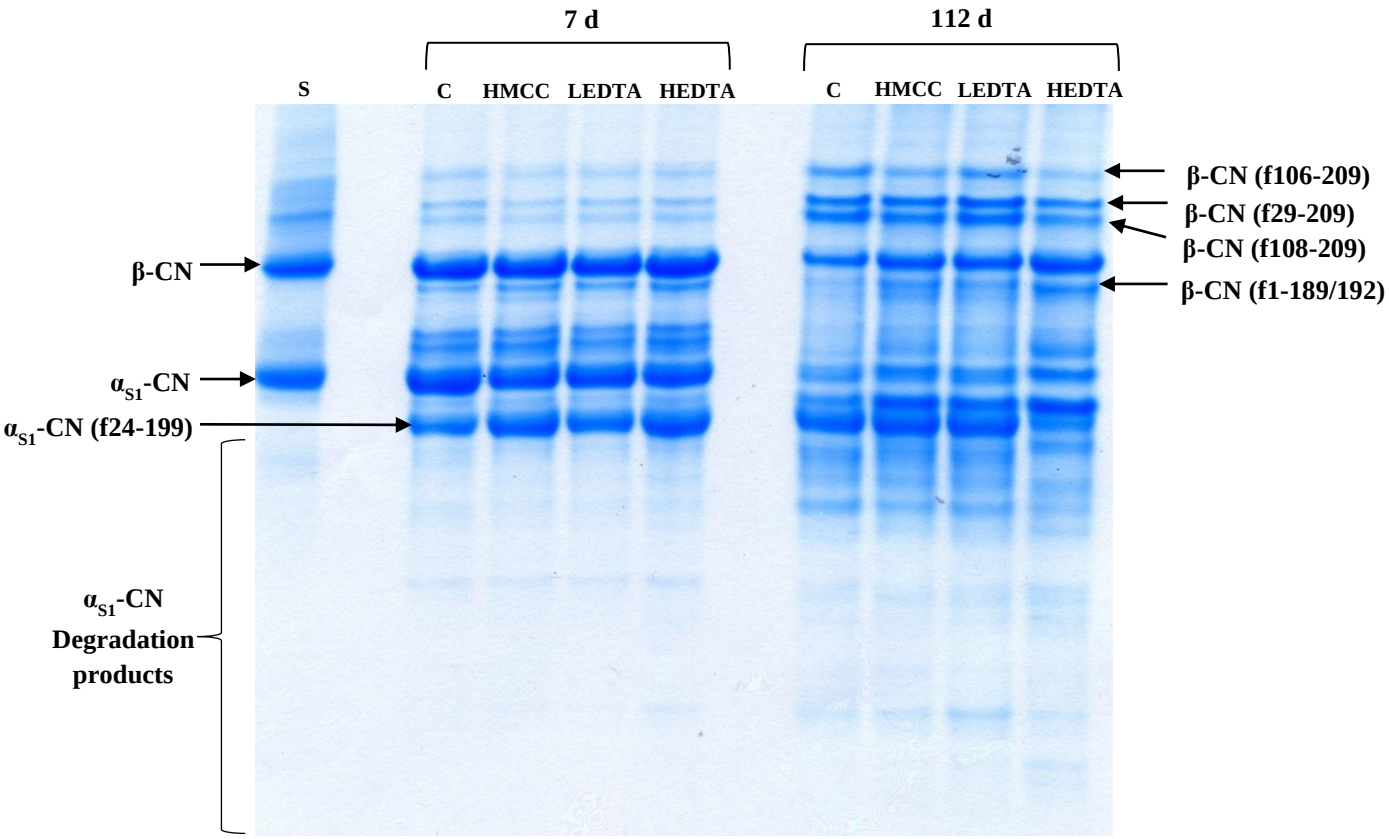

Figure 3.4 Urea-polyacrylamide gel electrophoretograms of the sodium caseinate standard (S) and the pH 4.6-insoluble fractions from the control (C), HMCC, LEDTA and HEDTA Gouda-type cheese at 7 and 112 days of ripening. Electroretograms are of Trial 2 and is representative of the other two trials.

The changes in levels of pH 4.6-SN%TN during ripening of Gouda-type control, HMCC, LEDTA and HEDTA Gouda-type are shown in Table 3.3. Levels of pH 4.6-SN%TN increased with increasing ripening time for all cheeses. Significant differences ($P \leq 0.01$) between treatments and ripening times were evident (Table 3.4). At both 7 and 112 d of ripening, the HMCC cheese exhibited the highest levels of proteolysis. Yun *et al*. (1993) reported reduced levels of proteolysis and increased moisture content of Mozzarella cheese produced at a

higher cooking temperature during 50 d of storage. Sheehan *et al*. (2007) demonstrated that cook temperature (47, 50 and 53 ˚C) and ripening time had a significant effect on the mean level of pH 4.6-SN, with levels in cheeses cooked at 50 and 53 ˚C being similar and significantly lower than those in cheeses cooked at 47 ˚C. These authors attributed this result to a greater residual chymosin activity, higher moisture content and lower pH which may be more favourable to proteolytic activity of residual chymosin in cheeses cooked at lower temperatures.

Table 3.4 Mean squares probabilities and R^2 values for pH 4.6-SN%TN, meltability, hardness, adhesiveness, springiness, cohesiveness, gumminess, chewiness and resilience during ripening of control, high moisture control, low EDTA and high EDTA Gouda-type cheeses.[1]

Factor	df[2]	pH 4.6-SN%TN	df[2]	Meltability	df[2]	Hardness (g)	Springiness	Gumminess	Chewiness	Chewiness
Whole Plot										
Day of cheese manufacture (D)	2	1.71 (0.22)	2	13.4 (0.84)	2	24790667 (0.11)	0.02* (0.02)	6775701 (0.10)	5727627 (0.08)	5727627 (0.08)
Treatment (T)	3	19.2** (0.00)	3	4399.3** (0.00)	3	340018976** (0.00)	0.23** (0.00)	46894751** (0.00)	31543685** (0.00)	31543685** (0.00)
D x T	6	0.87	6	3.53	6	7581833	0.02	1998754	1472429	1472429
Subplot										
Age (A)	1	1952.1** (0.00)	5	2485.7** (0.00)	4	56537809** (0.00)	0.07** (0.00)	11987272** (0.00)	6753011** (0.00)	6753011** (0.00)
T x A	3	6.26 (0.06)	15	98.4** (0.00)	12	9128071** (0.00)	0.00 (0.52)	2589839** (0.00)	1481127** (0.00)	1481127** (0.00)
Error	8	1.61	40	20.3	32	2211369	0.00	417762	221011	221011
R^2		0.99		0.97		0.95	0.96	0.95	0.96	0.96

[1]Split–plot design with the four treatments (control, HMCC, LEDTA and HEDTA) were analysed as a discontinuous variable and cheese manufacture day was blocked. Subplot included the effect of age and treatment and treatment × age as variables

[2]Degrees of freedom

*$0.01 < P \leq 0.05$; ** $P \leq 0.01$

Peptide profiles of the pH 4.6-soluble fraction were determined by reverse phase-high performance liquid chromatography (RP-HPLC), and results of cheeses from a single trial during ripening are shown in Figure 3.5; similar results (not shown) were found for cheeses from the other trials. The number and extent of peptides increased during 4 months of ripening for all the Gouda-type cheeses. Qualitative and quantitative differences were evident between all cheeses and these differences were more pronounced at month 4. Results indicate that both cooking temperature and EDTA addition cause a significant effect on the development of peptides in cheese. It appears that the major differences between cheeses at 7 d were evident in the retention time interval between, 1.5 and 3.5 min, whereas by 112 d of ripening differences were evident at the retention time interval between 3.5 and 6.5 min, possibly due to the production of hydrophobic peptides (O'Mahony *et al*., 2005). These results are consistent with results observed in the changes in levels of pH 4.6-SN and on urea-PAGE gel electrophoretograms.

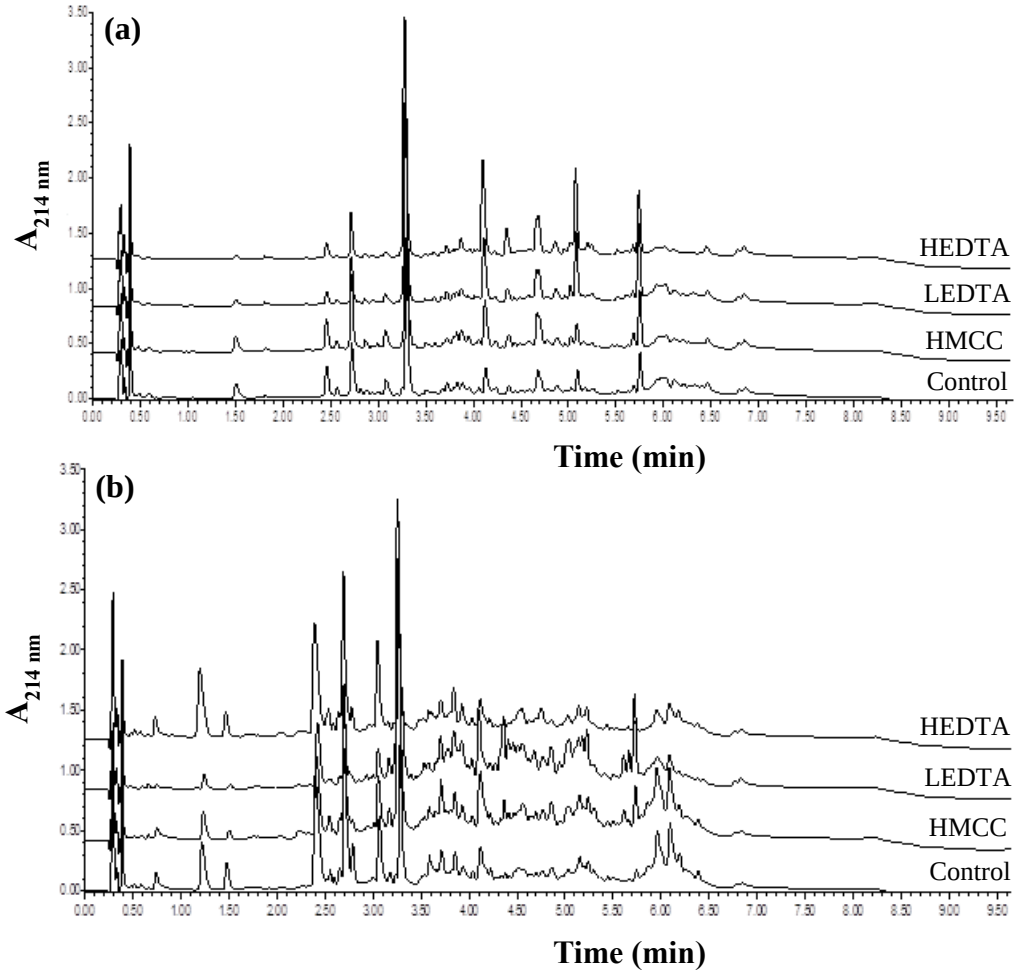

Figure 3.5 Peptide profiles at (**a**) week 1 and (**b**) month 4 for the control, high moisture control, low EDTA and high EDTA Gouda-type cheeses. Chromatograms are of cheeses from Trial 2 and are representative of the other two trials.

3.3.6 Determination of textural and rheological properties during ripening

3.3.6.1 Texture profile analysis

The changes in hardness, chewiness, springiness and gumminess during ripening of control, HMCC, LEDTA and HEDTA Gouda-type cheeses as determined by texture profile analysis are presented in Figure 3.6. Significant differences ($P \leq 0.01$) between treatments (control, HMCC, LEDTA and HEDTA) and ripening times (7, 14, 28, 56 and 112 d) were observed (Table 3.4). Significant and progressive reduction in hardness, chewiness, springiness and gumminess values were observed with increasing ripening time, from 7 to 112 d, for all Gouda-type cheeses. Hardness values (Figure 3.6a) were lower in all experimental cheeses compared with the control cheese. Significant differences in pH values (Table 3.3) between cheeses were evident throughout ripening, with pH values of 5.36, 5.10, 5.25 and 5.03, for control, HMCC, LEDTA and HEDTA Gouda-type cheeses, respectively, at 5 d. Several studies (Lawrence *et al*., 1987; Watkinson *et al*., 2001; Pastorino *et al*., 2003) have demonstrated that lowering the pH of cheese results in cheese with reduced hardness. It has been demonstrated that as cheese pH is reduced from 5.4 to 4.6, the caseins gradually separate into aggregates of smaller size and eventually into strands causing the cheese to change from springy at higher pH to non-cohesive at pH below about 4.8 (Lawrence *et al*., 1987). The HEDTA and HMCC cheeses had the lowest pH, 5.03-5.16 and 5.10-5.39, respectively, and hardness values, 56.6-39.9 N and 73.6-31.5 N, respectively at 7 and 112 d of ripening.

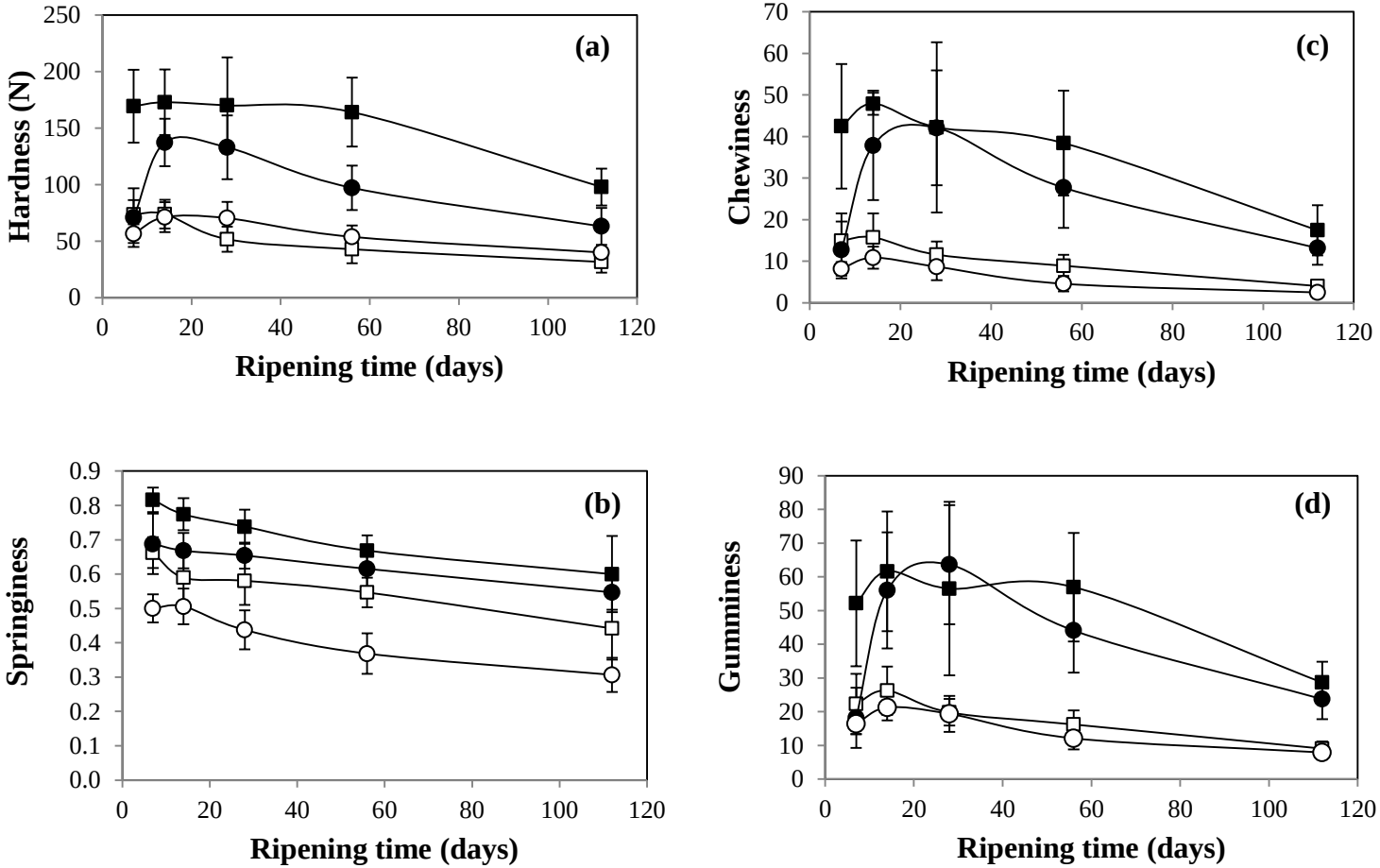

Figure 3.6 (**a**) Hardness, (**b**) springiness, (**c**) chewiness and (**d**) gumminess values expressed in newtons (N) for control (■), high moisture control (□), low EDTA (●) and high EDTA (○) Gouda-type cheeses at 7, 14, 28, 56 and 112 d of ripening as determined by texture profile analysis.

However, pH alone is not the only factor responsible for differences in cheese texture. Ca is removed from the casein particles as pH is reduced during cheese manufacture, resulting in cheese with lower total Ca levels, resulting in a softer cheese with increased melting properties (Lucey and Fox, 1993). It has been suggested however, that total Ca alone is not the best predictor of the physical properties of cheese (Lawrence *et al*., 1987) and that the amount of Ca associated with the casein (INSOL Ca) may play a more significant role in controlling cheese texture due to its direct influence on casein-casein interactions (Lucey *et al*., 2003; Lucey *et al*., 2005). Hardness values of cheeses in this study decreased with increasing concentration of EDTA throughout the entire ripening period. It has been demonstrated that addition of Ca chelating salts to cheese causes a reduction in the level of INSOL Ca, resulting in the solubilisation and hydration of caseins, leading to softer textural properties of cheese (Postorino *et al*., 2003; Hassan *et al*., 2004; Mizuno and Lucey, 2005; Lucey *et al*., 2005; Brickley *et al*., 2009). The total and INSOL Ca contents of cheeses indicated in Table 3.3 show that total Ca in cheese was significantly affected by both increasing EDTA concentration and decreasing pH.

Increased moisture levels (Table 3.3) were observed for the low cook temperature HMCC cheese and the EDTA-containing cheese (LEDTA and HEDTA) compared to the control cheese. The difference in cheese moisture content was greatest for the HMCC>HEDTA>LEDTA>Control. Decreased hardness values in cheese have been associated with increased moisture levels; most likely due to increased hydration of the protein matrix, leading to plasticisation, resulting in increased softening (Chen *et al*., 1979; McMahon *et al*.,

1996; Hennelly *et al*., 2005). The HMCC was found to have the lowest S/M content of all cheeses. Chevanan *et al*. (2006) reported higher hardness values in Cheddar cheese containing higher levels of Ca, P and S/M ratio. These authors attributed the increased hardness values to slower proteolysis, differences in pH or lower moisture contents. Therefore, the reduced hardness associated with the HMCC cheese may be attributed to its higher levels of proteolysis, lower pH and higher moisture content as well as lower total and INSOL Ca levels. Chewiness, springiness and gumminess values (Figure 3.6b, c and d) decreased for all Gouda-type cheeses throughout ripening. In all cases this decrease over time was greatest for the control cheese, 42.5-17.4, 0.82-0.59 and 52.1-28.6 for chewiness, springiness and gumminess values, respectively, at 7 and 112 d. However, the lowest values for each parameter were associated with the HEDTA cheese. In general, the springiness, chewiness and gumminess values decreased with decreasing levels of total and INSOL Ca levels and this trend continued throughout 112 d ripening. The lower springiness values associated with cheese containing lower levels of total and INSOL Ca may be due to the reduction in cross-linkage due to CCP solubilisation caused by the introduction of the Ca chelating agent, EDTA. The higher level of proteolysis, increased moisture content and lower total Ca levels of the HMCC cheese may be the causative factors associated with the reduction in springiness value of the HMCC cheese (Chevanan *et al*., 2006). No significant differences in chewiness and gumminess values were evident between the HEDTA and HMCC cheeses; however, significant differences were apparent when these cheeses were compared with the control and LEDTA cheese. Chewiness and gumminess are secondary TPA properties and depend on hardness, cohesiveness and springiness, respectively

(Gunasekaran and Ak, 2003**)**. The increased level of proteolysis and moisture content and lower levels of INSOL Ca observed in the EDTA-treated and HMCC cheeses compared with the control cheese may have caused the lower chewiness and gumminess values in these treatment cheeses (Chevanan *et al*., 2006; Upreti *et al*., 2006a, b).

Interestingly, the HEDTA cheese exhibited lower total and INSOL Ca levels and pH values throughout ripening compared to the HMCC cheese, but had similar moisture contents (48.3 and 47.2%, respectively) and similar hardness values (5765 and 7506 g at 5 d, respectively). This suggests that increasing the moisture levels of Gouda-type cheese influences the textural properties (hardness, chewiness and gumminess) to the same extent as changing the ratio of SOL to INSOL Ca by EDTA addition.

3.3.6.2 Cheese meltability

The increase in meltability (%) of the control, HMCC, LEDTA and HEDTA Gouda-type cheeses throughout ripening are shown in Figure 3.7. Significant ($P \leq 0.01$) differences between treatments (control, HMCC, LEDTA and HEDTA), ripening times (7, 14, 28, 56 and 112 d) and treatment × ripening time were observed (Table 3.4). Results show that EDTA addition to the curds/whey mixture of Gouda-type cheese resulted in cheese with increased melting properties. This result confirms that EDTA dissolved some CCP due to Ca chelation, indicated by the buffering curves (Figure 3.3), leading to a reduction in casein-casein interaction and a subsequent increase in meltability.

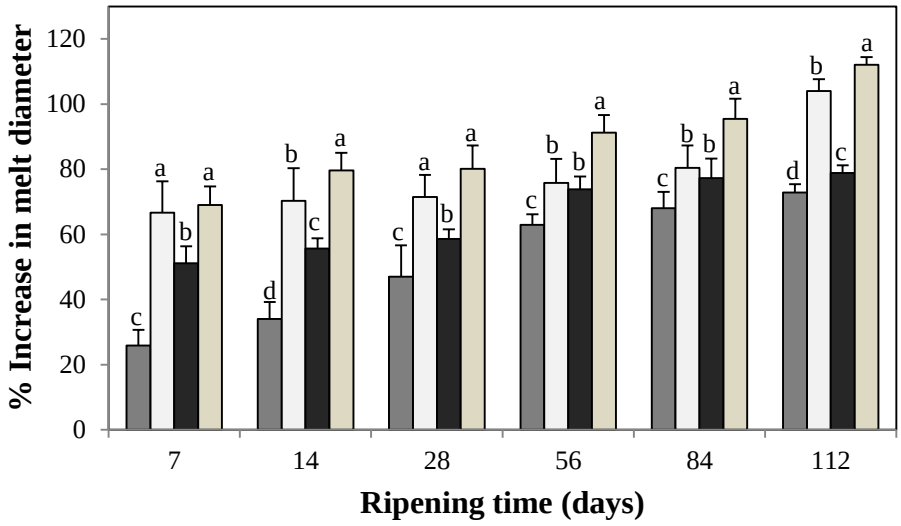

Figure 3.7 Cheese meltability as a function of ripening time, expressed as a % increase in melt diameter of cheese discs (35 mm diameter; 5 mm height), heated at 232 ˚C for 5 min, for control (▬), high moisture control (▭), low EDTA (▬) and high EDTA (▭) Gouda-type cheeses at 7, 14, 28, 56, 84 and 112 d of ripening.

Several authors have demonstrated that when heat is applied the flow and stretch of cheese increases with decreasing Ca content (Guinee *et al*., 2002; Joshi *et al*., 2003; Sheehan and Guinee, 2004). The HEDTA cheese showed the greatest % increase in cheese melt diameter throughout ripening of all cheeses, 50% higher than the control cheese. Results indicate that reducing the total and INSOL Ca level of Gouda-type cheese increases meltability to a greater extent than increasing the moisture content alone. Similarly, Chevanan and Muthukumarappan (2007) demonstrated that the meltability of low Ca and P, low lactose, and low S/M ratio Cheddar cheeses was 23.5%, 12.7%, and 7.3% higher compared to the corresponding high levels of Ca and P, residual lactose, and S/M ratio, respectively, after 8 months of ripening, indicating that the levels of Ca and P played a more significant role in meltability of Cheddar cheese compared to the levels of residual lactose and S/M ratio.

3.3.6.3 Small deformation rheological properties

The dynamic moduli (G' and G") values at 30 and 70 ˚C for control, HMCC, LEDTA and HEDTA Gouda-type cheeses at 28 and 112 d of ripening, as determined by controlled stress rheometry, are shown in Table 3.5. Significant differences between treatments (control, HMCC, LEDTA and HEDTA) and ripening times (28 and 112 d) were observed (Table 3.6).

During heating, both the storage (G') and loss (G") moduli significantly decreased and this reduction increased as ripening progressed for all cheeses. At 70 ˚C, the control cheese (highest concentration of INSOL Ca) exhibited the highest G' value at both ripening times. It can be seen that in cheeses containing lower concentrations of INSOL Ca (HMCC, LEDTA and HEDTA) the G" was greater than G', indicating a dominance of liquid-like rheological properties. However, at higher concentrations of INSOL Ca (Control), solid-like rheological properties dominated (i.e., G' > G"). O'Mahony *et al*. (2006) observed an increase in G' at 70 ˚C in cheese with increasing CCP concentration and attributed this to increased CCP bridging between casein molecules which increased the rigidity of the cheese matrix. Cooke *et al*. (2013) found that Cheddar cheese containing added strontium (Sr^{2+}) had a higher G' at 70 ˚C and attributed this to increased Sr^{2+} based CCP crosslinks, leading to increased strength and rigidity of the *para*-casein matrix.

Table 3.5 Small deformation rheological properties for control, high moisture control, low EDTA and high EDTA Gouda-type cheeses at 28 and 112 d of ripening as determined by a controlled stress rheometer.

Treatment	**Day**	**$G'_{30°C}$**	**$G'_{70°C}$**	**$G''_{30°C}$**	**$G''_{70°C}$**
		Pa	Pa	Pa	Pa
Control	**28**	29216^{a} (4696)	599^{a} (320)	9078^{a} (2151)	1029^{a} (341)
HMCC		15294^{c} (2984)	171^{b} (67)	5341^{b} (780)	344^{b} (125)
LEDTA		21993^{b} (1703)	122^{b} (16)	8519^{a} (942)	309^{b} (55)
HEDTA		22967^{b} (2878)	145^{b} (100)	7483^{ab} (1094)	275^{b} (222)
Control	**112**	23893^{a} (4501)	783^{a} (51)	8491^{a} (1337)	430^{a} (80)
HMCC		9650^{b} (3286)	44^{b} (25)	4079^{c} (764)	102^{b} (48)
LEDTA		12184^{b} (2470)	49^{b} (22)	5351^{bc} (667)	114^{b} (54)
HEDTA		20106^{a} (3217)	53^{b} (10)	6806^{b} (1187)	84^{b} (23)

Values are the means of triplicate trials with standard deviations in parentheses; means within columns (sharing the same day; 28 or 112) not sharing a common letter differ significantly ($P \leq 0.05$).

The maximum loss tangent (LT_{max}) values of the control, HMCC, LEDTA and HEDTA Gouda-type cheese throughout ripening are shown in Figure 3.8. Significant differences between treatments (control, HMCC, LEDTA and HEDTA), and treatment × ripening time (28, 56, 86 and 112 d) were observed (Table 3.6). There was a significant (P <0.001) and progressive increase in LT_{max} values for the control and HMCC cheeses throughout ripening. However, no significant increase in the LT_{max} values for the LEDTA and HEDTA cheeses during ripening were observed. The HEDTA cheeses had a significantly lower LT_{max} value, particularly after 2 months of ripening. Lucey *et al*. (2005) suggested that solubilisation of INSOL Ca was principally responsible for the increase in LT_{max} values during the early stages of ripening. The HEDTA cheese exhibited

the lowest INSOL Ca content of all cheeses throughout ripening; however, the extent of decrease was slight and inconsistent as ripening increased. Therefore, the absence of an increasing LT_{max} value for the HEDTA cheese may be as a result of limited increase in INSOL Ca solubilisation throughout ripening.

Table 3.6 Mean squares probabilities and R^2 values for maximum loss tangent, temperature at loss tangent = 1, storage modulus at 30 ˚C and 70 ˚C and loss modulus at 30 ˚C and 70 ˚C during ripening of control, high moisture control, low EDTA and high EDTA Gouda-type cheeses.[1]

Factor	**df**[2]	**LT_{max}**[3]	**T @ LT = 1**[4]	**G'_{30}**[5]	**G'_{70}**[6]	**G''_{30}**[7]	**G''_{70}**[8]
Whole Plot							
Day of cheese manufacture (D)	2	0.19** (0.00)	29.19** (0.00)	3836167 (0.73)	54521 (0.09)	1096521 (0.36)	126586* (0.01)
Treatment (T)	3	1.03** (0.00)	133.8** (0.00)	218184456** (0.00)	129543* (0.01)	16817415** (0.00)	415572** (0.00)
D x T	6	0.24	4.40	11482598	14289	886359	13498
Subplot							
Age (A)	3	0.02 (0.15)	18.4** (0.00)	209516595** (0.00)	187907** (0.01)	12153900* (0.03)	563262** (0.00)
T x A	9	0.08** (0.00)	2.53 (0.34)	12454792 (0.38)	38895 (0.09)	2162930 (0.36)	57611 (0.08)
Error	24	0.01	2.10	10507958	12857	1749476	17835
R^2		0.96	0.92	0.92	0.90	0.85	0.94

[1]Split–plot design with the four treatments (control, HMCC, LEDTA and HEDTA) were analysed as a discontinuous variable and cheese manufacture day was blocked. Subplot included the effect of age and treatment and treatment × age as variables
[2]Degrees of freedom
[3]Maximum loss tangent
[4]Temperature at loss tangent equals to 1
[5]Storage modulus at 30 ˚C, [6]Storage modulus at 70 ˚C
[7]Loss modulus at 30 ˚C, [8]Loss modulus at 70 ˚C
*$0.01 < P \leq 0.05$; ** $P \leq 0.01$

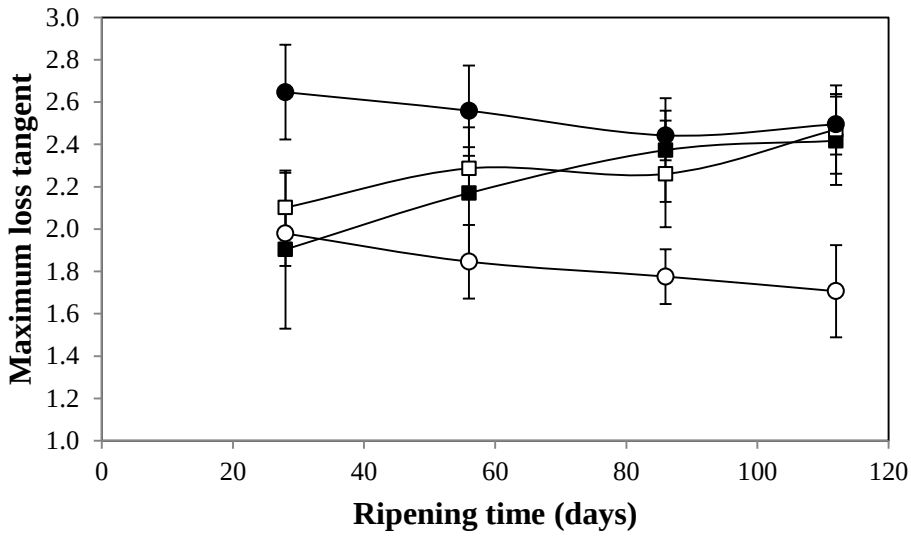

Figure 3.8 Maximum loss tangent (LT_{max}) values for control (■), high moisture control (□), low EDTA (●) and high EDTA (○) Gouda-type cheeses at 28, 56, 86 and 112 d of ripening, measured using a controlled stress rheometer.

Similarly, Brickley *et al*. (2009) and Cooke (2014) found that Cheddar cheese supplemented with TSC at the salting stage exhibited a lower LT_{max} than the control cheese throughout ripening. Brickley *et al*. (2009) attributed this result to the heat-induced precipitation of Ca phosphate from the serum phase of the cheeses helping to cross-link CNs. Lucey *et al*. (2003) stated that electrostatic repulsion between CN molecules is greatly reduced due to the proximity of the system to the isoelectric point of CN in cheeses with a low pH. This leads to a strengthening of the bonds between CN molecules and adversely affects melting ability. HEDTA cheese had a significantly lower pH value throughout ripening compared with the other cheeses (Table 3.3) and perhaps this is another reason why the LT_{max} value was reduced for this cheese.

LT_{max} is used as an index of meltability. However the Schreiber melting test results (Figure 3.7) did not correlate well with LT_{max} (Figure 3.8) values. Such was also the case with Cooke (2014). It is well established that melt analysis results are greatly influenced by type of heating system, the rate of heating and sample geometry (Lucey *et al*., 2003). Cooke (2014) attributed this inconsistency to the possibility that the slow increase in temperature through the region where hydrophobic attractions increase in strength and number and reach a maximum (~60-70 ˚C) may have altered the microstructure in a different way during the rheometer analysis compared to the Schreiber melting test before electrostatic repulsion forces became dominant (>70 ˚C).

3.4 Conclusions

Increasing EDTA addition levels and reducing the cook temperature of Gouda-type cheese resulted in a significant reduction in the levels of total Ca, INSOL Ca and pH, as well as an increase in moisture content. The differences in INSOL Ca concentration and moisture content significantly impacted on the small and large strain deformation properties and meltability of Gouda-type cheese. The HMCC and HEDTA cheeses had lower pH, total and INSOL Ca and higher moisture levels compared to the control and LEDTA cheeses; these factors combined contributed to increased softness and meltability observed in these cheeses. All cheeses exhibited progressive degradation of both α_{s1}-CN and β-CN throughout ripening, with the extent of α_{s1}-CN breakdown being greater than that of β-CN for all cheeses, except the HEDTA cheese which exhibited a greater breakdown in β-CN than α_{s1}-CN. Reduction of INSOL Ca levels in the cheese through the addition of EDTA and decreased cooking temperature resulted in an increase in cheese meltability, as well as a reduction in the textural (hardness, chewiness, gumminess) and rheological (dynamic moduli; G' and G") properties of brine salted Gouda-type cheese. Results indicated that the increase in meltability was influenced to a greater extent by a reduction in the INSOL Ca level in Gouda-type cheese and the textural properties were more dependent on the moisture content of the cheese, while the rheological properties were influenced equally by the reduction in the INSOL Ca level and a decrease in cook temperature, leading to an increase in moisture content. It can be concluded that changing the ratio of SOL to INSOL Ca by increasing the addition level of EDTA in the cheese, as well as, increasing the moisture content by increasing cooking temperature, lead to the

manipulation of the textural, functional and rheological characteristics of Gouda-type cheese. However, further studies focused on separating these effects would be beneficial in order to fully determine the precise factors (most dominant) at play (i.e. Ca equilibrium, pH or moisture content).

3.5 Acknowledgements

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References

Altan, A., Turhan, M. & Gunasekaran. S. (2005). Short communication: Comparison of covered and uncovered Schreiber test for cheese meltability evaluation. *Journal of Dairy Science*, *88*, 857-861.

Bourne M. C. (1978). Texture profile analysis. *Food Technology*. *32*, 62–66, 72.

Brickley, C. A., Lucey, J. A. & McSweeney, P.L.H. (2009). Effect of the addition of trisodium citrate and calcium chloride during salting on the rheological and textural properties of Cheddar-style cheese during ripening. *International Journal of Dairy Technology*, *62*, 527-534.

Chen, A. H., Larkin, J. W., Clark, C. J. & Irwin, W. E. (1979). Textural analysis of cheese. *Journal of Dairy Science*, *62*, 901-907.

Chevanan, N., Muthukumarappan, K., Upreti, P. & Metzger, L. E. (2006). Effect of calcium and phosphorus, residual lactose and salt-to-moisture ratio on textural properties of Cheddar cheese during ripening. *Journal of Texture Studies*, *37*, 711-730.

Chevanan, N. & Muthukumarappan, K. (2007). Effect of calcium and phosphorus, residual lactose, and salt-to-moisture ratio on the melting characteristics and hardness of Cheddar cheese during ripening. *Journal of Food Science*, *72*, E168-E176.

Choi, J., Horne, D. S., Johnson, M. E. & Lucey, J. A. (2008). Effects of the concentration of insoluble calcium phosphate associated with casein

micelles on the functionality of directly acidified cheese. *Journal of Dairy Science*, *91*, 513-522.

Cooke, D. R. & McSweeney, P. L. H. (2013). The influence of alkaline earth metal equilibria on the rheological, melting and textural properties of Cheddar cheese. *Journal of Dairy Research*, *80*, 418-428.

Cooke, D. R. (2014). Exploring the physicochemical basis of cheese texture, rheology and functionality, with emphasis on cation-casein interactions. *PhD Thesis*, University College Cork, Ireland.

Creamer, L. K., Lawrence, R. C. & Gilles, J. (1985). Effect of acidification of cheese milk on the resultant Cheddar cheese. *New Zealand Journal of Dairy Science and Technology*, *20*, 185–203.

Fathollahi, I., Hesari, J., Azadmard, S. & Oustan, S. (2010). Influence of proteolysis and soluble calcium levels on textural changes in the interior and exterior of Iranian UF white cheese during ripening. *World Academy Science, Engineering and Technology*, *66*, 844-849.

Fox, P. F. (1963). Potentiometric determination of salt in cheese. *Journal of Dairy Science*, *46*, 744–745.

Fröhlich-Wyder, M. T., Guggisberg, D. & Wechsler, D. (2009). Influence of low calcium and low pH on melting characteristics of model Raclette cheese. *Dairy Science and Technology*, *89*, 463-483.

Guinee, T. P., Feeney, E. P., Auty, M. A. E. & Fox, P. F. (2002). Effect of pH and calcium concentration on some textural and functional properties of Mozzarella cheese. *Journal of Dairy Science*, *85*, 1655-1669.

Gunasekaran, S. & Ak, M. M. (2003). Cheese Rheology and Texture. Florida, USA: CRC Press.

Hassan, A., Johnson, M. E. & Lucey, J. A. (2004). Changes in the proportions of soluble and insoluble calcium during the ripening of Cheddar cheese. *Journal of Dairy Science*, *87*, 854-862.

Hennelly, P. J., Dunne, P. G., O'Sullivan, M. & O'Riordan, D. (2005). Increasing the moisture content of imitation cheese: effects on texture, rheology and microstructure. *European Food Research and Technology*, *220*, 415-420.

Hou, J., Hannon, J. A., McSweeney, P. L. H., Beresford, T. P. & Guinee, T. P. (2014). Effect of curd washing on cheese proteolysis, texture, volatile compounds, and sensory grading in full fat Cheddar cheese. *International Dairy Journal*, *34*, 190-198.

IDF (1982). Cheese and processed cheese; Determination of the total solids content. Standard 4A:1982. Brussels, Belgium: International Dairy Federation.

IDF (1986). Determination of the nitrogen content (Kjeldahl method) and calculation of crude protein content. Standard 20A:1986. Brussels, Belgium: International Dairy Federation.

IIRS (1955). Determination of the percentage of fat in cheese. Irish Standard 69:1955. Institute for Industrial Research and Standards, Dublin.

Joshi, N. S., Muthukumarappan, K. & Dave, R. I. (2003). Understanding the role of calcium in functionality of part skim Mozzarella cheese. *Journal of Dairy Science, 86,* 1918-1926.

Kemmei, T., Kodama, S., Yamamoto, A., Inoue, Y. & Hayakawa, K. (2013). Determination of ethylenediaminetetraacetic acid in foods by reversed-phase high-performance liquid chromatography. *Food Chemistry, 138,* 866-869.

Kosikowski, F. V. & Mistry, V. V. (1997). *Cheese and Fermented Milk Foods, Vol 1: Origins and Principles,* 3rd Ed. F. V. Kosikowski & Associates: Great Falls, VA, USA.

Kuchroo C. N. & Fox P. F. (1982). Soluble nitrogen in Cheddar cheese: comparison of extraction procedures. *Milchwissenschaft, 37,* 331-335.

Lawrence, R. C., Creamer, L. K. & Gilles, J. (1987). Texture development during cheese ripening. *Journal of Dairy Science, 70,* 1748-1760.

Lee, M. R. & Lee, W. J. (2009). The role of calcium equilibrium on the functional properties of cheese: a review. *Korean Journal for Food Science of Animal Resources, 29,* 545-549.

Lee, M. R., Johnson, M. E. & Lucey, J. A. (2005). Impact of modifications in acid development on the insoluble calcium content and rheological properties of Cheddar cheese. *Journal of Dairy Science, 88,* 3798-3809.

Lee, M. R., Johnson, M. E., Govindasamy-Lucey, S., Jaeggi, J. J. & Lucey, J. A. (2011). Effect of different curd-washing methods on the insoluble

calcium content and rheological properties of Colby cheese during ripening. *Journal of Dairy Science*, *94*, 2692-2700.

Lucey, J. A. & Fox, P. F. (1993). Importance of calcium and phosphate in cheese manufacture: a review. *Journal of Dairy Science*, *76*, 1714-1724.

Lucey, J. A., Gorry, C. & Fox, P. F. (1993). Changes in the acid-base buffering curves during the ripening of Emmental cheese. *Milchwissenschaft*, *48*, 183-186.

Lucey, J. A., Johnson, M. E. & Horne, D. S. (2003). Perspectives on the basis of the rheology and texture properties of cheese. *Journal of Dairy Science*, *86*, 2725–2743.

Lucey, J. A., Mishra, R., Hassan, A. & Johnson, M. E. (2005). Rheological and calcium equilibrium changes during the ripening of Cheddar cheese. *International Dairy Journal*, *15*, 645-653.

Matheson, A. R. (1981). The immunochemical determination of chymosin activity in cheese. *New Zealand Journal of Dairy Science and Technology*, *16*, 33-41.

McAuliffe, L. N., Kilcawley, K. N., Sheehan, J. J. & McSweeney, P. L. H. (2016). Manufacture and incorporation of liposome-entrapped ethylenediaminetetraacetic acid into model miniature Gouda-type cheese and subsequent effect on starter viability, pH, and moisture content. *Journal of Food Science, 81, C2708-2712.*

McMahon, D. J., Alleyne, M. C., Fife, R. L. & Oberg, C. J. (1996). Use of fat replacers in low fat Mozzarella cheese. *Journal of Dairy Science, 79,* 1911-1921.

McMahon, D. J., Paulson, B. & Oberg, C. J. (2005). Influence of calcium, pH, and moisture on protein matrix structure and functionality in direct-acidified nonfat Mozzarella cheese. *Journal of Dairy Science, 88,* 3754-3763.

Metzger, L. E., Barbano, D. M., Rudan, M. A. & Kindstedt, P. S. (2000). Effect of milk pre-acidification on low fat Mozzarella cheese: 1. Composition and yield. *Journal of Dairy Science, 83,* 646–658.

Mizuno, R. & Lucey, J. A. (2005). Effects of two types of emulsifying salts on the functionality of nonfat *pasta filata* cheese. *Journal of Dairy Science, 88,* 3411-3425.

O'Mahony, J. A., Lucey, J. A. & McSweeney, P. L. H. (2005). Chymosin-mediated proteolysis, calcium solubilization and texture development during the ripening of Cheddar cheese. *Journal of Dairy Science, 88,* 3101–3114.

O'Mahony, J. A., McSweeney, P. L. H. & Lucey, J. A. (2006). A model system for studying the effects of colloidal calcium phosphate concentration on the rheological properties of Cheddar cheese. *Journal of Dairy Science, 89,* 892-904.

Pastorino, A. J., Hansen, C. L. & McMahon, D. J. (2003). Effect of pH on the chemical composition and structure-function relationships of Cheddar cheese. *Journal of Dairy Science, 86,* 2751-2760.

Rampilli, M., Raja, V. & Gatti, A. L. (1998). Evaluation of residual chymosin. *Scienza e Technica Lattiero-Casearia*, *49*, 29‑41.

Salaün, F., Mietton, B. & Gaucheron, F. (2005). Buffering capacity of dairy products. *International Dairy Journal*, *15*, 95-109.

Sheehan, J. J. & Guinee, T. P. (2004). Effect of pH and calcium level on the biochemical, textural and functional properties of reduced-fat Mozzarella cheese. *International Dairy Journal*, *14*, 161-172.

Sheehan, J. J. (2007). Syneresis. In: McSweeney, P. L. H. (Ed), *Cheese Problems Solved*, (pp. 77-78). Cambridge, England: Woodhead Publishing Limited.

Sheehan, J. J., Oliveira, J. C., Kelly, A. L. & Mc Sweeney, P. L. H (2007). Effect of cook temperature on primary proteolysis and predicted residual chymosin activity of a semi-hard cheese manufactured using thermophilic cultures. *International Dairy Journal*, *17*, 826-834.

Shirashoji, N., Jaeggi, J. J. & Lucey, J. A. (2006). Effect of trisodium citrate concentration and cooking time on the physicochemical properties of pasteurized process cheese. *Journal of Dairy Science*, *89*, 15-28.

Thomas, T. D. & K. N. Pearce. (1981). Influence of salt on lactose fermentation and proteolysis in Cheddar cheese. *New Zealand Journal of Dairy Science and Technology*, *16*, 253–259.

Upreti, P., McKay, L. L. & Metzger, L. E. (2006a). Influence of calcium and phosphorus, lactose, and salt-to-moisture ratio on Cheddar cheese quality: Changes in residual sugars and water-soluble organic acids during ripening. *Journal of Dairy Science*, *89*, 429-443.

Upreti, P., Metzger, L. E. & Hayes, K. D. (2006b). Influence of calcium and phosphorus, lactose, and salt-to-moisture ratio on Cheddar cheese quality: Proteolysis during ripening. *Journal of Dairy Science*, *89*, 444-453.

Upreti, P. & Metzger, L. E. (2007). Influence of calcium and phosphorus, lactose, and salt-to-moisture ratio on Cheddar cheese quality: pH changes during ripening. *Journal of Dairy Science*, *90*, 1-12.

Wang, F., Zhang, X., Luo, J., Guo, H., Zeng, S. S. & Ren, F. (2011). Effect of proteolysis and calcium equilibrium on functional properties of natural Cheddar cheese during ripening and the resultant processed cheese. *Journal of Food Science*, *76*, E248-E253.

Watkinson, P., Coker, C., Crawford, R., Dodds, C., Johnston, K., McKenna, A. & White, N. (2001). Effect of cheese pH and ripening time on model cheese textural properties and proteolysis. *International Dairy Journal*, *11*, 455-464.

Yun, J. J., Kiely, L. J., Barbano, D. M. & Kindstedt, P. S. (1993). Mozzarella cheese: Impact of cooking temperature on chemical composition, proteolysis and functional properties. *Journal of Dairy Science*, *76*, 3664-3673.

Yun, J. J., Barbano, D. M., Kindstedt, P. S. & Larose, K. L. (1995). Mozzarella cheese: impact of whey pH at draining on chemical, composition, proteolysis, and functional properties. *Journal of Dairy Science*, *78*, 1-7.

Chapter 4

Manufacture and incorporation of liposome-entrapped calcium chelating salt into model miniature Gouda-type cheese and subsequent effect on starter viability, pH, and moisture content

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Abstract

Liposome-entrapped ethylenediaminetetraacetic acid (EDTA) was incorporated into a model miniature Gouda-type cheese (20 g) in order to assess its effect on rennet gelation, starter viability, pH, and moisture content. EDTA was entrapped within two different food-grade proliposome preparations, Pro-Lipo Duo and Pro-Lipo C (50% and 40% unsaturated soybean phospholipids and 50% and 60% aqueous medium, respectively), using the following high-shear technologies: Ultra-Turrax (5000 rpm), two-stage homogenisation (345 bar), or microfluidisation (690 bar). Liposome size distribution was affected by the high-shear technology employed, with the proportion of large vesicles (>100 nm) decreasing in the order Microfluidisation>two-stage homogenisation>Ultra-Turrax. All EDTA-containing liposomes were stable during 28 d refrigerated storage, with no significant ($P \leq 0.05$) change in size distribution or EDTA entrapment efficiency (%EE). Liposome composition affected the entrapment of EDTA, with Pro-Lipo C having a significantly greater %EE compared to Pro-Lipo Duo, 63% and 54%, respectively. For this reason, Pro-Lipo C liposomes, with and without EDTA, were incorporated into model miniature Gouda-type cheese. Addition of liposome-entrapped EDTA to milk during cheese making did not impact pH or rennet gel formation. No differences in composition or pH were evident in liposome-treated cheeses. The results of this study show that the incorporation of liposome-entrapped EDTA into milk during cheese manufacture did not affect milk fermentation, moisture content, or pH suggesting that this approach may be suitable for studying the effects of calcium equilibrium on the texture of brine-salted cheeses.

Abbreviations

%EE, entrapment efficiency; 2-SH, two-stage homogeniser; Ca, calcium; CSLM, confocal scanning laser microscopy; DF, diafiltration; LUV, large unilamellar vesicles; MF, microfluidizer; MLV, multi-lamellar vesicles; PDI, polydispersity index; PLA_2, phospholipase; SUV, small unilameller vesicles; T-X, triton-X 100; UF, ultrafiltration; UT, Ultra-Turrax; VCF, volume concentration factor

4.1 Introduction

Liposomes are single or multi-layered vesicles capable of complete enclosure of an aqueous phase within their lipid bilayer (Law and King 1985; Risch and Reineccius 1995; Singh *et al.*, 2012). The lipid fraction is most commonly phospholipid. Liposomes form spontaneously when phospholipids are dispersed in an aqueous medium, although further manufacturing steps are required in order to obtain vesicles of a specific size (Risch and Reineccius 1995). The phospholipid bilayers are so arranged to reduce the interaction of lipophilic groups with water, thus forming a very stable particle. Liposomes may be composed of a single bilayer or hundreds of concentric bilayers depending on its composition and manufacturing method. Liposomes may have small unilameller vesicles (SUV: 25-100 nm), large unilameller vesicles (LUV: > 100 nm), or large multilamellar (MLV: five or more concentric lamellae, ranging from 100-1000 nm) layers (Ostro, 1983; Risch and Reineccius, 1995).

Proliposome formulations were developed (Payne *et al.*, 1986) which are stable phospholipid formulations that permit generation of liposomes upon addition of an aqueous phase. The Pro-Liposome Approach (Pro-Lipo) (Leigh 1991) is based on the use of a pre-prepared gel of phospholipid bilayers in a hydrophilic medium, usually ethanol. The pro-liposome gel does not contain adequate water to form a liposome and therefore remains stable until sufficient water is added. Dufour *et al.* (1996) successfully encapsulated enzymes with entrapment efficiencies of 96% using this method. Laloy *et al.* (1996) reported 64% entrapment efficiency of encapsulated liposomes within a cheese curd using this method.

Formation of liposomes of a specific size and lamellarity requires energy (Singh *et al.,* 2012). MLV form immediately when bilayer-forming polar lipids are dissolved in aqueous media under mild agitation. In order to produce LUV and SUV, considerable energy inputs are required that are sufficient to disrupt MLV structures and force the generation of monomodal vesicles (New, 1990). The energy applied to form the desired liposome is either (a) nonmechanical energy such as reverse-phase evaporation (Szoka and Papahadjopoulos 1978), dehydration-rehydration technique (Kirby and Gregoriadis 1984), and freeze-induced fusion (Uemura *et al.,* 1996), or (b) mechanical energy such as microfluidisation (Thompson and Singh 2006), high-pressure homogenisation (Alexander *et al.,* 2012) and sonication (Maa and Hsu 1999). Nonmechanical methods usually involve the use of organic solvents or detergents which are not desirable from a food manufacturing perspective as they may leave residues within the food product.

Recently, liposomes have found increasing application within the food industry. Due to the growing recognition of the functional properties of liposomes, improved manufacturing technologies as well as the reduced cost of raw materials, it has become more feasible to use liposomes to deliver functional components such as nutraceuticals, antimicrobials, and flavours to foods (Kirby *et al.,* 1991; Kheadr *et al.,* 2002; Kosaraju *et al.,* 2006; Imran *et al.,* 2015).

Enzymes encapsulated in liposomes have been successfully used in cheese manufacture to accelerate ripening (Kirby *et al.,* 1987; Anjani *et al.,* 2007; Nongonierma *et al.,* 2013), to increase shelf-life through encapsulation of antimicrobial agents such as nisin and lysozyme (Thapon and Brule 1986; Laridi

et al., 2003; de Silva Malheiros *et al.,* 2012), and to fortify cheese with liposome-entrapped vitamins and antioxidants (Kirby *et al.,* 1991; Bainville *et al.,* 2000; Rashidinejad *et al.,* 2014). Direct addition of these substances in the free form to milk containing rennet and starter culture presents a host of problems, namely, premature hydrolysis of caseins resulting in reduced cheese yield, losses during whey drainage, and uneven distribution in the curd. Encapsulation alleviates such problems because the active ingredient is protected from the outside environment, as well as allowing for controlled release.

This study was performed to develop EDTA-containing liposomes with the view to controlling the ratio of insoluble to soluble calcium (Ca) phosphate in brine-salted cheeses, in order to evaluate the effect of Ca equilibrium on the textural and rheological properties of the cheese. Previous studies (O'Mahony *et al.,* 2005; Brickley *et al.,* 2009) have indicated that controlling this ratio affects the texture of dry-salted cheeses and inclusion of Ca or sequestering agents, such as EDTA, is straightforward in such varieties as they can be added to the curd together with the salt. However, addition of EDTA during the manufacture of brine-salted cheeses has been found to cause unsatisfactory changes to pH and composition (Chapter 3), therefore, making it difficult to determine if the textural and rheological changes brought about by addition of Ca sequestering salts were as a result of changing the ratio of insoluble to soluble Ca or, due to increased moisture content of the cheese. Entrapment within liposomes might allow manufacture of Gouda-type cheese with added calcium chelator without causing differences in composition. The EDTA would then be released into the cheese matrix during ripening as the liposomes degraded.

The potential of two food-grade proliposomes (Duo and C) to encapsulate EDTA in soluble form using 3 high-shear mechanicalunit operations; Ultra-Turrax, 2-stage homogenisation and microfluidisation were investigated. The parameters evaluated included the influence of processing pressure, liposome formulation, and EDTA concentration on size distribution, polydispersity, zeta potential, entrapment efficiency (%EE), and liposome stability. Selected liposome preparations were incorporated into milk and cheese and their effects on rennet gelation, milk fermentation, starter viability, pH, and moisture content were determined.

4.2 Materials and methods

4.2.1 Preparation of EDTA solution

A concentrated solution of EDTA (Sigma-Aldrich, Dublin, Ireland) was prepared to facilitate encapsulation. EDTA was suspended in 25 mM Tris-HCl buffer at pH 7.4 to a final concentration of 12.5% (w/v).

4.2.2 Preparation of liposomes

Two different food-grade proliposome (Pro-Lipo) preparations were investigated, Pro-Lipo Duo and Pro-Lipo C (Lucas Meyer, Chelles, France), containing 50% and 40% unsaturated soybean phospholipids, suspended in 50% and 60% hydrophilic medium consisting of glycerol and ethanol, respectively. The composition of Pro-Lipo Duo and C differ in terms of the amount of phosphatidylinositol (PI) and phosphatidycholine (PC) present in each. The PI and PC contents of Duo were 3% and 52%, respectively, and C contained 14% and 23%, respectively, of the total phospholipid content. Liposomes were prepared by adding 100 g of Pro-Lipo Duo or C to 200 mL of EDTA solution. Control liposomes were prepared by adding 100 g of Pro-Lipo Duo or C to 200 mL of 25 mM Tris HCl buffer at pH 7.4. The mixtures were agitated using an Ultra-Turrax (model: t25 IKA-Werke GmbH and Co. KG, Staufen, Germany) operated at 500 rpm for 10 min at 21 ˚C, after which 600 mL of 25 mM Tris HCl buffer at pH 7.4 was added and further agitated at 500 rpm for 10 min at 21 ˚C. The liposome preparations were recovered and underwent 3 separate high-shear processing treatments:

1. *Ultra-Turrax (UT)* – Agitation provided by an Ultra-Turrax operated at 5000 rpm for 15 min at 21 ˚C.
2. *Two-stage homogenisation (2-SH)* – Processing performed with a single pass through a 2-stage homogeniser (APV GEA Niro-Soavi S.p.A., Parma, Italy) at 280/60 bar.
3. *Microfluidisation (MF)* – Shear provided by a single pass through a microfluidizer, M-110-EH-30 (Microfluidics, Chesham, UK) at 690 bar. The microfluidizer was equipped with a Y-shaped interaction chamber and operated with a refrigerant, which allowed the temperature of the mix to be controlled at 16 ˚C.

Liposome preparations were concentrated and washed using a pressure-driven, tangential-flow filtration device (Pellicon 2 mini-holder; Merck-Millipore, Tullagreen, Carrigtwohill, Ireland), as described by Crowley *et al.* (2015), except that no heat exchanger was used. All liposome preparations were concentrated to a volume concentration factor (VCF) of 2 by removing permeate, with the retentate being continuously recirculated back to the feed, the liposomes were washed by adding a volume of de-ionised water equivalent to the volume of permeate removed during the concentration step into the feed, followed by further concentration (VCF=2) by removing a second permeate. A constant trans-membrane pressure of ~ 0.5 bar was maintained throughout ultrafiltration (UF) and diafiltration (DF) for all liposome preparations.

4.2.3 Determination of EDTA entrapment efficiency

4.2.3.1 Liposome recovery

Following UF, 10 mL of concentrated washed liposomes was added to 20 mL of 25 mM Tris HCl buffer at pH 7.4 and subjected to ultracentrifugation at 100,000 g at 4 ˚C for 1 h. The resulting pellet containing the liposomes was separated from the supernatant and re-suspended in 10 mL of 25 mM Tris HCl buffer at pH 7.4 and vortexed.

4.2.3.2 Liposome preparation

The %EE is defined as the percentage of EDTA entrapped in the liposome relative to the total amount of EDTA initially present in the mixture (Dufour *et al.,* 1996). The %EE of EDTA within liposomes was determined using a modified HPLC method by Kemmei *et al.* (2013). The EDTA concentration was measured in the following samples:

- The supernatant, diluted 1:100 with 25 mM Tris HCl buffer, to determine unencapsulated EDTA concentration;
- The re-suspended liposomes, diluted 1:100 with 25 mM Tris HCl buffer, to determine EDTA present on surfaces of liposomes;
- The disrupted re-suspended liposomes, 0.5 mL of re-suspended vesicles was mixed with 0.5 mL of Triton-X 100 (T-X) (Sigma-Aldrich, Dublin, Ireland) vortexed for 10 s, placed in a 40 ˚C water bath for 1 h, and

vortexed for 10 s before being diluted 1:50 with 25 mM Tris HCl buffer, to determine the encapsulated EDTA.

Subtraction of the quantity of EDTA present in the pellet from the disrupted pellet provided the quantity of EDTA entrapped within the core.

The EDTA concentration in each phase was then expressed as a percentage of the total EDTA concentration determined in the liposome dispersion obtained directly after UF.

4.2.3.3 EDTA quantification by HPLC

A stock standard was prepared as described by Kemmei *et al.* (2013), except Fe (III) was not added to the standards or the samples before injection onto the column. EDTA was determined using a calibration curve ranging from 0 to 100 μM. All samples were passed through a 0.45 μm cellulose acetate filter (Sartorius GmbH, Gottingen, Germany) before injection onto the column.

4.2.3.4 Apparatus and chromatographic conditions for analysis of EDTA

The analysis was carried out using a Waters 600 HPLC system (Waters Corp., Milford, MA, USA) consisting of a Waters 2487 dual wavelength detector, a Waters 717^{+} autosampler, and a Waters 600 pump interfaced with Empower 3 software. The analysis was carried out at 40 ˚C and 260 nm using a Kinesis Telos C18 RP-HPLC column, 250 x 4.6 mm with 300 Å pore size and 5 μm particle size (Kinesis, St. Neots, Cambridgeshire, UK). The mobile phase was a mixture of 100

μM Fe (III) chloride and 5 mM sulfuric acid (pH 2.0), and the flow rate was 1.0 mL/min. The injection volume was 100 μL. Quantification was performed by integration of peak areas.

4.2.3.5 Stability of entrapped EDTA over time

The concentration of EDTA in the liposomes (Duo and C) manufactured by 2-SH was measured over 28 d as described by Nongonierma *et al.* (2009). The liposomes were stored at 8 ˚C as ultracentrifuged pellets. Pellets were re-suspended and disrupted using T-X over the 28 d period and analysed by HPLC for EDTA concentration as previously described.

4.2.4 Characterisation of liposomes

4.2.4.1 Z-average diameter, polydispersity index, and zeta potential

The hydrodynamic diameter and zeta potential of liposome preparations were measured at 21 ˚C by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments, Malvern, Worcestershire, UK) as described by Nongonierma *et al.* (2009). Detection was completed at a backscattering angle of 173˚. The refractive index of liposome and water were set at 1.45 and 1.33, respectively. The measurements were carried out on all liposome mixtures before UF; samples were diluted 1:100 with 25 mM Tris HCl buffer.

4.2.4.2 Influence of pH on liposome zeta potential

The influence of changing pH on the zeta potential of both Pro-Lipo Duo and C manufactured using all high-shear processes was determined according to the method described by Nongonierma *et al.* (2009). All liposomes were diluted 1:100 with 25 mM Tris HCl buffer, prior to UF. The pH was measured using a calibrated pH meter (Seven Go; Mettler-Toledo AG, Greifensee, Switzerland). The pH of the solution was adjusted using either 0.05 N HCl or 0.05 N NaOH aqueous solutions.

4.2.4.2 Confocal laser scanning microscopy

The morphology of the liposomes was determined using confocal laser scanning microscopy (Olympus FluoView® FV1000 confocal laser scanning microscope; CLSM, Olympus Corporation, Tokyo, Japan). Particles > 100 nm were detectable using this technique. CSLM was carried out on both Pro-Lipo Duo and C after UT and 2-SH processing, before UF. Samples were diluted 1:100 with Tris HCl buffer. The liposomes were immobilized using certified low-melt agarose (BIO-RAD, Hercules, CA, USA) and stained using Nile Red (Sigma-Aldrich, Dublin, Ireland). The liposomes were visualized using the differential interface contrast mode.

4.2.5 Stability of liposomes under Gouda time/temperature cycle

Only C EDTA liposomes manufactured using 2-SH, after UF were added to 100 mL of pasteurized milk. B11 starter culture (*Lactococcus lactis* subsp. *cremoris*,

Lactococcus lactis subsp. *lactis*, *Leuconostoc mesenteroides* subsp. *cremoris*, and citrate-positive *Lactococcus lactis*) (Chr. Hansen Ltd., Little Island, Co. Cork, Ireland) added at a level of 0.03% (w/v) was used for fermentation. Sterilised conical flasks (250 mL) were filled with 100 mL of full-fat, batch-pasteurized milk and subjected to the following treatments: (a) inoculated with starter and C EDTA-containing liposomes; (b) inoculated with starter only; (c) C EDTA only; (d) inoculated with starter, C EDTA, and T-X; (e) inoculated with starter and C EDTA; and (f) C EDTA and T-X. The flasks were transferred into a shaking water bath and subjected to the typical temperature cycle for Gouda cheese production: 31 ˚C for 90 min followed by 38 ˚C for 90 min. Samples were taken after inoculation and after 90 and 180 min of fermentation to determine the viable cell counts of starter bacteria. Viable counts of *Lactococcus lactis* were enumerated in duplicate as described by Terzaghi and Sandine (1975).

4.2.6 Rennet gelation properties of milk supplemented with Pro-Lipo Duo and C, with and without EDTA

Dynamic oscillatory analysis of renneted milks containing no liposomes (control), Pro-Lipo C (with and without EDTA manufactured by 2-SH), and Pro-Lipo Duo (with and without EDTA manufactured by 2-SH) were performed using a controlled shear stress rheometer (model: AR-G2, TA Instruments; Waters LLC, Leatherhead, Surrey, UK) equipped with a Peltier concentric cylinder system with a cylindrical rotor (aluminum; 42.01 mm (h) x 28.02 mm (d)). The storage (G') and loss (G") moduli were recorded at an angular frequency (ω) of 0.6283 rad s^{-1} and a shear strain of 0.01 for 90 min at 30 ˚C. Each sample was analyzed in triplicate. All samples were tempered at 30 ˚C for 30 min before direct addition of

rennet (200 μL of Maxiren-180, supplied by DSM Food Specialties, Delft, Holland).

4.2.7 Miniature Gouda-type cheese manufacture

Miniature Gouda-type cheeses were manufactured using a modification of the method of Shakeel-Ur-Rehman *et al.* (1998). Raw milk (1300 mL) was pasteurized in a sterile stainless steel beaker at 63 ˚C for 30 min by heating in a water bath at 65 ˚C with intermittent stirring. Milk (200 mL) was transferred into six 250 mL wide-mouth centrifuge bottles and the temperature was adjusted to 31˚C by holding in a water bath set at 31 ˚C. B11 starter (Chr. Hansen Ltd.,) (0.03%) and sodium nitrate (Sigma-Aldrich) (0.006%) were added to the milk which was left to ripen for 30 min. Rennet (Maxiren-180, supplied by DSM Food Specialties, Delft, Holland) (0.035%) and 1 M $CaCl_2$ (0.09%) were then added to the milk which was mixed with a glass rod. Following this, 4 mL Pro-Lipo Duo and C, with and without EDTA, manufactured by 2-SH and T-X (0.8 mL) was supplemented into milk before allowing 50 min for the milk to coagulate.

The coagulum was cut using horizontal and vertical cutters and the cubes were allowed to heal before stirring for 20 min. A third of the whey was removed and replaced with hot water (54 ˚C) until a final temperature of 38 ˚C was reached. The curds were “cooked” at 38 ˚C for 90 min, stirring intermittently to prevent them from sticking. The bottles were centrifuged at 1700 g for 60 min at 21 ˚C (Sorvall® RC-5C Plus; Kendro Laboratory Products, Newtown, CT, USA; fitted with a GSA rotor). The curds were removed from the centrifuge bottles and placed in cheese molds lined with cheese cloth. The molds were placed in 150 mL

beakers before the whey was poured in on top. The curds were pressed under the whey for 15 min. The curds were removed from the beaker containing the whey and pressed for a further 30 min. Then cheese was salted by placing in brine (20% NaCl, 0.05% $CaCl_2$) at room temperature for 30 min, after which it was removed from the brine, its surface dried and left over night at room temperature in 150 mL beakers covered with aluminium foil. Cheeses were vacuum-packed and ripened at 8 ˚C.

4.2.8 Enumeration of starter bacteria

Viable *Lactococcus lactis* cells were enumerated in duplicate as described by Terzaghi and Sandine (1975) at 1 day of ripening.

4.2.9 Chemical analysis

The moisture content of cheese was determined using an oven-drying method (IDF 1982). The pH was measured, using a calibrated pH meter, on cheese slurry made from 10 g cheese and 10 mL of de-ionised water. The EDTA concentrations of the cheese and whey samples were evaluated using the HPLC method described in *Chapter 3*. Samples were prepared as follows: the pH 4.6-soluble fraction was extracted from the cheese using the method described by Kuchroo and Fox (1982). T-X (0.5 mL) was added to 0.5 mL of the pH 4.6-soluble fraction in order to disrupt liposomes. Samples were diluted 1:50 using de-ionised water. T-X (0.5 mL) was added to 0.5 mL of whey, collected after cheese manufacture, in order to disrupt liposomes. Samples were diluted 1:50 using de-ionised water.

4.2.10 Microstructure of the cheese matrix

Cryogenic scanning electron microscopy of cheese samples was completed using the method described by O'Sullivan *et al*. (2016) with the following modifications: samples were etched for 1 min at a temperature of -95 ˚C inside a preparation chamber to remove ice that was formed during freezing in liquid nitrogen, and the electron beam had a voltage of 3 kV whilst imaging.

4.2.11 Statistical analysis

Each set of experiments was repeated in triplicate with at least triplicate analyses in each repetition. All statistical analyses were performed using Minitab® 16 (Minitab Inc., State College, PA, USA). Differences between treatments were tested by analysis of variance (ANOVA) using the general linear model (GLM). A significance level, α, of 0.05 was used for all statistical analyses ($P \leq 0.05$).

4.3. Results and Discussion

4.3.1 Characterisation of liposomes

4.3.1.1 Liposome size distribution

The hydrodynamic diameters of Pro-Lipo C and Duo, with and without EDTA, manufactured using UT, 2-SH, and MF, are illustrated in Figure 4.1 (a, b, c, d). Liposome size distribution was affected by both processing conditions and liposome composition. The proportion of larger-sized liposomes (>100 nm) decreased as the processing pressure increased (microfluidizer>2-stage homogeniser>Ultra Turrax) with a concomitant increase in the proportion of smaller-sized liposomes (<100 nm). All liposome preparations, manufactured using UT, contained 2 distinct particle size distribution peaks, the largest of which ranged between 800 and 6500 nm, indicating the presence of a significant number of larger liposomes. Liposomes manufactured using two 2-SH and MF, each appeared to have one main peak (monomodal) ranging between 16 and 450 nm and 30 and 60 nm, respectively, indicating good homogeneity. Similarly, Nongonierma *et al.* (2009) demonstrated that increasing processing pressure from 276 to 690 bar resulted in a decrease in the proportion of larger-sized liposomes and an increase in the proportion of smaller-sized liposomes produced. Thompson and Singh (2006) also reported a 40% decrease in the mean liposome size upon increasing pressure from 690 to 1034 bar.

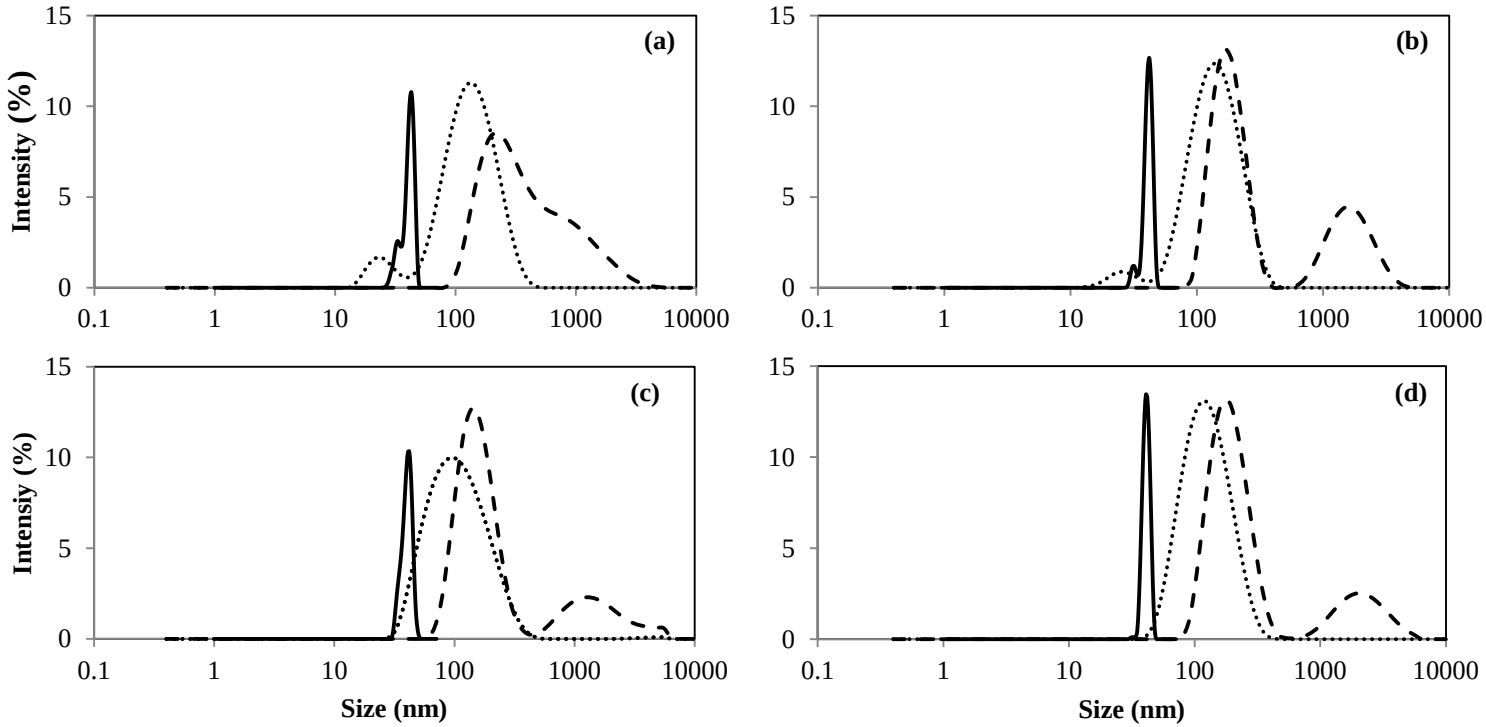

Figure 4.1 Influence of processing conditions: UT (▬ ▬), 2-SH (••••) and MF (▬) and EDTA addition on the size distribution profile of Pro-Lipo C No EDTA (**a**), Pro-Lipo Duo No EDTA (**b**), Pro-Lipo C EDTA (**c**), and Pro-Lipo Duo EDTA (**d**) directly after manufacture.

The z-average diameter values (Figure 4.2a) for each liposome preparation showed significant differences ($P \leq 0.05$) between each liposome type (C and Duo) processed using the same manufacturing technology. The z-average diameter values for liposomes manufactured using UT were 50% larger than liposomes produced using 2-SH or MF, indicating that higher pressure results in a more homogeneous liposome preparation in terms of size distribution. Liposome preparations (C and Duo) manufactured using 2-SH and MF had an average hydrodynamic diameter of 100 and 110 nm, respectively. These values are in accordance with previous studies, where the average diameter of liposomes was found to be in the region of 100 nm (Laloy *et al.*, 1998; Thompson and Singh 2006). The larger average diameter values obtained for Pro-Lipo Duo may be due to the increased phospholipid concentration compared with Pro-Lipo C (50 and 40%, respectively). Alexander *et al.* (2012) found that increasing soybean phospholipid concentration from 100 to 250 mg mL^{-1} resulted in an increase in liposome size from 103 to 137 nm.

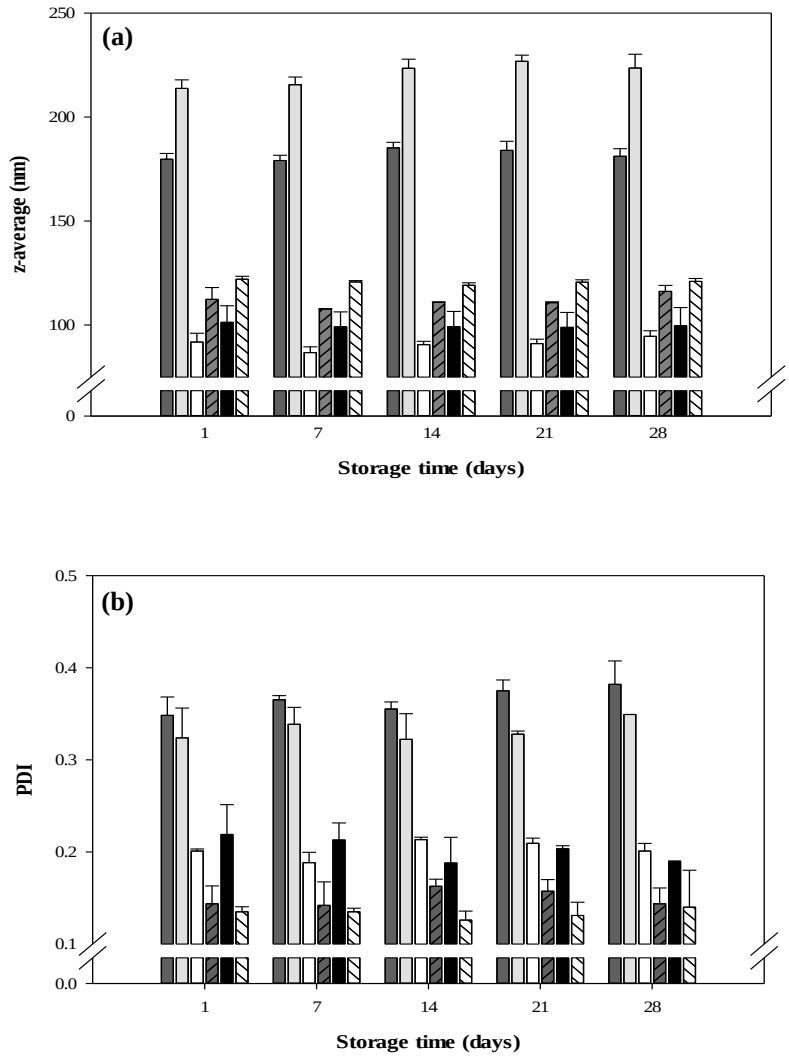

Figure 4.2 Z-average diameter (**a**) and polydispersity index (PDI) (**b**) values of liposomes manufactured using UT; Duo EDTA (▬) and C EDTA (▭), 2-SH; Duo EDTA (▭) and C EDTA (▨) and MF; Duo EDTA (▬) and C (▧) EDTA throughout 28 d refrigerated (4 ˚C) storage.

Entrapment of EDTA resulted in significant reduction of particle size z-average diameter. This effect was greatest for liposomes manufactured using UT, followed by 2-SH and then MF. The reduction in mean size distribution recorded for liposomes containing EDTA may be attributed to the effect of the EDTA on

membrane lipid organization and/or lipid hydrophobicity. EDTA is known to bind (divalent) cations, which are bound to the phospholipids. By removing these bound cations, the negative charges of the phosphate groups are more obvious. This higher charge density may explain the smaller liposome diameter in the presence of EDTA. Barisik *et al.* (2014) demonstrated that the surface charge density of spherical silica nanoparticles decreased with increased particle size and attributed this result to the change in the surface concentration of H^+ ions.

Confocal scanning laser microscopy (CSLM) images for Pro-Lipo C without EDTA manufactured using 2-SH and UT are shown in Figures 4.3a and 4.3b. Liposomes manufactured using UT were observed as small spherical particles, with a size ranging from 100 to several thousand nm, which is consistent with values found with the zetasizer (80 – 6500 nm). Only a few particles were visible in the micrograph of the liposomes manufactured using 2-SH, this is due to the fact that the liposomes produced using this process were too small (~ 100 nm, average) to be detected using CSLM, again supporting the values provided by the zetasizer (10 – 350 nm).

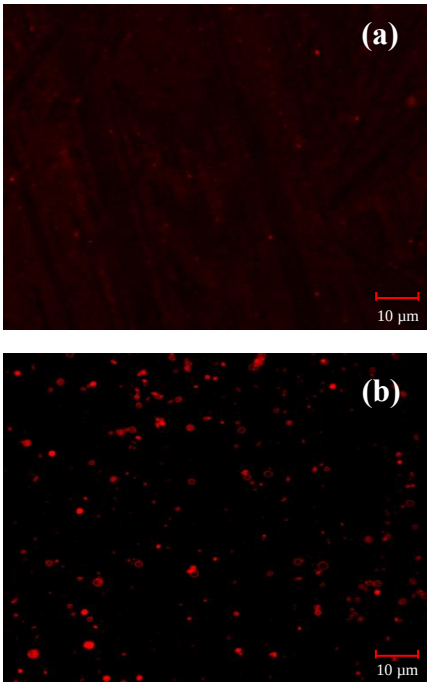

Figure 4.3 Confocal scanning laser microscopy images of liposomes; Pro-Lipo C No EDTA 2-SH (**a**) and Pro-Lipo C No EDTA UT (**b**), diluted 1:100 with 25 mM Tris HCl buffer, at day 28, stained with Nile Red, zoom 5x.

4.3.1.2 Polydispersity of liposomes

The polydispersity index (PDI) of liposome preparations (Figure 4.2b) decreased with increasing processing pressure. PDI values decreased from 0.32 to 0.14 for Pro-Lipo Duo EDTA and 0.35 to 0.21 for Pro-Lipo C EDTA, when the processing intensity was increased from 5000 rpm (UT) to 690 bar (MF). Similar results have been found by Talsma *et al.* (1989), and Barnadas-Rodríguez and Sabés (2001). No significant differences ($P \leq 0.05$) in PDI values were observed for either C EDTA or Duo EDTA when processing pressure was increased from 340 (2-SH) to 690 (MF) bar. There was, however, a significant difference ($P \leq 0.05$) in PDI values between liposome type (C EDTA and Duo EDTA)

manufactured using the same process. Duo EDTA had a significantly ($P \leq 0.05$) lower PDI value compared with Pro-Lipo C EDTA, 0.14 and 0.20, respectively, for 2-SH and 0.14 and 0.21, respectively, for MF-manufactured liposomes. PDI values are dependent on the bilayer composition of the liposome. Pereira-Lachataignerais *et al.* (2006) observed a decrease in PDI values for liposomes manufactured with different levels (3.75, 5.62, and 7.5 g/L) of soybean phosphatidylcholine (PC) at low sonication power, 0.36, 0.22, and 0.12, respectively.

4.3.1.3 Zeta potential

The zeta potential for all liposome preparations became less negative as the processing intensity increased (UT, low shear, 5000 rpm; 2-SH, 280:60 bar; MF, 690 bar) (Table 4.1). It has been suggested (Nongonierma *et al.*, 2009) that this change in zeta potential may be a result of changing the arrangement of the phospholipids at the liposomal surface, therefore modifying the exposition of certain charged groups. Regardless of the processing condition, the zeta potential of liposomes became significantly ($P \leq 0.05$) more negative upon the addition of EDTA, and this result was greatest for Pro-Lipo C. Pro-Lipo C had higher negative potential both in empty and EDTA-containing liposomes when compared with the same liposomes made with Pro-Lipo Duo, for all processing conditions. Greater zeta potential values have been associated with greater liposomal stability, as they can cause electrostatic repulsions, which in turn have been shown to prevent destabilization processes such as coalescence (Thompson and Singh 2006). Therefore, the most stable liposome preparation would appear to be Pro-Lipo C. These results are in agreement with those published by Nongonierma *et*

al. (2009), who found that the more negatively charged Pro-Lipo C was more stable than Pro-Lipo S.

There was a concomitant increase in negative zeta potential with increasing pH (4.0–7.0) for all liposomes studied (Table 4.1). The zeta potential increased only slightly for both liposomes (Duo and C) with and without entrapped EDTA. Similarly, Thompson and Singh (2006) and Nongonierma *et al.* (2009) did not find great variations in the zeta potential of liposomes produced from milk fat globule membrane material or two different liposome preparations (Pro-Lipo C and S) in the same pH range. Again, liposomes containing EDTA exhibited a greater negative zeta potential at each pH value, when compared to liposomes not containing EDTA. This result suggests that the zeta potential of all liposome preparations is more influenced by the presence of the negatively charged EDTA ions than by pH.

Table 4.1 Effect of processing treatment (UT, 2-SH, MF), liposome formulation (Duo or C), EDTA (with or without) and pH on the zeta potential (mV) of liposome preparations before ultrafiltration at 1 day after manufacture.

Processing Treatment		Duo - EDTA	Duo + EDTA	C - EDTA	C + EDTA
	pH	mV	mV	mV	mV
Ultra-Turrax	Unadjusted	-75.73^{Bb} (0.35)	-72.07^{Ba} (0.70)	-74.03^{Bab} (0.31)	-81.50^{Bc} (1.45)
	4.0	-58.27^{ab} (0.29)	-58.10^{a} (0.53)	-60.83^{b} (0.70)	-67.67^{c} (0.87)
	5.0	-74.40^{a} (0.56)	-74.43^{a} (0.75)	-72.27^{a} (0.68)	-81.00^{b} (0.86)
	6.0	-81.40^{b} (0.62)	-77.90^{b} (1.95)	-72.20^{a} (0.27)	-88.53^{c} (1.80)
	7.0	-82.30^{c} (0.20)	-76.30^{b} (0.30)	-73.97^{a} (1.01)	-88.67^{d} (0.06)
Two-Stage Homogenisation	Unadjusted	-61.44^{Aa} (0.76)	-64.90^{Aab} (2.00)	-62.53^{Aa} (2.71)	-68.73^{Ab} (0.57)
	4.0	-48.13^{a} (1.58)	-49.87^{ab} (0.25)	-51.67^{b} (1.33)	-58.90^{c} (0.36)
	5.0	-59.43^{a} (1.61)	-67.40^{b} (0.10)	-60.23^{a} (1.91)	-71.73^{b} (0.94)
	6.0	-62.67^{a} (2.26)	-70.87^{b} (1.09)	-59.03^{a} (0.50)	-77.72^{c} (0.78)
	7.0	-67.50^{a} (1.23)	-71.07^{a} (0.84)	-66.53^{a} (2.41)	-79.73^{b} (0.75)
Microfluidisation	Unadjusted	-61.83^{Aa} (0.80)	-62.17^{Aa} (0.35)	-68.30^{ABa} (4.32)	-79.87^{Bb} (1.82)
	4.0	-46.97^{a} (0.58)	-57.17^{ab} (4.93)	-55.17^{ab} (5.03)	-64.33^{b} (1.44)
	5.0	-55.53^{a} (1.56)	-69.80^{ab} (5.11)	-64.80^{ab} (4.31)	-72.30^{b} (3.16)
	6.0	-58.23^{a} (1.56)	-77.62^{c} (4.13)	-67.17^{b} (3.16)	-81.73^{c} (2.83)
	7.0	-59.20^{a} (0.91)	-78.16^{b} (4.64)	-65.17^{ab} (4.23)	-80.37^{b} (4.06)

Values presented are means of triplicate trials with standard deviation in parentheses. For each liposome preparation (C and Duo, with and without EDTA) with the same pH value and processing treatment (row), figures with different subscripts are significantly different (P>0.05). For each liposome preparation manufactured by a different method (UT, 2-SH or MF) with the same pH (unadjusted) (column), figures with different superscript letters are significantly different (P>0.05).

4.3.1.4 Physical stability of liposomes over time

The effect of liposome formulation and processing conditions on the physical stability of the liposomes over 28 d of storage at 4 ˚C was assessed and the results are shown in Figures 4.2 a and b. There was no significant ($P \leq 0.05$) change in the z-average diameter or PDI values for any of the liposomes during storage, indicating physical stability, which may be attributed to the high negative zeta potential values associated with each liposome preparation (Table 4.1). Crommelin (1984) found that negatively charged liposomes, both at low and high ionic strength, exhibited no increase in particle size during storage. No significant differences ($P \leq 0.05$) in %EE were found for either Pro-Lipo C or Duo during 28 d storage (results not shown). This was most likely due to the liposomes being stored as a pellet rather than as dispersion. Storing the liposomes as a pellet meant that the gradient between the inner and outer parts of the liposome would be unlikely to be adequate to drive the diffusion process of the EDTA outside the liposome (Nongonierma *et al.*, 2009).

4.3.2 Membrane filtration

Liposome preparations manufactured using 2-SH were concentrated to a VCF of 2 and washed with de-ionised water to remove unentrapped EDTA from the surface of the liposomes and surrounding aqueous phase using an UF membrane system, illustrated in Figures 4.4a and 4.4b. Results shown in Figures 4.5a and 4.5b indicated that increasing the VCF resulted in a concomitant decrease in permeate flux through the membrane for all liposome preparations.

For both UF and DF, liposomes containing no EDTA had a greater permeate flux than EDTA-containing samples, which was evident for both Duo and C. Liposome preparations without EDTA reached a VCF=2 in half the time required for the same liposome preparation containing EDTA, indicating that EDTA dramatically decreased the membrane efficiency (speed of permeate flow), perhaps as a result of increased membrane fouling due to the higher solute concentration of the EDTA-containing liposomes (de Balmann and Nobrega 1989). Another reason may be due to component interactions; the presence of larger molecules (liposomes) within the feed solution causes steric hindrance to the passage of smaller molecules (free EDTA in solution) through the membrane (Zhao *et al.,* 2000).

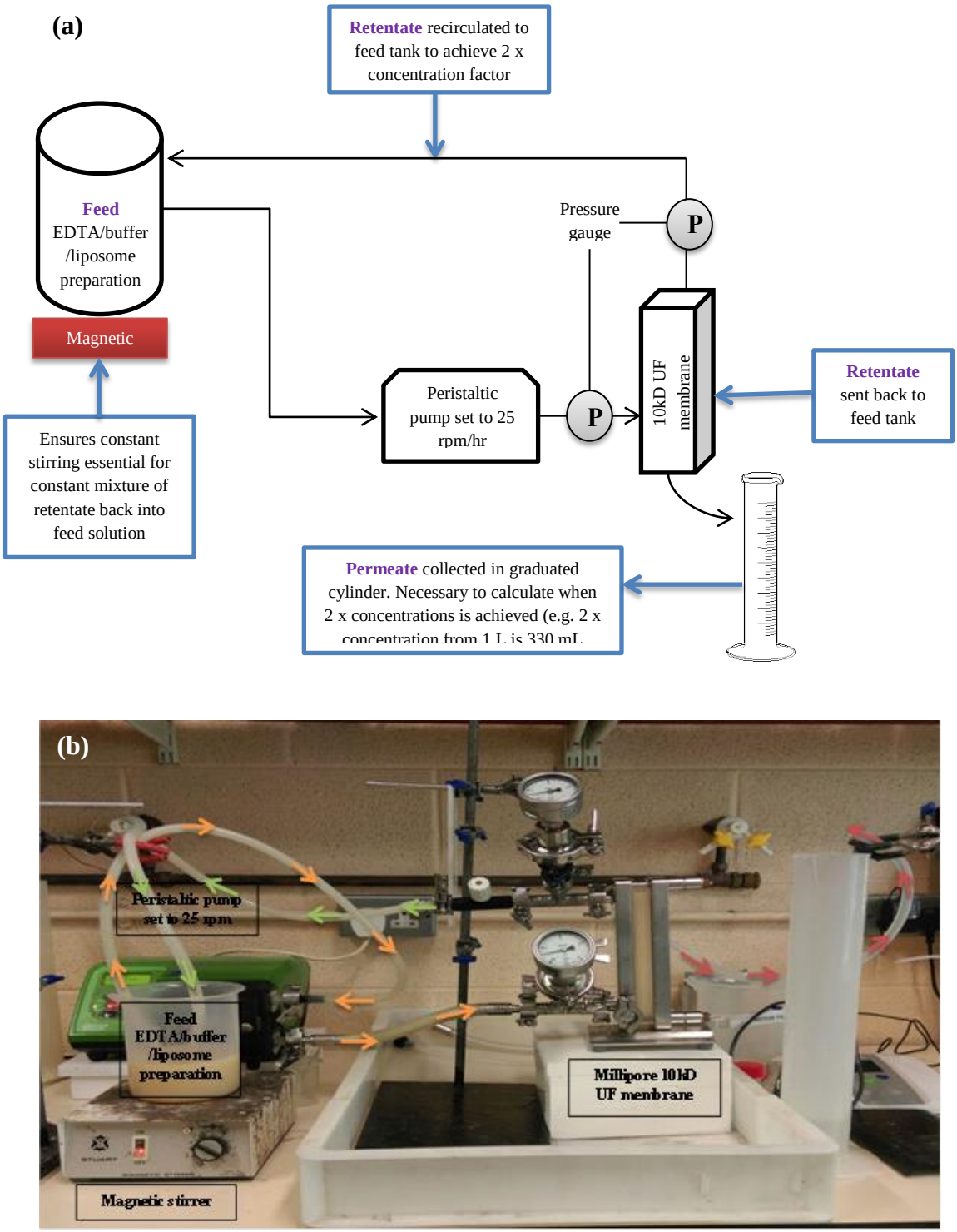

Figure 4.4 Schematic drawing (**a**) and photograph (**b**) of the ultrafiltration membrane system used for ultrafiltration and subsequent diafiltration of liposome-entrapped EDTA after homogenisation.

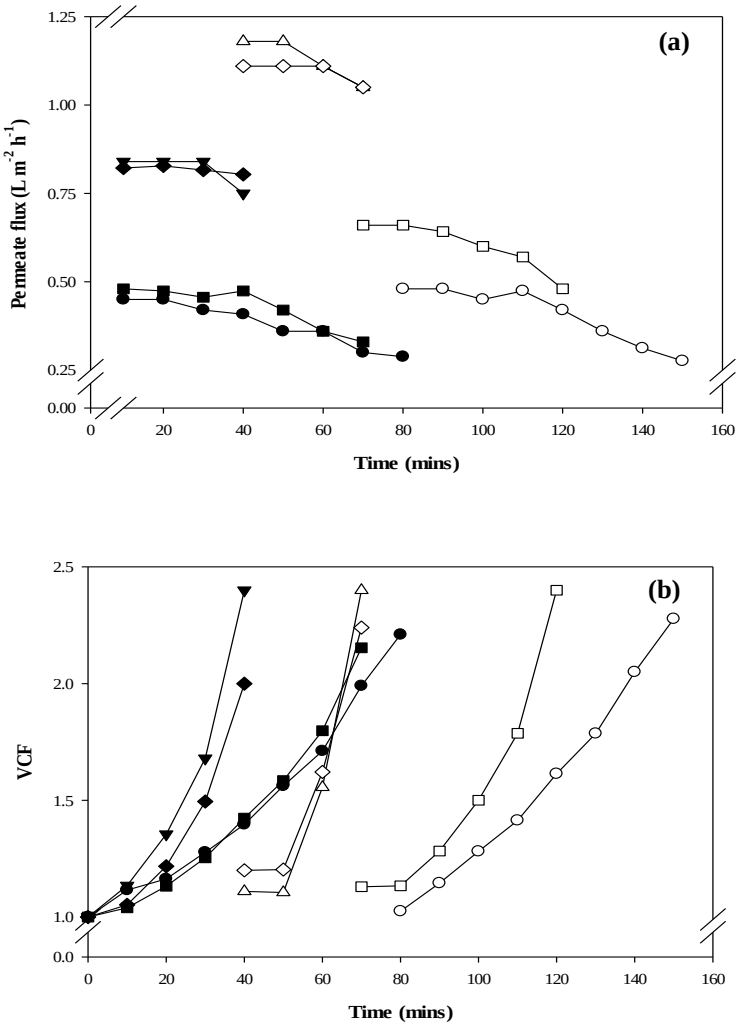

Figure 4.5 Permeate flux (**a**) and volume concentration factor (VCF) (**b**) obtained during ultrafiltration (UF) (closed symbols) and diafiltration (DF) (open symbols) for Pro-Lipo C EDTA (●,○), C No EDTA (▼,△), Duo EDTA (■,□) and Duo No EDTA (◆,◇).

4.3.3 Entrapment efficiency of EDTA within liposomes manufactured using 2-SH

The EDTA concentration was measured in each phase (supernatant, pellet, and disrupted pellet) using HPLC for both Duo and C manufactured using 2-SH (Table 4.2). Significant losses of EDTA were observed in both liposome dispersions after UF; 27.2% (C) and 21.4% (Duo) of the EDTA present in the original EDTA solution remaining in the liposome dispersions after UF. Following 2-SH liposome dispersions were diafiltered with de-ionised water in order to remove any free EDTA from the surface of the liposomes and the surrounding aqueous phase. De-ionised water has a lower osmotic strength than the 25 mM Tris HCl buffer in which the liposome dispersions were suspended. Therefore, it is possible that some of the EDTA-containing liposomes may have swelled and burst due to differences in osmotic pressure, which may explain the high loss of EDTA from both Pro-Lipo C and Duo after UF. The %EE of Pro-Lipo C was found to be significantly ($P \leq 0.05$) higher than that of Pro-Lipo Duo, 63% and 54%, respectively. Liposome composition and/or the net charge (Laridi *et al.,* 2003), as well as processing pressure (Nongonierma *et al.,* 2009) have been shown to affect entrapment capacity. Both Duo and C contain high levels of unsaturated soybean phospholipids. Liposomes with a higher PI content (C) exhibited greater negative potential (Table 4.1) and greater entrapment efficiency (Table 4.2). Similarly, Nongonierma *et al.* (2009) found that encapsulated enzymes may be interacting with the phospholipid membrane by both electrostatic and hydrophobic interactions and that those interactions were greatest in Pro-Lipo C which contained more highly charged phospholipids.

Table 4.2 Influence of the Pro-Lipo type (Duo and C) on the entrapment efficiency (%EE) of EDTA within liposomes manufactured using two-stage homogenisation (2-SH).

Liposome fraction	Liposome type	%EE
Unencapsulated	Duo	45.88^{cd} (1.23)
Encapsulated		54.12^{b} (1.23)
Surface		13.70^{f} (0.69)
Core		40.42^{de} (0.57)
Unencapsulated	C	34.12^{e} (1.44)
Encapsulated		63.15^{a} (4.52)
Surface		12.71^{f} (0.92)
Core		50.44^{bc} (3.61)

4.3.4 *Effect of liposomes on starter viability and pH during time/temperature profile of Gouda cheesemaking*

4.3.4.1 Viability of starter culture in the presence of EDTA-entrapped liposomes

The numbers of starter bacteria (*Lactococcus lactis*) in milk supplemented with starter, Pro-Lipo C, and/or T-X over 180 min are shown in Table 4.3. T-X was added to milk in order to disrupt liposomes, to assess the impact of released EDTA on starter viability. Starter cell numbers increased for all inoculated samples at the beginning of the time/temperature cycle. After 50 min, viable cell counts were significantly lower ($P \leq 0.05$) in milk samples inoculated with T-X and Pro-Lipo C EDTA. EDTA has been show to exhibit antibacterial properties. Reidmiller *et al.* (2006), demonstrated that, when the bacterial cultures *Streptococcus agalactiae* and *Streptococcus uberis* were exposed to EDTA (100 mM) it resulted in a 90% and 99% reduction, respectively. Puttalingamma *et al.* (2006) found that EDTA indirectly affected the growth of microbes by limiting the availability of important metals, due to its Ca chelating ability. Therefore, the

reduced starter numbers recorded may be a result of EDTA being released from the liposomes and interacting with the starter bacteria and/or limiting starter growth due to reduced essential metal availability.

4.3.4.2 Changes in pH

The pH of milk subjected to different treatments during the Gouda time/temperature cycle is shown in Table 4.3. The pH values decreased in samples inoculated with starter over 180 min, indicating that milk fermentation was not impacted by the incorporation of T-X and/or liposome-entrapped EDTA. Samples containing either liposome or liposome and T-X only (no starter) exhibited no change in pH over the 180 min, suggesting that pH is not affected by their presence in milk. Similarly, Nongonierma *et al.* (2013) found that the incorporation of liposomes containing lactic acid bacteria cell-free extract into Cheddar cheese had no adverse effect on the pH value of the cheese.

Table 4.3 The effect of Pro-Lipo C EDTA manufactured using two-stage homogenisation and Triton-X 100 on milk pH and viable cell counts of *Lactococcus lactis* enumerated on LM17 agar upon starter addition, after 50 min coagulation and after 90 min cooking.

Treatment	Starter added		After 50 min coagulation		After 90 min cooking	
	Log CFU/mL milk	pH	Log CFU/mL milk	pH	Log CFU/mL milk	pH
Starter/Pro-Lipo C	6.98^{ab} (0.01)	6.62^{a} (0.01)	7.05^{b} (0.04)	6.49^{b} (0.01)	7.18^{b} (0.04)	6.24^{c} (0.01)
Starter only	7.19^{a} (0.02)	6.63^{a} (0.01)	7.37^{a} (0.01)	6.51^{b} (0.01)	7.83^{a} (0.15)	6.32^{b} (0.01)
Pro-Lipo C only	0.00^{c} (0.00)	6.62^{a} (0.00)	0.00^{c} (0.00)	6.60^{a} (0.00)	0.00^{c} (0.00)	6.59^{a} (0.01)
Starter/Pro-Lipo C/T-X	6.94^{ab} (0.08)	6.63^{a} (0.01)	7.07^{b} (0.11)	6.44^{c} (0.01)	7.17^{b} (0.01)	6.16^{d} (0.01)
Starter/T-X	6.66^{b} (0.26)	6.62^{a} (0.01)	7.24^{ab} (0.03)	6.44^{c} (0.01)	7.40^{b} (0.12)	6.13^{d} (0.02)
Pro-Lipo C/T-X	0.00^{c} (0.00)	6.63^{a} (0.01)	0.00^{c} (0.00)	6.59^{a} (0.01)	0.00^{c} (0.00)	6.60^{a} (0.02)

Values presented are means from three replicate trials with standard deviations in parentheses; means within columns not sharing a common letter differ statistically ($P<0.05$).

4.3.5 *Effect of liposome addition on the rennet gelation properties of raw milk*

The effect of liposome addition on rennet gel formation was investigated and the results are shown in Figure 4.6. Results indicated that Pro-Lipo C did not affect rennet coagulation, while supplementation of Pro-Lipo Duo reduced the stiffness of the gel formed. Liposomes differ compositionally, with Duo containing 10% more phospholipid content than C. It has been suggested that an increased presence of lipid may weaken the structure or rennet gels by interrupting the matrix creating "soft spots" (Walstra and Jenness, 1984). Additionally, Piard *et al*. (1986) and Alkhalafe *et al*. (1989) demonstrated that charge affected liposome retention in curd and suggested it was due to the formation of electrostatic bonds with casein micelles during renneting. Laloy *et al*. (1998) found that negatively charged liposomes in particular appeared to interact with caseins during cheese manufacture and degraded into spiral structures during ripening. Similarly, Kirby *et al*. (1987) and Laridi *et al*. (2003) reported that liposomes in Cheddar cheese matrix are mainly confined to areas between fat globules and casein matrix.

4.3.6 *EDTA concentration, pH, and moisture content of miniature Gouda-type cheeses manufactured with liposomes*

The pH values and moisture contents of miniature Gouda-type cheeses manufactured by different treatments are shown in Table 4.4. No significant differences ($P \leq 0.05$) in pH were observed between cheeses. The pH of all cheeses ranged between 5.30 and 5.38, which conforms to the pH range of 5.0 to 5.6 given by van den Berg *et al.* (2004) for a Gouda-type cheese. Similar results

were found in Cheddar (Laloy *et al.*, 1998) and Manchego cheeses (Picon *et al.*, 1994), where pH was not affected upon addition of liposomes. The moisture contents of all cheeses were not significantly different, ranging between 41.7 and 42.7%. Similarly, Bainville *et al.* (2000) and Nongonermia *et al.* (2013) found that the addition of vitamin D encapsulated in Pro-Lipo Duo and *Lactococcus lactis* subsp. *lactis* 303 cell-free extract encapsulated within Pro-Lipo S and Duo, respectively, did not negatively impact the composition of Cheddar cheese.

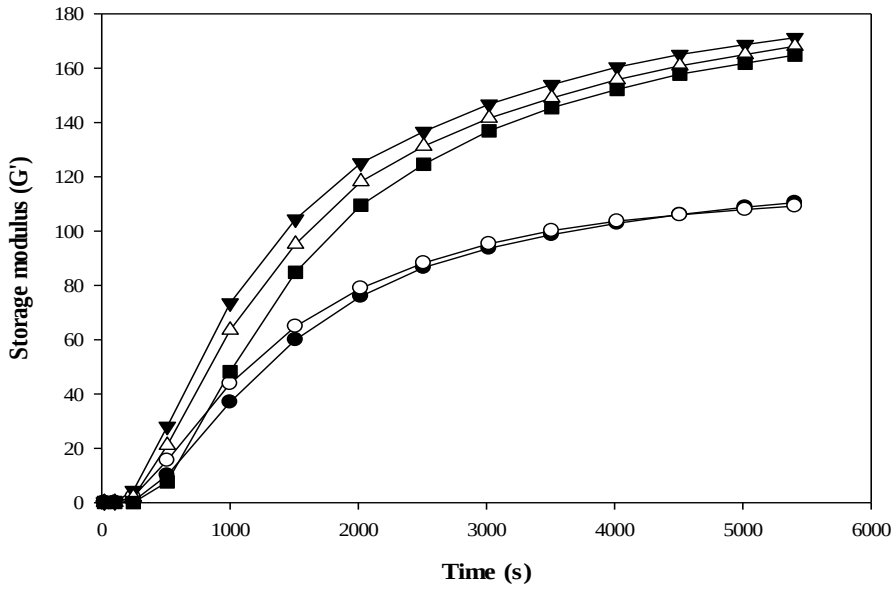

Figure 4.6 Effect of no liposome (raw milk) (■), Pro-Lipo Duo no EDTA (●), Pro-Lipo Duo EDTA (○), Pro-Lipo C no EDTA (△) and Pro-Lipo C EDTA (▼) addition on the storage modulus (G') of renneted milk gels measured using a controlled shear stress AR-G2 rheometer equipped with a Peltier concentric cylinder system and a conical rotor. Liposomes added to milk were manufactured by two-stage homogenisation.

Table 4.4 Moisture, pH, and EDTA concentration (mg EDTA/g cheese) of control and liposome containing miniature Gouda-type cheeses ripened for 7 d at 8 ˚C. Liposomes added to cheeses were manufactured by two-stage homogenisation.

Treatment	pH	Moisture (%)	mg EDTA/g cheese
Control	5.30[a] (0.13)	41.73[a] (1.53)	0.00[c] (0.00)
C EDTA	5.36[a] (0.08)	42.54[a] (2.18)	2.59[a] (0.31)
Duo EDTA	5.33[a] (0.07)	42.65[a] (3.23)	1.77[b] (0.15)

Values are the means of triplicate trials with standard deviations in parentheses; means within columns not sharing a common letter differ statistically ($P<0.05$).

EDTA concentrations of cheeses were determined by HPLC and results are shown in Table 4.4. Results indicated that cheeses supplemented with Pro-Lipo C EDTA had a greater concentration of EDTA trapped within the cheese matrix when compared with Pro-Lipo Duo EDTA cheeses (2.59 and 1.77 mg EDTA/g cheese, respectively). This is not surprising as Pro-Lipo C has already shown to have a greater EDTA entrapment efficiency compared to Pro-Lipo Duo (Table 4.2). Control cheese did not contain any EDTA, as expected. The concentration of EDTA retained in the curd was calculated to be 39.1% and 33.8% for Pro-Lipo C and Duo, respectively. Similarly, Piard *et al.* (1986) achieved liposome retention rates in cheese curd ranging from 35 to 65% depending on the type of liposome.

4.3.7 *Cryogenic-scanning electron micrographs of cheese*

Cryogenic-scanning electron microscopy (Cryo-SEM) was performed on cheese samples 5 d after manufacture in order to determine the effect of liposomes on the curd microstructure. The control (no liposomes) and 2-SH manufactured Pro-Lipo C EDTA containing cheese samples were analyzed and micrographs of samples are shown in Figure 4.7. The images suggest that the curd matrix was unaffected by the presence of liposomes as few distinguishable differences were visualized. The liposomes added into the cheese are generally the same size as the casein micelle and, therefore, it is hard to pinpoint their exact location within the curd microstructure.

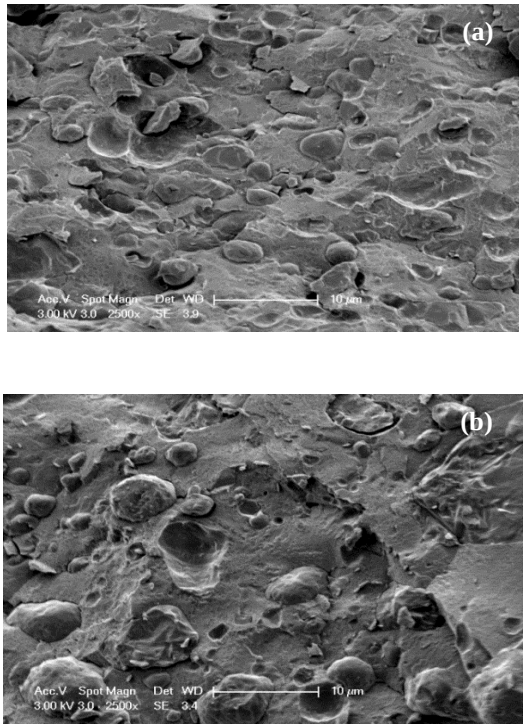

Figure 4.7 Cryogenic-scanning electron (Cryo-SEM) micrographs of control (no liposomes) (**a**) and two-stage homogenized Pro-Lipo C EDTA containing liposome (**b**) cheeses at day 5, viewed at 2500x magnification.

4.4 Conclusions

Entrapment of EDTA in liposomes was successfully achieved using Pro-Lipo C and Pro-Lipo Duo, manufactured using 3 different processing treatments, UT, 2-SH, and MF. Determination of the physicochemical properties of liposomes showed that the hydrodynamic diameter and PDI were influenced by the manufacturing process (UT, 2-SH, or UT), liposome composition (Duo or C), and the presence of EDTA. All liposomes were physically stable during 28 days of refrigerated storage, with no significant ($P \leq 0.05$) change in size distribution or %EE of EDTA during that period, highlighting the potential practical uses for liposome-entrapped EDTA when incorporated into cheese. Addition of EDTA-containing liposomes to milk during cheesemaking caused a reduction in viable cell count numbers; however, pH values decreased in all samples containing starter, indicating milk fermentation occurred, regardless of the presence of EDTA, therefore allowing for the manufacture of cheeses. Results of this study show that the incorporation of Pro-Lipo C-entrapped EDTA into milk during cheese manufacture did not affect rennet gelation, milk fermentation, moisture content, or pH. Thus, the potential for incorporation of EDTA entrapped in liposomes into brine-salted cheese has been demonstrated which may provide novel avenues for modifying cheese texture and functionality.

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References

Alexander, M., Lopez, A. A., Fang, Y. & Corredig, M. (2012). Incorporation of phytosterols in soy phospholipids nanoliposomes: encapsulation efficiency and stability. *LWT-Food Science and Technology*, *47*, 427-436.

Alkhalaf, W., El-Soda, M., Gripon, J. C. & Vassal, L. (1989). Acceleration of cheese ripening with liposomes entrapped proteinase influence of liposomes net charge. Journal of Dairy Science, *72*, 319–326.

Anjani, K., Kailasapathy, K. & Phillips, M. (2007). Microencapsulation of enzymes for potential application in acceleration of cheese ripening. *International Dairy Journal*, *17*, 79-86.

Bainville, C., Vuillemard, J. C. & Lacroix, C. (2000). Comparison of different methods for fortifying Cheddar cheese with vitamin D. *International Dairy Journal*, *10*, 375–382.

Barisik, M., Atalay, S., Beskok, A. & Qian, S. (2014). Size dependent surface charge properties of silica nanoparticles. *Journal of Physical Chemistry C*, *118*, 1836-1842.

Barnadas-Rodríguez, R. & Sabés, M. (2001). Factors involved in the production of liposomes with a high-pressure homogeniser. *International Journal of Pharmaceutics*, *213*, 175-186.

Brickley, C. A., Lucey, J. A. & McSweeney P. L. H. (2009). Effect of the addition of trisodium citrate and calcium chloride during salting on the

rheological and textural properties of Cheddar-style cheese during ripening. *International Journal of Dairy Technology*, *62*, 527-534.

Crommelin, D. J. (1984). Influence of lipid composition and ionic strength on the physical stability of liposomes. Journal of Pharmaceutical Science, 7, 1559-1563.

Crowley, S. V., O'Callaghan, T. F., Kelly, A. L., Fenlon, M. A. & O'Mahony, J. A. (2015). Use of ultrafiltration to prepare novel permeate for application in the functionality testing of infant milk ingredients. *Separation and Purification Technology*, *141*, 94-300.

da Silva Malheiros, P., Daroit, D. J. & Brandelli, A. (2012). Inhibition of *Listeria monocytogenes* in Minas Frescal cheese by free and nanovesicle-encapsulated nisin. *Brazilian Journal of Microbiology*, *43*, 1414-1418.

de Balmann, H. & Nobrega, R. (1989). The deformation of dextran molecules: Causes and consequences in ultrafiltration. *Journal of Membrane Science*, *40*, 311-327.

Dufour, P., Vuillemard, J. C., Laloy, E. & Simard, R. E. (1996). Characterization of enzyme immobilization in liposomes prepared from proliposomes. *Journal of Microencapsulation*, *13*, 185-194.

IDF (1982). Cheese and processed cheese; Determination of the total solids content. Standard 4A:1982. Brussels, Belgium: International Dairy Federation.

Imran, M., Revol-Junelles, A. M., Paris, C., Guedon, E., Linder, M. & Desobry, S. (2015). Liposomal nanodelivery systems using soy and marine

lecithin to encapsulate food biopreservative nisin. *LWT-Food Science and Technology*, *62*, 341-349.

Kemmei, T., Kodama, S., Yamamoto, A., Inoue, Y. & Hayakawa, K. (2013). Determination of ethylenediaminetetraaacetic acid in foods by reversed-phase high-performance liquid chromatography. *Food Chemistry*, *138*, 66-869.

Kheadr, E. E., Vuillemard, J. C. & El-Deeb, S. A. (2002). Acceleration of Cheddar cheese lipolysis by using liposome-entrapped lipases. *Journal of Food Science*, *67*, 485-492.

Kirby, C. & Gregoriadis, G. (1984). Dehydration-rehydration vesicles: a simple method for high yield drug entrapment in liposomes. *Nature Biotechnology*, *2*, 979-984.

Kirby, C. J, Brooker, B. E. & Law, B. A. (1987). Accelerated ripening of cheese using liposome-encapsulated enzyme. *International Journal of Food Science and Technology*, *22*, 355-375.

Kirby, C. J., Whittle, C. J., Rigby, N., Coxon, D. T. & Law, B. A. (1991). Stabilization of ascorbic acid by microencapsulation in liposomes. *International Journal of Food Science and Technology*, *26*, 437-449.

Kosaraju, S. L., Tran, C. & Lawrence, A. (2006). Liposomal delivery systems for encapsulation of ferrous sulfate: preparation and characterization. *Journal of Liposome Research*, *16*, 347-358.

Kuchroo, C. N. & Fox, P. F. (1982). Soluble nitrogen in Cheddar cheese: comparison of extraction procedures. *Milchwissenschaft*, *37*, 331-335.

Pereira-Lachataignerais, J., Pons, R., Panizza, P., Courbin, L., Rouch, J. & Lopez, O. (2006). Study and formation of vesicle systems with low polydispersity index by ultrasound method. *Chemistry and Physics of Lipids*, *140*, 88-97.

Laloy, E., Vuillemard, J. C., El Abboudi, M., Fliss, I., De Grace, E. & Simard, R. E. (1996). Development of an immunological method for studying the incorporation of ripening enzymes in cheese curd. *Journal of Dairy Research*, *63*, 141-149.

Laloy, E., Vuillemard, J. C. & Simard, R. (1998). Characterization of liposomes and their effect on the properties of Cheddar cheese during ripening. *Lait*, *78*, 401-412.

Laridi, R., Kheadr, E. E., Benech, R. O., Vuillemard, J. C., Lacroix, C. & Fliss, I. (2003). Liposome encapsulated nisin Z: optimization, stability and release during milk fermentation. *International Dairy Journal*, *13*, 325-336.

Law, B. A. & King, J. S. (1985). Use of liposomes for proteinase addition to Cheddar cheese. *Journal of Dairy Research*, *52*,183-188.

Leigh. S., inventor. (1991). Pro-liposome compositions. United States patent US 5,004,611. April 2, 1991.

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

Maa, Y. F. & Hsu, C. C. (1999). Performance of sonication and microfluidisation for liquid–liquid emulsification. *Pharmaceutical Development and Technology*, *4*, 233-240.

New, R. R. C. (1990). Preparation of liposomes. In: Liposomes: a practical approach, Vol 1, 1st Edition. New RRC, Ed., Oxford University Press, New York, NY, 33–104.

Nongonierma, A. B., Abrlova, M. & Kilcawley, K. N. (2009). Evaluation of two food grade proliposomes to encapsulate an extract of commercial enzyme preparation by microfluidisation. *Journal of Agricultural and Food Chemistry*, *57*, 3291-3297.

Nongonierma, A. B., Abrlova, M. & Kilcawley, K. N. (2013). Encapsulation of a lactic acid bacteria cell-free extract in liposomes and use in Cheddar cheese ripening. *Foods*, *2*, 100-119.

O'Mahony, J. A., Lucey, J. A. & McSweeney, P. L. H. (2005). Chymosin-mediated proteolysis, calcium solubilization and texture development during the ripening of Cheddar cheese. *Journal of Dairy Science*, *88*, 3101‑3114.

O'Sullivan, J., Murray, B., Flynn, C. & Norton, I. (2016). The effect of ultrasound treatment on the structural, physical and emulsifying properties of animal and vegetable proteins. *Food Hydrocolloids*, *53*, 141-154.

Ostro, M. H. (1983). Liposomes. New York: Marcel Dekker.

Payne, N. I., Timmins, P., Ambrose, C. V., Ward, M. D. & Ridgway, F. (1986). Proliposomes: a novel solution to an old problem. *Journal of Pharmaceutical Science*, *75*, 325-329.

Piard, J. C., El Soda, M., Alkhalaf, W., Rousseau, M., Desmazeaud, M., Vassal, L. & Gripon, J. C. (1986). Acceleration of cheese ripening with liposome-entrapped proteinase. *Biotechnology Letters*, *8*, 241-246.

Picon, A., Gaya, P., Medina, M. & Nunez, M. (1994). The effect of liposome encapsulation of chymosin derived by fermentation on Manchego cheese ripening. *Journal of Dairy Science, 77,* 16-23.

Puttalingamma, V., Begum, K. & Bawa, A. S. (2006). Antimicrobial peptides-new weapons against enteric pathogens. *Pakistan Journal of Nutrition, 5,* 432-435.

Rashidinejad, A., Birch, E. J., Sun-Waterhouse, D. & Everett, D. W. (2014). Delivery of green tea catechin and epigallocatechin gallate in liposomes incorporated into low-fat hard cheese. *Food Chemistry, 156,* 176-183.

Reidmiller, J. S., Smith, W. L., Sawyer, M. M., Osburn, B. I., Stott, J. L. & Cullor, J. S. (2006). Antimicrobial properties of the chelating agent EDTA on streptococcal bovine mastitis isolates. *Journal of Food Protection, 69,* 1460-1462.

Risch, S. J. & Reineccius, G. (1995). Encapsulation and controlled release of food ingredients. ACS Symposium Series. American Chemical Society.

Shakeel-Ur-Rehman, McSweeney, P. L. H. & Fox, P. F. (1998). Protocol for the manufacture of miniature cheeses. *Lait, 78,* 607-620.

Singh, H., Thompson, A., Liu, W. & Corredig, M. (2012). Liposomes as food ingredients and nutraceutical delivery systems, In: Encapsulation Technologies and Delivery Systems for Food Ingredients and Nutraceuticals. Garti N, McClements DJ, Eds., Woodhead Publishing Limited, Cambridge, UK, 287-318.

Szoka, F. & Papahadjopoulos, D. (1978). Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. *Proceedings of the National Academy of Sciences*, *75*, 4194-4198

Talsma, H., Özer, A. Y., Bloois, L. V. & Crommelin, D. J. A. (1989). The size reduction of liposomes with a high pressure homogeniser (Microfluidizer™). Characterization of prepared dispersions and comparison with conventional methods. *Drug Development and Industrial Pharmacy*, *15*, 197-207.

Thapon, J. L. & Brule, G. (1986). Effects of pH and ionic strength on lysozyme-casein affinity. *Lait*, *66*, 19-30.

Terzaghi, B. E. & Sandine, W. E. (1975). Improved medium for lactic streptococci and their bacteriopgages. *Applied Microbiology*, *29*, 807-813.

Thompson, A. K. & Singh, H. (2006). Preparation of liposomes from milk fat globule membrane phospholipids using a microfluidizer. *Journal of Dairy Science*, *89*, 410–419.

Uemura, M., Gilmour, S. J., Thomashow, M. F. & Steponkus, P. L. (1996). Effects of COR6. 6 and COR15am polypeptides encoded by COR (cold-regulated) genes of Arabidopsis thaliana on the freeze-induced fusion and leakage of liposomes. *Plant Physiology*, *111*, 313-327.

van den Berg, G., Meijer, W. C., Düsterhöft, E-M. & Smit, G. (2004). Gouda and related cheeses, In: Cheese: Chemistry, Physics and Microbiology. Vol.

2, Major Cheese Groups. 3rd Ed. Fox, P. F., McSweeney, P. L. H., Cogan, T. M., Guinee, T. P. (Eds.). Elsevier Publishers, London, 103-140.

Walstra, P. & Jenness, R. (1984). Dairy Chemistry and Physics. John Wiley and Sons, New York

Zhao, Y-J., Wu, K-F., Wang, Z-J., Zhao, L. & Li, S-S. (2000). Fouling and cleaning of membrane–a literature review. *Environmental Science*, *12*, 241-251.

Chapter 5

Calcium solubilisation, texture and functionality in a model Gouda-type cheese manufactured with liposome-entrapped calcium chelating salts

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Abstract

The effect of liposome entrapped ethylenediamine tetraacetic acid (EDTA) on calcium equilibrium, textural and rheological properties of a brine-salted Gouda-type cheese throughout ripening was investigated. EDTA-containing liposomes were manufacture using two-stage homogenisation (2-SH) with an entrapment efficiency of ~77% achieved. Three cheeses were manufactured in triplicate trials; a control containing only buffer (C), a liposome control containing liposome-entrapped buffer (LC) and a treatment cheese containing liposome-entrapped EDTA (LE). No differences in composition, pH and proteolysis patterns were observed between the control and treatment cheeses. Addition of liposome-entrapped EDTA (LE) into cheese resulted in a reduction in the concentration of insoluble calcium in the cheese. Hardness, chewiness and gumminess values for LE cheese were significantly ($P \leq 0.05$) lower at the early stages of ripening when compared to control cheeses. The LE cheese had significantly ($P \leq 0.05$) greater meltability values throughout ripening when compared with the control and LC cheese. The LE cheese had higher LT_{max} values than the control and LC cheese after 28 d ripening, indicating a greater propensity for LE cheese to flow and melt when heated. The results of this study highlight the potential of liposomes to incorporate calcium chelating salts during the production of brine-salted type cheese. Results also indicate that incorporation of liposome-entrapped EDTA into milk during Gouda-type cheese manufacture alters the distribution of Ca between insoluble and soluble forms in a similar fashion to dry-salted cheese varieties, thus modifying its structure, texture and functionality, without impacting negatively on the pH or composition of the cheese.

5.1 Introduction

Traditionally, proteolysis has been widely accepted as the major contributing factor associated with the development of textural and rheological properties of cheese (Creamer and Olson, 1982; Lawrence *et al*., 1987; Lawrence *et al*., 2004; Fox *et al*., 2000). However, in recent years, calcium within cheese has been highlighted as a major factor influencing the development of such characteristics (Lucey and Fox, 1993; Lucey *et al*., 2005).

Ca in cheese plays a major role in modulating cheese texture and rheology. Lucey *et al*. (2005) found that the proportion of soluble (SOL) Ca in Cheddar cheese increased throughout maturation, with most of this increase occurring during the first 60 days. These authors demonstrated that the changes in the insoluble (INSOL) Ca content during ripening were more significantly correlated with the rheological and melting properties than was the formation of pH 4.6-soluble nitrogen. The addition of calcium chloride ($CaCl_2$) and/or calcium chelating agents such as trisodium citrate (TSC) or ethylenediamine tetraacetic acid (EDTA) to dry-salted cheese varieties has been shown to alter both their textural and rheological properties. Brickley *et al*. (2009) added TSC and $CaCl_2$ to Cheddar cheeses during salting and found that cheeses supplemented with TSC had the softest texture while cheeses containing added $CaCl_2$ had the hardest texture. These authors attributed these differences to the changes in Ca equilibrium of the cheese (i.e., shift in proportion of INSOL to SOL forms as well as the increased total Ca content with the addition of $CaCl_2$). O'Mahony *et al*. (2005) added pepstatin (a potent chymosin inhibitor) at different levels to Cheddar cheese by adding it to the curds/whey mixture during ripening. These authors

reported that the softening of cheese texture was more highly correlated with the level of INSOL Ca than with the level of intact α_{s1}-casein (CN) in the control and treatment cheeses during the early stages of ripening (first 21 d).

Most research pertaining to the study of the effects of total Ca concentration, distribution of Ca between INSOL and SOL forms (Ca equilibrium), or both in determining textural and rheological properties of cheese has been largely focused on Cheddar (Creamer *et al*., 1985; Lee *et al*., 2005; O'Mahony *et al*., 2005) and Mozzarella (Metzger *et al*., 2000; Joshi *et al*., 2003b; Sheehan and Guinee, 2004) cheese varieties and to a much lesser extent on brine-salted varieties.

The addition of calcium sequestering salts such as EDTA to a brine-salted cheese variety such as Gouda causes more difficulties than their addition to a dry-salted variety (when EDTA may be added to the curds during salting). Direct addition of EDTA to the curds/whey mixture during manufacture of brine salted Gouda-type cheese has been found to cause a dramatic decrease in pH during production, resulting in cheese with a significantly lower pH and increased moisture content (Chapter 3). Entrapping such calcium chelating salts within liposomes may provide a solution to this problem as the EDTA will be protected by the liposome bilayer within the core, preventing it from coming in direct contact with the product during manufacture, but releasing the chelator during ripening as the liposomes degrade.

There are further advantages associated with supplementing EDTA to cheese entrapped within liposomes. A large proportion or EDTA has been found

to be lost in the whey when added directly to the curds/whey mixture (Chapter 3). Entrapping the EDTA within liposomes would reduce the loss of EDTA in the whey as the liposomes are trapped within the curd matrix during manufacture (Laloy *et al*., 1996). Entrapment of EDTA within liposomes should improve the distribution of EDTA (Wilkinson and Kilcawley, 2005). Previously, Anjani *et al.* (2007) demonstrated that encapsulating enzymes in liposomes led to even dispersal within the cheese matrix. Entrapment within liposomes will provide gradual release of EDTA within the cheese matrix during ripening. The controlled release of food ingredients at the right place and time within the cheese matrix has been achieved (Laloy *et al*., 1998) through encapsulation of ingredients within liposomes.

The objective of this study was to determine the effect of EDTA-entrapped liposomes on the total Ca concentration and the Ca equilibrium (distribution between SOL and INSOL forms) of a brine-salted Gouda-type cheese, as well as its subsequent effect on pH, composition, textural and rheological properties.

5.2 Materials and Methods

5.2.1 Preparation of EDTA solution

A concentrated solution of ethelyneditetraacetic acid (EDTA) (Sigma-Aldrich, Dublin, Ireland) was prepared to facilitate encapsulation. EDTA was suspended in 25 mM Tris-HCl buffer at pH 7.4 to a final concentration of 12.5% (w/v).

5.2.2 Liposome preparation

Liposomes were prepared by adding 100 g of Pro-Lipo C (Lucas Meyer, Chelles, France) to 200 mL of EDTA solution. Control liposomes were prepared by adding 100 g of Pro-Lipo C to 200 mL of 25 mM Tris HCl buffer at pH 7.4. The mixtures were agitated using an Ultra-Turrax t25 (IKA-Werke GmbH and Co. KG, Staufen, Germany) operated at 5000 rpm for 10 min at 21 ˚C, after which 600 mL of 25 mM Tris HCl buffer at pH 7.4 was added and agitated at 5000 rpm for 10 min at 21 ˚C. The liposome preparations were processed through a 2-stage homogeniser (2-SH) (APV GEA Niro-Soavi S.p.A., Parma, Italy) at 280/60 bar (5,000 psi).

The liposome preparations were concentrated and washed using a pressure-driven, tangential-flow filtration device (Pellicon 2 mini-holder; Merck-Millipore, Tullagreen, Carrigtwohill, Ireland), according to the method described by McAuliffe *et al*. (2016), except that all liposome preparations were concentrated to a volume concentration factor (VCF) of 3.

5.2.*3 Determination of EDTA entrapment efficiency*

The entrapment efficiency (%EE) is defined as the percentage of EDTA entrapped in the liposome relative to the total amount of EDTA initially present in the mixture (Dufour *et al*., 1996). The %EE of EDTA within liposomes was determined using the HPLC method described by McAuliffe *et al*. (2016). The EDTA concentration was measured in the supernatant, re-suspended liposome and disrupted re-suspended liposome samples. All samples were prepared according to the method described by McAuliffe *et al*. (2016).

5.2.4 Characterisation of liposomes

The hydrodynamic diameter and zeta potential of liposome preparations were measured at 21 ˚C by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments, Malvern, Worcestershire, UK). Detection was completed at a backscattering angle of 173˚. The refractive index of liposome and water were set at 1.45 and 1.33, respectively (McAuliffe *et al*., 2016). Each sample measurement was carried out five times for the hydrodynamic diameter and ten times for zeta potential. The measurements were carried out on all liposome mixtures before UF; samples were diluted 1:100 with de-ionised water.

5.2.5 Cheese manufacture

Three 2 kg scale Gouda-type cheeses were manufactured in the food processing facility at University College Cork. Raw milk (80 L) was heated to 50 ˚C, separated and standardised to a casein:fat ratio of 0.90:1.00. Standardised milk

was batch pasteurised, separated into three vats, each containing 20 L of milk and cooled to 31 ˚C. B11 starter culture (*Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp *lactis*, *Leuconostoc mesenteroides* subsp. *cremoris* and a citrate-positive *Lactococcus* ssp.) (Chr. Hansen Ltd., Little Island, Co. Cork, Ireland) was added at a level of 0.03% (w/v) and sodium nitrate (Sigma-Aldrich, Dublin, Ireland) at a level of 0.006% (w/v) and allowed to ripen for 30 min. Following ripening, 45 mL 1 M $CaCl_2$ (Sigma-Aldrich, Dublin, Ireland) and chymosin (CHY-MAX®M, Chr. Hansen Ltd., Little Island, Co. Cork, Ireland) at a rate of 17.5 mL per 20 L of milk were added to each vat. Following this, 400 mL of 25 mM Tris HCl buffer (C; to act as a control), Pro-Lipo C liposome without EDTA (LC) and Pro-Lipo C liposome with EDTA (LE) solutions were added to the appropriate vat, the milk was stirred gently for 2 min and given 50 min to coagulate. Once the coagulum had formed, the curds were cut, allowed to heal for 5 min, before stirring gently for 20 min. The curds were washed for the first time, ~ 1/3 whey was removed (5 L) followed by the addition of warm water into the vat until 38 ˚C was reached. Curds were cooked at 38 ˚C for 90 min with continuous stirring. The whey was drained and collected, curds were placed in 2 kg moulds lined with cheesecloth, the moulds were placed back into the vat with a 10 kg weight placed on top, the whey was reintroduced back into the vats and the curds were allowed to press under the whey for 30 min. After pressing under the whey, the moulds were removed from the vats and pressed at 1.03 bar overnight. The cheese was removed from the press and placed in a brine solution (10 kg H_2O 20 % (w/v) NaCl and 0.05 % (w/v) ($CaCl_2$) for 24 h. After brining, the cheeses were vacuum packed and ripened at 10 ˚C for 112 days. All Gouda-type cheeses were manufactured in triplicate.

5.2.6 Chemical analysis

The composition and pH of all cheeses supplemented with 25 mM Tris HCl buffer (C), Pro-Lipo C without EDTA (LC) and Pro-Lipo C with EDTA (LE) solutions was determined at 14 d post manufacture. The moisture content of the cheeses was determined using an oven-drying method (IDF, 1982). The pH was measured using a calibrated pH meter, on cheese slurry made from 10 g cheese and 10 ml of deionised water. Protein content of the cheeses was determined by the macro-Kjeldahl method (N x 6.38; IDF, 1986), fat by the Gerber method (IIRS, 1955) and salt was measured by a titrimetric method using potentiometric end-point determination (Fox, 1963). The proportion of total calcium in the insoluble form in Gouda-type cheeses was determined at 1, 14, 28, 56 and 112 d of ripening using the acid-base titration method described by Hassan *et al*. (2004). The total calcium content of the cheeses was determined using atomic absorption spectroscopy as described by O'Mahony *et al*. (2005). The EDTA concentration of the cheese and whey samples were evaluated using the modified HPLC method described previously (Kemmei *et al*., 2013). Samples were prepared according to the method described by McAuliffe *et al*. (2016).

5.2.7 Proteolysis

5.2.7.1 pH 4.6-SN%TN

The pH 4.6-soluble and insoluble fractions of cheese at 14, 56 and 112 d of ripening were prepared in triplicate as described Kuchroo and Fox (1982). The

nitrogen content of the pH 4.6-soluble fraction was determined by the macro-Kjeldahl method (N x 6.38; IDF, 1986).

5.2.7.2 Urea–polyacrylamide electrophoresis

Urea–polyacrylamide electrophoresis (urea-PAGE) was carried out on pH 4.6 insoluble fraction of cheeses using the procedure described by O'Mahony *et al*. (2005). Samples were run through the stacking gel at 280 V and the separating gel at 300 V. Gels were stained using Coomassie Brilliant Blue G250 (Blakesley and Boezi, 1977) and destained by several washes with distilled water.

5.2.7.3 Reverse phase high performance liquid chromatography

Peptide profiles of pH 4.6-soluble fractions were obtained by reverse-phase HPLC using an UPLC which consisted of a Waters Acquity UPLC H-Class Core System with an Acquity UPLC TUV Detector (dual wavelength) and Acquity Column Heater 30-A. The system was interfaced with Empower 3 software (Waters Corp., Milford, MA, USA). The core system includes an Acquity UPLC H-Class quaternary solvent manager, an H-Class Sample Manager-FTN and a CH-A column heater. The column used was an Acquity UPLC® Peptide BEH C18, 130 Å, 1.7 μm, 2.1 x 100 mm column. Elution was monitored at 214 nm and a mobile phase of two solvents, A, 0.1 % (v/v) trifluoroacetic acid (TFA, sequential grade, Sigma-Aldrich, St Louis, MO, USA) in deionized HPLC grade water (Milli-Q system, Millipore, Ireland) and B, 0.1 % (v/v) TFA in acetonitrile (HPLC grade, Sigma-Aldrich, Germany) was used. The pH 4.6-soluble fraction samples were filtered through 0.22 μm cellulose acetate filter (Sartorius GmbH, Gottingen,

Germany) and an aliquot (3.3 μL) of the filtrate was injected on the column at an eluent flow rate of 0.32 mL min^{-1}. The elution gradient used is shown in Table 3.2 (Chapter 3). Data acquisition was from 0 to 20.01 min. Time between injections was 2 min at 100 % solvent A. A blank (100% solvent A) was used at the start.

5.2.8 Determination of textural and rheological properties of cheese during ripening

5.2.8.1 Texture profile analysis

Texture profile analysis (TPA) was performed using a Texture Analyser TA-XT2i (Stable Micro Systems, Godalming, Surrey, UK) according to the method of Cooke and McSweeney (2013). Hardness (Bourne, 1978) was determined at 1, 14, 28, 56 and 112 d of ripening. Five replicate samples from each cheese were compressed at each ripening time point.

5.2.8.2 Small deformation rheological properties

Rheological analysis of cheese samples at 14, 28, 56 and 112 d of ripening was performed using a AR-G2 Controlled Stress Rheometer (TA Instruments, Leatherhead, UK), as described by Cooke and McSweeney (2013), with the following modifications: cheese discs were not glued to the base plate of the Rheometer; storage modulus (G'), loss modulus (G'') and loss tangent (LT) were recorded continuously at a low amplitude shear strain of 0.1% at a frequency of 1.0 Hz over 20 min during which the temperature was increased from 20 to 80 ˚C.

5.2.8.3 Cheese meltability

Cheese meltability was performed using the Schreiber melt test procedure (Altan *et al*., 2005). Cheese samples were prepared and stored according to the procedure described by Cooke and McSweeney (2013). Results were expressed as a % increase in diameter of the cheese discs. Analysis on each cheese sample was performed in triplicate at 14, 28, 56 and 112 d of ripening.

5.2.9 Statistical analysis

Analysis of variance (ANOVA) was performed on data from liposomes and cheese composition and proteolysis at a significance level of $P \leq 0.05$. A split-plot design was used to evaluate the effect of treatment (control, LC and LE), ripening time and their interactions on hardness, chewiness, gumminess, meltability, G', G'' and LT_{max} values. The ANOVA for the split-plot designs was performed using a general linear model (GLM) procedure. When significant differences ($P \leq 0.05$) were found, the treatments means were analysed by Tukey's multiple comparison test. All analyses were performed using MiniTab® 16 (MiniTAB Inc., State College, PA, USA).

5.3 Results and Discussion

5.3.1 Characterisation of liposomes

5.3.1.1 Zeta-potential

The vesicles obtained from Pro-Lipo C liposomes with (LE) and without (LC) EDTA had negative zeta-potential values, -74.36 and -60.93 mV, respectively (Table 5.1). This result suggests the presence of strong electrostatic repulsion between the vesicles which is indicative of good stability of a colloidal system (Thompson and Singh, 2006; Nongonierma *et al*., 2009; Michelon *et al*., 2016; McAuliffe *et al*., 2016). These highly negative values are typical of liposomes produced with phosphatidylcholine (PC), a neutral, amphiphilic molecule containing a polar group that interacts with water molecules, resulting in a negative net surface charge (Nongonierma *et al*., 2009; Toniazzo *et al*., 2014; McAuliffe *et al*., 2016).

Pro-Lipo C with EDTA (LE) had a significantly ($P \leq 0.05$) greater negative zeta potential compared to Pro-Lipo C without EDTA (LC). EDTA is an anionic compound and its addition to the liposome preparation is likely to increase the negative charge of the system. Laridi *et al*. (2003) demonstrated that nisin (cationic compound) encapsulated within liposomes with lower levels of negatively charged phospholipids were more stable as a result of decreased susceptibility to nisin-membrane destabilising actions. If the same assumption is valid for anionic compounds (EDTA) encapsulated within positively charged liposome vesicles, it can be expected that EDTA-containing liposomes will be

stable. The zeta potential, which is an indirect predictor of the stability of a colloidal dispersion, did not significantly change during 28 d storage for either LC or LE liposomes (results not shown). Moraes *et al*. (2013) stated that surface charge values below -30 mV are typical of stable dispersions and therefore LC and LE liposome preparations can be considered extremely stable.

5.3.1.2 Size distribution

The effect of EDTA on the size distribution of liposomes prepared using Pro-Lipo C is shown in Figure 5.1 and Table 5.1. Both Pro-Lipo C liposomes with (LE) and without (LC) EDTA appear to have a narrow monomodal particle size distribution range with an mean diameter of 121 and 127 nm, respectively. These results are comparable with previous studies by Lariviere *et al*. (1991) and Kosaraju *et al*. (2006), who achieved mean particle size of 110 and 153 nm, for trypsin-encapsulated and iron-encapsulated liposomes, respectively, prepared by microfluidisation. Entrapment of EDTA resulted in a significant ($P \leq 0.05$) reduction in liposome size. For EDTA-containing liposomes (LE), the size distribution ranged from 68 and 295 nm with a mean diameter of 121 nm, compared with a size distribution of 44 and 458 nm with a mean diameter of 127 nm for empty vesicles (LC). The reduction in mean size distribution found for liposomes containing EDTA (LE) may be attributed to their higher negative zeta potential when compared to non-EDTA-containing liposomes (LC), -71.02 and -60.93 mV, respectively (Table 5.1) (McAuliffe *et al*., 2016).

Table 5.1 Particle size (z-average), polydispersity index (PDI), zeta-potential and EDTA entrapment efficiency of liposomes manufactured using two-stage homogenisation (2-SH) at 1 d.

Treatment	**z-average**	**PDI**	**zeta potential**	**EDTA encapsulation efficiency (% liposome fraction)**			
	(d.nm)		(mV)	Surface (%)	Core (%)	Encapsulated (%)	Un-encapsulated (%)
LC	126.89[a] (3.02)	0.24[a] (0.03)	-60.93[a] (4.75)	-	-	-	-
LE	120.54[b] (3.17)	0.11[b] (0.01)	-74.36[b] (3.74)	40.29 (1.08)	59.71 (1.08)	76.63 (1.84)	23.27 (2.00)

Values presented are means from three replicate trials with standard deviations in parentheses; means within columns not sharing a common letter differ statistically ($P \leq 0.05$)

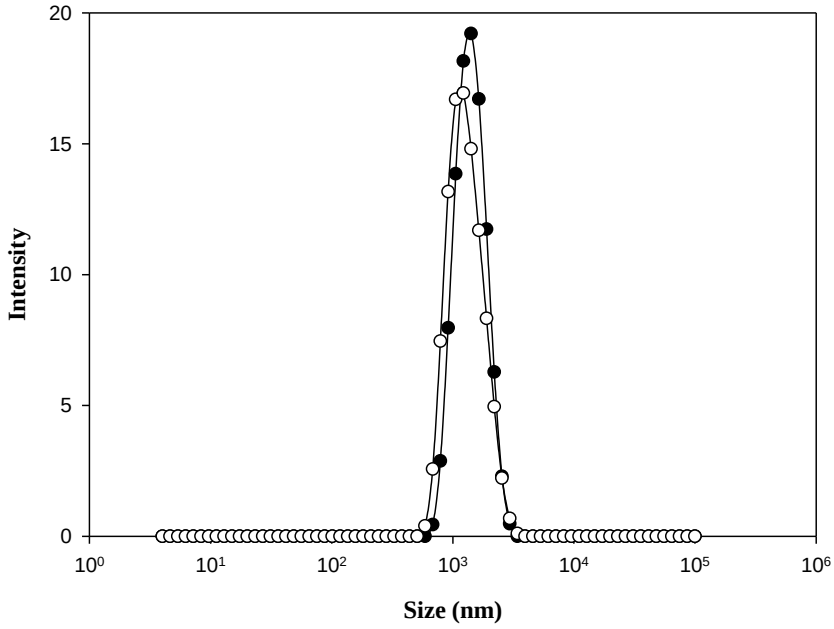

Figure 5.1 Size distributions of Pro-Lipo C liposomes with EDTA (LE) (○) and without EDTA (LC) (●) directly after two stage homogenisation processing.

Hattori and Hashida (2014) found that as the net charge of the cationic carrier/DNA complexes approached zero, interactions between complexes due to weak surface charge increased, causing the formation of larger sized particles. The EDTA contained in the LE liposomes may also affect the membrane lipid organization and/or lipid hydrophobicity (Laridi *et al*., 2003; Moraes *et al*., 2013). EDTA is known to fix (divalent) cations, which are bound to the phospholipids. By removing these bound cations, the negative charges of the phosphate groups are more obvious. This higher charge density may explain the smaller liposome diameter in the presence of EDTA.

5.3.1.3 Polydispersity index (PDI)

The polydispersity indices (PDI) obtained from dynamic light scattering (DLS) readings for Pro-Lipo C liposomes manufactured with (LE) and without (LC) EDTA are shown in Table 5.1. The PDI values are generally small for both liposome preparations LC and LE, 0.20 and 0.11, respectively, indicating that the liposomes were relatively monodispersed within the solution. These results are typical of SUVs obtained by microfluidisation (Thompson *et al*., 2007; Imran *et al*., 2015). Liposome preparations containing EDTA (LE) were, however, significantly ($P \leq 0.05$) more monodisperse when compared with liposomes devoid of EDTA (LC). Again, this result is most likely due to the greater surface charge of LE preventing coalescence of the liposomes through electrostatic repulsion, resulting in a more monodisperse solution. Liposomes composed of charged polar lipids with higher electrical charges can be expected to be more stable than liposomes composed of neutral polar lipids, since high (negative or positive) zeta-potentials increase the repulsive interactions, reducing the frequency of liposome collisions.

5.3.2 Ultrafiltration membrane efficiency

Liposome preparations were concentrated to a VCF of 3 and washed with de-ionised water to remove unentrapped EDTA from the surface of the liposomes and surrounding aqueous phase using a 10 kDa UF membrane system. A constant transmembrane pressure (TMP) of ~0.5 bar was maintained throughout UF and DF of all liposome preparations. For both UF and DF processes, liposome preparations containing no EDTA (LC) had a greater permeate flux (L m^{-2} h^{-1})

than EDTA-containing (LE) preparations (Figure 5.2a). Increasing the VCF from 1.0 to 3.0 resulted in a concomitant decrease in permeate flux (L m^{-2} h^{-1}) through the membrane for both liposome preparations (Pro-Lipo C with and without EDTA). As well as this, the liposome preparations without EDTA reached the VCF=3 in 15 min, while it took the EDTA-containing preparations 40 min to reach the same concentration (Figure 5.2b). These results indicate that the presence of EDTA dramatically decreases the efficiency (speed of permente flow) of the UF membrane, perhaps as a result of increased membrane fouling due to the higher solute concentration of the LE liposomes compared to the LC liposomes. de Balmann and Nobrega (1989) found that increasing dextran solution concentrations generally resulted in a decrease in permeate flux across a skinned polysulfone hollow fiber membrane. It is also possible that component interactions due to the presence of larger phospholipid vesicles, in this case the liposomes, within the feed solution may be causing steric hindrance to the passage of smaller molecules (EDTA) through the membrane, resulting in the decreased flow rate of the EDTA-containing liposomes (Yan-jun *et al*., 2000).

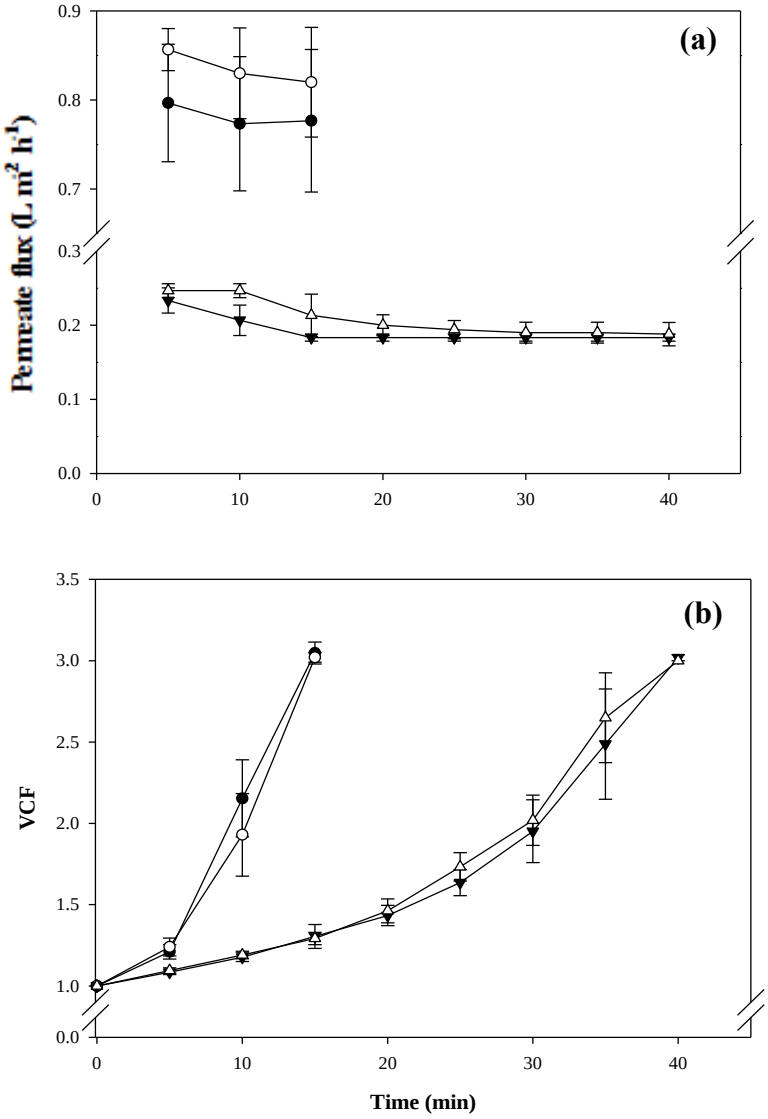

Figure 5.2 (**a**) Permeate flux and (**b**) volume concentration factor (VCF) obtained during ultrafiltration (closed symbols) and diafiltration (open symbols) for Pro-Lipo C - (without EDTA) (○,●) and Pro-Lipo C + (with EDTA) (▼,Δ) as a function of processing time.

5.3.3 Entrapment efficiency of EDTA within Pro-Lipo C manufactured using 2-SH

The concentration of EDTA present in each phase (supernatant, pellet and disrupted pellet) of the liposome was determined using HPLC and the results are indicated in Table 5.1. Almost half of the EDTA (~49.9 %) present in the original solution was lost during UF. Similar results were observed by McAuliffe *et al*. (2016) who suggested that a proportion of the EDTA-containing liposomes may have swelled and burst due to differences in osmotic pressure between the de-ionised water used to wash the liposomes and the 25 mM Tris HCl in which the liposomes were suspended. Of the total amount of EDTA entrapped within Pro-Lipo C, ~60% of the entrapped EDTA was situated within the liposome core. It has been demonstrated that the concentration of compounds capable of entrapment is a function of lipid composition and may be attributed to electrostatic and hydrophobic interactions between compounds and phospholipids (Laridi *et al*., 2003; Were *et al*., 2003). It is not surprising that a greater concentration of EDTA was situated in the vesicle core; EDTA is an anionic hydrophilic aminopolycarboxylic acid and therefore is most likely to be entrapped within the inner aqueous phase of the liposome, and less likely to be immobilised into the liposome membrane, which may occur with a hydrophobic compound.

5.3.4 Compositional analysis

Means of three independent trials for pH and composition from Gouda-type cheeses made with buffer (C), Pro-Lipo C without EDTA (LC) and Pro-Lipo C with EDTA (LE) are shown in Table 5.2. Analysis of variance (ANOVA) of the

composition data showed no significant ($P \leq 0.05$) differences between treatment cheeses in terms of pH, moisture, protein, NaCl, salt in moisture (S/M), fat, moisture in non-fat solids (MNFS) and fat in dry matter (FDM). These results support the findings of previous authors who reported that addition of liposomes did not adversely affect the pH (Kheadr *et al*., 2003) or cheese composition (Bainville *et al*., 2000; Nongonierma *et al*., 2013; McAuliffe *et al*., 2016). The concentration of EDTA entrapped within the cheese curd was measured using HPLC and the results (Table 5.2) were as expected; no EDTA was detected in the control and LC cheeses. EDTA was present at concentrations of 1.68 mg EDTA/g cheese in LE cheese. Previous studies which investigated the effects of EDTA on the Ca equilibrium of cheese did not quantify the levels of EDTA present in the final cheese, and therefore it is not possible to compare the levels of EDTA in brine-salted Gouda-type cheese with previously studied dry-salted varieties (Choi *et al*., 2008; McMahon *et al.*, 2005). Results showed no significant difference ($P \leq 0.05$) in the total Ca concentration between the control and treatment cheeses, with levels in the range expected for Gouda-type cheese (Kosikowski and Mistry, 1997). However, the proportion of Ca in the INSOL form was significantly ($P \leq 0.05$) lower in the LE-treated cheese. Additionally, the levels of INSOL Ca (mg/100 g protein) in the control, LC and LE cheese decreased during ripening from 215, 207 and 189 mg on day 1 to 159, 161 and 145 mg by day 28 respectively, and decreased only slightly throughout the remaining 112 d ripening. These results are in agreement with previous studies (Hassan *et al*., 2004; Lee *et al*., 2005; Lucey *et al*., 2005; O'Mahony *et al*., 2005; Cooke and McSweeney, 2013) which found that the level of INSOL Ca decreased rapidly during the early stages (first 21 d) of Cheddar cheese ripening and plateaued thereafter.

Table 5.2 Composition, level of proteolysis and pH values of Gouda-type cheeses made with buffer (C), Pro-Lipo C without EDTA (LC) and Pro-Lipo C with EDTA (LE) at 14 d of ripening.

Treatment	pH	Moisture	NaCl	Protein	Fat	MNFS[2]	FDM[3]	S/M[4]	pH 4.6 SN%TN	EDTA conc.	Total Ca	Insoluble Ca			
												Day			
												1	14	28	112
		%	%	%	%	%	%	%	%	mg/g cheese	mg/100g cheese	mg/100g protein			
Control	5.37[a] (0.06)	44.75[a] (0.38)	1.76[a] (0.05)	24.65[a] (0.10)	22.17[a] (0.47)	57.49[a] (0.16)	40.10[a] (0.58)	3.92[a] (0.15)	19.54[a] (0.32)	0.00[a] (0.00)	983[a] (41.0)	214.7[a] (10.43)	176.0[a] (10.05)	159.2[a] (13.29)	150.5[a] (16.74)
LC	5.36[a] (0.06)	44.94[a] (0.04)	1.77[a] (0.11)	24.15[a] (0.04)	22.33[a] (0.62)	57.87[a] (0.41)	40.56[a] (1.10)	3.95[a] (0.25)	19.22[a] (0.28)	0.00[a] (0.00)	981[a] (34.7)	207.2[a] (7.97)	171.4[a] (10.74)	160.7[a] (8.72)	142.6[a] (13.62)
LE	5.35[a] (0.06)	44.01[a] (0.14)	1.49[a] (0.07)	24.86[a] (0.17)	22.67[a] (0.85)	56.92[a] (0.75)	40.49[a] (1.59)	3.37[a] (0.15)	19.61[a] (0.41)	1.68[b] (0.69)	931[a] (27.1)	187.7[b] (9.27)	150.2[b] (7.98)	144.5[b] (12.11)	143.4[a] (11.87)

[1]Values are the means of triplicate trials with standard deviations in parentheses; means within columns not sharing a common letter differ statistically ($P \leq 0.05$)

[2]Moisture in nonfat solids of the cheese

[3]Fat in dry matter of the cheese

[4]Salt in moisture phase of cheese

The level of INSOL Ca present in the LE cheese was the lowest throughout ripening compared with the control and LC cheeses. Several studies have demonstrated that the addition of EDTA to milk reduces its CCP content (Udabage *et al*., 2001; Choi *et al*., 2007, 2008). The addition of liposome-entrapped EDTA to the cheese during manufacture and their subsequent rupture during early ripening most likely resulted in the displacement of Ca from CCP to the aqueous phase caused by its calcium chelating ability. The displacement of Ca from CCP to the aqueous phase caused by the EDTA is likely to have caused the considerable reduction in INSOL Ca levels in the LE cheese during early ripening. Similarly, Cooke (2014) found that addition of EDTA to Cheddar cheese at the salting stage, resulted in cheese with significantly lower % INSOL Ca level ($P \leq 0.05$) compared to the control at 1 d of ripening; however, by 28 d of ripening the control and EDTA cheeses had statistically similar levels of % INSOL Ca ($P \leq 0.05$). These authors also attributed this result to the ability of EDTA to sequester Ca from the CCP leading to the decrease in % INSOL Ca observed during early ripening.

5.3.5 Proteolysis

The changes in proteolysis patterns, as measured by pH 4.6-SN/TN, urea-PAGE gels and UPLC peptide profiles, during ripening of control (C) and liposome-treated cheeses (LC and LE) are illustrated in Table 5.2 and Figures 5.3 and 5.4, respectively. The level of pH 4.6-SN/TN in each of the three cheeses showed no significant ($P > 0.05$) differences at 112 d of ripening (Table 5.2) suggesting that the extent of proteolysis was similar for all cheeses. Results

indicate that the addition of liposomes both with (LE) and without EDTA (LC) did not impact on the proteolysis of the cheese. Similar results have been found by Brickley *et al*. (2009) and Cooke (2014), who found no difference in the levels of primary or secondary proteolysis upon the addition of Ca-binding salts (TSC and EDTA) to Cheddar cheese at salting.

The extent of proteolysis increased as ripening progressed for all Gouda-type cheeses (Figure 5.3). Degradation of α_{s1}-CN was more extensive than that of β-CN for all cheeses. This result was expected as α_{s1}-CN hydrolysis is catalysed by residual chymosin in the cheese at the onset of maturation (Creamer, 1970; Sousa *et al*., 2001; Van den Berg *et al*., 2004), whereas β-CN hydrolysis is predominantly linked to the action of plasmin, whose activity is limited in Gouda-type cheeses. The pH optimum for chymosin and plasmin activity is 5.1–5.3 (Humme, 1972) and 8.0 (Kaminogawa *et al*., 1972), respectively. The pH of all cheeses (C, LC and LE) at 1 and 112 d of ripening was ~5.31 and ~5.59, respectively, and therefore chymosin was the major contributing agent associated with proteolysis.

The UPLC peptide profiles of the pH 4.6-soluble fractions of Gouda-type cheeses made with buffer (C), Pro-Lipo C without EDTA (LC) and Pro-Lipo C with EDTA (LE) are shown in Figure 5.4. There were few quantitative or qualitative differences between the peptide profiles of the three cheeses at 112 d of ripening. These results are consistent with pH 4.6-SN/TN values and urea-PAGE electrophoretograms, which support the conclusion that the addition of liposomes both with and without EDTA did not impact on cheese proteolysis.

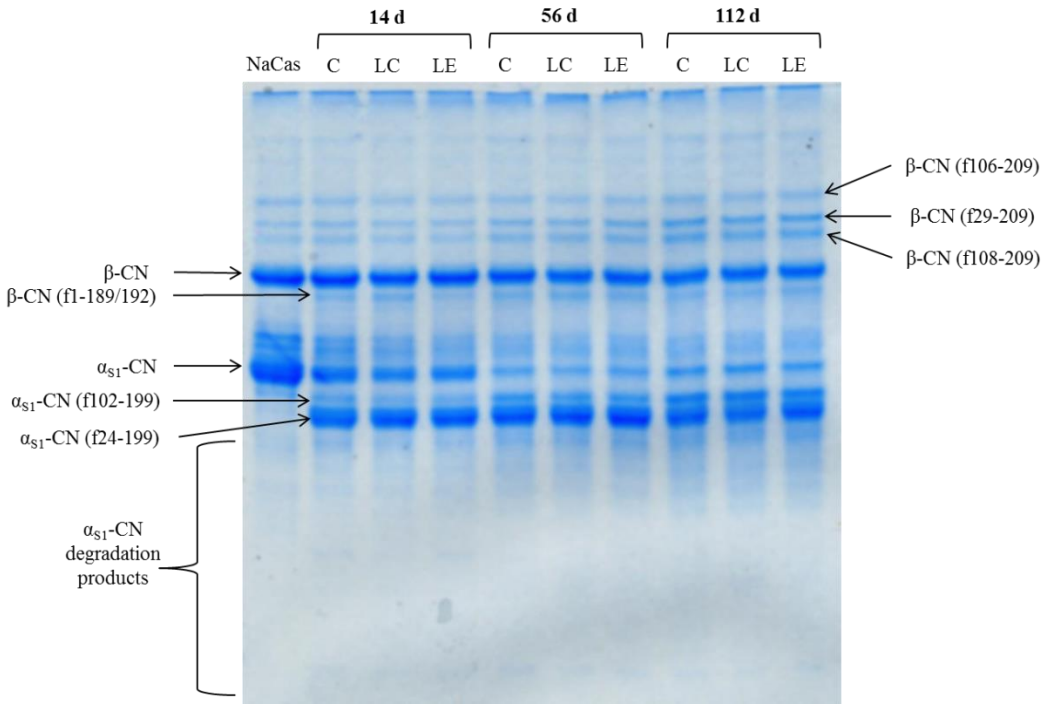

Figure 5.3 Urea-polyacrylamide gel electrophoretograms of sodium caseinate (S), and the pH 4.6-insoluble fractions from Gouda-type cheeses manufactured with buffer only (C), Pro-Lipo C without EDTA (LC) and Pro-Lipo C with EDTA (LE) at 14, 56 and 112 d of ripening. Electroretograms are of cheeses of Trial 2 and are representative of all three trials.

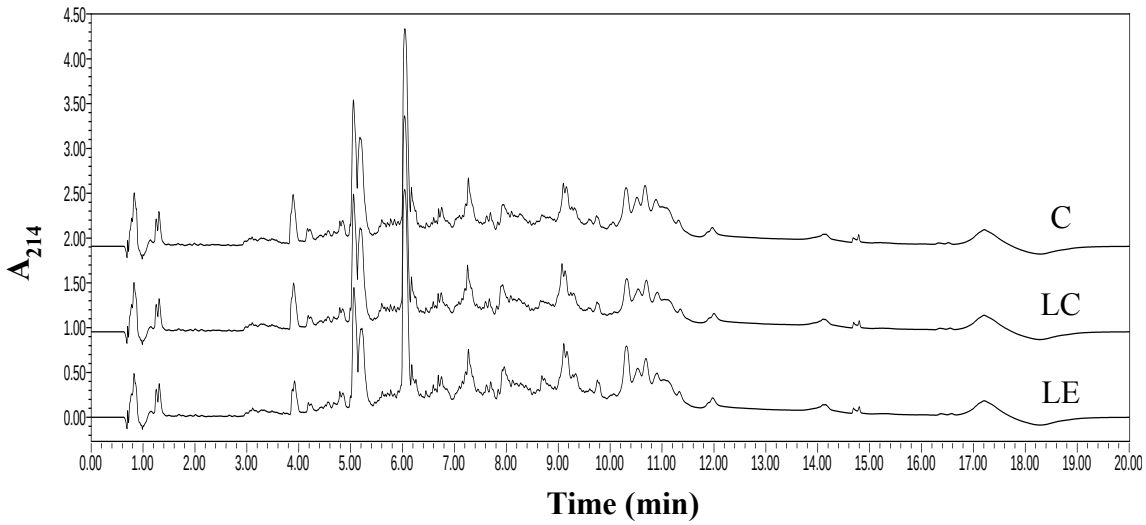

Figure 5.4 Reversed-phase UPLC separation of peptides in the pH 4.6 soluble fraction of Gouda-type cheeses made with buffer only (C), Pro-Lipo C without EDTA (LC) and Pro-Lipo C with EDTA (LE) with detection at wavelength 214 nm at 112 d of ripening.

5.3.6 Determination of textural and rheological properties of cheese during ripening

5.3.6.1 Texture profile analysis

Changes in the textural properties (hardness, chewiness and gumminess) during ripening of control (C) and experimental cheeses (LC and LE) are shown in Figure 5.5 and Table 5.3. The textural parameters; hardness, chewiness and gumminess were significantly ($P \leq 0.05$) affected by the addition of EDTA-containing liposomes (LE), particularly in the early stages of ripening, while adhesiveness, springiness, cohesiveness and resilience appeared to be unaffected. During maturation, the values for all three parameters decreased with the major reduction occurring during the first 56 d and the progression thereafter slowing down.

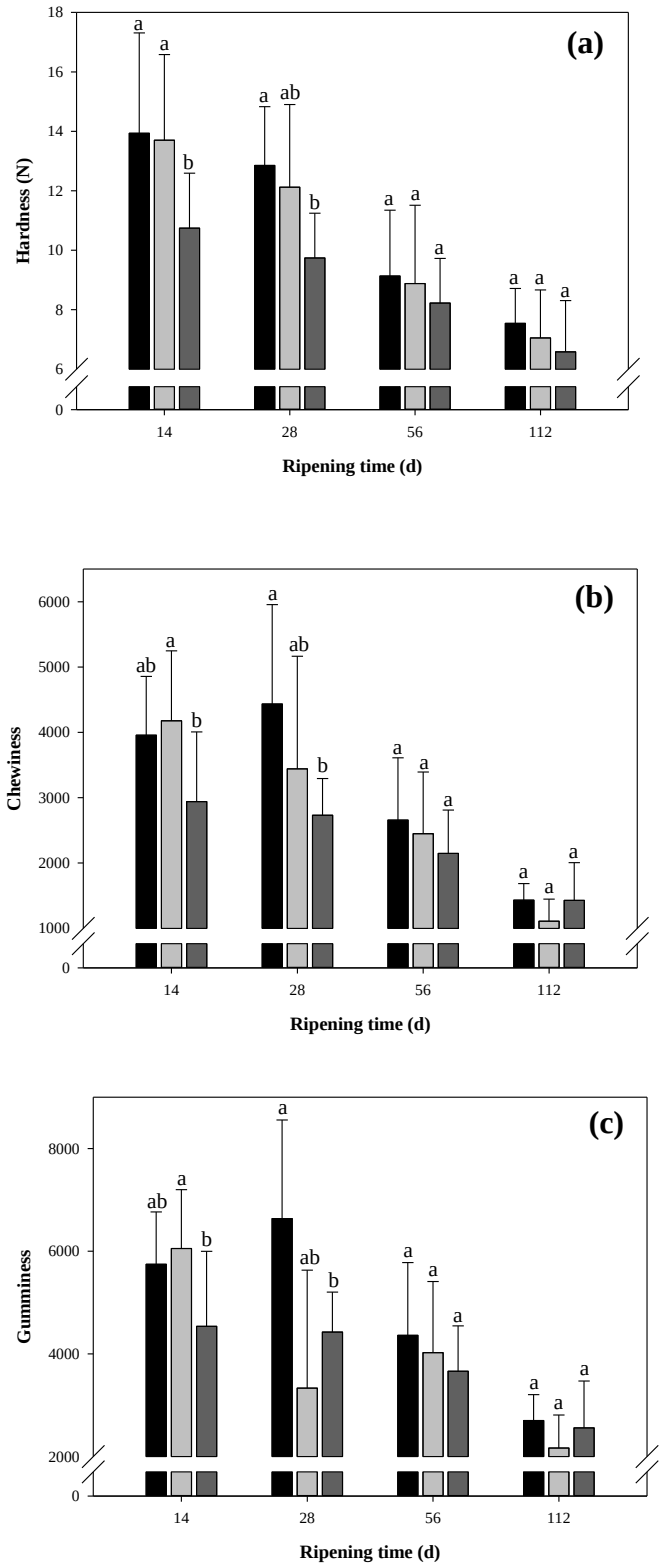

Figure 5.5 (**a**) Hardness, (**b**) chewiness and (**c**) gumminess values of Gouda-type cheeses made with buffer only (C) (■), Pro-Lipo C without EDTA (LC) (□) and Pro-Lipo C with EDTA (LE) (■) at 14, 28, 56 and 112 d of ripening as determined by texture profile analysis.

Table 5.3 Probabilities and R^2 values for hardness, adhesiveness, springiness, cohesiveness, gumminess, chewiness and resilience as determined by texture profile analysis of Gouda-type cheeses made with buffer (C), Pro-Lipo C without EDTA (LC) and Pro-Lipo C with EDTA (LE) throughout 112 d ripening.[1]

Factor	df[2]	Hardness	Adhesiveness	Springiness	Cohesiveness	Gumminess	Chewiness	Resilience
Whole plot								
Day of cheese manufacture (D)	2	72550999** (0.00)	8760 (0.16)	0.008 (0.13)	0.001 (0.06)	23125409** (0.00)	12009173** (0.00)	0.005 (0.05)
Treatment (T)	2	13936928** (0.02)	392 (0.88)	0.002 (0.49)	0.001 (0.51)	3687240 (0.05)	2180805 (0.07)	0.001 (0.49)
Error (D x T)	4	1049685 (0.57)	2938 (0.82)	0.002 (0.20)	0.002 (0.41)	548777 (0.61)	371717 (0.60)	0.001 (0.31)
Subplot								
Age (A)	3	61563831** (0.00)	40970** (0.00)	0.030593** (0.00)	0.023** (0.00)	17915659** (0.00)	10890946** (0.00)	0.011** (0.00)
T x A	6	1621389 (0.37)	6087 (0.60)	0.001 (0.71)	0.012 (0.32)	842941 (0.43)	540420 (0.44)	0.001 (0.25)
Error	18							
R^2		0.94	0.58	0.83	0.80	0.89	0.88	0.83

[1]Split-plot design with three treatments (control, LC and LE) were analysed as a discontinuous variable and cheese manufacture day was blocked. Subplot included the effect of age and treatment and treatment × age as variables

[2]Degrees of freedom

*$0.01 < P \leq 0.05$; ** $P \leq 0.01$

Several authors (Guinee *et al*., 2002; Joshi *et al*., 2003b) have attributed the reduction in cheese firmness to a weakening of the CN-CN interactions due to proteolysis. However, few differences in proteolysis were evident between C, LC and LE treated cheeses. Therefore, the significantly ($P \leq 0.05$) lower hardness values demonstrated by the LE cheese, particularly in the first 28 d, are most likely due to the difference in the INSOL Ca content (Table 5.2) compared with the control and LC cheeses. These results are in agreement with Hassan *et al*. (2004), Lee *et al*. (2005), O'Mahony *et al*. (2006) and Brickley *et al*. (2009) who found that Cheddar cheese containing lower levels of INSOL Ca exhibited softer textural properties. Addition of Ca chelating agents to cheese has been found to cause a reduction in the availability of INSOL Ca, resulting in the solubilisation and hydration of caseins, which in turn results in cheese with softer textural and rheological properties (Mizuno and Lucey, 2005; Brickley *et al*., 2009).

The chewiness values of all cheeses decreased as ripening progressed. However, significant ($P \leq 0.05$) differences in chewiness values were evident between cheeses, particularly in the first 28 d of maturation. Cheese supplemented with EDTA-containing liposomes (LE), exhibited significantly lower chewiness values up until 1 month and thereafter no significant changes were evident. Mizuno and Lucey (2005) reported that chewiness values of *pasta filata* cheese decreased with increasing levels of TSC. Beal and Mittal (2000) found a direct correlation between cheese hardness and chewiness; harder cheese is more difficult to chew. Therefore, the lower chewiness values for LE cheese is likely to be attributed to the increase in EDTA which decreased the number of CCP cross-links and increased electrostatic repulsion.

Gumminess values show a decreasing trend for all cheeses as ripening time progressed from 14 to 112 d. Ayyash *et al*. (2011) attributed the decrease in gumminess of Halloumi cheese after 14 d storage to two reasons; (1) the decrease in INSOL Ca during storage, leading to a reduction in CN cross-linkage resulting in an increase in cheese softening and (2) the increase in proteolysis throughout ripening resulting in protein hydrolysis and ultimately a softer cheese matrix. However, because proteolysis between cheeses did not differ significantly throughout ripening, it is likely that the effect on gumminess is attributed to the difference in the INSOL Ca content between cheeses.

5.3.6.2 Cheese meltability analysis (Schreiber test)

The meltability results of Gouda-type cheeses made with buffer (C), Pro-Lipo C without EDTA (LC) and Pro-Lipo C with EDTA (LE) at 14, 28, 56 and 112 d ripening are shown in Figure 5.6. Results indicate that LE cheeses had significantly ($P \leq 0.05$) greater meltability values throughout ripening when compared with the control and LC cheeses (Table 5.4). There was no significant ($P \leq 0.05$) difference in meltability between the control and LC cheeses throughout 112 d of ripening. The CCP content of the LE treated cheese at 1 d of ripening was greatly reduced (188 mg of INSOL Ca) when compared with the control and LC cheeses, which had INSOL Ca contents of 215 and 207 mg, respectively.

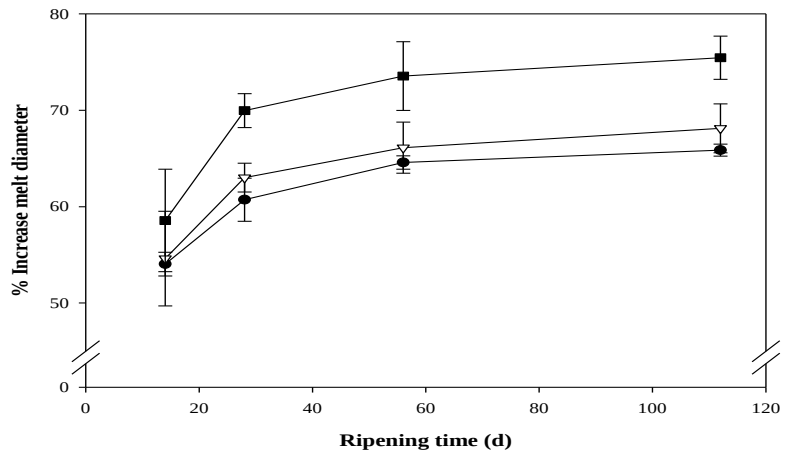

Figure 5.6 Cheese meltability as a function of ripening time, expressed as % diameter increasing cheese discs (35 mm diameter; 5 mm height), heated at 232˚C for 5 min for Gouda-type cheeses made with buffer only (C) (●), Pro-Lipo C without EDTA (LC) (▽) and Pro-Lipo C with EDTA (LE) (■) at 14, 28, 56 and 112 d of ripening.

It has been well established that reducing the levels of INSOL Ca in Cheddar cheese results in cheese with increased flowability and melting characteristics (Lucey *et al.*, 2005). These results suggest that decreasing the levels of INSOL calcium in Gouda-type cheese considerably changes the melting properties of the cheese also. It is likely that the strong sequestering ability of EDTA resulted in the removal of Ca from the CCP cross-links causing a reduction in the number of CN-CN interactions and a subsequent increase in CN hydration within the cheese matrix. Joshi *et al*. (2004) reported that cheeses containing lower levels of total calcium contain more hydrated protein within the network along with better emulsified fat; which lends itself to a cheese with increased meltability. Several other authors have also reported an increase in cheese meltability with decreasing total and INSOL Ca levels in Mozzarella (Joshi *et al*.,

2003a), Cheddar (Chevanan, and Muthukumarappan, 2007) and non-fat *pasta filata*-type cheeses (Mizuno and Lucey, 2005).

5.3.8.3 Small deformation rheological properties

The dynamic moduli (G' and G") values at 30 and 70 ˚C for Gouda-type cheeses made with buffer (C), Pro-Lipo C without EDTA (LC) and Pro-Lipo C with EDTA (LE) at 14 and 112 d ripening are shown in Table 5.5. Increasing temperature from 20 to 80 ˚C resulted in a decrease in both G' and G" values for all cheeses. No major differences in G' values were evident between control and EDTA-containing cheese at 30 ˚C. However at 70 ˚C, the G' value for the LE cheese decreased more than the control cheeses, although the degree of this reduction was small and not statistically significant. Choi *et al.* (2008) reported that cheeses with identical pH and composition but with lower CCP concentration exhibited decreased G' values at 70 ˚C compared to control cheeses at 1 d of ripening.

Table 5.4 Probabilities and R^2 values for meltability as determined using the Schreiber method, maximum loss tangent (LT_{max}), storage modulus at 30˚C (G'_{30}) and 70˚C (G'_{70}) and loss modulus at 30˚C (G''_{30}) and 70˚C (G''_{70}) of Gouda-type cheeses made with buffer (C), Pro-Lipo C without EDTA (LC) and Pro-Lipo C with EDTA (LE) throughout 112 d ripening.[1]

Factor	df[2]	Meltability	df[2]	LT_{max}[3]	G'_{30}[4]	G'_{70}[5]	G''_{30}[5]	G''_{70}[7]
Whole plot								
Day of cheese manufacture (D)	2	31.92 (0.08)	2	0.31 (0.07)	604288957** (0.01)	99555 (0.10)	22176081** (0.00)	181683 (0.07)
Treatment (T)	2	218.59** (0.00)	2	0.17 (0.16)	41177021 (0.29)	7465 (0.74)	1418950 (0.19)	25499 (0.51)
Error (D x T)	4	6.33 (0.69)	4	0.06 (0.19)	24293158 (0.59)	22712 (0.42)	556741 (0.91)	32069 (0.75)
Subplot								
Age (A)	3	353.83** (0.00)	1	0.02 (0.67)	817220419** (0.00)	653020** (0.00)	203412433** (0.00)	3454126** (0.00)
T x A	6	4.52 (0.87)	2	0.05 (0.23)	5050090 (0.86)	2728 (0.87)	340322 (0.87)	21370 (0.74)
Error	18		6					
R^2		0.90		0.72	0.92	0.89	0.95	0.91

[1]Split-plot design with three treatments (control, LC and LE) were analysed as a discontinuous variable and cheese manufacture day was blocked. Subplot included the effect of age and treatment and treatment × age as variables
[2]Degrees of freedom
[3]Maximum loss tangent
[4]Storage modulus at 30 ˚C
[5]Storage modulus at 70 ˚C
[6]Loss modulus at 30 ˚C
[7]Loss modulus at 70 ˚C
*$0.01 < P \leq 0.05$; ** $P \leq 0.001$

Table 5.5 Small deformation rheological properties of Gouda-type cheeses made with buffer (C), Pro-Lipo C without EDTA (LC) and Pro-Lipo C with EDTA (LE) at 14 and 112 d of ripening, determined by controlled stress rheometer.

Treatment	Day	G'_{30}	G'_{70}	G''_{30}	G''_{70}
		Pa	Pa	Pa	Pa
Control	14	23147^{a} (9361)	516^{a} (178)	7887^{a} (3012)	1035^{a} (450)
LC		27073^{a} (9474)	547^{a} (255)	9152^{a} (2856)	976^{a} (427)
LE		23214^{a} (4923)	460^{a} (170)	9071^{a} (2341)	795^{a} (234)
Control	112	13647^{b} (5793)	192^{b} (106)	1697^{b} (371)	60^{b} (21.0)
LC		14064^{b} (15200)	167^{b} (210)	2279^{b} (1252)	66^{b} (28.0)
LE		11294^{b} (11967)	122^{b} (162)	1964^{b} (874)	52^{b} (23.0)

Values are the means of triplicate trials with standard deviations in parentheses; means within columns not sharing a common letter differ statistically ($P \leq 0.05$).

The loss tangent (LT) values for all cheeses at 14 and 112 d of ripening are shown in Figures 5.7a and 5.7b. There was little change evident in the LT values below 40 ˚C; however, at temperatures >40˚C, the LT values for all cheeses increased greatly. This increase in the LT values is indicative of a phase transition from an unheated cheese, which appeared more elastic, to a more viscous flowing cheese when heat is applied. As milk fat is entirely liquefied at 40 ˚C (Norris *et al*., 1973) this observed increase in LT values is most likely due to both the reduction in protein-protein interactions and the reduction in CN cross-linkage resulting from a decrease in INSOL Ca which in turn increases the propensity for bond breakage and structural rearrangements giving rise to cheese with a greater ability to melt and flow. Similar findings have been observed in Cheddar and other dry-salted cheese varieties (Mizuno and Lucey, 2005; Lucey *et al*., 2005; Brickley *et al*., 2009; Wang *et al*., 2011). Authors attributed the increase in LT values of these cheeses during heating to a reduction in cross-linking material due

to the solubilisation of the INSOL Ca as well as a decrease in protein-protein interactions. The LT values increased significantly for all Gouda-type cheeses throughout ripening from 14 to 112 d, which is in agreement with previous Cheddar cheese studies (Lucey *et al*., 2003; Lucey *et al*., 2005). No significant differences in LT were observed between cheeses at 14 d; however, at 112 d of ripening LE cheese had a significantly ($P \leq 0.05$) greater LT value at temperatures greater than 60 ˚C. Choi *et al.* (2008) reported that cheeses with no pH or compositional differences but with reduced INSOL calcium exhibited increased LT values at 70 ˚C compared to control cheeses at 1 d of ripening.

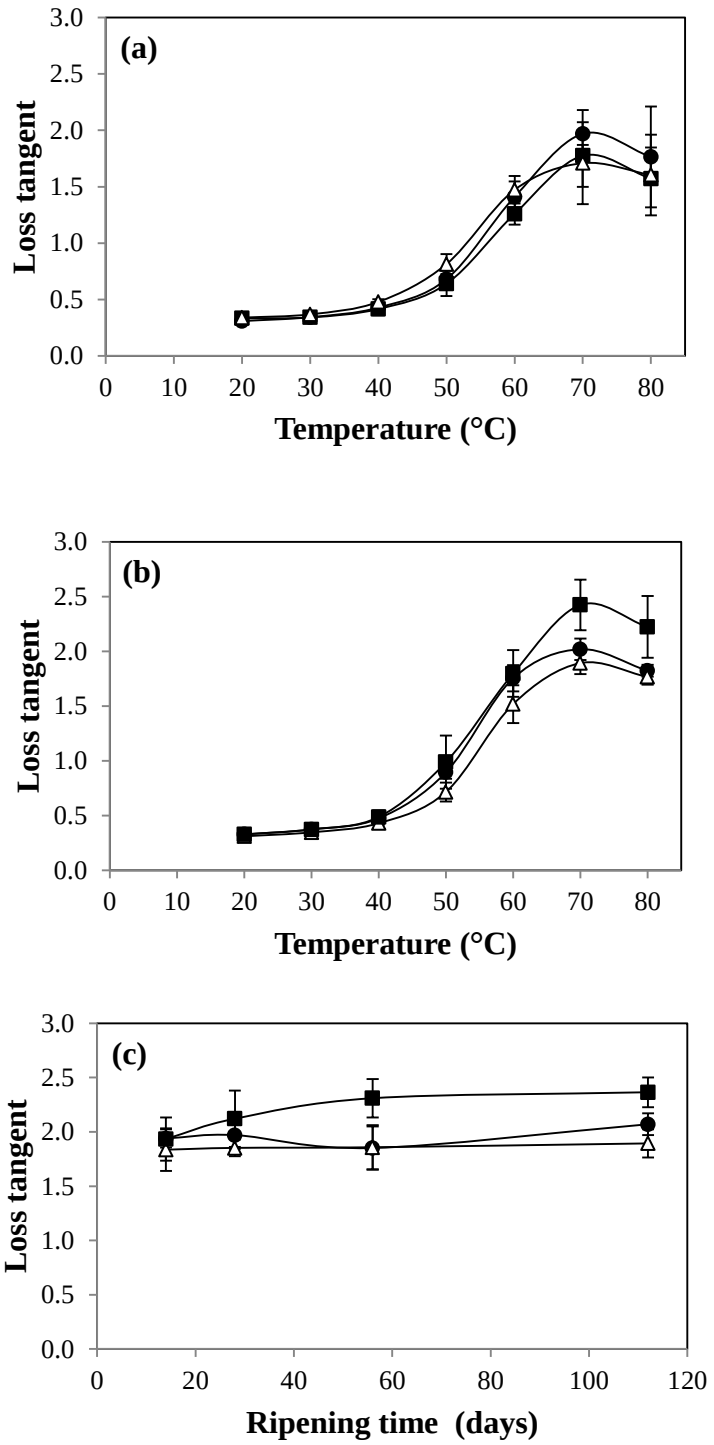

Figure 5.7 Loss tangent values from the dynamic small amplitude oscillatory rheology analysis at (**a**) 14 and (**b**) 112 d and (**c**) maximum loss tangent (LT_{max}) for all Gouda-type cheeses manufactured with buffer only (C) (●), Pro-Lipo C without EDTA (LC) (△) and Pro-Lipo C with EDTA (LE) (■).

The maximum loss tangent (LT_{max}) values for Gouda-type cheeses made with buffer (C), Pro-Lipo C without EDTA (LC) and Pro-Lipo C with EDTA (LE) throughout 112 d ripening are shown in Figure 5.7c. There was a considerable increase in the LT_{max} in all cheeses from 14 to 112 d of ripening. The LE cheese had a higher LT_{max} value than the control and LC cheese after 28 d ripening. Higher LT_{max} values indicate a greater propensity for cheese to melt and flow when heated (O'Mahony *et al*., 2006). These results suggest that the LE cheese had the greatest meltability, particularly after 1 month ripening, correlating well with the results of the Schreiber test. The increased LT_{max} values exhibited by the LE cheese again demonstrate the importance of Ca, in particularly the level of INSOL Ca, within the cheese matrix on textural and rheological properties of cheese. LE cheese contained lower levels of INSOL Ca due to the Ca sequestering ability of EDTA resulting in a reduction in strength of the interactions between caseins leading to increased melt. Mizuno and Lucey (2005) found that *pasta filata* cheese containing higher concentrations of added TSC, exhibited a higher LT_{max} value. The LT_{max} values for control and cheese with 5% TSC added were ~2.7 and 3.5, respectively. Delayed rupture of liposomes and release of EDTA into the cheese may explain the significant increase in LT_{max} only after 1 month of ripening observed in this study. O'Mahony *et al*. (2006) demonstrated that increasing the proportion of INSOL Ca in Cheddar cheese significantly ($P \leq 0.05$) decreased LT_{max} values.

5.4 Conclusions

Addition of liposome-entrapped EDTA (LE) into cheese did not cause significant differences in composition, pH and proteolysis patterns when compared with control and LC cheeses. However, LE cheese had reduced levels of INSOL Ca particularly during the early stages of ripening, compared to control and LC cheeses, most likely attributed to the displacement of Ca from the INSOL to the aqueous phase caused by the calcium chelating ability of EDTA. Hardness, chewiness and gumminess values for LE cheese were significantly ($P \leq 0.05$) lower at the early stages of ripening when compared to control cheeses. Since there were few differences in proteolysis between C, LC and LE treated cheeses, the lower hardness, chewiness and gumminess values demonstrated by the LE cheese, particularly in the first 28 d, was most likely due to the difference in the INSOL Ca content. The LE cheese had significantly ($P \leq 0.05$) greater meltability values throughout ripening when compared with the control and LC cheese. As well as this, LE cheese exhibited higher LT_{max} values than the control and LC cheeses after 28 d ripening, indicating a greater propensity for LE cheese to flow and melt when heated. The results of this study highlight the potential of liposomes to optimise the retention of calcium chelating salts within the cheese curd during the production of brine-salted type cheese. Results also indicate that liposome-entrapped EDTA incorporated into milk during cheese manufacture alters the distribution of Ca between the insoluble and soluble forms in brine-salted continental-type cheese, thus modifying its structure, texture and functionality, without impacting negatively on the pH or composition of the cheese.

5.5 Acknowledgements

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References

Altan, A., Turhan, M. & Gunasekaran, S. (2005). Short communication: Comparison of covered and uncovered Schreiber test for cheese meltability evaluation. *Journal of Dairy Science*, *88*, 857-861.

Anjani, K., Kailasapathy, K. & Phillips, M. (2007). Microencapsulation of enzymes for potential application in acceleration of cheese ripening. *International Dairy Journal*, *17*, 79-86.

Ayyash, M. M., Sherkat, F., Francis, P., Williams, R. P. W. & Shah, N. P. (2011). The effect of sodium chloride substitution with potassium chloride on texture profile and microstructure of Halloumi cheese. *Journal of Dairy Science*, *94*, 37-42.

Bainville, C.; Vuillemard, J. C. & Lacroix, C. (2000). Comparison of different methods for fortifying Cheddar cheese with vitamin D. *International Dairy Journal*, *10*, 375–382.

Beal, P. & Mittal, G. S. (2000). Vibration and compression responses of Cheddar cheese at different fat content and age. *Milchwissenschaft*, *55*, 139-142.

Blakesley, R. W. & Boezi, J. A. (1977). A new staining technique for proteins in polyacrylamide gels using Coomassie Brilliant Blue G250. *Analytical Biochemistry*, *82*, 580-582.

Bourne M. C. (1978). Texture profile analysis. *Food Technology*. *32*, 62–66, 72.

Brickley, C. A., Lucey, J. A. & McSweeney, P. L. H. (2009). Effect of the addition of trisodium citrate and calcium chloride during salting on the

rheological and textural properties of Cheddar-style cheese during ripening. *International Journal of Dairy Technology*, *62*, 527-534.

Chevanan, N. & Muthukumarappan, K. (2007). Effect of calcium and phosphorus, residual lactose, and salt-to-moisture ratio on the melting characteristics and hardness of Cheddar cheese during ripening. *Journal of Food Science*, *72*, 168-176.

Choi, J., Horne, D. S. & Lucey, J. A. (2007). Effect of insoluble calcium concentration on rennet coagulation properties of milk. *Journal of Dairy Science*, *90*, 2612-2623.

Choi, J., Horne, D. S., Johnson, M. E. & Lucey, J. A. (2008). Effects of the concentration of insoluble calcium phosphate associated with casein micelles on the functionality of directly acidified cheese. *Journal of Dairy Science*, *91*, 513-522.

Cooke, D. R. & McSweeney, P. L. H. (2013). The influence of alkaline earth metal equilibria on the rheological, melting and textural properties of Cheddar cheese. *Journal of Dairy Research*, *80*, 418-428.

Cooke, D. R. (2014). *Exploring the physicochemical basis of cheese texture, rheology and functionality, with emphasis on cation-casein interactions.* PhD Thesis, University College Cork, Ireland. Retrieved from https://cora.ucc.ie/handle/10468/1887.

Creamer, L. K. (1970). Protein breakdown in Gouda cheese. *New Zealand Journal of Dairy Science and Technology*, *5*, 152-154.

Creamer, L. K. & Olson, N. F. (1982). Rheological evaluation of maturing Cheddar cheese. *Journal of Food Science, 47,* 631-636.

Creamer, L. K., Lawrence, R. C. & Gilles, J. (1985). Effect of acidification of cheese milk on the resultant Cheddar cheese. *New Zealand Journal of Dairy Science and Technology, 20,* 185-203.

de Balmann, H. & Nobrega, R. (1989). The deformation of dextran molecules: Causes and consequences in ultrafiltration. *Journal of Membrane Science, 40,* 311-327.

Dufour, P., Vuillemard, J. C., Laloy, E. & Simard, R. E. (1996). Characterisation of enzyme immobilization in liposomes prepared from proliposomes. *Journal of Microencapsulation, 13,* 185-194.

Fox, P. F. (1963). Potentiometric determination of salt in cheese. *Journal of Dairy Science, 46,* 744-745.

Fox, P. F., Guinee, T. P., Cogan, T. M. & McSweeney, P. L. H. (2000). *Fundamentals of Cheese Science.* Aspen Publishers, Gaithersburg, MD.

Guinee, T. P., Feeney, E. P., Auty, M. A. E. & Fox, P. F. (2002). Effect of pH and calcium concentration on some textural and functional properties of Mozzarella cheese. *Journal of Dairy Science, 85,* 1655-1669.

Hassan, A., Johnson, M. E. & Lucey, J. A. (2004). Changes in the proportions of soluble and insoluble calcium during the ripening of Cheddar cheese. *Journal of Dairy Science, 87,* 854-862.

Hattori, Y. & Hashida, M. (2014). Evaluation of size and zeta potential of DNA/carrier complexes. In Taira, K., Kataoka, K. & Niidome, T. (Eds.), *Non-viral Gene Therapy*, (pp. 293-299). Tokyo, Japan: Springer Publishers.

Humme, H. E. (1972). The optimum pH for the limited specific proteolysis of κ-casein by rennin (primary phase of milk clotting). *Netherlands Milk Dairy Journal*, *26*, 180-185.

IDF (1982). Cheese and processed cheese; determination of the total solids content. Standard 4A:1982. Brussels, Belgium: International Dairy Federation.

IDF (1986). Determination of the Nitrogen Content (Kjeldahl Method) and calculation of crude protein content. Standard 20A:1986. Brussels, Belgium: International Dairy Federation.

IIRS (1955). Determination of the percentage of fat in cheese. Irish Standard 69:1955. Institute for Industrial Research and Standards, Dublin.

Imran, M., Revol-Junelles, A. M., Paris, C., Guedon, E., Linder, M. & Desobry, S. (2015). Liposomal nanodelivery systems using soy and marine lecithin to encapsulate food biopreservative nisin. *LWT-Food Science and Technology*, *62*, 341-349.

Joshi, N. S., Muthukumarappan, K. & Dave, R. I. (2003a). Effect of calcium on physicochemical properties of fat-free Mozzarella cheese. *Journal of Food Science*, *68*, 2289-2294.

Joshi, N. S., Muthukumarappan, K. & Dave, R. I. (2003b). Understanding the role of calcium in functionality of part-skim Mozzarella cheese. *Journal of Dairy Science*, *86*, 1918-1926.

Joshi, N. S., Muthukumarappan, K. & Dave, R. I. (2004). Effect of calcium on microstructure and meltability of part skim Mozzarella cheese. *Journal of Dairy Science*, *87*, 1975-1985.

Kaminogawa, S., Mizobuchi, H. & Yamauchi, K. (1972). Comparison of bovine milk protease with plasmin. *Agricultural and Biological Chemistry*, *36*, 2163-2167.

Kemmei, T., Kodama, S., Yamamoto, A., Inoue, Y. & Hayakawa, K. (2013). Determination of ethylenediaminetetraacetic acid in foods by reversed-phase high-performance liquid chromatography. *Food Chemistry*, *138*, 866-869.

Kheadr, E. E., Vuillemard, J. C. & El-Deeb, S. A. (2003). Impact of liposome-encapsulated enzyme cocktails on Cheddar cheese ripening. *Food Research International*, *36*, 241-252.

Kosaraju, S. L., Tran, C. & Lawrence, A. (2006). Liposomal delivery systems for encapsulation of ferrous sulfate: preparation and characterisation. *Journal of Liposome Research*, *16*, 347-358.

Kosikowski, F. V. & Mistry, V. V. (1997). *Cheese and Fermented Milk Foods, Vol 1: Origins and Principles*, 3rd Ed. F. V. Kosikowski & Associates: Great Falls, VA, USA.

Kuchroo, C. N. & Fox, P. F. (1982). Soluble nitrogen in Cheddar cheese: comparison of extraction procedures. *Milchwissenschaft*. *37*, 331-335.

Laloy, E., Vuillemard, J. C., El Abboudi, M., Fliss, I., De Grace, E. & Simard, R. E. (1996). Development of an immunological method for studying the incorporation of ripening enzymes in cheese curd. *Journal of Dairy Research*, *63*, 141-149.

Laloy, E., Vuillemard, J. C., Dufour, P. & Simard, R. (1998). Release of enzymes from liposomes during cheese ripening. *Journal of Controlled Release*, *54*, 213-222.

Laridi, R., Kheadr, E. E., Benech, R. O., Vuillemard, J. C., Lacroix, C. & Fliss, I. (2003). Liposome encapsulated nisin Z: optimization, stability and release during milk fermentation. *International Dairy Journal*, *13*, 325-336.

Lariviere, B., El Soda, M., Soucy, Y., Trépanier, G., Paquin, P. & Vuillemard, J.C. (1991). Microfluidized liposomes for the acceleration of cheese ripening. *International Dairy Journal*, *1*, 111-124.

Lawrence, R. C., Creamer, L. K. & Gilles, J. (1987). Texture development during cheese ripening. *Journal of Dairy Science*, *70*, 1748-1760.

Lawrence, R. C., Gilles, J., Creamer, L. K., Crow, V. L., Heap, H. A., Honore, C. G., Johnston, K. A. & Samal, P. K. (2004). Cheddar cheese and related dry-salted cheese varieties. In Fox, P. F., McSweeney, P. L. H., Cogan, T. M. & Guinee, T. P (Eds.), *Cheese: Chemistry, Physics and Microbiology. 2. Major Cheese Groups*, 3rd ed., (pp. 71-102). San Diego, USA: Academic Press.

Lee, M. R., Johnson, M. E. & Lucey, J. A. (2005). Impact of modifications in acid development on the insoluble calcium content and rheological properties of Cheddar cheese. *Journal of Dairy Science*, *88*, 3798-3809.

Lucey, J. A. & Fox, P. F. (1993). Importance of calcium and phosphate in cheese manufacture: a review. *Journal of Dairy Science*, *76*, 1714-1724.

Lucey, J. A., Johnson, M. E. & Horne, D. S. (2003). Perspectives on the basis of the rheology and texture properties of cheese. *Journal of Dairy Science*, *86*, 2725-2743.

Lucey, J. A., Mishra, R., Hassan, A. & Johnson, M. E. (2005). Rheological and calcium equilibrium changes during the ripening of Cheddar cheese. *International Dairy Journal*, *15*, 645-653.

McAuliffe, L. N., Kilcawley, K. N., Sheehan, J. J. & McSweeney, P. L. H. (2016). Manufacture and incorporation of liposome-entrapped ethylenediaminetetraacetic acid into model miniature Gouda-type cheese and subsequent effect on starter viability, pH, and moisture content. *Journal of Food Science* (in press).

McMahon, D. J., Paulson, B. & Oberg, C. J. (2005). Influence of calcium, pH, and moisture on protein matrix structure and functionality in direct-acidified nonfat Mozzarella cheese. *Journal of Dairy Science*, *88*, 3754-3763.

Metzger, L. E., Barbano, D. M., Rudan, M. A. & Kindstedt, P. S. (2000). Effect of milk preacidification on low fat Mozzarela cheese: 1. Composition and yield**.** *Journal of Dairy Science*, *83*, 646-658.

Michelon, M., Mantovani, R. A., Sinigaglia-Coimbra, R., de la Torre, L. G. & Cunha, R. L. (2016). Structural characterization of *β*-carotene-incorporated nanovesicles produced with non-purified phospholipids. *Food Research International*, *79*, 95-105.

Mizuno, R. & Lucey, J. A. (2005). Effects of two types of emulsifying salts on the functionality of nonfat *pasta filata* cheese. *Journal of Dairy Science*, *88*, 3411-3425.

Moraes, M., Carvalho, J. M. P., Silva, C. R., Cho, S., Sola, M. R. & Pinho, S. C. (2013). Liposomes encapsulating beta-carotene produced by the proliposomes method: characterisation and shelf life of powders and phospholipid vesicles. *International Journal of Food Science and Technology*, *48*, 274-282.

Nongonierma, A. B., Abrlova, M., Fenelon, M. A. & Kilcawley, K. N. (2009). Evaluation of two food grade proliposomes to encapsulate an extract of a commercial enzyme preparation by microfluidisation. *Journal of Agricultural and Food Chemistry*, *57*, 3291-3297.

Nongonierma, A. B., Abrlova, M. & Kilcawley, K. N. (2013). Encapsulation of a lactic acid bacteria cell-free extract in liposomes and use in Cheddar cheese ripening. *Foods*, *2*, 100-119.

Norris, G. E., Gray, I. K. & Dolby, R. M. (1973). Seasonal variations in the composition and thermal properties of New Zealand milk fat. *Journal of Dairy Research*, *40*, 311-21.

O'Mahony, J. A., Lucey, J. A. & McSweeney, P. L. H. (2005). Chymosin-mediated proteolysis, calcium solubilization and texture development during the ripening of Cheddar cheese. *Journal of Dairy Science*, *88*, 3101-3114.

O'Mahony, J. A., McSweeney, P. L. H. & Lucey, J. A. (2006). A model system for studying the effects of colloidal calcium phosphate concentration on the rheological properties of Cheddar cheese. *Journal of Dairy Science*, *89*, 892-904.

Sheehan, J. J. & Guinee, T. P. (2004). Effect of pH and calcium level on the biochemical, textural and functional properties of reduced-fat Mozzarella cheese. *International Dairy Journal*, *14*, 161-172.

Sousa, M. J., Ardö, Y. & McSweeney, P. L H. (2001). Advances in the study of proteolysis during cheese ripening. *International Dairy Journal*, *11*, 327-345.

Thompson, A. K. & Singh, H. (2006). Preparation of liposomes from milk fat globule membrane phospholipids using a microfluidizer. *Journal of Dairy Science*, *89*, 410-419.

Thompson, A. K., Mozafari, M. R. & Singh, H. (2007). The properties of liposomes produced from milk fat globule membrane material using different techniques. *Lait*, *87*, 349-360.

Toniazzo, T., Berbel, I. F., Cho, S., Fávaro-Trindade, C. S., Moraes, I. C. & Pinho, S. C. (2014). *β*-Carotene-loaded liposome dispersions stabilized with xanthan and guar gums: Physico-chemical stability and feasibility of application in yogurt. *LWT-Food Science and Technology*, *59*, 1265-1273.

Udabage, P., McKinnon, I. R. & Augustin, M. A. (2001). Effects of mineral salts and calcium chelating agents on the gelation of renneted skim milk. *Journal of Dairy Science*, *84*, 1569-1575.

Van den Berg, G., Meijer, W. C., Düsterhöft, E. M. & Smit, G. (2004). Gouda and related cheeses. In Fox, P. F., McSweeney, P. L. H., Cogan, T. M. & Guinee, T. P (Eds.), *Cheese: Chemistry, Physics and Microbiology. 2. Major Cheese Groups*, 3rd ed., (pp.103-140). San Diego, USA: Academic Press.

Wang, F., Zhang, X., Luo, J., Guo, H., Zeng, S. S. & Ren, F. (2011). Effect of proteolysis and calcium equilibrium on functional properties of natural Cheddar cheese during ripening and the resultant processed cheese. *Journal of Food Science*, *76*, E248-E253.

Were, L. M., Bruce, B. D., Davidson, P. M. & Weiss, J. (2003). Size, stability, and entrapment efficiency of phospholipid nanocapsules containing polypeptide antimicrobials. *Journal of Agricultural and Food Chemistry*, *51*, 8073-8079.

Wilkinson, M. G. & Kilcawley, K. N. (2005). Mechanisms of incorporation and release of enzymes into cheese during ripening. *International Dairy Journal*, *15*, 817-830.

Yan-jun, Z., Kai-fen, W., Zheng-jun, W., Liang, Z. & Shu-shen, L. (2000). Fouling and cleaning of membrane-a literature review. *Environmental Science*, *12*, 241-251.

Chapter 6

Chymosin-mediated proteolysis, calcium solubilisation and texture development during the ripening of Gouda-type cheese

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Abstract

The relationship between chymosin-mediated proteolysis, insoluble calcium (INSOL Ca) level and texture development of Gouda-type cheese throughout 112 d of ripening was investigated. Four 2 kg Gouda-type cheeses were manufactured with near identical gross chemical compositions, but wide variations in proteolysis levels, obtained by increasing the concentration of pepstatin (a potent competitive inhibitor of chymosin), added to the curds and whey mixture before cooking. Gouda-type cheese manufactured with 0.0 (control), 0.1, 1.0 and 10.0 μmol/L added pepstatin contained residual chymosin levels at 1 d that were 100, 73, 43 and 7% of the activity of the control cheese, respectively, and these levels remained constant throughout ripening. pH 4.6-soluble nitrogen levels of cheese containing pepstatin were significantly lower than that of the control cheese throughout ripening, with almost complete inhibition of α_{s1}-CN hydrolysis at Phe_{23}-Phe_{24} for the cheese made with 10.0 μmol/L pepstatin. Irrespective of the level of pepstatin, INSOL Ca levels decreased significantly ($P \leq 0.05$) at the same rate during ripening in all cheeses, with a concurrent decrease in textural properties, particularly hardness, occurring during the first month. Softening of Gouda-type cheese was found to be more correlated with the levels of INSOL Ca than with the level of intact α_{s1}-CN during early ripening, and thereafter the level of intact α_{s1}-CN emerged as the major factor responsible for continued softening. Therefore, it was concluded that the changes in the texture during early ripening, most notably, hardness, were principally due to the partial solubilisation of colloidal calcium phosphate associated with the *para*-CN matrix of the curd, and not, α_{s1}-CN hydrolysis at Phe_{23}-Phe_{24}.

6.1 Introduction

Cheese ripening consists of a complex series of biochemical and microbiological changes (McSweeney, 2004; McSweeney *et al*., 2006; Beresford and Williams, 2004; Fox *et al*., 2017). The primary biochemical changes which occur during ripening involve the metabolism of residual lactose and of lactate and citrate, lipolysis and proteolysis. These extremely complex biochemical changes occur through the catalytic action of several agents: namely, coagulants; indigenous milk enzymes, particularly plasmin and lipoprotein lipase; starter bacteria and their enzymes; secondary microflora and their enzymes (Fox *et al*., 2000). The microbiological changes that occur during the ripening of Gouda cheese involve the death of starter culture and the growth of secondary microflora (Beresford and Williams, 2004). As a consequence of these biochemical and microbiological changes, the texture of Gouda cheese also changes. A softening of cheese texture (reduced firmness, hardness, fracture strain and fracture stress) is generally observed as ripening progresses due to several factors including changes in pH and water binding (McSweeney, 2004), hydrolysis of the casein matrix due to proteolysis (Lawrence *et al*., 1987; Fox, 1989; McSweeney, 2004; Upadhyay *et al*., 2004) and the alteration of calcium (Ca) equilibrium in the cheese (Lucey and Fox, 1993; Pastorino *et al*., 2003; Lucey *et al*., 2003, 2005; Hassan *et al*., 2004; Lee *et al*., 2005; O'Mahony *et al*., 2006; Wang *et al*., 2011).

Proteolysis has long been considered the most complex, and often most important, biochemical event responsible for the textural and rheological changes occurring in cheese and, hence, has been extensively reviewed (Lawrence *et al*., 1987; Fox, 1989; McSweeney, 2004; Upadhyay *et al*., 2004; Ardo *et al*., 2016).

Textural development of cheese during ripening is a dynamic process which can be partitioned into two distinct phases (Lawrence *et al*., 1987; Lucey *et al*., 2003). The first phase involves the transformation of cheese texture from that of a rubbery state, commonly associated with young cheese, to a much smoother, homogeneous form (Lucey *et al*., 2003). These changes are associated with the concurrent weakening of the *para*-CN matrix, as well as slow solubilisation of some of the residual INSOL calcium phosphate (CCP), leading to the reduction in cheese hardness, and generally occur within the first ~ 2–4 weeks (Lawrence *et al*., 1987; Lucey *et al*., 2003). In the second phase, pH changes, continuing proteolysis and changes in protein hydration (Lawrence *et al*., 1987; Guinee, 2003) are the major contributing factors responsible for the gradual changes in cheese texture occurring during the remainder of ripening.

Chymosin-mediated hydrolysis of α_{s1}-CN at Phe_{23}-Phe_{24} had long been considered the principal factor responsible for the characteristic softening of cheese texture during the early stages of ripening (phase 1 proteolysis) (Creamer *et al*., 1982; Lawrence *et al*., 1987; Fox *et al*., 1993; Guinee, 2003; Lucey *et al*., 2003). It had been hypothesised that hydrolysis of α_{s1}-CN at Phe_{23}-Phe_{24} causes a reduction in molecular hydrophobicity, leading to a deficiency of hydrophobic interaction sites on the α_{s1}-CN molecule between residues 14 and 24, leading to softening of cheese texture during phase 1 ripening (Creamer *et al*., 1982).

More recently, the effect of CCP solubilisation on cheese texture and rheology during ripening has been investigated (Lucey and Fox, 1993; Pastorino *et al*., 2003; Lucey *et al*., 2003, 2005; Hassan *et al*., 2004; Lee *et al*., 2005; O'Mahony *et al*., 2006; Wang *et al*., 2011). CCP is a fundamental structural

component of the casein micelle (Horne, 1998; Lucey *et al*., 2003), and any reduction on the strength or number of CCP cross-links within the *para*-CN system of the cheese would be expected to influence the structural, textural and rheological properties of the cheese. O'Mahony *et al*. (2005) assessed the effects of both CCP solubilisation and chymosin-mediated proteolysis on the textural properties of a dry-salted Cheddar cheese during ripening, independently of one another. These authors hypothesised that hydrolysis by chymosin of α_{s1}-CN at Phe_{23}-Phe_{24} and Cheddar cheese softening during ripening are concurrent, rather than interdependent, processes, and that some physicochemical changes (particularly reduction in the level of Ca associated with the CN matrix), may be responsible for this initial decrease in hardness. These authors demonstrated that the softening of cheese texture is more highly correlated with the slow solubilisation of residual CCP associated with the *para*-CN matrix of the cheese rather than the level of intact α_{s1}-CN.

To date, the exact mechanisms responsible for the development of texture of brine-salted cheese varieties throughout ripening have not been elucidated. In this study, we investigated the effects of proteolysis and INSOL Ca concentration on the textural and rheological properties of Gouda-type cheese throughout 112 d of ripening. Gouda-type cheeses (2 kg) were manufactured with 0.0 (control), 0.1, 1.0 and 10.0 μmol of pepstatin, added per litre of curds/whey mixture at the beginning of cooking to inhibit α_{s1}-CN hydrolysis at Phe_{23}-Phe_{24}. Proteolysis, differences in type of Ca (concentration of SOL and INSOL Ca) and the textural and rheological properties of cheese were determined to evaluate if textural and

rheological changes still occur when chymosin-mediated hydrolysis of α_{s1}-CN is completely inhibited, as is the case in dry-salted cheeses.

Abbreviations

CCP; INSOL calcium phosphate, CN; casein, G*; complex modulus, G'; storage modulus, G"; loss modulus, INSOL; insoluble, LT; loss tangent, SOL; soluble, TSC; tri-sodium citrate

6.2 Materials and Methods

6.2.1 Pepstatin preparation

Pepstatin (Pepstatin A, synthetic; Merck Millipore Ireland, Carrigtwohill, Co. Cork, Ireland) solutions were prepared by solubilising the powder in 150 mL of methanol and diluting to 300 mL with de-ionised water purified with a PURELAB Option-Q (ELGA LabWater, Celbridge, Co. Kildare, Ireland) prior to addition to the curds and whey mixture.

6.2.2 Cheese manufacture

Four 2 kg scale Gouda cheeses were manufactured in the food processing facility at University College Cork, Ireland. Raw milk was heated to 50 ˚C, separated and standardised to a casien:fat ratio of 0.90:1.00. Standardised milk was batch pasteurised, separated into 3 vats, each containing 20 L of milk and cooled to 31 ˚C. A concentrated direct vat set starter culture (B11 containing *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, *Leuconostoc mesenteroides* subsp. *cremoris* and a citrate-positive *Lactococcus* ssp.; Chr. Hansen Ltd., Little Island, Co. Cork, Ireland) was added at a level of 0.03% (w/v) and sodium nitrate (Sigma-Aldrich, Dublin, Ireland) at a level of 0.006% (w/v) and allowed to ripen for 30 min. Following this, 45 mL 1 M $CaCl_2$ (Sigma-Aldrich, Dublin, Ireland) and chymosin (CHY-MAX®M, Chr. Hansen Ltd., Little Island, Co. Cork, Ireland) at a rate of 17.5 mL per 20 L of milk were added to each vat. Cheesemilk was allowed to coagulate for 50 min. Once the coagulum had formed, the curds were cut, allowed to heal for 5 min, before stirring gently

for 20 min. After stirring, the curds were washed, a third of the whey was removed (4 L) followed by the addition of 4 L warm water (54 ˚C). Pepstatin was added at 3 levels (0.1, 1.0, or 10.0 μmol/L) to the curds/whey mixture at the start of cooking and evenly distributed by continuous stirring during cooking. Curds were then cooked at 38 ˚C for 90 min with continuous stirring. Following stirring, the whey was drained and collected, curds were placed in 2 kg moulds lined with cheese cloth, the moulds were placed back in the vat with a 10 kg weight placed on top, the whey was reintroduced back into the vat and the curds were allowed to press under the whey for 30 min. After pressing under the whey, the moulds were removed from the vat and placed on the press (1.03 bar) overnight. The cheese was removed from the press and placed in a brine solution (10 kg H_2O, 20% (w/v) NaCl and 0.05% (w/v) $CaCl_2$) for 24 h. After brining, the cheeses were vacuum packed and ripened at 10˚C for 112 d. All Gouda-type cheeses were manufactured in triplicate.

6.2.3 Chemical analysis

The composition and pH of all cheeses made with 0.0 (control), 0.1, 1.0 and 10.0 μmol/L pepstatin was determined at 14 d after manufacture. The moisture contents of the cheeses were determined using an oven-drying method (IDF, 1982). The pH was measured using a calibrated pH meter, on cheese slurry made from 10 g cheese and 10 mL of deionised water. Protein content of the cheeses was determined by the macro-Kjeldahl method (N x 6.38; IDF, 1986), fat by the Gerber method (IIRS, 1955) and salt was measured by a titrimetric method using potentiometric end-point determination (Fox, 1963). The proportion of total calcium in the insoluble form (i.e., CCP) in Gouda-type cheese made with 0.0,

0.1, 1.0 or 10.0 μmol/L pepstatin was determined at 1, 14, 28 and 112 d of ripening using the acid-base titration method described by Hassan *et al*. (2004) and the total calcium content of cheese was determined using atomic absorption spectroscopy as described by O'Mahony *et al*. (2005). All analysis was performed in triplicate.

6.2.4 Proteolysis

The pH 4.6-soluble and insoluble fractions of the cheeses at 1, 14, 56 and 112 d of ripening were prepared as described Kuchroo and Fox (1982). The nitrogen content of the pH 4.6-soluble fraction was determined by the macro-Kjeldahl method (N x 6.38; IDF, 1986). Urea–polyacrylamide electrophoresis was carried on the pH 4.6-insoluble fraction of cheeses using the procedure described by O'Mahony *et al*. (2005). Individual FAAs were determined using an ion-exchange chromatography method described by Fenelon *et al*. (2000). Peptide profiles of pH 4.6-soluble fractions were obtained by reverse-phase HPLC using an UPLC which consisted of a Waters Acquity UPLC H-Class Core System with an Acquity UPLC TUV Detector (dual wavelength) and Acquity Column Heater 30-A. The system was interfaced with Empower 3 software (Waters Corp., Milford, MA, USA). The core system includes an Acquity UPLC H-Class quaternary solvent manager, an H-Class Sample Manager-FTN and a CH-A column heater. The column used was an Acquity UPLC® Peptide BEH C18, 130 Å, 1.7 m, 2.1 x 100 mm column. Elution was monitored at 214 nm and a mobile phase of two solvents, A, 0.1 % (v/v) trifluoroacetic acid (TFA, sequential grade, Sigma-Aldrich, St Louis, MO, USA) in deionized HPLC grade water (Milli-Q system, Millipore, Ireland) and B, 0.1 % (v/v) TFA in acetonitrile (HPLC grade, Sigma-

Aldrich, Germany) was used. The pH 4.6-soluble fraction samples were filtered through 0.22 μm cellulose acetate filter (Sartorius GmbH, Gottingen, Germany) and an aliquot (3.3 μL) of the filtrate was injected on the column at an eluent flow rate of 0.32 mL min^{-1}. The elution gradient used is shown in Table 3.2 (Chapter 3). Data acquisition was from 0 to 20.01 min. Time between injections was 2 min at 100 % solvent A. A blank was used at the start of 100% A.

6.2.5 Determination of residual chymosin

The levels of residual chymosin activity in each of the four cheeses were determined using a synthetic heptapeptide as substrate (Pro-Thr-Glu-Phe-[NO_2-Phe]-Arg-Leu; Bachem GmbH, Weil am Rhein, Germany) as described by O'Mahony *et al*. (2005). The area of the peak corresponding to the product peptide was used to express the residual chymosin activity of the pepstatin-treated cheeses as a percentage of that in the control cheese (0.0 μmol/L pepstatin). The stability of the chymosin-pepstatin complex in experimental cheeses during ripening was evaluated by determining the residual chymosin activity in the control and pepstatin-treated cheeses at 1 and 112 d of ripening. All analyses were conducted in triplicate.

6.2.6 Determination of textural and rheological properties of cheese during ripening

6.2.6.1 Texture profile analysis

Texture profile analysis (TPA) was performed using a Texture Analyser TA-XT2i (Stable Micro Systems, Godalming, Surrey, UK) according to the method of Cooke and McSweeney (2013). Hardness was defined according to Bourne (1978) and measured at 1, 14, 28, 56 and 112 d of ripening. Five replicate samples from each cheese were compressed at each ripening time point.

6.2.6.2 Small deformation rheological properties

Rheological analysis of cheese samples at 14, 28, 56 and 112 d of ripening was performed using a AR-G2 Controlled Stress Rheometer (TA Instruments, Leatherhead, UK), as described by Cooke and McSweeney (2013), with the following modifications: cheese discs were not glued to the base plate of the rheometer; storage modulus (G′), loss modulus (G″) and loss tangent (LT) were recorded continuously at a low amplitude shear strain of 0.1% at a frequency of 1.0 Hz at an axial force of 0.8 Pa over 20 min during which the temperature was increased from 20 to 80 ˚ C.

6.2.6.3 Cheese meltability analysis

Cheese meltability was performed using the Schreiber melt test procedure (Altan *et al*., 2005) at 14, 28, 56, 112 d of ripening. Cheese samples were prepared and

stored according to the procedure described by Cooke and McSweeney (2013). Results were expressed as a % increase in diameter of the cheese discs. Analysis on each cheese sample was performed in triplicate at each time point.

6.2.7 Statistical analysis

Analysis of variance (ANOVA) was performed on composition, pH, insoluble calcium and total FAA at a significant level of $P \leq 0.05$. A split-plot design was used to evaluate the effect of treatment (0.0 (control), 0.1, 1.0 and 10.0 μmol/L added pepstatin), ripening time and their interactions on levels of pH 4.6-soluble nitrogen (SN), INSOL Ca levels (expressed as a % of total Ca), residual chymosin activity, hardness, chewiness, gumminess, springiness, cohesiveness, meltability, G', G" and LT_{max} values. The ANOVA for the split-plot designs was performed using a general linear model (GLM) procedure. When significant differences ($P \leq 0.05$) were found, the treatment means were analysed by Tukey's multiple comparison test. All analyses were performed using MiniTab® 16 (MiniTAB Inc., State College, PA, USA). Pearson's correlation coefficients were determined between the response variables (i.e., intact α_{S1}-CN, insoluble calcium, loss tangent at 60 ˚C and hardness) using MiniTab® 17 (MiniTAB Inc., State College, PA, USA).

6.3 Results and Discussion

6.3.1 Cheese composition and pH

The composition and pH of Gouda-type cheeses made with 0.0 (control), 0.1, 1.0 and 10.0 μmol/L pepstatin at 14 d ripening are shown in Table 6.1. There was no significant ($P \leq 0.05$) difference in moisture, protein, fat, FDM, MNFS or total Ca between the control and treatment cheeses, with values ranging from 43.3-43.5, 24.1-24.4, 18.7-19.7, 33.23-34.7, 53.6-53.9% and 837-877 mg/100 g cheese, respectively. The pH and composition of all cheeses were within the normal range expected for Gouda cheese (van den Berg *et al*., 2004). These results are in accordance with Shakeel-Ur-Rehman *et al*. (1998) and Milesi *et al*. (2008) who found no gross compositional difference between miniature Cheddar cheeses containing 7.5, 15.0 or 30.0 μmol/L pepstatin and 15.0 μmol/L pepstatin, respectively. In contrast, O'Mahony *et al*. (2005) reported decreasing moisture levels in Cheddar cheese manufactured with increasing levels of pepstatin (0.1, 1.0 and 10.0 μmol/L pepstatin) and attributed these changes to greater syneresis of the curd in the presence of pepstatin. The salt content and the S/M values of the 0.1 μmol/L pepstatin treated cheese was significantly ($P \leq 0.05$) lower than the control and other treatment cheeses, perhaps as a result of poor distribution and diffusion of salt into the cheese during brining and/or insufficient sampling of cheese from both the rind and inner part of the cheese. In brine-salted cheese varieties such as Gouda, salt is unevenly distributed initially, being high at the outer rind and lower in the inner core (Lee *et al*., 1980; Kindstedt, 2001).

Table 6.1 Composition and pH of Gouda-type cheeses made with 0.0 (control), 0.1, 1.0 and 10.0 μmol/L added pepstatin at 14 d ripening.

Parameter	**μmol pepstatin/L curds and whey**			
	0.0	0.1	1.0	10.0
Moisture (%)	43.46[a] (1.54)	43.49[a] (01.24)	43.39[a] (1.44)	43.25[a] (0.79)
Protein (%)	24.43[a] (1.57)	24.10[a] (1.10)	24.35[a] (1.95)	24.11[a] (1.39)
Fat (%)	19.17[a] (2.51)	18.72[a] (3.07)	18.94[a] (2.56)	19.67[a] (2.26)
FDM[1] (%)	34.01[a] (5.30)	33.23[a] (6.12)	33.58[a] (5.45)	34.70[a] (4.34)
MNFS[2] (%)	53.86[a] (3.47)	53.60[a] (3.49)	53.63[a] (3.50)	53.90[a] (2.33)
NaCl (%)	1.68[a] (0.02)	1.46[b] (0.05)	1.69[a] (0.45)	1.68[a] (0.14)
S/M[3] (%)	3.87[a] (0.14)	3.36[b] (0.17)	3.90[a] (0.12)	3.90[a] (0.38)
pH	5.28[a] (0.09)	5.26[a] (0.04)	5.31[a] (0.15)	5.28[a] (0.08)
Total Ca (mg/100g)	855[a] (109.8)	837[a] (85.8)	840[a] (98.2)	877[a] (93.1)

[a,b,c,d] Means within a row with different superscripts are significantly different (Tuckey's HSD; $P \leq 0.05$).
[1]Fat in dry matter of the cheese
[2]Moisture in nonfat solids of the cheese
[3]Salt in moisture phase of cheese

No significant differences ($P \leq 0.05$) were observed in the rates of acidification during manufacture (results not shown) or the final pH of all cheese. The pH values of cheeses ranged from 5.26 to 5.31 at 14 d of ripening (Table 6.1).

6.3.2 Residual chymosin activity and stability of chymosin-pepstatin complex

Levels of residual chymosin activity decreased significantly ($P \leq 0.01$) as the level of pepstatin addition to Gouda-type cheese increased (Table 6.2). No significant difference ($P \leq 0.05$) was observed between trials (Table 6.3), indicating good dispersion and/or diffusion of pepstatin into the curd particles before whey drainage on different days of manufacture. At day 1, Gouda-type cheeses made with 0.1, 1.0 and 10.0 μmol/L pepstatin had levels of residual chymosin activity

that were 73, 43 and 7% of the activity of the control cheese, respectively (Table 6.2). Similar results were found by O'Mahony *et al*. (2005), who manufactured Cheddar cheeses with pepstatin at the same levels used in this study (0.0, 0.1, 1.0 and 10.0 μmol/L) added at the beginning of cooking and obtained residual chymosin levels of 100, 89, 55 and 16% of the activity of the control cheese, respectively. Milesi *et al*. (2008) manufactured miniature Cheddar cheeses with 15 μmol/L pepstatin added to the curd-whey mixture at the start of cooking and found that residual chymosin activity was reduced by 95% compared to the control. These authors attributed this effect to the ability of pepstatin to form a 1:1 complex with chymosin by binding to the active site of the enzyme.

The residual chymosin activity of the control and pepstatin-treated cheeses was evaluated at 1 and 112 d of ripening and results (Table 6.2) indicated that there was no significant difference ($P \leq 0.05$) in activity levels throughout ripening; suggesting that the chymosin-pepstatin complex formed was extremely stable during ripening. This result supports the findings of O'Mahony *et al*. (2005), who found no changes in the levels of residual chymosin of the control and pepstatin-treated cheeses throughout 180 d ripening of Cheddar cheese.

Table 6.2 Residual chymosin activity (expressed as a % of that in the control cheese) for Gouda-type cheeses made with 0.0 (control), 0.1, 1.0 and 10.0 μmol/L added pepstatin at 14 d ripening.

Level of pepstatin	**Day**	**Product peptide peak area**		**Residual chymosin activity**
(μmol/L)		Mean	SD	(% of control)
0.0 (Control)	**1**	133979[a]	49115	100.0[a] (0.00)
0.1		110931[a]	32285	72.73[b] (14.5)
1.0		59896[b]	6242	42.55[c] (13.4)
10.0		11065[c]	2178	7.47[d] (2.10)
0.0 (Control)	**112**	157307[a]	33844	100.0[a] (0.00)
0.1		130987[a]	12318	70.18[b] (4.99)
1.0		79971[b]	2234	42.95[c] (12.1)
10.0		14445[c]	1889	7.76[d] (1.07)

[a,b,c,d] Means within columns with different superscripts are significantly different (Tuckey's HSD; $P \leq 0.05$).
[1]SD = Standard deviation (n = 3).

Table 6.3 Probabilities and R^2 values for residual chymosin activity (expressed as % of control cheese), pH4.6 SN%TN, hardness, cohesiveness, springiness, gumminess and chewiness values for Gouda-type cheeses made with 0.0 (control), 0.1, 1.0 and 10.0 μmol/L added pepstatin at 14 d ripening.[1]

Factor	df[2]	Residual chymosin (% of control)	df[2]	INSOL Ca (%)	df[2]	pH4.6 SN%TN	Meltability	Hardness	Cohesiveness	Springiness	Gumminess	Chewiness
Whole plot												
Day of cheese manufacture (D)	2	40.55 (0.33)	2	99.33* (0.01)	2	14.19** (0.01)	1219.76** (0.00)	103302992* (0.02)	0.05* (0.02)	0.05** (0.01)	48667669* (0.01)	29702104* (0.01)
Treatment (T)	3	9380.71** (0.00)	3	36.53 (0.09)	3	88.33** (0.00)	116.31** (0.01)	97259054* (0.01)	0.00 (0.90)	0.01 (0.09)	10160984 (0.21)	5818171 (0.22)
Error (D x T)	6	29.82 (0.77)	6	10.53 (0.51)	6	0.97 (0.38)	11.37 (0.70)	11732002** (0.00)	0.01* (0.01)	0.00* (0.02)	4986548** (0.00)	2940603** (0.00)
Subplot												
Age (A)	1	1.31 (0.88)	4	468.45** (0.00)	3	144.46** (0.00)	46.91 (0.07)	39042157** (0.00)	0.012233** (0.00)	0.04** (0.00)	15239611** (0.00)	11177287** (0.00)
T x A	3	2.90 (0.98)	12	12.22 (0.44)	9	8.24** (0.00)	20.91 (0.35)	4506008 (0.10)	0.00 (0.81)	0.00 (0.67)	809335 (0.56)	363557 (0.70)
Error	8	56.01	32	11.82	24	0.86	17.74	2390141	0.00	0.00	928912	516797
R^2		0.98		0.86		0.98	0.88	0.93	0.81	0.91	0.90	0.91

[1]Split-plot design with four treatments (0.0 (control), 0.1, 1.0 and 10.0 μmol/L added pepstatin) were analysed as a discontinuous variable and cheese manufacture day was blocked. Subplot included the effect of age and treatment and treatment × age as variables. [2]Degrees of freedom *$0.01 < P \leq 0.05$; ** $P \leq 0.01$

6.3.3 Proteolysis

4.3.3.1 pH 4.6-soluble nitrogen as a % of total nitrogen (pH 4.6-SN%TN)

The levels of pH 4.6-SN%TN of Gouda-type cheeses made with 0.0 (control), 0.1, 1.0 and 10.0 μmol/L pepstatin throughout 112 d ripening are shown in Figure 6.1 and Table 6.3. In all cheeses manufactured with added pepstatin, the level of pH 4.6-SN was significantly ($P \leq 0.05$) lower than that of the control cheese at all stages of ripening. Similar results were reported by Shakeel-Ur-Rehman *et al*. (1998), O'Mahony *et al*. (2005) and Milesi *et al*. (2008), who all found significant reductions in the level of pH 4.6-SN in Cheddar cheese with increasing pepstatin addition and attributed this result to the extensive inactivation of chymosin. The level of pH 4.6-SN for all cheeses increased with increasing ripening time, with the extent of increase greatest for the control cheese. Throughout ripening the level of pH 4.6-SN of the control cheese was two-fold greater than cheese manufactured with 10.0 μmol/L added pepstatin. In agreement with the results obtained by O'Mahony *et al*. (2005), the greatest divergence in terms of development of pH 4.6-SN occurred during the first month of ripening and was critical in governing the final (112 d) level of pH 4.6-SN in the cheeses. The levels of pH 4.6-SN increased in each of the four cheeses throughout 112 d ripening, suggesting that the chymosin-pepstatin complex formed during manufacture was extremely stable irrespective of the level of pepstatin added to the cheese (O'Mahony *et al*., 2005).

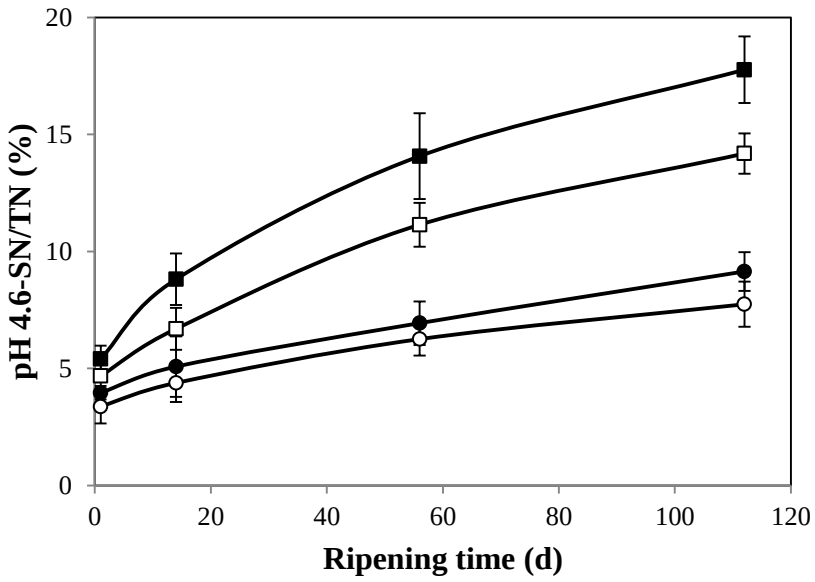

Figure 6.1 Levels of pH 4.6-soluble nitrogen (expressed as a % of total nitrogen) for Gouda-type cheeses made with 0.0 (control) (■), 0.1 (□), 1.0 (●) and 10.0 (○) μmol/L added pepstatin at 1, 14, 56 and 112 d of ripening.

6.3.3.2 Urea-polyacrylamide gel electrophoresis (urea-PAGE)

The levels of intact α_{s1}-CN and β-CN in Gouda-type cheeses supplemented with 0.0 (control), 0.1, 1.0 and 10.0 μmol/L pepstatin throughout 112 d ripening are shown in Figure 6.2. Altering the level of residual chymosin activity by the addition of pepstatin produced large quantitative differences in the hydrolysis of α_{s1}-CN (Figure 6.2a), while the breakdown of β-CN (Figure 6.2b) remained relatively unchanged. Degradation of α_{s1}-CN led to the accumulation of the breakdown product α_{s1}-CN (f 24-199) and α_{s1}-CN (f102–199).

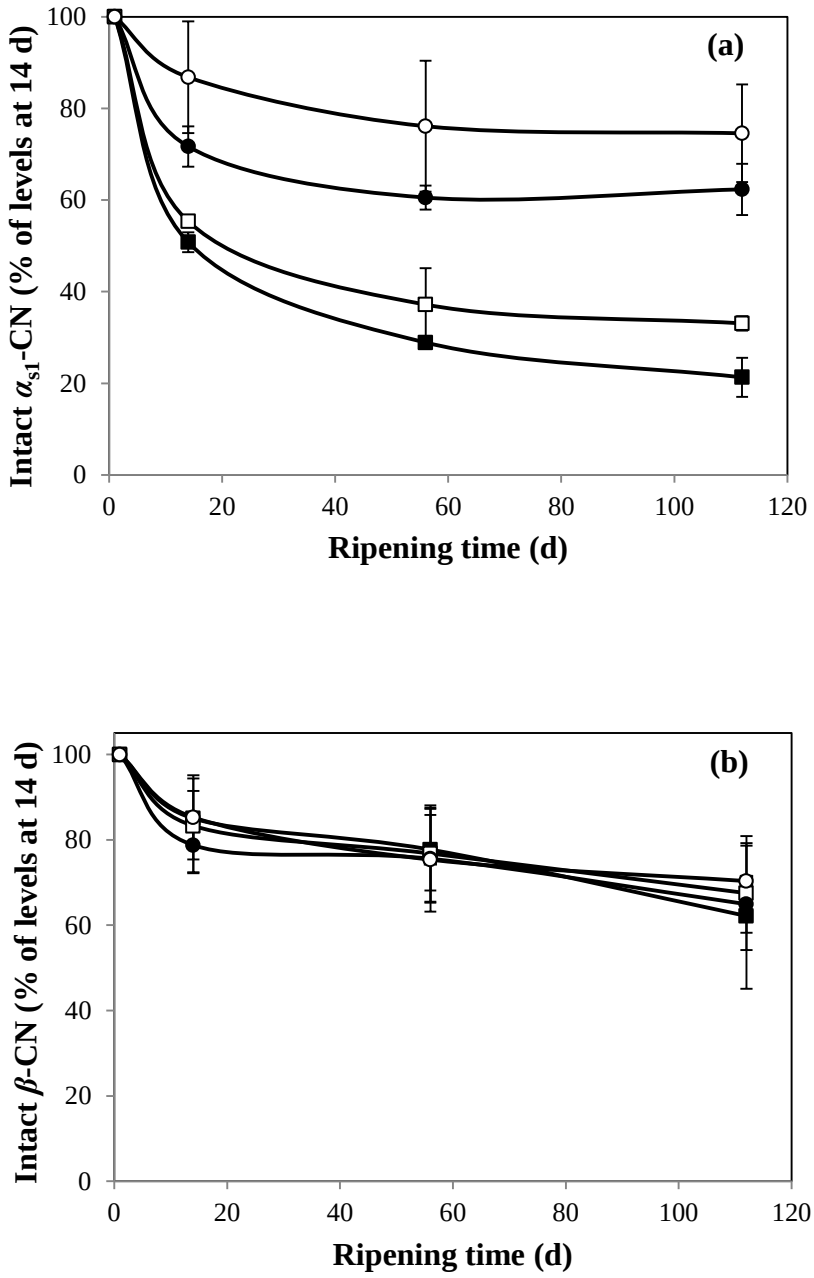

Figure 6.2 Levels of intact α_{s1}-CN (**a**) and β-CN (**b**) in Gouda-type cheeses made with 0.0 (control) (■), 0.1 (□), 1.0 (●) and 10.0 (○) μmol/L added pepstatin at 14, 56 and 112 d of ripening.

The extent of α_{s1}-CN hydrolysis was considerably reduced with increased level of pepstatin addition to cheese, with a large amount of peptides absent from

the 1.0 and 10.0 μmol/L pepstatin treated cheeses at all stages of ripening. At 112 d of ripening, the level of intact α_{s1}-CN for the 0.0 (control) and 10.0 μmol/L pepstatin-treated cheeses were 21 and 75%, respectively. Such degradation in cheese has been primarily associated with residual proteolytic activity of the coagulant (Fox *et al*., 2000; Sousa *et al*., 2001). Results confirm that pepstatin at the level used effectively inhibited residual chymosin activity and, therefore, primary proteolysis. This result supports previous studies (O'Mahony *et al*., 2005; Milesi *et al*., 2008) where α_{s1}-CN remained relatively intact throughout ripening, upon addition of pepstatin (10 and 15 μmol/L curds and whey) to Cheddar cheese. Hydrolysis of β-CN resulted in the production of γ-caseins (β-CN f106-209, f29-201, f108-209) at relatively the same extent for all cheeses; 62 to 70% intact β-CN for cheeses made with 0.0 and 10.0 μmol/L pepstatin at 112 d ripening. This degradation in cheese has been primarily associated with plasmin (Fox *et al*. 2000), the activity of which is unaffected by pepstatin.

6.3.3.3 UPLC peptide profiles

The peptide profiles of the pH 4.6-soluble fraction of the control and pepstatin-treated cheeses at 14 and 112 d of ripening are illustrated in Figure 6.3a and b. An increase in peak height was observed for all cheeses throughout ripening. However, the extent of the increase was greatest for the control (0.0 μmol/L pepstatin) cheese compared with the pepstatin-treated cheeses. It was noticeable that peak height was reduced with increasing levels of pepstatin addition. The lack of α_{s1}-CN degradation in pepstatin-treated cheeses may have been responsible for differences between the peptide profiles of these cheeses.

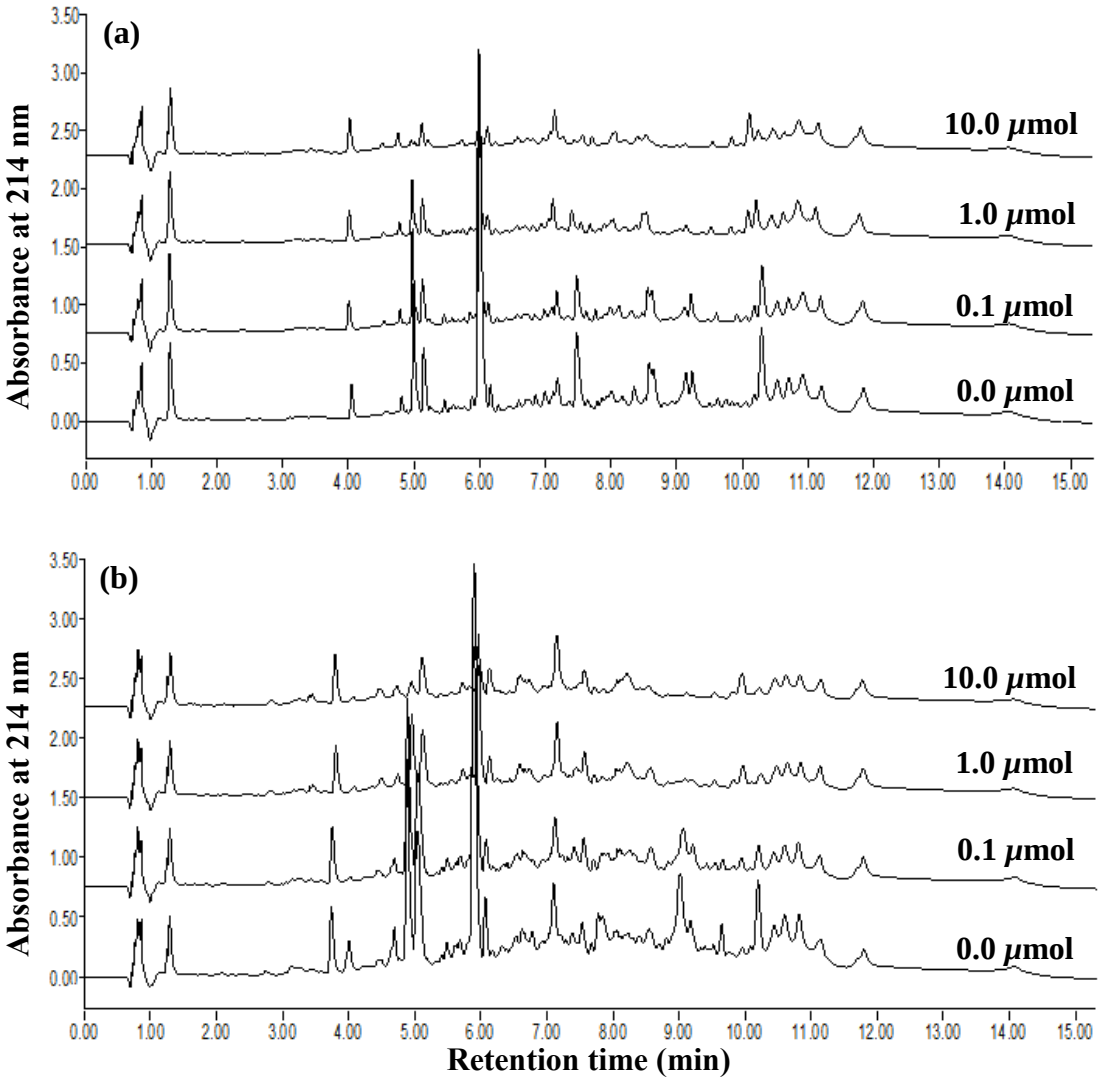

Figure 6.3 UPLC chromatograms of pH 4.6-soluble extracts of Gouda-type cheeses manufactured with 0.0 (control), 0.1, 1.0 and 10.0 μmol/L added pepstatin at 14 (**a**) and 112 d (**b**) of ripening. Chromatogram is of Trial 2 and is representative of Trials 1 and 3 also.

Both quantitative and qualitative differences in the peptide profiles were observed in Gouda-type cheeses made with 0.0 (control), 0.1, 1.0 and 10.0 μmol/L added pepstatin at both 14 and 112 d of ripening. It appears that the major differences between cheeses at 14 d were evident in the retention time interval between, 5 and

10.2 min, whereas by 112 d of ripening the greatest difference were evident at the retention time interval between 4 and 10.2 min. The peaks that varied most between the control and pepstatin-treated cheeses were those which eluted at approximately 4.0, 4.8, 5.0, 5.9, 6.1, 9.0, 9.9 and 10.2 min. The concentration of both hydrophilic and hydrophobic peptides produced increased with decreasing levels of pepstatin addition to the cheese (i.e. control cheese contained the greatest concentration of peptides. The greater concentrations of hydrophobic peptides with higher retention times may cause a slightly bitter taste. Greater levels of hydrophobic fragments of caseins have been associated with an increased concentration of bitter peptides in Hispanico cheese (Gomez *et al*., 1997).

6.3.3.4 Free amino acids

The total concentration (mg g^{-1} cheese) of free amino acids (FAAs) for cheeses manufactured with 0.0 (control), 0.1, 1.0 and 10.0 μmol/L added pepstatin at 112 d of ripening was 47.6, 45.8, 44.6 and 44.5 mg g^{-1} cheese, respectively (Figure 6.4). Pepstatin-treated cheeses exhibited significantly ($P < 0.05$) lower levels of total FAA than the control cheese. These results are in accordance with those reported by Shakeel-Ur-Rehman *et al*. (1998), O'Mahony *et al*. (2005) and Milesi *et al*. (2008) who found that the FAA content decreased with increasing levels of pepstatin in Cheddar cheese. This result was attributed to the lower level of intermediate sized peptides available to starter proteinases and peptidases to produce small peptides and free amino acids, which is also true in this study according to urea-PAGE results previously described.

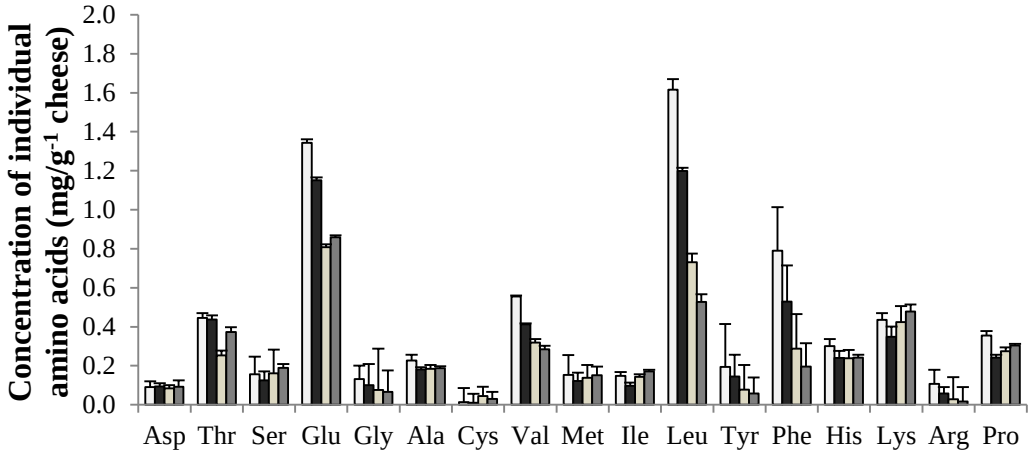

Figure 6.4 Concentration of individual free amino acids in Gouda-type cheeses made with 0.0 (control) (□), 0.1 (■), 1.0 (□) and 10.0 (■) μmol/L added pepstatin at 112 d of ripening.

The concentration of Glu, Gly, Val, Leu, Tyr, Phe and Arg decreased with increasing concentration of pepstatin added to the cheese. These results are in accordance with Milesi *et al*. (2008) who reported significantly lower concentration of Ser, Gly, Arg, Val, Leu and Phe in pepstatin-treated cheeses compared with cheese not containing pepstatin. Among individual amino acids, eight amino acids were more concentrated in all cheese samples. The principal FAAs present in all cheeses in the highest concentrations were Leu, Glu, Phe, Val, Lys, Thr, His and Tyr, all of which have been previously identified in Gouda cheese (Fox and Wallace, 1997). Similar results were obtained by Milesi *et al*. (2008) who found that Glu, His, Thr, Pro, Lys and Leu were most prominant in control and pepstatin-treated cheeses. Also in agreement with these results, O'Mahony *et al*. (2003, 2005) reported Glu, Leu, Val, Phe and Lys to be the most abundant FAA in both miniature and 2 kg Cheddar cheese at 2 months and 180 d.

FAAs unaffected by pepstatin addition to Gouda-type cheese included Asp, Ser, Ala, Cys, Met, Ile, His, Lys and Pro.

6.3.4 Insoluble calcium content of cheeses

The insoluble (INSOL) Ca content (expressed as % of the total Ca) of the cheeses (0.0 (control), 0.1, 1.0 and 10.0 μmol/L added pepstatin) throughout 112 d of ripening are shown in Figure 6.5. No significant differences in levels of INSOL Ca were observed between the control and pepstatin-treated cheeses at each ripening point. However, there was a significant ($P \leq 0.01$) reduction in the INSOL Ca level of each of the four cheeses during the first month of ripening, and this remained relatively constant throughout the remainder of the 112 d ripening period. The INSOL Ca content of the 0.0 (control) and 10.0 μmol/L pepstatin-treated cheeses decreased from 89% and 87% at 1 d, to 71% and 69% at 112 d, respectively. These results are in agreement with previous studies (Hassan *et al*., 2004; Lee *et al*., 2005; Lucey *et al*., 2005; O'Mahony *et al*., 2005; Cooke and McSweeney, 2013) which found that the level of INSOL Ca decreased rapidly during the early stages (first 21 d) of Cheddar cheese ripening and levelled out thereafter. These authors attributed the observed partial solubilisation of INSOL Ca (CCP; colloidal calcium phosphate) evident during early ripening to the attainment of pseudoequlibrium between the INSOL and soluble (SOL) forms of Ca present in cheese. Calcium equilibrium in brine-salted cheese varieties has not been fully elucidated to date. It is interesting to observe a similar decreasing trend in the levels of INSOL Ca in the brine-salted cheeses investigated in this study, similar to that of dry-salted varieties.

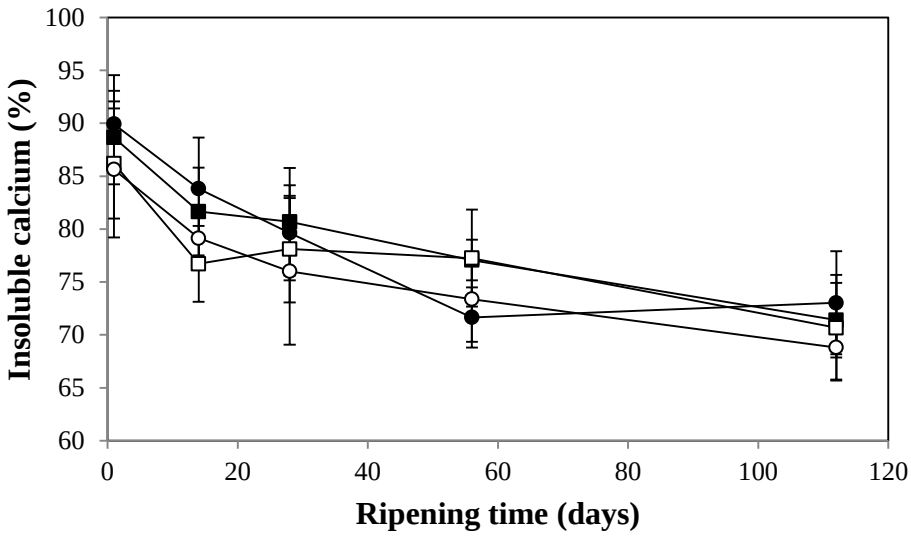

Figure 6.5 Insoluble calcium (expressed as a % of total calcium) content of Gouda-type cheeses made with 0.0 (control) (■), 0.1 (□), 1.0 (●) and 10.0 (○) μmol/L added pepstatin.at 1, 14, 28, 56 and 112 d of ripening.

6.3.5 Meltability (Schreiber test)

The meltability results of Gouda-type cheeses made with 0.0 (control), 0.1, 1.0 and 10.0 μmol/L added pepstatin at 14, 28, 56 and 112 d ripening are shown in Figure 6.6. The % increase in melt diameter of the control (0.0) and 0.1 μmol/L pepstatin-treated cheeses increased throughout ripening, although not significantly (Table 6.3). Whereas, cheeses treated with 1.0 and 10.0 μmol/L added pepstatin did not significantly change throughout 112 d of ripening. Candioti *et al*. (2007) showed that despite differences in proteolysis during the first month of ripening of Cremoso cheese, the melting ability did not differ ($P > 0.05$) between control and coagulant-inactivated cheeses. These authors suggested that meltability of Cremoso cheeses was dependant on factors other than proteolysis, such as

changes in pH or CCP content, which occur concurrently during cheese ripening. Kim *et al*. (2004) demonstrated that Cheddar cheese meltability was more highly correlated with the extent of β-CN hydrolysis than with the hydrolysis of α_{s1}-CN, for cheeses manufactured with different levels of chymosin and protease from *Cryphonectria parasitica*.

In the current study, all treatment cheeses had similar INSOL Ca and β-CN levels and varying α_{s1}-CN levels throughout ripening, with the extent of α_{s1}-CN hydrolysis greatest for the control cheese. At 112d of ripening, the control (0.0 μmol/L pepstatin) had a significantly higher melt diameter than the pepstatin-treated cheeses, signifying that the increase in meltability was most likely attributed to the extent of α_{s1}-CN hydrolysis rather than with the hydrolysis of β-CN or with CCP solubilisation. Similarly, Madsen and Qvist (1998) demonstrated that acceleration of proteolysis in UF Mozzarella by enzyme addition generally improved the meltability due to increased casein degradation. Results of this study suggest that α_{s1}-CN degradation became a determining factor in influencing the meltability of Gouda-type cheese but only in later ripening. Likewise, Hayaloglu *et al*. (2014) reported that the meltability of Halloumi cheese manufactured with varying levels of coagulant (1× and 4× the normal level) was almost identical at 1 d of ripening but increased with age and increasing levels of coagulant.

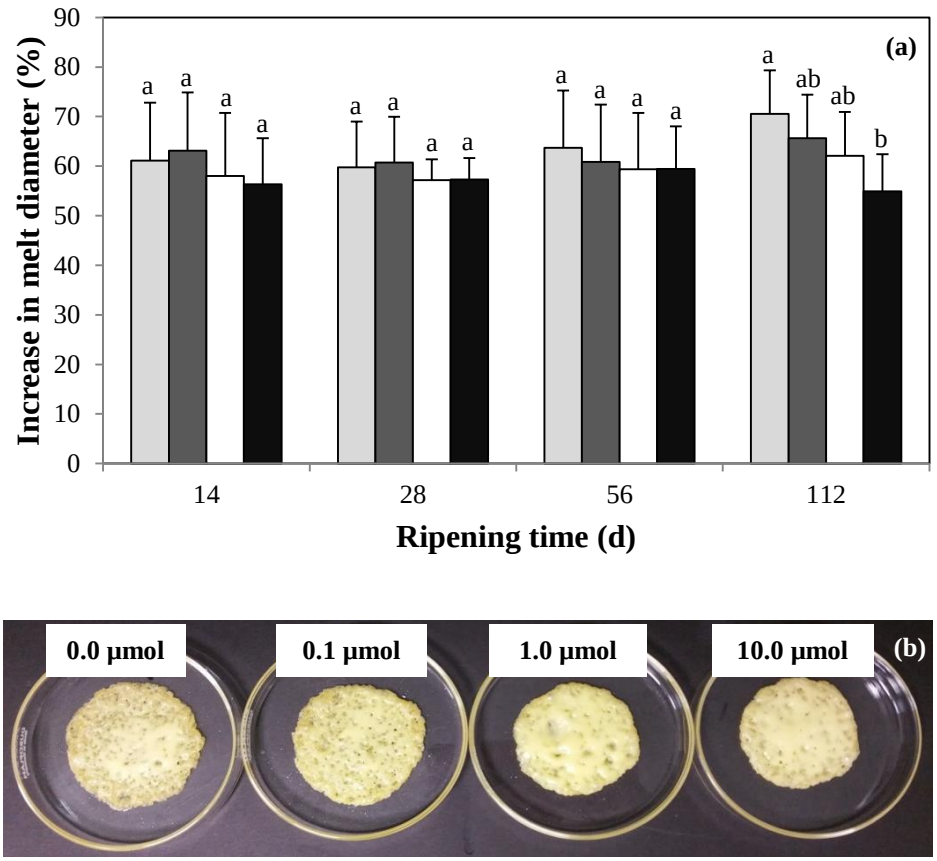

Figure 6.6 (**a**) Cheese meltability as a function of ripening time, expressed as % diameter increasing on cheese discs (35 mm diameter; 5 mm height), heated at 232˚C for 5 min for Gouda-type cheeses made with 0.0 (control) (■), 0.1 (□), 1.0 (●) and 10.0 (○) μmol/L added pepstatin.at 14, 28, 56 and 112 d of ripening. (**b**) Images of cheeses at 112 d of ripening after melting occurred.

6.3.6 Texture profile analysis

Hardness, cohesiveness, springiness, gumminess and chewiness values, as measured by the texture profile analyser (TPA), decreased in all cheeses (0.0, 0.1, 1.0 and 10.0 μmol/L added pepstatin) throughout 112 d of ripening (Figure 6.7 and Table 6.4). The extent of the reduction of each parameter was greatest for the control (0.0 μmol/L pepstatin) cheese at all stages of ripening.

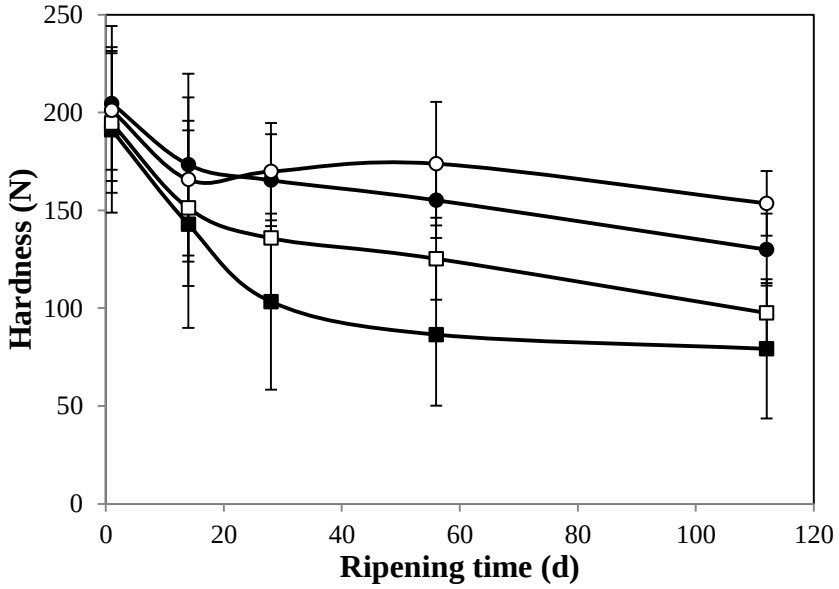

Figure 6.7 Hardness values measured using texture profile analyser (TPA) for Gouda-type cheeses made with 0.0 (control) (■), 0.1 (□), 1.0 (●) and 10.0 (○) μmol/L added pepstatin at 14, 28, 56 and 112 d of ripening for Trials 1 (a), 2 (b) and 3 (c).

Table 6.4 Texture profile analysis parameters cohesiveness, springiness, gumminess and chewiness for Gouda-type cheeses made with 0.0 (control), 0.1, 1.0, or 10.0 μmol/L added pepstatin at 1, 14, 28, 56 and 112 d of ripening.[1]

Parameter	Ripening time (d)	Level of pepstatin addition (μmol/L)			
		0.0	0.1	1.0	10.0
Cohesiveness	14	0.43^{a} (0.13)	0.39^{a} (0.07)	0.39^{a} (0.11)	0.43^{a} (0.11)
	28	0.43^{a} (0.07)	0.39^{a} (0.07)	0.37^{a} (0.08)	0.36^{a} (0.09)
	56	0.39^{a} (0.09)	0.39^{a} (0.07)	0.37^{a} (0.09)	0.39^{a} (0.09)
	112	0.32^{a} (0.07)	0.35^{a} (0.08)	0.34^{a} (0.09)	0.34^{a} (0.08)
Springiness	14	0.66^{b} (0.07)	0.65^{ab} (0.05)	0.69^{ab} (0.04)	0.71^{a} (0.03)
	28	0.61^{a} (0.08)	0.62^{a} (0.06)	0.66^{a} (0.05)	0.66^{a} (0.06)
	56	0.57^{a} (0.08)	0.60^{a} (0.06)	0.62^{a} (0.08)	0.64^{a} (0.06)
	112	0.48^{b} (0.09)	0.53^{ab} (0.10)	0.60^{a} (0.09)	0.59^{a} (0.08)
Gumminess (N)	14	65.9^{a} (33.2)	60.9^{a} (24.1)	70.6^{a} (33.8)	71.4^{a} (23.9)
	28	46.1^{a} (23.1)	53.9^{a} (17.7)	63.0^{a} (22.0)	63.4^{a} (24.2)
	56	35.7^{b} (18.3)	50.3^{ab} (15.6)	58.6^{a} (21.5)	69.5^{a} (27.8)
	112	27.5^{c} (17.0)	34.5^{bc} (11.1)	44.6^{ab} (14.8)	52.9^{a} (16.8)
Chewiness (N)	14	45.7^{a} (24.9)	40.5^{a} (18.2)	49.5^{a} (26.0)	51.1^{a} (17.7)
	28	29.6^{a} (16.5)	34.5^{a} (13.3)	42.2^{a} (16.9)	42.9^{a} (19.1)
	56	21.5^{b} (13.2)	30.9^{ab} (11.8)	37.5^{b} (17.9)	45.5^{b} (21.4)
	112	14.6^{c} (11.1)	19.1^{bc} (8.62)	27.5^{ab} (12.8)	32.1^{a} (14.3)

[a,b,c,d] Means within a row with different superscripts are significantly different (Tuckey's HSD; $P \leq 0.05$).

[1]Values represent means (±SD; n = 5)

It was evident that as the level of pepstatin increased, the extent of the reduction in each of these values decreased. The greatest decrease in hardness for all cheeses occurred within the first 28 d of ripening and reduced thereafter. However, despite complete inhibition of α_{s1}-CN hydrolysis (10.0 μmol/L added pepstatin-treated cheese), there was still a significant decrease in Gouda-type cheese hardness, and indeed, cohesiveness, springiness, gumminess and chewiness values during the early stages (first 28 d) of cheese ripening. These results are in agreement with O'Mahony *et al*. (2005) who reported a significant ($P \leq 0.05$) reduction in the hardness, cohesiveness, chewiness and springiness values of the control and pepstatin-treated Cheddar cheeses, within the first 21 d or ripening. These authors found that the hardness of Cheddar cheeses was more highly correlated with the levels of INSOL Ca than with the level of intact α_{s1}-CN during the first month of ripening. It was demonstrated by Hayaloglu *et al*. (2014) that increasing coagulant addition from 1× to 4× the normal level, significantly reduced hardness of Halloumi cheese. Authors also observed significant ($P \leq 0.05$) age-related changes (decrease in hardness values) for all cheese and attributed these to the changes in proteolysis (first 30 d) and the solubilisation of Ca (after 30 d). The opposite however is true in the study, as Pearson correlation (Table 6.5) indicates that INSOL Ca levels are more correlated to Gouda-type cheese softening during early ripening, while the levels of intact α_{s1}-CN are highly correlated ($P \leq 0.05$) to decreased hardness values in later ripening (after 28 d).

Table 6.5 Pearson's correlation coefficients between hardness and intact α_{s1}-CN, hardness and insoluble calcium levels, loss tangent at 60 ˚C and intact α_{s1}-CN, and loss tangent at 60 ˚C and insoluble calcium levels of Gouda-type cheeses at 1, 28, 56 and 112 d of ripening. Data presented are combined from all three trials.

Ripening time (d)	Hardness		Loss tangent (60 ˚C)	
	Intact a_{s1}-CN	Insoluble calcium	Intact α_{s1}-CN	Insoluble calcium
14	-0.053 (0.95)	0.757 (0.04)*		
28	0.089 (0.11)	0.447 (0.55)	-0.832 (0.17)	0.035 (0.97)
56	0.974 (0.03)*	-0.524 (0.48)		
112	0.098 (0.02)*	0.201 (0.80)	-0.984 (0.02)*	0.362 (0.64)

*6.3.7 Rheological analysis of chees*es

The dynamic moduli (G' and G") values at 30 and 60 ˚C for Gouda-type cheeses made with 0.0 (control), 0.1, 1.0 and 10.0 μmol/L added pepstatin at 14 and 112 d ripening are shown in Table 6.6. The dynamic moduli of all cheeses decreased significantly ($P \leq 0.01$) with increasing ripening time. No significant difference between the control and treatment cheeses and were observed at each time point for G' and G" at 30 ˚C (Table 6.7). However, when the temperature was increased to 60 ˚C, significant ($P \leq 0.05$) differences between cheeses were observed at 112 d of ripening (Figure 6.8a, b). The control (0.0 μmol/L pepstatin) cheese exhibited significantly lower values compared with 1.0 and 10.0 μmol/L pepstatin-treated cheeses, most likely due to increased α_{s1}-CN degradation. Similarly, Dave *et al.* (2003) reported an increase in softening during storage of Mozzarella cheese, and attributed this decrease in complex modulus (G*) to the level of coagulant addition to the cheese (0.25×, 1× and 4× the normal level used for cheesemaking). For cheeses made with 0.25× the normal level of coagulant, authors found very

little decrease in G* after 60 d, while at 1× and 4× the normal coagulant levels the softening of the cheese was more pronounced, due to higher levels α_{s1}-CN hydrolysis. Authors reported that 50% intact α_{s1}-CN was hydrolysed at < 1, 2 and 13 d ripening, for cheeses treated with 0.25×, 1× and 4× the normal coagulant level, respectively.

The loss tangent (LT) values for all cheeses at 112 d of ripening are shown in Figures 6.8c. There was little change evident in the LT values below 40 ˚C; however, at temperatures > 40 ˚C, the LT values for all cheeses increased greatly. No significant differences in LT were observed between cheeses at 14 d; however, at 112 d of ripening the control (0.0 μmol/L pepstatin) cheese had a significantly ($P \leq 0.001$) greater LT value at temperatures between 40 and 70 ˚C, when compared with pepstatin-treated cheeses (Table 6.6 and 6.7). Pearson's correlation (Table 6.5) show that the LT was significantly ($P \leq 0.05$) more highly correlated with the level of intact α_{s1}-CN than with the levels of INSOL Ca at the latter stages of ripening, supporting Schreiber meltability results described above.

Table 6.6 Small deformation rheological property values for Gouda-type cheeses made with 0.0 (control), 0.1, 1.0, or 10.0 μmol/L added pepstatin at 14 and 112 d of ripening as determined by controlled stress rheometer.

Pepstatin addition level (μmol/L)	Day	G'_{30} [1]	G'_{60} [2]	G''_{30} [3]	G''_{60} [4]	LT_{30} [5]	LT_{60} [6]	LT_{max} [7]	T @ LT = 1 [8]
0.0	**14**	21958^{a} (5525)	1511^{a} (802)	7203^{a} (1950)	1894^{a} (902)	0.32675^{a} (0.02)	1.3086^{a} (0.20)	1.97^{a} (0.23)	57.77^{a} (1.02)
0.1		25462^{a} (4367)	2075^{a} (718)	8388^{a} (1575)	2281^{a} (670)	0.32872^{a} (0.01)	1.1208^{a} (0.11)	1.91^{a} (0.12)	58.89^{a} (1.22)
1.0		28368^{a} (5004)	3155^{a} (2018)	9367^{a} (1390)	2966^{a} (1354)	0.33170^{a} (0.01)	1.0599^{a} (0.24)	1.83^{a} (0.09)	60.09^{a} (2.42)
10.0		26690^{a} (5486)	2773^{a} (1294)	8829^{a} (1863)	2823^{a} (1053)	0.33085^{a} (0.01)	1.0717^{a} (0.15)	1.76^{a} (0.55)	59.43^{a} (1.55)
0.0	**112**	4355^{a} (1485)	115^{b} (58)	1527^{a} (516)	182^{b} (88)	0.35132^{a} (0.01)	1.6091^{a} (0.10)	1.97^{a} (0.20)	54.44^{c} (1.21)
0.1		5503^{a} (1159)	177^{b} (34)	1737^{a} (439)	231^{b} (43)	0.31262^{b} (0.03)	1.3119^{b} (0.13)	1.94^{a} (0.22)	57.95^{b} (1.38)
1.0		5779^{a} (840)	665^{a} (175)	1752^{a} (236)	558^{a} (63)	0.30421^{b} (0.02)	0.8742^{c} (0.16)	1.93^{a} (0.15)	61.84^{a} (2.06)
10.0		5802^{a} (888)	692^{a} (140)	1768^{a} (230)	610^{a} (80)	0.30588^{b} (0.01)	0.8928^{c} (0.08)	1.88^{a} (0.13)	61.66^{a} (1.23)

Values are the means of triplicate trials with standard deviations in parentheses; means within columns (sharing the same day; 14 or 112) not sharing a common letter differ statistically ($P \leq 0.05$)

[1]Storage modulus at 30 ˚C; [2]Storage modulus at 60 ˚C

[3]Loss modulus at 30 ˚C; [4]Loss modulus at 60 ˚C

[5]Loss tangent at 30 ˚C; [6]Loss tangent at 60 ˚C

[7]Maximum loss tangent

[8]Temperature at loss tangent equals to 1

Table 6.7 Probabilities and R^2 values for maximum loss tangent, temperature at loss tangent equals to 1, storage modulus at 30 ˚C and 60 ˚C and loss modulus at 30 ˚C and 60 ˚C, loss tangent at 30 ˚C and 60 ˚C during ripening for Gouda-type cheeses made with 0.0 (control), 0.1, 1.0 and 10.0 μmol/L added pepstatin.[1]

Factor	**df**[2]	**LT_{max}**[3]	**T @ LT = 1**[4]	**G'_{30}**[5]	**G'_{60}**[6]	**G''_{30}**[7]	**G''_{60}**[8]	**LT_{30}**[9]	**LT_{60}**[10]
Whole Plot									
Day of cheese manufacture (D)	2	0.11*	1.84	31598060	2864801**	3475428	2181884**	0.00	0.03
		(0.05)	(0.14)	(0.07)	(0.01)	(0.07)	(0.00)	(0.51)	(0.100)
Treatment (T)	3	0.03	29.97**	17047710	1579999*	1577499	751804*	0.00	0.32**
		(0.39)	(0.00)	(0.17)	(0.03)	(0.22)	(0.03)	(0.15)	(0.00)
D x T	6	0.021	0.67	7115053	266563	490219	117293	0.00	0.01
Subplot									
Age (A)	1	0.02	0.03	2462705821**	23194234**	273427561**	26355818**	0.00	0.01
		(0.15)	(0.93)	(0.00)	(0.00)	(0.00)	(0.00)	(0.14)	(0.68)
T x A	3	0.01	9.98	6483575	308749	1003889	130016	0.00	0.09
		(0.58)	(0.15)	(0.76)	(0.84)	(0.66)	(0.89)	(0.08)	(0.11)
Error	8	0.01	4.24	16274728	1094986	1793129	616155	0.00	0.03
R^2		0.87	0.79	0.95	0.81	0.95	0.87	0.77	0.84

[1]Split-plot design with four treatments (0.0 (control), 0.1, 1.0 and 10.0 μmol/L added pepstatin) were analysed as a discontinuous variable and cheese manufacture day was blocked. Subplot included the effect of age and treatment and treatment × age as variables
[2]Degrees of freedom; [3]Maximum loss tangent, [4]Temperature at loss tangent equals to 1
[5]Storage modulus at 30 ˚C, [6]Storage modulus at 60 ˚C
[7]Loss modulus at 30 ˚C, [8]Loss modulus at 60 ˚C
[9]Loss tangent at 30 ˚C, [10]Loss tangent at 60 ˚C, *$0.01 < P \leq 0.05$; ** $P \leq 0.01$

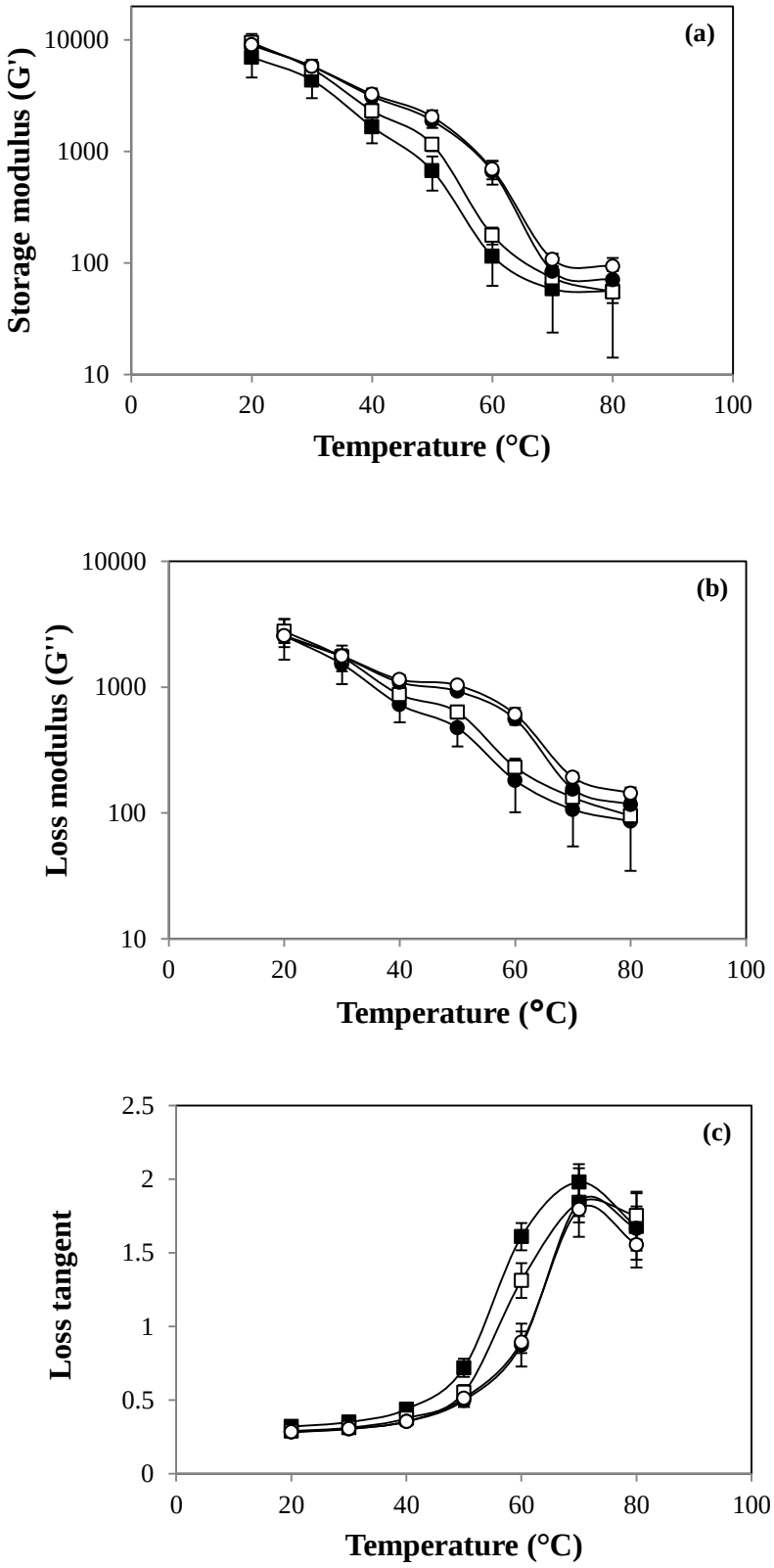

Figure 6.8 (**a**) Storage modulus (G'), (**b**) loss modulus (G'') and (**c**) loss tangent values for Gouda-type cheeses made with 0.0 (control) (■), 0.1 (□), 1.0 (●) and 10.0 (○) μmol/L added pepstatin at 112 d of ripening.

The maximum loss tangent (LT_{max}) values of Gouda-type cheeses made with 0.0 (control), 0.1, 1.0 and 10.0 μmol/L added pepstatin at 14 and 112 d ripening are shown in Table 6.6. No significant ($P \leq 0.05$) differences in LT_{max} values were observed between cheeses or ripening times (Table 6.7). However, the LT_{max} values for the control (0.0 μmol/L pepstatin) cheese were considerably higher than pepstatin-treated cheeses. A greater LT_{max} value indicates a greater propensity for cheese to flow and melt when heated (O'Mahony *et al*., 2006). The trend of these results relates well with values obtained from the Schreiber melt test, with the control cheese (0.0 μmol/L pepstatin) and 10.0 μmol/L pepstatin-treated cheese having the highest and lowest LT_{max} values, respectively, throughout ripening, indicating the greatest and least flow and melt properties, respectively.

Generally, an increase in LT_{max} is observed as ripening progress due to loss of attractive crosslinking interactions, caused by the reduction of INSOL Ca content in the cheese and the loss of intact casein due to proteolysis, leading to increased meltability (Lucey *et al*., 2005; O'Mahony *et al*., 2006; Choi *et al*., 2008). Interestingly, in this study no significant ($P \leq 0.05$) difference in the LT_{max} values between the cheeses were observed at 14 and 112 d of ripening. This perhaps suggests that that solubilisation of INSOL Ca is principally responsible for the increase in the LT_{max} and that perhaps the reduction in INSOL Ca levels was not sufficient to influence LT_{max} values.

During early ripening (14 d), no significant differences in the temperature when LT = 1 (cross over temperature; CO) were observed between control (0.0 μmol/L) and treatment cheeses (0.1, 1.0 and 10.0 μmol/L added pepstatin).

However, a significant ($P \leq 0.0001$) difference in the CO temperature was observed between cheeses at 112 d of ripening. During heating, the point at which G' and G" cross-over can be used to identify the stage at which melting transitions occur (Gunasekaran and Ak, 2000), and therefore is widely considered a good index of the temperature at which cheese initially begins to melt. A considerable decrease in the CO temperature of the 0.0 (control) and 0.1 μmol/L added pepstatin cheese was observed at 112 d of ripening, 54 and 58 ˚C, compared with 58 and 59 ˚C, recorded at 14 d, respectively. Interestingly, no significant ($P \leq 0.05$) difference in the CO temperature of the 1.0 and 10.0 μmol/L added pepstatin cheeses was observed during ripening. It is therefore likely that the loss of intact casein due to increased proteolysis in the 0.0 (control) and 0.1 μmol/L added pepstatin cheese was responsible for the reduction in CO temperature rather than the effects of solubilisation of some INSOL Ca, which was the same in all treatment cheeses.

6.4 Conclusions

Pepstatin addition to the curds/whey mixture at the start of cooking effectively reduced the level of residual chymosin activity in the resultant Gouda-type cheese, leading to the inhibition of chymosin-mediated hydrolysis of α_{s1}-CN. Increasing pepstatin addition levels in Gouda-type cheese hindered both primary and secondary proteolysis during ripening. The shift from INSOL to SOL (solubilisation of CCP) form of Ca in the cheeses was unaffected by the level of pepstatin addition at all stages of maturation. It was determined that α_{s1}-CN hydrolysis at Phe_{23}-Phe_{24} was not a prerequisite for the early softening of Gouda-type cheese texture. The reduction in cheese hardness during early ripening was more strongly correlated with the concentration of insoluble calcium than with the level of intact α_{s1}-CN. Changes to the rheological properties of cheeses upon heating during ripening were more highly correlated with the level of intact α_{s1}-CN than the concentration of INSOL Ca. It is concluded that the changes to Gouda-type cheese texture (i.e., softening) that occur during early ripening (first 28 d) can occur without hydrolysis of α_{s1}-CN at Phe_{23}-Phe_{24}. Therefore, we suggest that this softening of texture in brine-salted cheeses is due largely to solubilisation of some of the residual CCP associated with the *para*-CN matrix of the cheese, as also occurs in dry-salted varieties.

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References

Altan, A., Turhan, M. & Gunasekaran, S. (2005). Short communication: Comparison of covered and uncovered Schreiber test for cheese meltability evaluation. *Journal of Dairy Science*, *88*, 857–861.

Ardö, Y., McSweeney, P. L. H. Magboul, A. A. A., Upadhyay, V. K. & Fox, P. F. (2017). Biochemistry of cheese ripening: proteolysis. In: McSweeney, P. L. H., Cotter, P., Fox, P. F. & Everett, D. W. (Eds.). *Cheese: Chemistry, Physics and Microbiology*, Vol 1, *General Aspects*, 4th ed. Elsevier, San Diego (in press).

Beresford, T. & Williams, A. (2004). The microbiology of cheese ripening. In: Fox, P. F., Guinee, T. P., Cogan, T. M. & McSweeney, P. L. H., Eds. *Cheese: Chemistry, Physics and Microbiology*, Vol 1, *General Aspects*, 3rd ed. Elsevier Applied Science, Amsterdam, pp 287-317.

Bourne M. C. (1978). Texture profile analysis. *Food Technology*, *32*, 62–66, 72.

Candioti, M. C., Alonso, M. J. & Hynes, E. (2007). Influence of residual milk-clotting enzyme and proteolysis on melting properties of soft cheese. *International Journal of Dairy Technology*, *60*, 175-181.

Choi, J., Horne, D. S., Johnson, M. E. & Lucey, J. A. (2008). Effects of the concentration of insoluble calcium phosphate associated with casein micelles on the functionality of directly acidified cheese. *Journal of Dairy Science*, *91*, 513-522.

Cooke, D. R. & McSweeney, P. L. H. (2013). The influence of alkaline earth metal equilibria on the rheological, melting and textural properties of Cheddar cheese. *Journal of Dairy Research*, *80*, 418-428.

Creamer, L. K., Zoerb, H. F., Olson, N. F. & Richardson, T. (1982). Surface hydrophobicity of α_{s1}-I, α_{s1}-casein A and B and its implications in cheese structure. *Journal of Dairy Science*, *65*, 902-906.

Dave, R. I., McMahon, D. J., Oberg, C. J. & Broadbent, J. R. (2003). Influence of coagulant level on proteolysis and functionality of Mozzarella cheeses made using direct acidification. *Journal of Dairy Science*, *86*, 114-126.

Fox P. F. (1963). Potentiometric determination of salt in cheese. *Journal of Dairy Science*, *46*, 744–745.

Fox, P. F. (1989). Proteolysis during cheese manufacture and ripening. *Journal of Dairy Science*, *72*, 1379-1400.

Fox, P. F. & Wallace, J. M. (1997). Formation of flavour compounds in cheese. *Advances in Applied Microbiology*, *45*, 17-86.

Fox, P. F., Law, J., McSweeney, P. L. H. & Wallace, J. (1993). Biochemistry of Cheese Ripening. In: Fox P. F., Ed. *Cheese: Chemistry, Physics and Biochemistry*. Chapman and Hall, London, UK, 389–438.

Fox, P. F., Cogan, T. M., Guinee, T. P. & McSweeney, P. L. H. (2000). *Fundamentals of Cheese Science*. Aspen Publishers Inc., Gaithersburg, MD.

Fox, P. F., Guinee, T. P., Cogan, T. M. & McSweeney, P. L. H. (2017). *Fundamentals of Cheese Science*, 2nd Ed. (pp. 333-390) Springer: New York, US.

Gomez, M. J., Garde, S., Gaya, P., Medina, M. & Nuñez, M. (1997). Relationship between level of hydrophobic peptides and bitterness in cheese made from pasteurized and raw milk. *Journal of Dairy Research*, **64**, 289-297.

Guinee, T. P. (2003). Role of protein in cheese and cheese products In: Fox, P. F. and McSweeney, P. L. H., Eds. *Advanced Dairy Chemistry*, Vol 1, 3rd ed. Kluwer Academic/Plenum Publishers, New York, 1083-1174.

Gunasekaran, S. & Ak, M. M. (2000). Dynamic oscillatory shear testing of foods – selected applications. *Trends in Food Science and Technology*, 11, 115–127.

Hassan, A., Johnson, M. E. & Lucey, J. A. (2004). Changes in the proportions of soluble and insoluble calcium during the ripening of Cheddar cheese. *Journal of Dairy Science*, *87*, 854-862.

Hayaloglu, A. A., Karatekin, B. & Gurkan, H. (2014). Thermal stability of chymosin or microbial coagulant in the manufacture of Malatya, a Halloumi type cheese: Proteolysis, microstructure and functional properties. *International Dairy Journal*, *38*, 136-144.

Horne, D. S. (1998). Casein interactions: Casting light on the black boxes, the structure in dairy products. *International Dairy Journal*, *8*, 171–177.

IDF (1982). Cheese and processed cheese; determination of the total solids content. Standard 4A:1982. Brussels, Belgium: International Dairy Federation.

IDF (1986). Determination of the nitrogen content (Kjeldahl Method) and calculation of crude protein content. Standard 20A:1986. Brussels, Belgium: International Dairy Federation.

IIRS (1955). Determination of the percentage of fat in cheese. Irish Standard 69:1955. Institute for Industrial Research and Standards, Dublin.

Kim, S. Y., Gunasekaran, S. & Olson, N. F. (2004). Combined use of chymosin and protease from *Cryphonectria parasitica* for control of meltability and firmness of Cheddar cheese. *Journal of Dairy Science*, *87*, 274-283.

Kindstedt, P. S. (2001). Moisture variations in brine-salted *pasta filata* cheese. *Journal of AOAC International*, *84*, 605-612.

Kuchroo, C. N. & Fox, P. F. (1982). Soluble nitrogen in Cheddar cheese: comparison of extraction procedures. *Milchwissenschaft*, *37*, 331-335.

Lawrence, R. C., Creamer, L. K. & Gilles, J. (1987). Texture development during cheese ripening. *Journal of Dairy Science*, *70*, 1748-1760.

Lee, H. J., Olson, N. F. & Lund, D. B. (1980). Diffusion of salt, fatty acids, and esterases in Mozzarella cheese. *Journal of Dairy Science*, *63*, 513-522.

Lee, M. R., Johnson, M. E. & Lucey, J. A. (2005). Impact of modifications in acid development on the insoluble calcium content and rheological properties of Cheddar cheese. *Journal of Dairy Science*, *88*, 3798-3809.

Lucey, J. A. & Fox, P. F. (1993). Importance of calcium and phosphate in cheese manufacture: a review. *Journal of Dairy Science*, *76*, 1714-1724.

Lucey, J. A., Johnson, M. E. & Horne, D. S. (2003). Perspectives on the basis of the rheology and texture properties of cheese. *Journal of Dairy Science, 86,* 2725–2743.

Lucey, J. A., Mishra, R., Hassan, A. & Johnson, M. E. (2005). Rheological and calcium equilibrium changes during the ripening of Cheddar cheese. *International Dairy Journal, 15,* 645-653.

Madsen, J. S. & Qvist, K. B. (1998). The effect of added proteolytic enzymes on meltability of Mozzarella cheese manufactured by ultrafiltration. *Lait,78,* 259-272.

McSweeney, P. L. H. (2004). Biochemistry of cheese ripening. *International Journal of Dairy Technology, 57,* 127-144.

McSweeney, P. L. H., Hayaloglu, A. A., O'Mahony, J. A. & Bansal, N. (2006). Perspectives on cheese ripening. *Australian Journal of Dairy Technology, 61,* 69-77.

Milesi, M. M., McSweeney, P. L. H. & Hynes, E. R. (2008). Impact of chymosin and plasmin-mediated primary proteolysis on the growth and biochemical activities of lactobacilli in miniature Cheddar-type cheeses. *Journal of Dairy Science, 91,* 3277-3290.

Mounier, J., Rea, M. C., O'Connor, P. M., Fitzgerald, G. F. & Cogan, T. M. (2007). Growth characteristics of *Brevibacterium*, *Corynebacterium*, *Microbacterium* and *Staphylococcus* spp. isolated from surface-ripened cheese. *Applied and Environmental Microbiology, 73,* 7732-7739.

O'Mahony, J. A., Sousa, M. J., & McSweeney, P. L. H. (2003). Proteolysis in miniature Cheddar-type cheeses made using blends of chymosin and *Cynara cardunculus* proteinases as coagulant. *International Journal of Dairy Technology*, *56*, 52–58.

O'Mahony, J. A., Lucey, J. A. & McSweeney, P. L. H. (2005). Chymosin-mediated proteolysis, calcium solubilization and texture development during the ripening of Cheddar cheese. *Journal of Dairy Science*, *88*, 3101–3114.

O'Mahony, J. A., McSweeney, P. L. H. & Lucey, J. A. (2006). A model system for studying the effects of colloidal calcium phosphate concentration on the rheological properties of Cheddar cheese. *Journal of Dairy Science*, *89*, 892-904.

Pastorino, A. J., Hansen, C. L. & McMahon, D. J. (2003). Effect of pH on the chemical composition and structure-function relationships of Cheddar cheese. *Journal of Dairy Science*, *86*, 2751-2760.

Shakeel-Ur-Rehman, Feeney, E. P., McSweeney, P. L. H. & Fox. P. F. (1998). Inhibition of residual coagulant in cheese using pepstatin. *International Dairy Journal*, *8*, 987–992.

Sheehan, J. J., Fenelon, M. A., Wilkinson, M. G. & McSweeney, P. L. H. (2007). Effect of cook temperature on starter and non-starter lactic acid bacteria viability, cheese composition and ripening indices of a semi-hard cheese manufactured using thermophilic cultures. *International Dairy Journal*, *17*, 704-716.

Sousa, M. J. Ardö, Y. & McSweeney, P. L. H. (2001). Advances in the study of proteolysis during cheese ripening. *International Dairy Journal*, *11*, 327-345.

Upadhyay, V. K., McSweeney, P. L. H., Magboul, A. A. A. & Fox, P. F. (2004). Proteolysis in cheese during ripening. In: Fox, P. F., McSweeney, P. L. H., Cogan, T. M. & Guinee, T.P., Eds. *Cheese: Chemistry, Physics and Microbiology*, Vol 1, 3rd ed. Elsevier Academic Press, London, 319-433.

van den Berg, G., Meijer, W. C., Düsterhöft, E-M. & Smit, G. (2004). Gouda and Related Cheeses, In: Fox, P. F., McSweeney, P. L. H., Cogan, T. M. & Guinee, T. P., Eds. *Cheese: Chemistry, Physics and Microbiology*, Vol. 2, *Major Cheese Groups*, 3rd ed., Elsevier Publishers, London, 103-140.

Wang, F., Zhang, X., Luo, J., Guo, H., Zeng, S. S. & Ren, F. (2011). Effect of proteolysis and calcium equilibrium on functional properties of natural Cheddar cheese during ripening and the resultant processed cheese. *Journal of Food Science*, *76*, E248-E253.

Wang, J., Zheng, Z., Zhao, X., Yang, Y. & Yang, Z. (2015). Effect of starter cultures on the ripening properties of Yak milk cheese. *Food Science and Technology Research*, *21*, 419-430.

Chapter 7

Characterisation of a Swiss-Cheddar-hybrid cheese during ripening

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Abstract

A Swiss-Cheddar-hybrid cheese was manufactured in triplicate using *Streptococcus thermophilus* and *Lactobacillus helveticus* as starter and its ripening was characterised. Cheese pH increased significantly ($P \leq 0.05$) throughout 224 d of ripening at 9 ˚C. The distribution of calcium between the soluble and INSOL phases in cheese was evaluated. The concentration of INSOL Ca decreased as ripening progressed up to 112 d, as expected, and then appeared to increase slightly for the remainder of ripening (224 d), indicating the possible reformation of INSOL Ca. Chymosin and plasmin were both found to contribute to primary proteolysis of Swiss-Cheddar-hybrid cheese. The FAA levels present in the cheese increased significantly and were similar to Swiss and Swiss-Cheddar varieties (Dubliner). The principal FAAs were Glu, Val, Leu, Lys, Phe and Pro. The FFA production was low in Swiss-Cheddar-hybrid cheese studied during ripening, due likely to the poor growth of propionic acid bacteria. The development of changes to the textural, rheological and functional properties of Swiss-Cheddar cheese was very limited. There was no significant decrease in the firmness of the Swiss-Cheddar-hybrid during ripening. Cheese meltability increased slightly throughout 224 d of ripening, with the greatest increase occurring between 56 and 224 d of ripening. This increase is likely owing to the effects of increased proteolysis rather than the changes in INSOL Ca levels. Overall, this study highlights the changes (pH, Ca equilibrium and proteolysis) occurring in Swiss-Cheddar-hybrid cheese during ripening and their influence on the textural and functional properties.

7.1 Introduction

The abolition of milk quotas in Ireland in 2015 has led to a dramatic increase in milk production, 215.1 to 294.8 million litres in 2015 and 2016, respectively, an increase of 37% (Forde, 2016). This increase in raw material (milk) has provided cheese manufacturers with the opportunity to expand and diversify their cheese product portfolio. There is an increased demand worldwide for diversity of sensory and textural attributes of cheese (Sheehan and Wilkinson, 2002; Wilkinson *et al*., 2000). Diversification of the range of cheese produced in existing manufacturing plants may proceed via two routes: (1) production of soft, semi-soft, mould, smear-ripened and other specialty cheeses, or (2) production of diverse, innovative and novel cheese types in existing Cheddar or Emmental-type cheese plants with a resulting broader product portfolio (Wilkinson *et al*., 1997; Sheehan, 2013). Sheehan *et al*. (2008) highlighted, however, that the first route requires major capital investment in specialised curd manufacture or ripening facilities and therefore, the second approach provides a much more attractive option to cheese producers.

The production of novel cheese types on existing equipment involves the manipulation of process and ripening variables, e.g., starter type, cook temperature, draining pH and ripening temperature, to generate a cheese with unique flavours, textures and functionalities. Cheeses manufactured in this way can ultimately develop characteristics from a diverse range of established cheese varieties such as Cheddar and Swiss. Novel or hybrid cheese types already produced in an existing plant include

‘‘Short method’’ Cheddar (Czulak *et al*., 1954; Hammond, 1979; Radford and Hull, 1982), Egmont (Gillies, 1974) and Dubliner (Wilkinson *et al*., 1997).

Several studies exist which focus on the production of novel cheese varieties through the manipulation of manufacturing process and ripening conditions (e.g., Sheehan *et al*., 2007; 2008). However, these authors only considered the volatiles composition and sensory attributes of the cheeses, with little emphasis given to the textural and functional properties.

Physical properties of cheese such as hardness, melt, stretch and sliceability are of great importance to both consumers and industry alike. Bourne (2004) stated that cheese texture is an important attribute for differentiating cheese varieties and for consumer acceptance. Additionally, the use of cheese as a food ingredient has increased over the past few decades due to its numerous functional properties (Lucey, 2008). Therefore, it is essential to determine the textural and functional properties of all new cheese hybrids and the factors (proteolysis, pH, calcium equilibrium) that influence these characteristics at all stages of ripening. The objective of this study was to characterise the textural and functional properties of a Swiss-Cheddar-hybrid cheese, and to elucidate the factors (pH, proteolysis, distribution of calcium with the cheese) responsible for the changes in these properties throughout 224 d of ripening.

7.2 Materials and methods

7.2.1 Starter strains

Thermophillic starter cultures *S. thermophilus* (TH3) and *L. helveticus* (LHB02) were a gift from Chr. Hansen Ltd. (Little Island, Co. Cork, Ireland) and were stored at -85 ˚C until cheese manufacture. *P. freudenreichii* DPC 6451 (propionic acid bacteria: PAB) from the MFRC culture collection, was grown for 3 d at 30 ˚C in sodium lactate broth as described by Piveteau *et al*. (1995).

7.2.2 Cheese manufacture

Three replicate cheesemaking trials were carried out over a 4 week period. Raw milk was standardised to a casein to fat ratio of 0.78:1.0. Milk was pasteurised at 72 ˚C for 15 s, pumped at 32 ˚C into a 50 L cylindrical, jacketed, stainless steel vat with automated variable-speed cutting and stirring equipment (Stainless Services, Blackstone Bridge, Cork) in the food processing facility at University College Cork, Ireland. Cheese milk was inoculated with 0.125% TH3 (12.5 g/100 L) and 0.065% LHB02 (6.5 g/100 L). After a 20 min ripening period (pH reached 6.55), chymosin (Chymax plus, Chr. Hansen Ltd., Little Island, Co. Cork, Ireland), diluted 1:6 with deionised water, was added at a level of 0.026%. A 50 min coagulation period was allowed before cutting. Curds were allowed to heal for ~10 min before they were stirred and cooked at a rate of 0.5 ˚C min^{-1} from 32 to 45 ˚C and at 1 ˚C min^{-1} from 45 to 53 ˚C. Curds and whey were stirred until drainage at pH 6.15. Curds were

cheddared at ~40 ˚C and milled at pH 5.45. The curds were then dry-salted at a rate of 1.2 % (w/w) using NaCl. The salted curds were transferred into triangular 5 kg moulds lined with cheesecloth, pressed over night at 2.65 bar, vacuum packed and ripened at 9 ˚C.

7.2.3 Enumeration of starter and non-starter cultures

Cheese samples were removed aseptically at 14, 56, 112 and 224 d of ripening. The samples were placed in a stomacher bag, diluted 1:10 with sterile trisodium citrate (TSC) (2%, w/v) and homogenised in a stomacher (Seward Stomacher 400; Seward Ltd., London, UK) for 5 min. Further dilutions were prepared as required. Samples were analysed in duplicate.

Viable *S. thermoplilus* and *L. helveticus* cells and non-starter lactic acid bacteria (NSLAB) were enumerated in duplicate as described by Sheehan *et al*. (2007). Propionic acid bacteria (PAB) were enumerated on sodium lactate agar after incubation at 30 ˚C for 7 d. Vancomycin (Sigma Aldrich, Steinheim, Germany) was added to the sodium lactate agar (1.5 mg/L) in order to prevent the growth of starter and NSLAB.

7.2.4 Compositional analysis

The moisture content of the cheeses was determined using an oven-drying method (IDF, 1982). The pH was measured using a calibrated pH meter, on cheese slurry

made from 10 g cheese and 10 ml of deionised water. Protein content of the cheeses was determined by the macro-Kjeldahl method (N x 6.38; IDF, 1986), fat by the Gerber method (IIRS, 1955) and salt was measured by a titrimetric method using potentiometric end-point determination (Fox, 1963). The total calcium (Ca) content was determined using atomic absorption spectroscopy as described by O'Mahony *et al*. (2005), and the insoluble (INSOL) Ca level in cheese was determined using the acid-base titration method described by Hassan *et al*. (2004) at 3, 7, 28, 112 and 224 d of ripening.

7.2.5 Proteolysis

The pH 4.6-soluble and -insoluble fractions of cheese at 7, 112 and 224 d of ripening were prepared in triplicate as described Kuchroo and Fox (1982). The nitrogen content of the pH 4.6-soluble fraction was determined by the macro-Kjeldahl method (N x 6.38; IDF, 1986). Urea–polyacrylamide electrophoresis (urea-PAGE) was carried out on pH 4.6-insoluble fraction of cheese using the procedure described by O'Mahony *et al*. (2005). Samples were run through the stacking gel at 280 V and the separating gel at 300 V. Gels were stained using Coomassie Brilliant Blue G250 and de-stained by several washes with distilled water. Individual free amino acid (FAA) contents were determined in the pH 4.6-soluble extracts of cheeses after 112 and 224 d of ripening as described by Mounier *et al*. (2007).

Peptide profiles of the soluble fractions of each of the cheeses were determined by UPLC using a Waters Acquity UPLC H-Class Core System with an Acquity UPLC

TUV Detector (dual wavelength) and Acquity Column Heater 30-A, according to the method described in Chapter 6 with the following modifications: a flow rate of 0.16 mL min^{-1} was used and the gradient used was 100% solvent A for 0-23.2 min, 50% solvent A and 50% solvent B from 23.2 to 25.6 min, 5% solvent A and 95% solvent B from 27.2 to 31.2 min, 100% solvent B from 31.2 to 34.0 min and 100% sovent A from 34.0 to 41.2 min.

7.2.6 Volatile profile analysis by GC-MS

Volatile compounds present in cheese samples were identified using gas chromatography-mass spectrometry (GC-MS) as described by Walsh *et al*. (2016), with the following modifications: 4 g of the sample was added to 20 ml screw capped solid-phase microextraction (SPME) vials and equilibrated to 40 ˚C for 10 min with pulsed agitation of 5 s at 500 rpm. The SPME fiber was exposed to the headspace above the samples for 20 min at a depth of 1 cm at 40 ˚C. Samples were analysed in triplicate.

7.2.7 Individual free fatty acid analysis

Identification and quantification of free fatty acid (FFA) was carried out using the method described by De Jong and Bading (1990), with a slight modification: fat was extracted in ethanol and the FFA separated by amino-propyl solid phase extraction. Analysis was carried out on 4 g of grated cheese, using a flame ionization detector

with on-column injection using a 30 m FFAP column (0.32 mm x 25 mm x 3 μm). Samples were analysed in triplicate.

7.2.8 Determination of textural and rheological properties

7.2.8.1 Texture profile analysis

Texture profile analysis (TPA) was performed using a Texture Analyser TA-XT2i (Stable Micro Systems, Godalming, Surrey, UK) according to the method of Cooke and McSweeney (2013). Hardness, defined according to Bourne (1978), was determined at 14, 42, 56, 112 and 224 d of ripening. Five replicate samples from each cheese were compressed at each ripening time point.

7.2.8.2 Small deformation rheological properties

Rheological analysis of cheese samples at 14, 42, 63, 112 and 224 d of ripening was performed using a AR-G2 Controlled Stress Rheometer (TA Instruments, Leatherhead, UK), as describe by Cooke and McSweeney (2013), with the following modifications: cheese discs were not glued to the base plate of the rheometer; storage modulus (G'), loss modulus (G") and loss tangent (LT) were recorded continuously at a low amplitude shear strain of 0.1% at a frequency of 1.0 Hz and an axial force of 0.9 over 20 min during which the temperature was increased from 20 to 80 ˚C at a rate of 3 ˚C/min. Each cheese sample was analysed in triplicate.

7.2.8.3 Cheese meltability analysis

Cheese meltability was performed using the Schreiber melt test procedure (Altan *et al*. 2005) at 14, 28, 56, 112 and 224 d of ripening. Cheese samples were prepared and stored according to the procedure described by Cooke and McSweeney (2013). Results were expressed as a % increase in diameter of the cheese discs. Analysis of each cheese sample was performed in triplicate at each time point.

7.2.9 Statistical analysis

Each set of experiments was repeated in triplicate with at least triplicate analyses in each repetition. All statistical analyses were performed using Minitab® 16 (Minitab Inc., State College, PA, USA). Differences between trials and ripening times were tested by analysis of variance (ANOVA). A significance level, α, of 0.05 was used for all statistical analyses (P value $\leq$ 0.05). Principal component analysis was also applied to data from volatile compounds.

7.3 Results and Discussion

7.3.1 Cheese composition

The gross composition and pH values of Swiss-Cheddar-hybrid cheeses at 14 d ripening are shown in Table 7.1. There were no significant differences in cheese composition between trials and therefore results presented are means and standard deviations from the three replicate trials. The moisture, protein, NaCl, fat, MNFS, S/M and FDM content of Cheddar-hybrid cheeses were 36.26, 25.67, 1.12, 31.66, 53.06, 3.08 and 49.66%, respectively, and correlate well with Swiss and Swiss-Cheddar-type cheeses manufactured using similar manufacturing conditions (Sheehan *et al*., 2007, 2008). Cheese pH increased throughout ripening from 5.41 to 5.88 at 1 and 224 d, respectively. The pH values recorded were within the pH range previously described for Swiss, Cheddar and Swiss-Cheddar-hybrid cheese varieties (Lawrence *et al*., 1984; Bachmann *et al*., 2002; Sheehan *et al*., 2007, 2008), with all showing an increasing trend upon ageing. It is common for the pH of Swiss-type cheese to increase during ripening, but the pH of Cheddar changes little during ripening; this is due to the strong buffering peak at ~ pH 5.2 and, consequently, its low initial pH is difficult to alter (Fox, 2002). Sheehan *et al*. (2008) found that increased cheese pH was significantly correlated with increased propionic acid bacteria (PAB) counts during ripening. The concentration of total calcium in the Swiss-Cheddar cheese was 971 mg/100 g cheese, which is higher than levels previously reported for Cheddar, Swiss and Swiss-Cheddar-hybrid cheeses, 720 (O'Brien and O'Connor, 2004), 825 (Sheehan *et al*., 2008) and 778 mg/100 g cheese (Sheehan *et al*., 2007), respectively.

Table 7.1 Composition of Swiss-Cheddar cheese at 14 d, pH values, pH 4.6-SN/TN and insoluble calcium levels during 224 d of ripening.

Day	Moisture	Protein	NaCl	Fat	MNFS	S/M	FDM	Total calcium	pH 4.6 SN/TN
	(%)	(%)	(%)	(%)	(%)	(%)	(%)	mg/100g cheese	
1									
14	36.26 (0.47)	25.67 (0.76)	1.12 (0.03)	31.66 (1.09)	53.06 (0.85)	3.08 (0.07)	49.66 (1.60)	971 (47.8)	8.46[c] (2.34)
28									
112									14.4[b] (0.62)
224									22.4[a] (1.91)

Values presented are means from three replicate trials with standard deviations in parentheses; means within columns not sharing a common letter differ statistically ($P \leq 0.05$)

The proportion of insoluble calcium (INSOL Ca) in the cheese decreased with increasing ripening time up until 112 d and thereafter began to increase slightly from 112 to 224 d of ripening, as shown in Figure 7.1. ANOVA confirmed that the observed increase in the concentration INSOL Ca from 112 to 224 d was not statistically significant ($P > 0.05$). It is well established that the calcium in cheese solubilises from the residual INSOL calcium phosphate (CCP) in the *para*-casein matrix during ripening to become part of the aqueous phase of cheese in order to attain a so-called 'pseudoequilibrium' of calcium phosphate between the soluble and insoluble phases of cheese (Hassan *et al*., 2004). Most studies have reported that the majority of the changes in calcium equilibrium of Cheddar cheese actually occur within the first month of ripening (Hassan *et al*., 2004; Lucey *et al*., 2005; O'Mahony *et al*., 2005). However, the results of this study raise the question that perhaps the reformation of Ca phosphate is occurring in Swiss-Cheddar-type cheese, maybe due to the increasing pH of the cheese during ageing. pH is an effective means of monitoring acidification which leads to the loss of insoluble calcium during cheese manufacture and influences the characteristics of the final cheese (Johnson and Lucey, 2006). Ge *et al*. (2002) investigated the effects associated with reversing the pH-induced changes on calcium distribution and melting characteristics of Mozzarella cheese. These authors observed an increase in INSOL Ca levels with increasing pH in the range 5.2-6.2, and attributed this to the probable reassociation of soluble (SOL) Ca with the casein matrix.

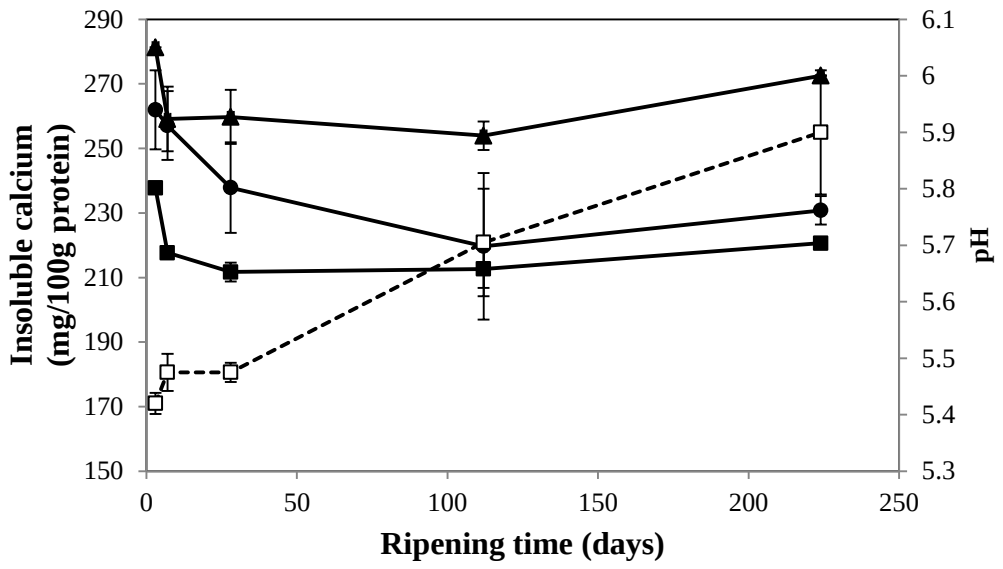

Figure 7.1 Concentration of insoluble calcium (mg/100g protein) in Swiss-Cheddar cheese from Trials 1 (■), 2 (●) and 3 (▲) and mean pH values (□) throughout 224 d of ripening.

7.3.2 Starter, PAB and NSLAB viability during ripening

During early ripening starter cells are the dominant flora in the young curd, and are usually present in relatively high numbers, ~10^9 cfu g^{-1}, and these high numbers represent considerable biocatalytic potential during cheese ripening (Cogan and Beresford, 2002). Starters were only enumerated up until 56 d of ripening because the NSLAB also grow on the LM17 medium used for counting the starter and thus make the starter counts inaccurate later in ripening (Cogan and Beresford, 2002; Sheehan *et al*., 2007). In this study, Figure 7.2 shows mean viable cell counts of *S. thermophilius* and *L. helveticus* decreased from ~$10^{7.5}$cfu g^{-1} and ~10^7cfu g^{-1} at

14 d to ~10^6cfu g^{-1} and ~$10^{5.5}$cfu g^{-1} at 56 d, respectively. Fenelon *et al*. (2002) reported similar starter cell populations for reduced-fat Cheddar cheeses manufactured with different bacterial cultures. Similarly, Sheehan *et al*. (2008) observed a comparable reduction in the levels of both *S. thermophilius* and *L. helveticus* in both Swiss and Swiss-Cheddar-hybrid cheeses ripened at 9 ˚C.

Cogan and Beresford (2002) stated that cheeses made from pasteurized milk normally contain low levels of NSLAB, probably < 10^3cfu g^{-1} at the beginning of ripening, but they grow relatively rapidly during ripening to levels of ~10^8cfu g^{-1} within 2 to 4 months, depending on the species, the cheese, and the cooling rate of the cheese block and the subsequent ripening temperature. In the current study, NSLAB numbers (Figure 7.2) increased significantly throughout ripening from ~$10^{0.8}$ cfu g^{-1} at 14 d to ~10^8 cfu g^{-1} at 224 d. There was a significant effect of ripening time on the mean viable NSLAB cell count populations which initially increased rapidly to ~$10^{5.8}$ cfu g^{-1} at 56 d of ripening and more slowly to ~10^8 cfu g^{-1} at 224 d. Similar trends were observed in Cheddar (Fenelon *et al*., 2002), Swiss (Valence *et al*., 2000) and Swiss-Cheddar-hybrid cheese varieties (Sheehan *et al*., 2007; Sheehan *et al*., 2008).

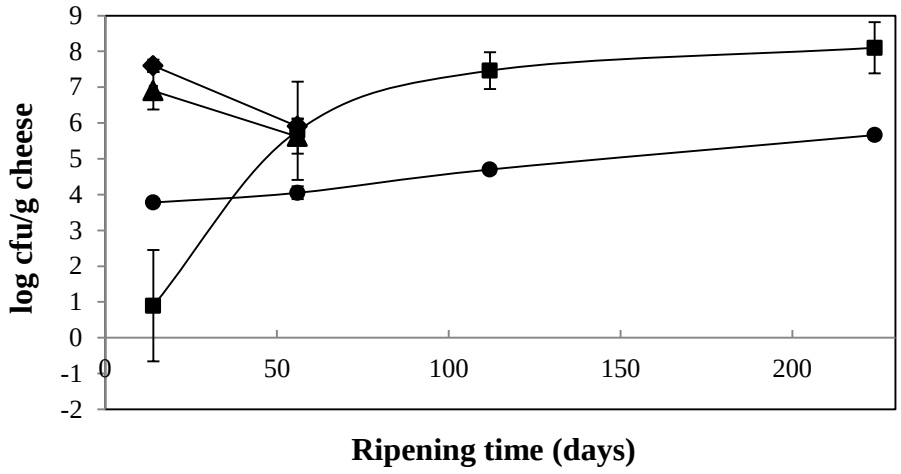

Figure 7.2 The viable cell counts of non-starter lactic acid bacteria enumerated on Rogosa agar (■), *Lactobacillus helveticus* enumerated on MRS pH 5.4 agar (▲), *Streptococcus thermophilius* enumerated on LM17 agar (♦) and propionic acid bacteria on SLA agar with vancomycin (●) during 224 d of ripening. Values presented are means from three replicate trials**.**

Propionic acid bacteria (PAB) grow in many cheese varieties during ripening and contribute to the characteristic microflora of Swiss-type cheeses where they metabolize lactate (Cogan and Beresford, 2002) and are responsible for the characteristic eye development associated with this type of cheese. Typically, Swiss-type cheeses are ripened at much higher temperatures (18-24 ˚C) than Cheddar varieties (6-8 ˚C) (Fox *et al.*, 2000) and this higher temperature facilitates the production of gas and subsequent formation of eyes. A slight increase in the numbers of PAB was evident in cheeses ripened for 224 d. PAB numbers increased from ~$10^{3.8}$ cfu g^{-1} at 14 d to ~$10^{5.7}$ cfu g^{-1} after 9 months of ripening at 9 ˚C (Figure 7.2). The growth of PAB may have been inhibited by the high salt content of the cheese

(1.12 %) and may explain the low increase in PAB numbers present in the cheese. These results are in accordance with Sheehan *et al*. (2008) who reported viable PAB populations of ~ $10^{5.5}$ to ~$10^{6.5}$ cfu g^{-1} for Swiss-Cheddar-hybrid cheeses ripened at 9 and 12 ˚C, respectively at 180 d of ripening. In this study, no eye development occurred in the Swiss-Cheddar-hybrid chesses and this is likely owing to the ripening temperature (9 ˚C) and NaCl content of the cheeses which provided an unsuitable environment for PAB growth and production of gas. Additionally, the milled curd structure of the cheese due to "cheddaring" may have also encouraged gas loss through the cracks of the curd.

7.3.4 Proteolysis

7.3.4.1 Urea-polyacrylamide gel electrophoresis

Urea-PAGE gel electrophoretograms of Cheddar-hybrid cheeses form all three trials during ripening are shown in Figure 7.3. There was progressive and significant degradation of both α_{s1}-CN and β-CN during ripening of cheeses from all three trials. Degradation led to the accumulation of the breakdown product of both α_{s1}-CN (f 24-199) and γ-caseins (β-CN f106-209, f29-201, f108-209), respectively. Such degradation in cheese has been is primarily associated with residual proteolytic activity of the coagulant and of plasmin from the milk (Fox *et al*., 2000; Sousa *et al*., 2001). However, in high cook cheeses such as these, chymosin is usually denatured extensively and makes relatively little contribution to cheese ripening. Therefore, it is

plasmin, the naturally occurring heat-stable enzyme that is likely to be the primary proteolytic agent responsible for proteolysis (Upadhyay *et al*., 2004).

That being said, several authors (Hayes *et al*., 2002; Hynes *et al*., 2004; Sheehan *et al*., 2007, 2008) have reported that coagulant inactivation is only partial and may also be reversible in some instances, and thus chymosin may contribute to primary proteolysis during ripening. The significant proportion of α_{s1}-CN (f124-199) present in the Swiss-Cheddar-hybrid cheeses in this study suggests that chymosin also contributed to primary proteolysis as well as plasmin. The extent of hydrolysis for both α_{s1}-CN and β-CN appear to be far less than that reported for Cheddar (Fenelon and Guinee, 2000) but similar to that in Swiss cheese (Bastian *et al*., 1997).

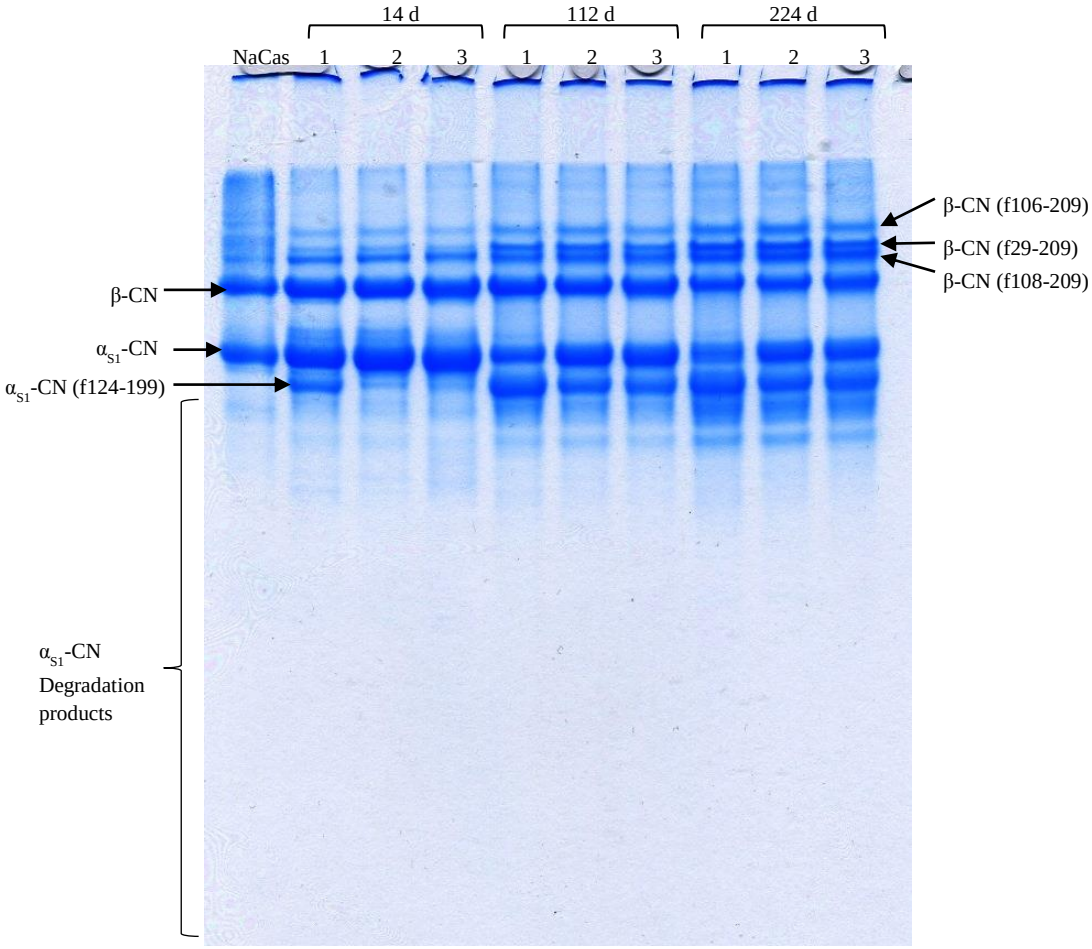

Figure 7.3 Urea-polyacrylamide gel electrophoretograms of sodium caseinate standard (NaCas) and the pH 4.6-insoluble fractions from the Cheddar-hybrid cheeses at 14, 112 and 224 d of ripening for Trials 1, 2 and 3.

7.3.4.2 Levels of pH 4.6-SN/TN

The pH 4.6-SN/TN levels of Cheddar-hybrid cheeses at 14, 112 and 224 d of ripening are shown in Table 7.1. The pH 4.6-SN/TN levels increased significantly ($P \leq 0.05$) during ripening and this trend was similar for all three trials, therefore results

presented are means from the three replicate trials with standard deviations in parentheses. This increase in pH 4.6-SN/TN is in agreement with the progressive hydrolysis of both α_{s1}-CN and β-CN as shown by urea-PAGE (Figure 7.3). The levels of pH 4.6-SN in Swiss-Cheddar-hybrid cheese ripened at 9 ˚C were lower than those previously described by Fenelon and Guinee (2000) and Fenelon *et al*. (2000) for full-fat Cheddar cheese but similar to Swiss-type and Swiss-Cheddar-hybrid cheeses (Sheehan *et al*., 2008) at 180 d of ripening.

7.3.4.3 Levels of individual free amino acids

The levels of total and individual FAAs (mg/g of cheese) in the cheeses at 112 and 224 d of ripening are shown in Figures 7.4a and 7.4b. The total concentration (mg g^{-1} cheese) of FAA increased from 13.13 to 29.73 mg g^{-1} cheese from 112 to 224 d respectively. These levels were much greater than those reported, at comparable ages, in full fat Cheddar (Guinee *et al*., 2000) and Gouda (Fox and Wallace, 1997), but were similar to those observed by Lawlor *et al*. (2002) and Sheehan *et al*. (2007) in mature Swiss-type and Swiss-Cheddar-hybrid cheeses, respectively. Similar to Sheehan *et al*. (2007) and Lawlor *et al*. (2002) the cheeses characterized in this study were produced using *Lactobacillus* strains that are more proteolytic (Fenelon *et al*., 2002) and therefore are capable of producing greater quantities of amino acids than lactococci which are used in the manufacture of Gouda- and Cheddar-type cheese.

The principal FAAs found in all cheeses at both ripening times were Glu, Val, Leu, Lys, Phe and Pro which reflects the typical amino acid profiles observed in

Cheddar (Fenelon *et al*., 2000) and Swiss-type cheeses (Fox and Wallace, 1997; Lawlor *et al.*, 2002). Wood *et al*. (1985) noted that the relative proportions of FAAs present in Swiss-type cheese were similar to those in Cheddar with the exception of Pro, which is high in Swiss cheese due to its importance as a flavour compound. Langsrud (1974) suggested that PAB present in Swiss-type cheese is the source of peptidases which release proline, the amino acid giving the cheese its characteristic sweet flavour. All FAAs increased throughout ripening, except for Arg, whose levels decreased during the latter stages of ripening in all three trials. This is not surprising as several authors have reported a decrease in Arg during the latter stages of ripening (Broome *et al*., 1990; Fox and Wallace, 1997). Arg was detected at very low concentrations at both ripening points, 0.37 and 0.23 mg g^{-1}cheese at 112 and 224 d respectively, indicating that the hybrid was closer in this respect to Swiss Emmental cheese rather than to Cheddar (Lawlor *et al*., 2002). His and Ile were also dominant particularly at 224 d. Very low quantities of Cys were detected at both time points. Lawlor *et al*. (2002) also only recorded Cys values of 22-40 μg g^{-1} cheese in Dubliner and Emmental cheeses.

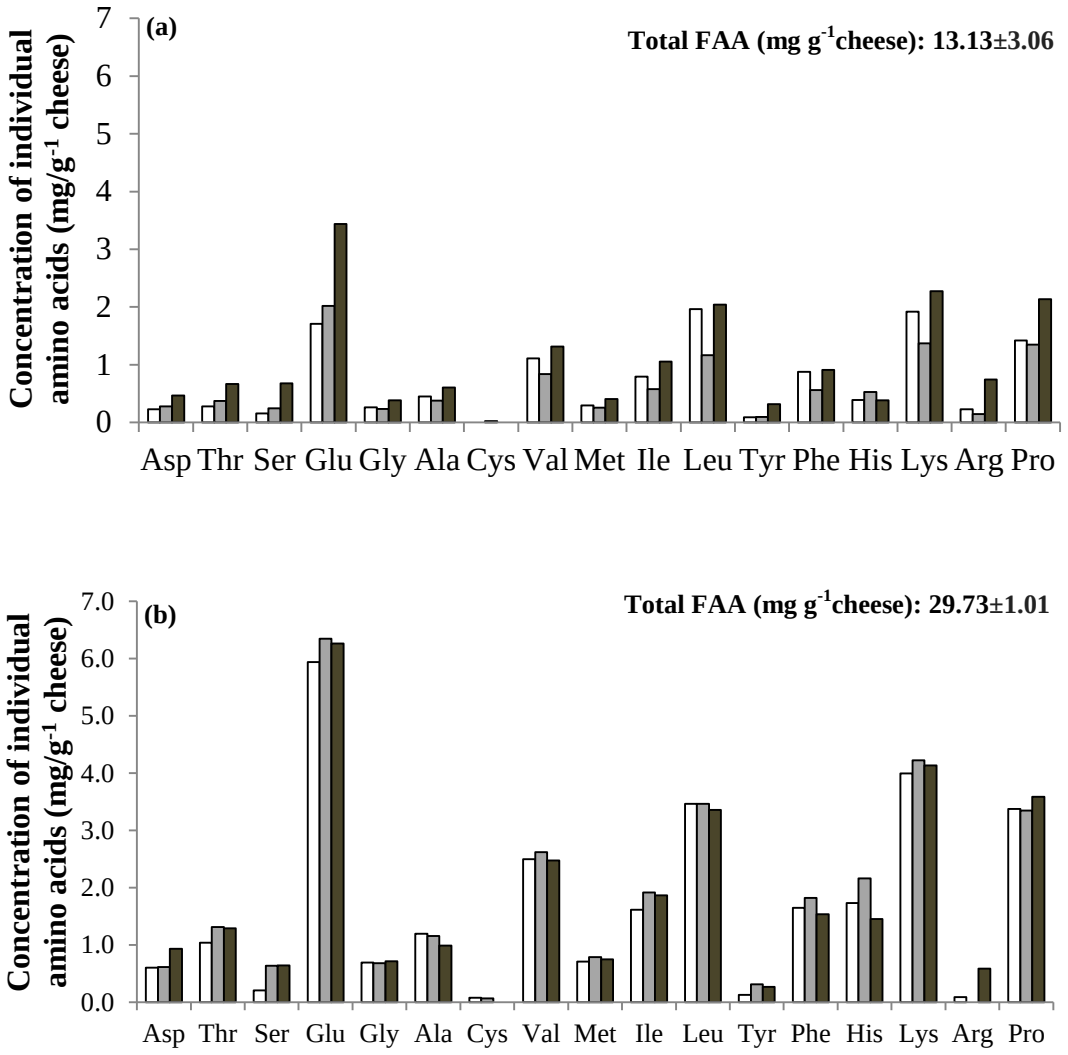

Figure 7.4 Concentration of individual free amino acids in pH 4.6-soluble extract of cheese from Trial 1 (□), Trial 2 (■) and Trial 3 (■) at (a) 112 and (b) 224 d of ripening. Values are a result of single analysis of samples. Total FAA values are averages of the three trials each time point.

7.3.4.4 RP-UPLC peptide profiles

The peptide profiles of pH 4.6-soluble fractions from cheeses at 14, 112 and 224 d of ripening are shown in Figure 7.5. Both quantitative and qualitative differences in the peptide profiles were observed in Swiss-Cheddar-hybrid cheese as ripening progressed. The greatest quantitative changes occurred in the peptides eluting between 2-5, 9-10 and 15-21 min. The peaks that varied most during ageing of Swiss-Cheddar-hybrid cheese were those which eluted at approximately 2.2, 3.8, 9.8, 15.8 and 16.9 min. This result indicates that proteolysis increased progressively upon ageing; this is in agreement with the progressive hydrolysis of both α_{s1}-CN and β-CN as shown by urea-PAGE and the increased levels of pH 4.6-SN/TN observed.

7.3.5 Lipolysis

Enzymatic hydrolysis (lipolysis) of triglycerides to fatty acids and glycerol, mono- or di-glycerides is an important biochemical event during cheese ripening and is considered to be essential for flavour development in cheese (Collins *et al*., 2003b, 2004). The concentration of each of the 11 individual free fatty acids (FFA) present in the cheeses from the three trials at 14, 112 and 224 d of ripening are shown in Table 7.2.

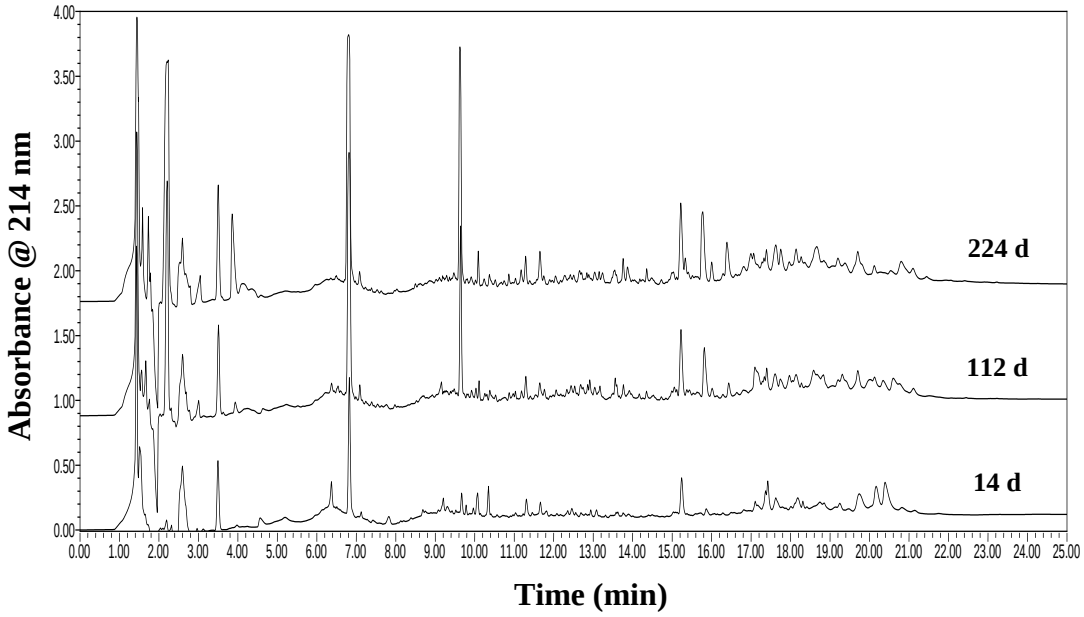

Figure 7.5 UPLC chromatograms of pH 4.6-soluble extracts of Cheddar-hybrid cheeses at 14, 112 and 224 d of ripening. Samples were read at 214 nm absorbance. Chromatograms are of Trial 1 and are representative of Trials 2 and 3 also. Samples were analysed in triplicate but only one chromatogram is shown.

Table 7.2 Individual free fatty acid (FFA) profiles of Swiss-Cheddar-hybrid cheeses from three trials at 14, 112 and 224 d of ripening. Analysis was carried out in triplicate; values in brackets are standard deviations.

Day	Trial	FAA (mg/kg cheese)											
		C4:0	**C6:0**	**C8:0**	**C10:0**	**C12:0**	**C14:0**	**C16:0**	**C18:0**	**C18:1**	**C18:2**	**C18:3**	**Total**
14	1	12(1)	4(0)	5(0)	14(0)	19(0)	59(0)	231(7)	119(7)	189(7)	0(0)	0(0)	655(25)
	2	18(1)	4(1)	5(1)	15(1)	21(1)	61(2)	239(13)	126(14)	198(1)	0(0)	0(0)	686(33)
	3	9(1)	3(2)	4(0)	14(0)	20(0)	55(1)	213(0)	109(2)	189(2)	0(0)	0(0)	619(9)
112	1	26(1)	7(0)	6(0)	16(1)	22(1)	70(3)	249(10)	118(0)	211(13)	0(0)	0(0)	729(11)
	2	28(2)	6(0)	6(0)	17(0)	23(0)	74(1)	258(1)	126(5)	225(0)	0(0)	0(0)	767(11)
	3	16(2)	4(0)	4(0)	14(2)	20(3)	61(6)	218(21)	109(13)	186(22)	0(0)	0(0)	636(69)
224	1	60(3)	14(1)	9(0)	22(0)	30(4)	94(5)	295(0)	127(2)	303(7)	12(8)	15(11)	972(41)
	2	47(4)	11(3)	6(0)	20(1)	27(2)	85(4)	277(16)	125(17)	286(21)	0(0)	0(0)	889(68)
	3	38(3)	8(0)	6(0)	18(1)	24(1)	74(3)	251(14)	112(5)	231(12)	0(0)	0(0)	766(40)

It is well established that excessive lipolysis is undesirable in many varieties (Collins *et al*., 2004), of which Cheddar and Emmental are included, and cheeses containing even a moderate concentration of FFAs may be considered as rancid by some consumers (McSweeney, 2004). However, it is desirable for Swiss-type and Cheddar-type cheeses to undergo intermediate and low levels of lipolysis, respectively (Wilkinson, 2007). The total FFA concentration ($C_{4:0}$ to $C_{18:3}$) of cheeses increased throughout 224 d of ripening, from ~ 657 to ~ 879 mg kg^{-1} cheese at 14 and 224 d, respectively. The total FFA levels recorded are low but are within the range previously described for Cheddar and Swiss-type cheese (Woo *et al.*, 1984; Reddy and Marth, 1993; Fox and Wallace, 1997; Kilcawley *et al*., 2001; Collins *et al*., 2004). In Swiss-type cheese, moderate levels of FFAs, in the range of 2-7 g kg^{-1} cheese are typically released during ripening and make a significant contribution to the characteristic aroma and flavour of the cheese (Collins *et al*., 2004). However, the levels recorded in cheeses from this study are lower, perhaps due to the conditions under which the cheeses were ripened. Frohlich-Wyder and Bachmann (2007) stated that lipolysis in Swiss-type cheese is mainly caused by the growth of PAB in the warm room (~20-24 ˚C), leading to the release of FFAs, and is generally recognized as necessary to produce characteristic Swiss cheese flavour. In this study however, cheeses had unfavorable conditions for PAB growth and FFA production.

Hexadecanoic ($C_{16:0}$) and octadecanoic ($C_{18:0}$) have been described as the most abundant FFAs present in most cheese varieties (Wilkinson, 2007) and results from this study are in agreement. The FFAs present at the greatest concentrations

throughout ripening were palmatic ($C_{16:0}$) and oleic ($C_{18:1}$) acids, which reached average values of 274 mg g^{-1} and 273 mg g^{-1} cheese, respectively. These results are in accordance with Collins *et al*. (2003a) who found that throughout 238 d of ripening, palmitic and oleic acids were the most abundant FFAs in all Cheddar cheese. Similarly, Kilcawley *et al*. (2001) found that the predominant FFAs in most enzyme-modified Cheddar cheeses studied were $C_{16:0}$, $C_{18:1}$, $C_{14:0}$, and $C_{18:0}$, which represented ~30, ~19, ~13, and ~11% of the total FFA, respectively. While in natural Cheddar cheese, the same FFAs predominated, but not in the same order, with $C_{18:1}$ representing ~27% of the total FFA, $C_{16:0}$ ~20%, $C_{14:0}$ ~12%, and $C_{18:0}$ ~9%.

The concentration of all individual FFA increased during ripening, except $C_{18:2}$ and $C_{18:3}$, which were not detected in any cheese (expect Trial 1, $C_{18:2}$ and $C_{18:3}$ detected at 224 d) throughout ripening. In a previous study undertaken by Collins *et al*. (2003a), linolenic acid ($C_{18:3}$) was not detected in any of the Cheddar cheese under investigation. These authors suggested that this FFA may have been present below the detection level of the analytical method used, which may also be the case in this study.

7.3.6 Volatile flavour compounds

Flavour compounds in cheese are produced during ripening by a complex series of microbiological changes and biochemical events, and these pathways have been extensively studied (McSweeney and Sousa, 2000; McSweeney, 2004). Biochemical transformations of residual lactose, and lactate and citrate, and

proteolysis of the caseins to peptides and ultimately to free amino acids, which are subsequently catabolized to a range of volatile flavour compounds, are important in most cheeses (McSweeney and Sousa, 2000; Guinee and McSweeney, 2006). The peptides and the amino acids released during proteolysis contribute to, and act as precursors for, flavour development in cheese (Beresford and Williams, 2004; Guinee and McSweeney, 2006). Likewise, fatty acids, when liberated from triacylglycerols by the action of lipases, are important precursors of several volatile compounds which contribute to flavour either directly or indirectly (Collins *et al.*, 2003a, b; Collins *et al*., 2004; Guinee and McSweeney, 2006; Wilkinson, 2007).

In this study a total of 39 volatile compounds were identified and semi-quantified (Table 7.3), these consisted of 9 alcohols, 9 ketones, 8 aldehydes, 6 acids, 4 sulphur compounds, 2 pyrazines, and 1 benzene compound. Interestingly, no esters were found in the cheeses at any time point, which is quite uncommon for semi-hard cheeses; especially as ethanol and short chain fatty acids were present. Collins *et al*. (2004) has suggested that ethanol produced from lactose metabolism or through the catabolism of FFAs, can be the limiting reactant in the production of esters in cheese. However, all of the compounds identified have been previously found in both Swiss and Cheddar cheese varieties (Collins *et al*., 2004).

Table 7.3 Volatile compounds present in Swiss-Cheddar-hybrid cheeses at 14, 112 and 224 d of ripening.

Volatile compound	Tentative precursor	Odour description	Ripening time (d)								
			14			112			224		
			1	2	3	1	2	3	1	2	3
Aldehydes											
Acetaldehyde	Asp, Thr, Citrate, Lactose	Yoghurt, green, nutty, pungent, sweet, fruity	0	0	0	0	96644	0	88316	99750	124522
2-Methylpropanal	Val (Leu)	Malty, cocoa, chocolate-like, banana	51093	42685	32004	156960	119528	56183	67218	181496	105093
3-Methylbutanal	Leu	Malty, cheese, green, dark chocolate, cocoa	296009	234509	160620	1270878	953442	486397	804041	5152533	867542
2-Methylbutanal	Ile	Malty, dark chocolate, almond, cocoa, coffee	71789	46915	37853	442394	282717	153126	258486	1480244	260711
Pentanal	LO	Pungent, almond-like, chemical, malty, apple, green	192648	210391	381865	0	114380	42171	361042	117601	203728
Heptanal	LO	Slightly fruity (Balsam), fatty, oily, green, woody	45603	44288	39488	0	16110	0	27432	88496	35845
Nonanal	LO	Green, citrus, fatty, floral	35787	27575	25043	26921	39556	18029	31135	31209	758762
Benzaldehyde	Phe, Trp	Bitter almond, sweet cherry	1096656	1557193	724602	196224	128799	367142	434681	180099	368400
Alcohols											
Ethanol	Thr, Asp, Citrate, Lactose	Dry, dust	0	196530	133560	0	474533	2308401	1550302	1310418	3091166
2-Butanol	LO	Fruity	0	0	0	0	0	0	25621764	8539763	2030758
2-Pentanol	LO	Green, fusel oil, fruity, fresh	0	0	0	0	0	0	489274	6334808	1821157
3-Methyl-1-butanol	Leu	Fresh cheese, breathtaking, alcoholic, fruity, grainy, solvent-like, floral, malty	1578112	575330	0	5534154	3823499	2218322	952882	8999948	2037687
2-Methyl-1-butanol	Ile	Fermented, malty	117648	0	53136	471314	337196	215069	128073	1322439	191480
1-Pentanol	Pentanal	Fruity, alcoholic, green, balsamic, fusel oil, woody	474045	418844	180228	437538	370638	216640	567783	292821	350214
2,3-Butanediol	Citrate, Lactose	Fruity	0	0	0	0	51629	0	2894750	4694441	992281
1-Hexanol	Hexanal	Green, floral	0	0	0	0	0	0	0	248710	29401
2-Heptanol	LO	Fruity, green, earthy, sweetish, dry, dusty carpet	0	0	0	0	0	0	0	765679	734867
Ketones											
3-hydroxybutanone (Acetoin)	Asp, Citrate, Lactose	Buttery, sour mlk, caramel	0	34703656	49596053	40862007	40470208	19837318	22626617	50027781	57337930
2,3-Butanedione (Diacetyl)	Asp, Citrate, Lactose	Buttery, strong	13818930	14407821	9295657	3414412	6069311	6810195	3808725	4430465	5521094
Acetone	LO	Pungent, wood pulp, hay	4946863	5098805	4972384	4067117	3733708	5802822	1806126	4038065	4498092
2-Butanone	Citrate, Lactose, LO	Etheric, sour milk	0	4124908	5214598	790981	2769403	3824544	41710066	35446361	27196150
2-Pentanone	LO	Orange peel, sweet, fruity	630108	616224	574700	1099929	1047801	4857523	4441625	865157	42627930
2,3-Pentanedione	Thr	Creamy, cheesy, sweet buttery, carmellic	492561	278468	212589	0	104989	328774	0	1093816	0
2-Heptanone	LO	Blue cheese, spicy, roquefort	1060600	1187066	1114386	1373865	1484859	3319026	26587729	18824925	13980333
2-Octanone	LO	fruity, musty, unripe apple, green	0	0	0	0	0	0	0	125166	54392
2-Nonanone	LO	Malty, fruity, hot milk, smoked cheese	135154	116997	117856	137288	126997	388633	256177	4350305	878997
Sulphur compounds											
Dimethyl sulphide	Met	Aspargus, cabbage-like, pomegranate, sulphur, cooked-cabbage	79284	78991	57976	106893	87882	64910	101561	74233	68020

Table continued

Volatile compound	Tentative precursor	Odour description	Ripening time (Day)								
			14			112			224		
			1	2	3	1	2	3	1	2	3
Carbon disulphide	?	Sweet, pleasing, ethereal	408536	462997	1236663	470235	446166	1135299	746731	1394404	1485152
Methional	Met	Boiled or baked potato	0	0	0	0	0	0	25763	26970	15499
Dimethyl sulphone	Met, Cys	Sulphurous, hot milk, burnt	609815	362483	340860	445906	314659	284828	417883	315956	313613
Acids											
Acetic Acid	Lactose, FAA, LO	Vinegar, peppers, green, fruity floral, sour	0	23645155	15290669	14878416	15232498	10635415	73025968	17117706	21628992
Butanoic acid	Lipid, LO, BCFA	Sweaty, butter, cheese, strong, acid, fecal, rancid, dirty sock	7776113	8706236	2806202	0	12207743	3067473	37161980	15909422	23628232
Isovaleric acid	BCFA	Cheesy, sweaty, old socks, rancid, fecal, rotten fruit, goat	0	41942	0	0	257815	0	0	467160	294762
2-Methylbutanoic acid	Ile	Fruity, waxy, sweaty-fatty acid	0	0	0	0	121994	0	49857	211434	75150
Octanoic acid	Lipid	Cheesy, rancid, pungent, sweat	158654	154539	141671	72429	78869	59290	324799	142539	136241
Nonanoic acid	Lipid	Goaty, waxy, green	65206	40108	13854	26637	34950	4613	21701	24329	13413
Pyrazines											
2,6-Dimethyl-pyrazine	MB	Freshly roasted nuts, chocolate	750357	8772398	590683	465907	472258	681699	201940	387630	350177
Trimethyl-pyrazine	MB	Chocolate, earthy	398371	474424	303208	445871	401773	265411	362873	579177	386347
Benzene											
Toluene	Contamination/Grass	Nutty, bitter, almond, plastic	9468254	9503746	7597531	8585864	8008436	7517701	9425032	7225824	7242937

Asp; aspartic acid, BCFA; branched chain fatty acids, Cys; cysteine, FAA; free amino acids, Ile; isoleucine, Leu; leucine, LO; lipid oxidation, Met; methionine, MB; myoglobin binding fatty acids, Phe; phenylalanine, Thr; threonine, Trp; tryptophan, Val; valine.

The PCA plot shown in Figure 7.6 highlights discrimination between the cheeses based on their volatile profiles at 14, 112 and 224 d of ripening. It is evident that most of the discrimination relates to the PC1 axis (45%). It is very evident that on an individual basis at 14 d, cheeses from all three trials were quite similar and not that different to cheese at 112 d of ripening, all of which are most strongly associated with benzaldehyde, 2,3-butanedione and nonanoic acid. The main discrimination occurred as the cheeses ripen, with cheeses at 224 d differing from the 14 d and 112 d cheeses, but also from each other. Trial 1 224 d cheese was most strongly associated with acetic, octanoic and butanoic acids and 2-butanol, Trial 3 224 d cheese was strongly associated, ethanol, heptanal, tri-methyl pyrazine, 2-methyl butanoic acid, 2-pentanone, 2-methyl propanol and carbon disulphide and Trial 2 224 d was on the extreme right of the biplot and not strongly associated with any individual compound but quite different to the other 224 day cheeses. It is typical in cheese that discrepancies between samples increase with ripening time (Delgado *et al*., 2010; Calzada *et al.*, 2014).

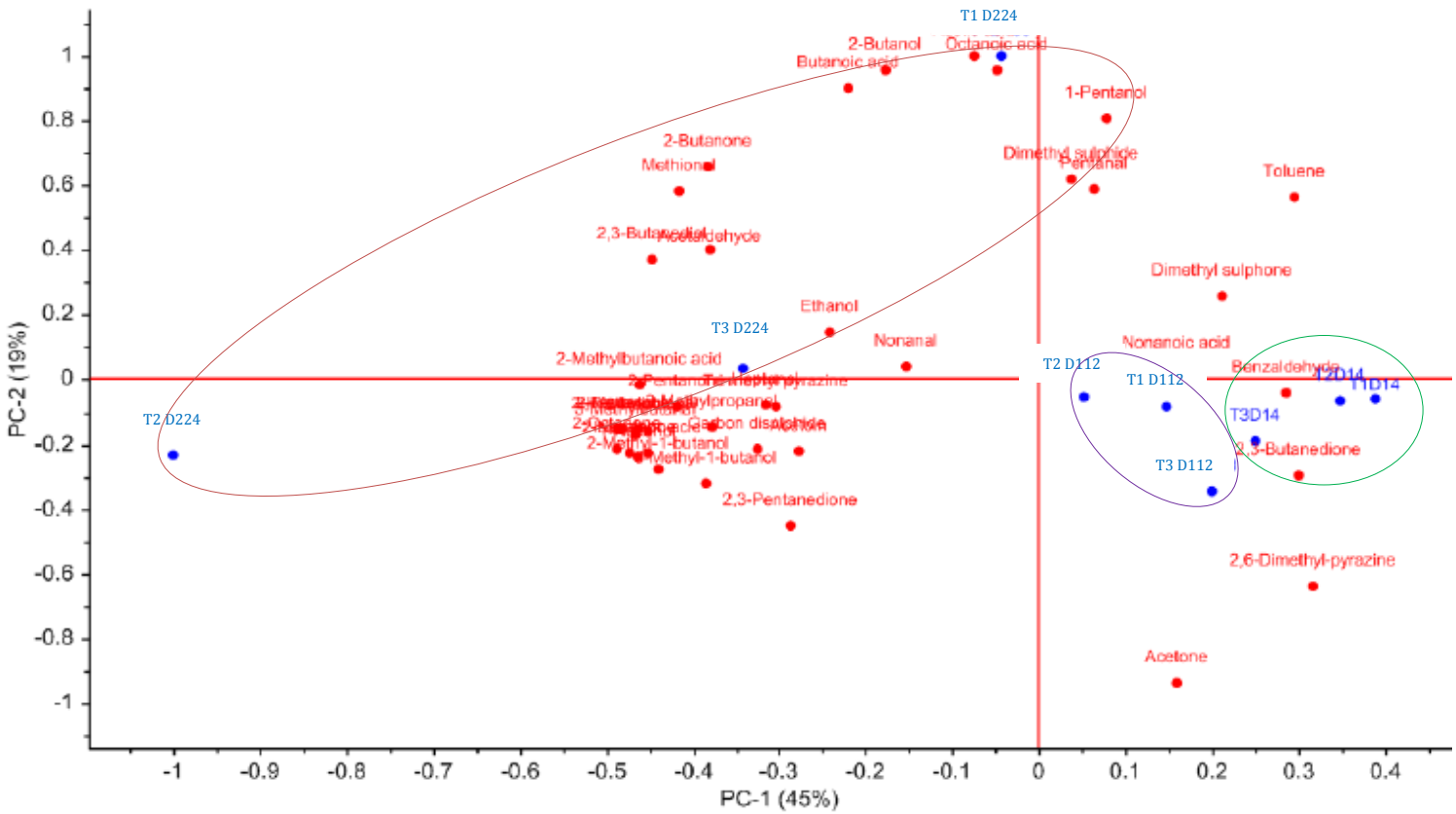

Figure 7.6 Results of principal component analysis on the volatile compounds of Swiss-Cheddar-hybrid-type cheese at d 14, 60 and 180 of ripening (blue font).

7.3.7 Textural and rheological properties of cheese

The extensive proteolytic breakdown of α_{s1}-CN within the protein matrix (Creamer and Olson, 1982), as well as the slow solubilisation of CCP (O'Mahony *et al*., 2005), which results in the loosening of the CCP crosslinks between the caseins, leading to a reduction in the structural integrity of the *para*-CN matrix (Lucey *et al*., 2003) have been associated with the reduction in hardness values of cheese during ripening. Additionally, the pH of cheese curds distinctly affects texture; low pH curds tend to be more crumbly compared to high pH curds, which exhibit a more elastic structure (Lucey and Fox, 1993). This relates to conformation of the proteins at various pH values; protein aggregates in high pH cheese are larger, with a well-defined structure compared to low pH cheese, which has smaller aggregates with less structural uniformity (Lucey and Fox, 1993; Pastorino *et al*., 2003).

7.3.6.1 Meltability

Swiss-Cheddar-hybrid cheeses from the three trials showed a progressive and significant ($P \leq 0.05$) increase in meltability during 224 d of ripening (Table 7.4); with the greatest increase occurring at the later stages of ripening (56 to 224 d). Cheese meltability is influenced by several inter-related factors, but it is most likely owing to the degradation of the *para*-casein matrix and/or changes to its calcium equilibrium. The level of INSOL Ca and pH are key factors relating to cheese meltability. It is well established in Cheddar cheese that lower levels of INSOL Ca result in cheese with increased flowability and melting characteristics (Lucey *et al*,

2005). However, results suggest that the reduction in the levels of INSOL Ca observed in this study were not sufficient to influence the flow properties of the cheese significantly during early ripening. Therefore, it is likely that the effects of progressive proteolysis were the determining factor associated with the increase in cheese meltability particularly in later ripening.

7.3.6.1 Texture profile analysis

The mean values for texture profile analysis (TPA) results for Swiss-Cheddar-hybrid cheeses shown in Table 7.4. Results indicate a progressive and significant ($P \leq 0.05$) reduction in gumminess, chewiness, springiness, cohesiveness and resilience values during 224 d of ripening. Interestingly, however, the hardness values of Swiss-Cheddar-hybrid cheese did not significantly decrease during ageing. As previously described, several inter-related factors (proteolysis, pH, calcium solubilisation, etc.) are responsible for the characteristic increase in cheese softening (reduction in hardness/firmness) throughout cheese maturation.

Table 7.4 Texture profile analysis parameters (hardness, gumminess, chewiness, springiness, cohesiveness and resilience) and cheese meltability (expressed as % increase in cheese disc diameter) as a function of ripening time for Cheddar-hybrid cheese.

Day	**TPA parameters**						**Schreiber test**	**Rheometer**	
	Hardness	**Gumminess**	**Chewiness**	**Springiness**	**Cohesiveness**	**Resilience**	**Meltability**	**LT_{max}**	**T @ LT = 1**
	(g)						(% increase in melt diameter)		
14	21424^{a} (4921)	7143^{a} (2340)	4498^{a} (1465)	0.636^{a} (0.07)	0.327^{a} (0.06)	0.133^{a} (0.03)	47.95^{d} (3.77)	2.43^{b} (0.25)	59.7^{a} (1.14)
28	20769^{a} (3621)	6365^{ab} (1623)	3653^{ab} (1166)	0.568^{b} (0.08)	0.302^{ab} (0.03)	0.109^{b} (0.01)	48.75^{cd} (3.95)	n.d	n.d
42	n.d	n.d	n.d	n.d	n.d	n.d	n.d	2.67^{ab} (0.19)	59.2^{a} (1.67)
56	19706^{a} (2958)	5298^{bc} (1311)	2925^{bc} (675)	0.548^{b} (0.06)	0.271^{b} (0.04)	0.095^{b} (0.01)	54.30^{bc} (5.46)	n.d	n.d
112	n.d	n.d	n.d	n.d	n.d	n.d	n.d	2.69^{ab} (0.18)	58.9^{a} (1.48)
224	19795^{a} (2380)	4248^{c} (846)	1971^{c} (492)	0.464^{c} (0.06)	0.214^{c} (0.03)	0.067^{c} (0.01)	71.56^{a} (0.89)	2.78^{a} (0.07)	58.8^{a} (2.08)

Values presented are means from three replicate trials with standard deviations in parentheses; means within columns not sharing a common letter differ statistically ($P \leq 0.05$)

n.d; not determined

pH has a strong effect on solubilisation of CCP; decreasing pH solubilises CCP and affects its solubilisation rate (Lucey and Fox, 1993; Pastorino *et al*., 2003; Johnson and Lucey, 2006). Pastorino *et al*. (2003) found that decreasing cheese pH leads to decreased hardness due to calcium solubilisation leading to decreased protein-protein interactions weakening the cheese matrix. Conversely, Cortez *et al*. (2008) showed that increased pH values of Mozzarella cheese resulted in a harder cheese that required longer aging to develop desirable melting characteristics. In addition, Hou *et al*. (2014) used curd washing in cheese with standardised calcium levels and found that increased pH led to increased firmness and fracture stress which was attributed to higher levels of casein bound calcium. In this study, Swiss-Cheddar-hybrid cheese pH values increased, proteolysis increased and INSOL Ca levels decreased (day 3 to 112) followed by a slight increase (day 112 to 224) during ripening. Therefore, the absence of a reduction in cheese hardness may be credited to the counteracting effects of each of the aforementioned processes. Both proteolysis and solubilisation of CCP cause a loosening of the CCP crosslinks between the caseins (Lucey *et al*., 2005, 2003), leading to a reduction in the structural integrity of the *para*-CN matrix, resulting in the reduction of cheese hardness, while cheese with a high pH value tends to contain larger protein aggregates with good structural integrity (Lucey and Fox, 1993; Pastorino *et al*., 2003), contributing to increased hardness. Therefore, it is possible that the effects of proteolysis and solubilisation of CCP on increasing cheese softening were cancelled out by the effects of increasing pH throughout ripening.

7.3.6.1 Rheological analysis

The mean maximum loss tangent (LT_{max}) values of Swiss-Cheddar-type cheese at 14, 42, 112 and 224 d of ripening, as measured by dynamic small amplitude oscillatory rheometry (DSAOR), are shown in Table 7.4. The LT_{max} value has been strongly correlated with cheese melt and therefore is used as an index of meltability; results showed that there was a significant ($P \leq 0.05$) and progressive increase in LT_{max} values throughout ripening, thus indicating an increase in cheese meltability. This result correlates well with Schreiber melting test results (Table 7.4). Insoluble calcium has been found to be positively correlated with LT_{max} and degree of flow of cheese. Joshi *et al*. (2003) found that reducing calcium using preacidification but maintaining the same cheese pH value affected the melt properties of Mozzarella cheese. Reduced-calcium cheese had a higher melt, flow rate, extent of flow and softened at a lower time and temperature. Lucey *et al*. (2005) suggested that solubilisation of INSOL Ca was principally responsible for the increase in LT_{max} values during the early stages of ripening. It is plausible that the increased meltability observed in the first 4 months of ripening was due to the reduction in the levels of INSOL Ca in the cheese. However, results suggest that the INSOL Ca levels in Swiss-Cheddar-hybrid cheese appeared to increase (Figure 7.1) after 112 d of ripening (increase only statistically significant for Trial 3 cheese). Johnson and Lucey (2006) stated that the attractive forces between caseins associated with high levels of insoluble calcium results in a cheese that will not flow at higher temperatures. Therefore, it is likely that that continued increase in meltability was due to the effects of increased proteolysis rather than to changes in the INSOL Ca levels in the cheese.

The temperature at which the loss tangent is equal to one (T @ LT = 1) is an index of the initial melting temperature of the cheese. Results in Table 7.4 show that the T @ LT = 1 decreased during ripening although this reduction was not significant ($P \leq 0.05$). It is generally regarded that less thermal energy is required to induce melt in aged cheese due to CCP solubilisation and proteolysis (Lucey *et al*., 2005). Due to the relatively insignificant decrease in T @ LT = 1 demonstrated by the Swiss-Cheddar-hybrid cheese in this study, it appears that the well-established effects of CCP solubilisation (loosening of the CCP crosslinks between the caseins, leading to a reduction in the structural integrity of the *para*-CN matrix) are being hindered or overshadowed by another factor, most likely increased pH throughout ripening.

7.4 Conclusions

This study characterised the composition and ripening of a Swiss-Cheddar-hybrid cheese capable of production in existing manufacturing plant. Chymosin and plasmin were both found to contribute to primary proteolysis of Swiss-Cheddar-hybrid cheese. The FAA levels present in the cheese increased significantly and were similar to Swiss and Swiss-Cheddar varieties. The principal FAAs were Glu, Val, Leu, Lys, Phe and Pro. The FFA production was low in Swiss-Cheddar-hybrid cheese studied during ripening, due likely to the poor growth of PAB. Volatile compounds (39) were identified and semi-quantified in the Swiss-Cheddar-hybrid cheeses and these consisted of 9 alcohols, 9 ketones, 8 aldehydes, 6 acids, 4 sulphur compounds, 2 pyrazines, and 1 benzene compound. The distribution of calcium between the soluble and insoluble phases in cheese was evaluated. The concentration of INSOL Ca decreased as ripening progressed up to 112 d, as expected, and then appeared to increase slightly for the remainder of ripening (224 d), indicating the reformation of INSOL Ca. There was no significant decrease in the firmness of Swiss-Cheddar cheese during ripening. This result supports the suggestion that INSOL Ca may be reforming causing an increase in the strength of the CCP crosslinks, counteracting the effects of proteolysis. Cheese meltability increased (Schreiber and DSAOR) throughout 224 d of ripening, with the greatest increase between 56 and 224 d of ripening. This increase is likely owing to the effects of increased proteolysis rather than the changes in INSOL Ca levels and pH values. Overall, this study highlights the changes (pH, Ca equilibrium and proteolysis) occurring in Swiss-Cheddar-hybrid

cheese during ripening and their influence on the textural and functional properties of the cheeses under investigation.

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References

Altan, A., Turhan, M. & Gunasekaran, S. (2005). Short communication: Comparison of covered and uncovered Schreiber test for cheese meltability evaluation. *Journal of Dairy Science*. *88*, 857‑861.

Bachmann, H. P., Buëtikofer, U. & Isolini, D. (2002). Swiss-type cheese. In: Fox, P. F., McSweeney, P. L. H., Cogan, T. M. & Guinee, T. P. (Eds). *Cheese: Chemistry, Physics and Microbiology: General Aspects*, 3rd Ed., *Vol 1*, pp. 363-371. Elsevier Ltd., London, UK.

Bastian, E. D., Lo, C. G. & David, K. M. M. (1997). Plasminogen activation in cheese milk: Influence on Swiss cheese ripening. *Journal of Dairy Science*, *80*, 245-251.

Beresford, T. & Williams, A. (2004). The microbiology of cheese ripening. In: Fox, P. F., McSweeney, P. L. H, Cogan, T. M. & Guinee, T. P. (Eds.), *Cheese: Chemistry, Physics and Microbiology*, *Vol 1*, 3rd Ed., General Aspects, pp. 287-317. Elsevier Academic Press, San Diego, CA.

Bourne M. C. (1978). Texture profile analysis. *Food Technology*, *32*, 62–66, 72.

Bourne, M. (2004). Relation between texture and mastication. *Journal of Texture Studies*, *35*, 125-143.

Broome, M. C., Krause, D. A. & Hickey, M. W. (1990). The use of non-starter lactobacilli in Cheddar cheese manufacture. *Australian Journal of Dairy Technology, 45*, 67-73.

Calzada, J., del Olmo, A., Picon, A. & Nuñez, M. (2014). Effect of high-pressure-processing on lipolysis and volatile compounds of Brie cheese during ripening and refrigerated storage. *International Dairy Journal, 39*, 232-239.

Cogan, T. M., & Beresford, T. P. (2002). Microbiology of hard cheese. In: Robinson, R. K. (Ed), *Dairy Microbiology Handbook: The Microbiology of Milk and Milk Products*, 3rd Ed., pp. 515-560. John Wiley and Sons, New York, NY.

Collins, Y. F., McSweeney, P. L. H. & Wilkinson, M. G. (2003a). Evidence of a relationship between autolysis of starter bacteria and lipolysis in Cheddar cheese during ripening. *Journal of Dairy Research, 70*, 105-113.

Collins, Y. F., McSweeney, P. L. H. & Wilkinson, M. G. (2003b). Lipolysis and free fatty acid catabolism in cheese: a review of current knowledge. *International Dairy Journal, 13*, 841-866.

Collins, Y. F., McSweeney, P. L. H. & Wilkinson, M. G. (2004). Lipolysis and catabolism of fatty acids in cheese. In: Fox, P. F., McSweeney, P. L. H., Cogan, T. M. & Guinee, T. P. (Eds). *Cheese: Chemistry, Physics and Microbiology: General Aspects*, 3rd Ed., *Vol 1*, pp. 373-389. John Wiley and Sons, New York, NY.

Cooke, D. R. & McSweeney, P. L. H. (2013). The influence of alkaline earth metal equilibria on the rheological, melting and textural properties of Cheddar cheese. *Journal of Dairy Research*, *80*, 418-428.

Cortez, M. A. S., Furtado, M. M., Gigante, M. L. & Kindstedt, P. S. (2008). Effect of pH on characteristics of low-moisture Mozzarella cheese during refrigerated storage. *Journal of Food Science*, *73*, S443-S448.

Creamer, L. K. & Olson, N. F. (1982). Rheological evaluation of maturing Cheddar cheese. *Journal of Food Science*, *47*, 631-636.

Czulak, J., Hammond, L. A. & Meharry, H. J. (1954). A new method for Cheddar cheese manufacture. *Australian Dairy Review*, *22*, 18–23.

De Jong, C. & Badings, H. T. J. (1990). Determination of free fatty acids in milk and cheese. Procedures for extraction, clean up and capillary gas chromatography analysis. *Journal of High Resolution Chromatography*. *13*, 94-98.

Delgado, F. J., González-Crespo, J., Cava, R., García-Parra, J. & Ramírez, R. (2010). Characterisation by SPME–GC–MS of the volatile profile of a Spanish soft cheese PDO Torta del Casar during ripening. *Food Chemistry*, *118*, 182-189.

Fenelon, M. A. & Guinee, T. P. (2000). Primary proteolysis and textural changes during ripening in Cheddar cheeses manufactured to different fat contents. *International Dairy Journal*, *10*, 151-158.

Fenelon, M. A., O'Connor, P. & Guinee, T. P. (2000). The effect of fat content on the microbiology and proteolysis in Cheddar cheese during ripening. *Journal of Dairy Science*, *83*, 2173-2183.

Fenelon, M. A., Beresford, T. P. & Guinee, T. P. (2002). Comparison of different bacterial culture systems for the production of reduced-fat Cheddar cheese. *International Journal of Dairy Technology*, *55*, 194-203.

Forde, A. (2016). 12 months on from milk quotas – what's changed? Retrieved from http://www.agriland.ie/farming-news/12-months-on-from-milk-quotas-whats-changed/ on 29.10.16.

Fox, P. F. (1963). Potentiometric determination of salt in cheese. *Journal of Dairy Science*. *46*, 744‑745.

Fox, P. F. (2002). Biochemistry of cheese ripening. In: Fox, P. F., McSweeney, P. L. H., Cogan, T. M. & Guinee, T. P. (Eds). *Cheese: Chemistry, Physics and Microbiology: General Aspects*, 3rd Ed., *Vol 1*, pp. 320-326. Elsevier Ltd., London, UK.

Fox, P. F. & Wallace, J. M. (1997). Formation of flavour compounds in cheese. *Advances in Applied Microbiology*, *45*, 17-86.

Fox, P. F., Guinee, T. P., Cogan, T. M. & McSweeney, P. L. H. (2000). *Fundamentals of Cheese Science*. Gaithersburg, MD: Aspen publishers.

Frohlich-Wyder, M. T. & Bachmann, H. P. (2007). What are the causes of the most common flavour defects of Swiss cheese? In: McSweeney, P. L.H. (Ed).

Cheese Problems Solved, pp. 263-264. Woodhead Publishing Limited., Cambridge, England.

Ge, Q., Almena-Aliste, M. & Kindstedt, P. S. (2002). Reversibility of pH-induced changes in the calcium distribution and melting characteristics of Mozzarella cheese. *Australian Journal of Dairy Technology*, *57*, 3-9.

Guinee, T. P., Auty, M. A. & Fenelon, M. A. (2000). The effect of fat content on the rheology, microstructure and heat-induced functional characteristics of Cheddar cheese. *International Dairy Journal*, *10*, 277-288.

Guinee, T. P. & McSweeney, P. L. H. (2006). Significance of milk fat in cheese. In: P. F. Fox and P. L. H. McSweeney (Eds.). *Advanced Dairy Chemistry, Volume 2, Lipids*, pp. 377-440. Springer: New York, USA.

Hammond, L. A. (1979). Revival of a short method of manufacturing Cheddar cheese. In *First Biennial Marschall International Cheese Conference* (pp. 495–502). Madison, WI, USA.

Hassan, A., Johnson, M. E. & Lucey, J. A. (2004). Changes in the proportions of soluble and insoluble calcium during the ripening of Cheddar cheese. *Journal of Dairy Science*, *87*, 854-862.

Hayes, M. G., Oliveira, J. C., McSweeney, P. L. H. & Kelly, A. L. (2002). Thermal inactivation of chymosin during cheese manufacture. *Journal of Dairy Research*, *69*, 269-279.

Hou, J., McSweeney, P. L. H., Beresford, T. P., & Guinee, T. P. (2014). Effect of curd washing on the properties of reduced-calcium and standard-calcium Cheddar cheese. *Journal of Dairy Science*, *97*, 5983-5999.

Hynes, E. R., Aparo, L. & Candioti, M. C. (2004). Influence of residual milk-clotting enzyme on α-$_{s1}$ casein hydrolysis during ripening of Reggianito Argentino cheese. *Journal of Dairy Science*, *87*, 565-573.

IDF (1982). Cheese and processed cheese. Determination of the total solids content. Standard 4A:1982. Brussels, Belgium: International Dairy Federation.

IDF (1986). Determination of the nitrogen content (Kjeldahl Method) and calculation of cude protein content. Standard 20A:1986. Brussels, Belgium: International Dairy Federation.

IIRS (1955). Determination of the percentage of fat in cheese. Irish Standard 69:1955. Institute for Industrial Research and Standards, Dublin.

Johnson, M. E. & Lucey, J. A. (2006). Calcium: A key factor in controlling cheese functionality. *Australian Journal of Dairy Technology*, *61*, 147-153.

Joshi, N. S., Muthukumarappan, K. & Dave, R. I. (2003). Understanding the role of calcium in functionality of part skim Mozzarella cheese. *Journal of Dairy Science*, *86*, 1918-1926.

Kilcawley, K. N., Wilkinson, M. G. & Fox, P. F. (2001). A survey of lipolytic and glycolytic end-products in commercial Cheddar enzyme-modified cheese. *Journal of Dairy Science*, *84*, 66-73.

Kuchroo C. N. & Fox P. F. (1982). Soluble nitrogen in Cheddar cheese: comparison of extraction procedures. *Milchwissenschaft*, *37*, 331-335.

Langsrud, T. (1974). *Proline production in propionibacteria*. PhD Thesis, Iowa State University, Iowa, USA. Retrieved from http://lib.dr.iastate.edu/cgi/viewcontent.cgi?article=7351&context=rtd on 11.10.16.

Lawlor, J. B., Delahunty, C. M., Wilkinson, M. G. & Sheehan, J. J (2002). Relationships between the gross, non-volatile and volatile compositions and the sensory attributes of eight hard-type cheeses. *International Dairy Journal*, *12*, 493-509.

Lawrence, R. C., Heap, H. A. & Gilles, J. (1984). A controlled approach to cheese technology. *Journal Dairy Science*, *67*, 1632-1645.

Lucey, J. A. & Fox, P. F. (1993). Importance of calcium and phosphate in cheese manufacture: a review. *Journal of Dairy Science*, *76*, 1714-1724.

Lucey, J. A., Johnson, M. E. & Horne, D. S. (2003). Invited review: perspectives on the basis of the rheology and texture properties of cheese. *Journal of Dairy Science*, *86*, 2725-2743.

Lucey, J. A., Mishra, R., Hassan, A. & Johnson, M. E. (2005). Rheological and calcium equilibrium changes during the ripening of Cheddar cheese. *International Dairy Journal*, *15*, 645-653.

Lucey, J. A. (2008). Some perspectives on the use of cheese as a food ingredient. *Dairy Science and Technology*, *88*, 573–594

McSweeney, P. L. H. (2004). Biochemistry of cheese ripening: Introduction and overview. In: Fox, P. F., McSweeney, P. L. H., Cogan, T. M. & Guinee, T. P. (Eds). *Cheese: Chemistry, Physics and Microbiology: General Aspects*, 3rd Ed., *Vol 1*, pp. 347-360. Elsevier Ltd., London, UK.

McSweeney, P. L. H. & Sousa, M. J. (2000). Biochemical pathways for the production of flavour compounds in cheeses during ripening: A review. *Le Lait*, *80*, 293-324.

Mounier, J., Rea, M. C., O'Connor, P. M., Fitzgerald, G. F. & Cogan, T. M. (2007). Growth characteristics of *Brevibacterium*, *Corynebacterium*, *Microbacterium* and *Staphylococcus* spp. isolated from surface-ripened cheese. *Applied and Environmental Microbiology*. *73*, 7732-7739.

O'Mahony, J. A., Lucey, J. A. & McSweeney P. L. H. (2005). Chymosin-mediated proteolysis, calcium solubilisation, and texture development during the ripening of Cheddar cheese. *Journal of Dairy Science*. *88*, 3101‑3114.

Piveteau, P. G., Condon, S. & Cogan, T. M. (1995). Interactions between lactic and propionic acid bacteria. *Lait*, *75*, 331–343.

Pastorino, A. J., Hansen, C. L. & McMahon, D. J. (2003). Effect of pH on the chemical composition and structure-function relationships of Cheddar cheese. *Journal of Dairy Science*, *86*, 2751-2760.

Radford, D. R. & Hull, R. R. (1982). Utilization of carbohydrates in "short method" Cheddar cheese by mesophilic starters. *Australian Journal of Dairy Technology*, *37*, 104–106.

Reddy, K. A. & Marth, E. H. (1993). Lipolysis in Cheddar cheese made with sodium chloride, potassium chloride or mixtures of sodium and potassium chloride. *Milchwissenschaft*, *48*, 488-493.

Sheehan, J. J. & Wilkinson, M. G. (2002). Novel and specialty cheeses - Broadening the national industrial cheese base. Sandymount Avenue, Dublin, Ireland: Teagasc.

Sheehan, J. J., Fenlon, M. A., Wilkinson, M. G. & McSweeney, P. L. H. (2007). Effect of cook temperature on starter and non-starter lactic acid bacteria viability, cheese composition and ripening indices of a semi-hard cheese manufactured using thermophilic cultures. *International Dairy Journal*. *17*, 704-716.

Sheehan, J. J., Wilkinson, M. G. & McSweeney, P. L. H. (2008). Influence of processing and ripening parameters on starter, non-starter and propionic acid bacteria and on the ripening characteristics of semi-hard cheeses. *International Dairy Journal*, *18*, 905-917.

Sheehan, J. J. (2013). Milk quality and cheese diversification. *Irish Journal of Agricultural and Food Research*, 243-253.

Sousa, M. J., Ardo, Y. & McSweeney, P. L. H. (2001). Advances in the study of proteolysis in cheese during ripening. *International Dairy Journal*, *11*, 327-345.

Upadhyay, V. K., McSweeney, P. L. H., Magboul, A. A. A. & Fox, P. F. (2004). Proteolysis in cheese during ripening In: Fox, P. F., McSweeney, P. L. H., Cogan, T. M. & Guinee, T. P. (Eds). *Cheese: Chemistry, Physics and Microbiology: General Aspects*, 3rd Ed., *Vol 1*, pp. 391-433. Elsevier Ltd., London, UK.

Valence, F., Deutsch, S. M., Richoux, R., Gagnaire, V. & Lortal, S. (2000). Autolysis and related proteolysis in Swiss cheese for two *Lactobacillus helveticus* strains. *Journal of Dairy Research*, *67*, 261-271.

Walsh, A. M., Crispie, F., Kilcawley, K., O'Sullivan, O., O'Sullivan, M. G., Claesson, M. J. & Cotter, P. D. (2016). Microbial succession and flavour production in the fermented dairy beverage kefir. *mSystems*, *1*, e00052-16. doi:10.1128/mSystems.00052-16.

Wilkinson, M. G., Sheehan, D. & Kelly, M. C. (1997). Production of non-Cheddar varieties of cheese—The Moorepark experience. In T. M. Cogan, P. F. Fox, & R. P. Ross (Eds.), *Proceedings of the Fifth Cheese Symposium* (pp. 133–140). Sandymount Avenue, Dublin, Ireland: Teagasc.

Wilkinson, M. G., Meehan, H. F. & Guinee, T. P. (2000). Cheese diversification into specialty cheeses: Approaches and models in Ireland and New Zealand. *Farm and Food*, *10*, 19–24.

Wilkinson, M. G. (2007). Lipolysis and cheese flavour development. In: Weimer, B. C. (Ed), *Improving the Flavour of Cheese*, pp. 102-120. Woodhead Publishing Limited, Cambridge, England.

Woo, A. H., Kollodge, S. & Lindsay, R. C. (1984). Quantification of major free fatty acids in several cheese varieties. *Journal of Dairy Science*, *67*, 874-878.

Wood, A. F., Aston, J. W. & Douglas, G. K. (1985). The determination of free amino acids in cheese by capillary column gas liquid chromatography. *Australian Journal of Dairy Technology*, *40*, 166–169.

Chapter 8

Conclusions and Recommendations

8.1 Overall Conclusions

Cheese ripening involves numerous microbiological, biochemical and physicochemical changes, many of which are interrelated. Collectively, these changes are responsible for the conversion of the rubbery, bland young cheese into a mature cheese with characteristic flavour, texture and aroma (Fox and McSweeney, 1998; Lucey *et al*., 2003). It is well established that calcium (Ca) significantly contributes to the overall textural and rheological attributes of cheese. It has been well recognised that total calcium levels in cheese play a major role in modulating the textural, functional and rheological properties of several cheese varieties (Hassan *et al*., 2004; Lucey *et al*., 2005; O'Mahony *et al*., 2005). Ca content varies between cheese varieties due to their unique manufacturing procedures; in particular, the pH at whey drainage has a major influence on the final Ca content of cheese (Lucey and Fox, 1993). However, it has been suggested that total Ca alone is not the most accurate predictor of the textural and functional properties of cheese, but rather the distribution of insoluble (INSOL) and soluble (SOL) calcium in the cheese during ripening (Lucey and Fox, 1993).

Much of the work pertaining to the study of the effects of total Ca concentration, distribution of Ca between INSOL and SOL forms (Ca equilibrium), or both in determining textural and rheological properties of cheese has been largely focused on dry-salted Cheddar (Creamer *et al*., 1985; Lee *et al*., 2005; O'Mahony *et al*., 2005), Colby (Lee *et al*., 2010, 2011) and Mozzarella (Metzger *et al*., 2000; Joshi *et al*., 2003; Sheehan and Guinee, 2004) cheese varieties, and to a much lesser extent on

brine-salted varieties. Consequently, little knowledge exists with regards to the influence of these factors on the textural properties of brine-salted continental cheese varieties such as Gouda or Swiss-Cheddar varieties.

The abolition of Irish milk quotas in 2015 has provided dairy researchers and cheese manufactures alike with the opportunity to explore new avenues and ideas for the diversification of existing cheese product portfolios on already existing cheese plants. The manipulation of Ca equilibrium within the cheese matrix under controlled conditions offers considerable potential in the development of novel cheese varieties with enhanced textural and functional properties. The improvement in the rheological, melting and textural properties of cheese (largely Cheddar and Mozzarella varieties only) has been attributed to several mechanisms including composition (moisture, salt, S/M ratio, fat and protein), pH during manufacture and ripening, total and INSOL Ca content, proteolysis, curd washing technique and acidification methods. The objectives of the research reported in this thesis were to develop and comprehensively characterise the effects of manipulating cheese pH, moisture content and Ca equilibrium on the textural, functional and rheological properties of brine-salted continental-type and Swiss-Cheddar hybrid-type cheese varieties.

The studies described within have facilitated comprehensive, novel investigations into the impact of changing manufacturing protocol (cooking temperature), composition (moisture content), pH and addition of Ca chelating salt (EDTA) on the textural and functional properties of Gouda and Swiss-Cheddar hybrid cheese

varieties. Some of the key findings from the work completed as part of this thesis may be summarised as follows;

In the first part of this thesis the effects of direct addition of the calcium chelating salt EDTA and decreased cooking temperature, on the textural and rheological properties of Gouda-type cheese (*Chapter 3*) were investigated. Preliminary trials demonstrated that direct addition of EDTA to the curds/whey mixture during Gouda-type cheese manufacture resulted in a dramatic decrease in the pH value of the cheese and this decrease increased with increasing levels of EDTA addition. Consequently, cheeses containing addition levels > 7.5 g EDTA/L whey removed, presented an inability for curd pieces to fuse together, and this was attributed to the low pH values of these cheeses. The addition of EDTA to Gouda-type cheese resulted in a significant decrease in the levels of CN-bound calcium as did decreasing the temperature at which the cheese was cooked (31 ˚C rather than 38 ˚C); however, this effect was greatest for the cheese treated with the highest level of EDTA. Significant losses of EDTA in the whey were observed (~ 90%), suggesting that a more appropriate method of EDTA addition should be employed in order to avoid losses and reduce cost. Cheese treated with the highest level of EDTA exhibited the greatest % increase in meltability throughout ripening. While, textural properties appeared to be influenced more by the moisture content on the cheese; higher the moisture content the softer the cheese. It is proposed that the textural and functional properties of brine-salted Gouda-type cheeses in this study are influenced to an equal extent by the changing levels of INSOL calcium as well as the moisture levels of the cheese. However, increasing the moisture content of cheese may not be the best approach for

modifying the textural and functional properties of cheese for a number of reasons; (1) legal limitations regarding the amount of water present in the food is necessary for producing specific products (i.e., < 40% for Cheddar cheese); (2) microbial activity is increased in food materials that have high levels of available moisture, therefore making the product more susceptible to the microbial attack (reduced shelf life, poor ripening).

A novel method was developed for the incorporation of EDTA into brine-salted cheese (*Chapter 4*) in a bid to avoid changing the pH and composition which occurs during direct attrition of EDTA to Gouda-type cheese (*Chapter 3*). EDTA was successfully entrapped within two different liposome preparations (ProLipo C and ProLipo Duo) using three different processing technologies (Ultra-Turrax, two-stage homogenisation and microfluidisation), with all preparations exhibiting good stability over 28 d storage (4 ˚C). EDTA entrapped within ProLipo C demonstrated superior entrapment efficiencies compared with ProLipo Duo manufactured under the same conditions; thus ProLipo C was supplemented into milk during the manufacture of miniature Gouda-type cheese. A higher concentration of EDTA was found to be trapped within the curd in this study (~39%) when compared with cheeses manufactured with direct addition of EDTA to the curds/whey mixture (*Chapter 3*), where only ~ 10% of the EDTA originally added was retained in the curd. Addition of liposome-entrapped EDTA did not affect the pH value or composition of cheese, unlike in *Chapter 3* when the EDTA was added directly to the curd/whey mixture; resulting in dramatic compositional changes to the cheese.

As a follow on from *Chapters 3* and *4*, *Chapter 5* investigated the influence of liposome-entrapped EDTA on the Ca equilibrium of brine-salted Gouda-type cheese and its subsequent effect on the textural and rheological properties of the cheese. The addition of liposome-entrapped EDTA resulted in a reduction in the concentration of INSOL Ca within cheese, while pH, gross composition and proteolysis patterns of cheese remained unaffected (unlike *Chapter 3*). The textural properties hardness, chewiness and gumminess were significantly lower in cheese containing liposome-entrapped EDTA, particularly during the early stages of ripening (first 28 d). Cheese containing liposome-entrapped EDTA exhibited a greater propensity to flow and melt when heated; indicated by both the Schreiber and DSAOR (higher LT_{max}) analysis. The results of this study highlight the potential of liposomes to incorporate calcium chelating salts such as EDTA to cheese milk during the production of brine-salted Gouda-type cheese; and it is therefore proposed that incorporation of liposomes containing EDTA into milk during Gouda-type cheese making alters the distribution of Ca between the INSOL and SOL forms, thus modifying its structure, texture and functionality, without impacting negatively on the pH or composition of the cheese.

Proteolysis, differences in type of Ca (concentration of SOL and INSOL Ca) and the textural and rheological properties of cheese were determined in *Chapter 6* to evaluate if textural and rheological changes still occur when chymosin-mediated hydrolysis of α_{s1}-CN is completely inhibited, as is the case in dry-salted cheeses. At the highest addition level of pepstatin used, 10.0 μmol/L curds and whey mixture, chymosin-mediated proteolysis was almost completely inhibited; allowing for the effects of proteolysis and calcium equilibrium on the textural and rheological

properties of Gouda-type cheese to be evaluated independently of one another. It was determined that hydrolysis of α_{s1}-CN at Phe_{23}-Phe_{24} was not a prerequisite for the early softening of Gouda-type cheese texture. Instead, the reduction in cheese hardness during early ripening was more associated with the decrease in the levels of insoluble calcium than with the levels of intact α_{s1}-CN, although this correlation was not as strong as that described by O'Mahony *et al*. (2005) for Cheddar cheese. The observed changes to the rheological properties (DSAOR) of cheese during ripening were more highly correlated with the levels of intact α_{s1}-CN, in particular during the latter stages of ripening (after 28 d). Additionally, the observed changes to Gouda-type cheese texture (i.e., softening) that occurs during the first month of ripening occur without hydrolysis of α_{s1}-CN at Phe_{23}-Phe_{24} and therefore we can conclude that this softening of texture is largely due to the solubilisation of some residual CCP associated with the *para*-CN matrix of the cheese. This being said, it is not our desire to definitively dismiss the role of α_{s1}-CN hydrolysis in the development of the textural properties of Gouda-type cheese, but merely to encourage research interest in the role of physicochemical processes leading to the development of cheese texture and rheology.

A Swiss-Cheddar hybrid-type cheese has the potential to be easily implemented into most existing cheese plants in order to diversify a cheese manufactures portfolio, with little need for major investment (i.e., can be produced in an existing plant, no new equipment/staff needed). Therefore, it is worthwhile investigating the development of proteolysis, lipolysis and Ca equilibrium in a Swiss-Cheddar-hybrid cheese and its influence on the volatile flavour profile and textural changes throughout ripening

(*Chapter 7*). The characteristic eye development associated with Swiss-type cheese did not occur, owing to the low ripening temperature (9 ˚C) and high NaCl concentration of the cheese which does not encourage the growth of PAB which are responsible for the acid development needed to produce the eye. Additionally, the milled curd structure of the cheese due to "cheddaring" may have also encouraged CO_2 gas loss through the cracks of the curd. The concentration of INSOL Ca decreased as ripening progressed up to 112 d, as expected, and then appeared to increase slightly for the remainder of ripening (224 d), indicating the possible reformation of INSOL Ca, likely due to the increase in pH values of cheese throughout ripening. The development of changes to the textural, rheological and functional properties of Swiss-Cheddar-hybrid cheese was very limited; no significant decrease in the firmness of Swiss-Cheddar cheese during ripening and meltability increased slightly throughout 224 d of ripening, with the greatest increase occurring between 56 and 224 d of ripening; suggesting that INSOL Ca may be reforming causing an increase in the strength of the CCP crosslinks, counteracting the effects of proteolysis.

In summary, the studies reported in this thesis have generated new insights into the relationships between mineral equilibria of cheese and the proposed mechanisms of rheological properties of bine-salted continental-type cheese varieties. Prior to these studies, little was known about the distribution of calcium between the SOL and INSOL Ca phases in brine-salted cheeses. This research has shown that manipulating the calcium equilibrium of brine-salted cheese is an important method of modulating its textural, rheological and functional properties, similar to that of dry-salted

variants. This work also highlights the potential for the use of liposomes as suitable carrier systems for the incorporation of Ca chelating salts into brine-salted cheese systems. Such outputs provide a better understanding of how manipulation of the Ca equilibrium which exists in Gouda and Swiss-Cheddar hybrid cheese varieties may be applied industrially in order to maximise cheese production and profitability.

8.2 Recommendations for Future Research

Follow-up studies that would be complimentary to the work presented in this thesis include:

8.2.1 Novel methods for the incorporation of Ca chelating salts into brine-salted cheese

In order to alleviate the effects of direct addition of the Ca chelating agent EDTA on the pH and composition of brine-salted cheese, future work is needed to develop an alternative method for its incorporation into cheese. The use of liposomes presented in this thesis (*Chapters 4* and *5*) has provided evidence that the encapsulation/entrapment of EDTA allows brine-salted cheese to be manufactured with varying levels of INSOL Ca, without impacting on the pH or composition of the cheese. However, the viability of such a method at an industrial/commercial level is questionable, owing to the significant increase in cost associated with the liposomes. Therefore, further scope exists to investigate and determine if other encapsulation/entrapment methods would be suitable for this application (addition of

Ca chelating salts to natural cheese), for example, emulsion-based entrapment systems. As well as this, further research into the use of milk proteins (Augustin and Oliver, 2104) for the encapsulation/entrapment of EDTA or other Ca chelating salts would be beneficial.

8.2.2 Independent evaluation of the multiple factors influencing the textural and rheological factors of brine-salted cheese varieties

It is essential to attempt to separate the multiple factors (pH, moisture, calcium and proteolysis) influencing the textural and rheological properties of brine-salted cheese in order to determine precisely which factor, or what combination of factors, are responsible for the changing texture of cheese throughout ripening (*Chapters 3, 5* and *6*). A targeted approach to identifying such factors individually may allow researchers and manufactures alike to conclude new hypotheses and in turn develop novel methods for controlling such factors. It is proposed that brine-salted Gouda-type cheese should be manufactured (1) in which both chymosin- and plasmin-mediated proteolysis are totally inhibited in a bid to elucidate fully the relationships between solubilisation of Ca, proteolysis and the development of the textural and rheological properties of brine-salted cheese; (2) with varying levels of INSOL Ca, through the addition of Ca chelating salts but with constant moisture content (adjusted using different cook temperatures) and pH values (adjusted with dilute lactic acid) (Choi *et al*., 2008).

8.2.3 Optimise UF and DF of liposome preparations

The entrapment efficiency (%) of EDTA within liposomes after ultrafiltration (UF) and diafiltration (DF) with de-ionised have been found to be as low as 21-49% (*Chapters 4* and *5*). It has been suggested (McAuliffe *et al*., 2016) that the difference in osmotic pressure between the de-ionised water used during UF and DF, and the 25 mM HCl in which the liposomes were prepared, may have caused the liposomes to swell and burst. It is therefore recommended that further investigation regarding this phenomenon be undertaken; during UF and DF of liposomes that instead of using de-ionised water, the buffer in which the liposomes were prepared should be used. This may lead to increased entrapment efficiencies of EDTA within the liposomes as the risk of osmotic swelling and subsequent bursting could be avoided, providing researchers and manufactures with better knowledge of this system.

8.2.4 Establish liposome release kinetics in cheese throughout ripening

It is essential from a commercial perspective to definitively know the precise release kinetics of an active ingredient from liposome preparations, firstly during storage of the preparation prior to incorporation into cheese, and secondly during the manufacture and ripening of cheese. During this research, there were limitations with regards to fully elucidating exactly how and when the liposomes ruptured, releasing the EDTA into the cheese matrix and the milk fat globule membrane (MFGM). Further scope exists to determine the exact location (i.e., partitioned between casein) (Laridi *et al*., 2003) and number of liposomes within the cheese matrix using

microscopy techniques (i.e., dynamic light scattering (DLS), transmission electron microscopy (TEM), scanning electron microscopy (SEM) and atomic force microscopy (AFM) at several time points throughout ripening. Another possible avenue of research to consider would be to determine the release kinetics of the liposomes under different storage/ripening conditions. Liposomes may have specific applications in several different varieties of cheeses ripened at different ripening conditions; Swiss-type cheese is ripened at ~ 20-24 ˚C, while a typical Cheddar cheese is ripened at conditions of ~ 9-12 ˚C,

8.2.5 Comprehensive study on the microbiological effects of EDTA on cheese and consumer acceptability

EDTA has proven its ability to influence the texture of cheese (Brickley *et al.*, 2009) but it has also been found to contain antimicrobial properties (Reidmiller *et al*., 2006). Substantial microbiological evaluation of cheese supplemented with EDTA should be completed. Any change (slight or major) to cheese microbiology can have a serious impact on proteolysis and flavour development within the cheese. From a consumer perspective taste is the most import factor (Glanz *et al*., 1998; Verbeke, 2005), while manufactures consider food safety paramount. For these reasons, further commercial scope exists to fully elucidate the microbiological effects of EDTA addition to brine-salted Gouda-type cheese.

As a possible follow on study, consumer acceptance panels evaluating brine-salted Gouda-type cheese manufactured with food-grade EDTA-entrapped liposomes

warrants investigation, in order to determine their influence on sensory properties of the cheese and consumer acceptability of the product.

8.2.6 Further in-depth study on Swiss-Cheddar hybrid cheese varieties

From a commercial perspective, there is little knowledge on the exact pathways responsible for the production of volatile flavour compounds in Swiss-Cheddar hybrid cheese throughout ripening. This research (*Chapter 7*) has highlighted that discrepancies in cheese flavour appear to become an issue in late ripening. Further scope exists to attempt to fully elucidate the factors responsible for these discrepancies and sensory analysis is critical to determine consumer acceptability. Additionally, current research (*Chapter 7*) has brought to light the apparent reformation of INSOL Ca during extended ripening likely due to increasing pH; the changes in the distribution of calcium between the SOL and INSOL phase of Swiss-Cheddar cheese therefore needs further investigation to fully clarify the exact mechanism behind this reformation of INSOL Ca in Swiss-Cheddar hybrid cheese.

References

Augustin, M. A. & Oliver, C. M. (2014). Use of milk proteins for encapsulation of food ingredients. *Microencapsulation in the Food Industry: A Practical Implementation Guide*, 211-226.

Brickley, C. A., Lucey, J. A. & McSweeney, P. L. H. (2009). Effect of the addition of trisodium citrate and calcium chloride during salting on the rheological and textural properties of Cheddar-style cheese during ripening. *International Journal of Dairy Technology*, *62*, 527-534.

Choi, J., Horne, D. S., Johnson, M. E. & Lucey, J. A. (2008). Effects of the concentration of insoluble calcium phosphate associated with casein micelles on the functionality of directly acidified cheese. *Journal of Dairy Science*, *91*, 513-522.

Creamer, L. K., Lawrence, R. C. & Gilles, J. (1985). Effect of acidification of cheese milk on the resultant Cheddar cheese. *New Zealand Journal of Dairy Science and Technology*, *20*, 185–203.

Glanz, K., Basil, M., Maibach, E., Goldberg, J. & Snyder, D. A. N. (1998). Why Americans eat what they do: taste, nutrition, cost, convenience, and weight control concerns as influences on food consumption. *Journal of the American Dietetic Association*, *98*, 1118-1126.

Iwata, T., Kimura, Y., Tsutsumi, K., Furukawa, Y. & Kimura, S. (1993). The effect of various phospholipids on plasma lipoproteins and liver lipids in

hypercholesterolemic rats. *Journal of Nutritional Science and Vitaminology*, *39*, 63-71. doi:10.3177/jnsv.39.63.

Jimenez, M. A., Scarino, M. L., Vignolini, F. & Mengheri, E. (1990). Evidence that polyunsaturated lecithin induces a reduction in plasma cholesterol level and favorable changes in lipoprotein composition in hypercholesterolemic rats. *Journal of Nutrition*, *120*, 659-667.

Joshi, N. S., Muthukumarappan, K. & Dave, R. I. (2003). Understanding the role of calcium in functionality of part-skim Mozzarella cheese. *Journal of Dairy Science*, *86*, 1918–1926.

Laridi, R., Kheadr, E. E., Benech, R. O., Vuillemard, J. C., Lacroix, C. & Fliss, I. (2003). Liposome encapsulated nisin Z: optimization, stability and release during milk fermentation. *International Dairy Journal*, *13*, 325-336.

Lee, M. R., Johnson, M. E. & Lucey, J. A. (2005). Impact of modifications in acid development on the insoluble calcium content and rheological properties of Cheddar cheese. *Journal of Dairy Science*, *88*, 3798–3809.

McAuliffe, L. N., Kilcawley, K. N., Sheehan, J. J. & McSweeney, P. L. (2016). Manufacture and incorporation of liposome-entrapped ethylenediaminetetraacetic acid into model miniature Gouda-type cheese and subsequent effect on starter viability, pH, and moisture content. *Journal of Food Science*, *81*, C2708-C2701.

Metzger, L. E., Barbano, D. M., Rudan, M. A. & Kindstedt, P. S. (2000). Effect of milk preacidification on low fat Mozzarela cheese: 1. Composition and yield**.** *Journal of Dairy Science*, *83*, 646–658.

O'Mahony, J. A., Lucey, J. A. & McSweeney, P. L. H. (2005). Chymosin-mediated proteolysis, calcium solubilization and texture development during the ripening of Cheddar cheese. *Journal of Dairy Science*, *88*, 3101–3114.

Reidmiller, J. S., Smith, W. L., Sawyer, M. M., Osburn, B. I., Stott, J. L. & Cullor, J. S. (2006). Antimicrobial properties of the chelating agent EDTA on Streptococcal bovine mastitis isolates. *Journal of Food Protection*, *69*, 1460-1462.

Sheehan, J. J. & Guinee, T. P. (2004). Effect of pH and calcium level on the biochemical, textural and functional properties of reduced-fat Mozzarella cheese. *International Dairy Journal*, *14*, 161–172.

Verbeke, W. (2005). Consumer acceptance of functional foods: socio-demographic, cognitive and attitudinal determinants. *Food Quality and Preference*, *16*, 45-57.

Appendix