Assessment of structure and component mobility within Mozzarella cheese

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Abstract

The objective of this study was to identify mechanisms responsible for component mobility relating to structural change within Mozzarella cheese. The use of new techniques alongside well established methods allowed insights to be gained beyond the current understanding of the dynamics within Mozzarella. A number of processing and storage trials were conducted utilising a range of techniques to gain a multi-scale indication of changes in component mobility and structural reorganisation.

Dielectric spectroscopy was explored as a method to characterise both ion and water mobility. Initially a model system was utilised as a means of evaluating the technique prior to being applied to Mozzarella. The model system allowed the composition of the cheese to be systematically controlled, especially the calcium. However, subsequent trials (in real Mozzarella cheese systems) indicated that water movement within the cheese during both maturation and heating confound the dielectric response, indicating the method is not ideally suited to measuring a dynamic Mozzarella system.

Nuclear magnetic resonance was used to probe changes in component mobility within Mozzarella. Initially well-established relaxation methods were used to monitor the decrease in free water within Mozzarella following manufacture. However, after raising the question of the effect of temperature on free water in cheese, relaxation and diffusion measurements were employed as tools to gain an understanding of the dynamics of water movement within the porous cheese structure. This work was extended further by using these techniques to follow a newly manufactured Mozzarella through a storage trial. The relaxation and diffusion measurements were taken at a range of temperatures at each point of the trial. Phosphorus NMR was explored as a novel approach to monitor changes in the arrangement of calcium and phosphorus (as phosphate) within Mozzarella during storage. This in combination with additional techniques characterising structural changes allowed potential mechanisms for the solubilisation of CCP to be discussed.

Collectively these techniques found that Mozzarella undergoes a number of structural changes during storage. Two primary drivers for change were identified from which the other processes cascaded: changing strength in hydrophobic interactions and proteolytic breakdown. Initially the development of the cheese structure was driven primarily by a relaxation in protein matrix (caused by weakening hydrophobic interactions), resulting in the moisture equilibration processes through the associated impact on colloidal calcium phosphate solubility and thus protein-protein interactions. Further structural changes occurred as a result of the proteolytic breakdown of the casein and a possible relaxation in the protein structure. These proteolytic mechanisms dominated maturation behaviour after the moisture equilibration processes were substantially completed (typically >20 days).

This thesis revealed new information relating to the movement of components within Mozzarella, particularly at elevated temperatures. These insights will aid in building a more detailed understanding of the dynamics within Mozzarella. It also highlighted several techniques that show promise as potential tools for assessing the structural changes within Mozzarella cheese.

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Contents

Symbol / Abbreviation	Meaning
ε*	Complex permittivity
ε_0	Permittivity of free space
ε'	Dielectric constant
ε”	Dielectric loss factor
G’	Storage modulus
G”	Loss modulus
Tanδ	Loss tangent
η*	Complex viscosity
ω_0	Larmor frequency
ɣ	Gyromagnetic ratio
B_0	Magnetic field
I	Proton intensity
A	Proton intensity of casein associated water
B	Proton intensity proportional to free water
T	time
T_1	Longitudinal (spin-lattice) relaxation
T_2	Transverse (spin-spin) relaxation
NMR	Nuclear Magnetic Resonance
MRM	Magnetic resonance microscopy
D_0	Free diffusion
A	Tortuosity
T	Time
$(\frac{S}{V_p})$	Surface to volume ratio
Θ	Time constant (Pade fitting parameter)
D(t)	Diffusion as a function of time
MAS ^{31}P NMR	Magic angle spinning phosphorous (31) nuclear magnetic resonance
CLSM	Confocal laser scanning microscope
TPA	Texture profile analysis
SEM	Scanning electron microscopy
Δ	Observation times
Δ	Pulse duration

1 Introduction

The dairy industry in New Zealand makes up a significant proportion of the export revenue for the country. Cheese plays an essential part of the product mix, contributing to the export revenue of the country. Mozzarella cheese is produced in large quantities, over 50,000 tonnes, within New Zealand constituting a large proportion of the cheese manufactured in the country.

Gaining an intricate knowledge of the structure of Mozzarella and how components move within the structure of the cheese will allow manufacturing and storage conditions to be manipulated. This may enable the optimisation of processing conditions as well as the functional properties of the cheese.

The main aim of this thesis is to gain an understanding of how the structure of low moisture part skim (LMPS) Mozzarella evolves during storage. A multi-scale approach to evaluate the structure of Mozzarella at different length scales was conducted using a wide range of techniques, both well established and relatively novel. Although the functional properties of Mozzarella are of great importance to the dairy industry, this thesis focuses on the structure of the cheese rather than functionality. Mozzarella is a dynamic system which undergoes structural rearrangement following manufacture and in its end use as a pizza topping. Gaining insights into these changes is critical in the development of a deeper understanding of the cheese system. Such knowledge is key to commercially relevant activities such as quality control and product development.

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The research questions arising that are addressed in this work are:

- Can techniques capable of studying component mobility, including dielectric spectroscopy & NMR, be applied to Mozzarella during storage and at elevated temperatures to expand on the current understanding of structural changes within the cheese?

- What are the key drivers responsible for the changes in structure in Mozzarella during the maturation process?

- What effect does heating have on water mobility within Mozzarella cheese?

- Do variations in calcium content within Mozzarella affect water mobility?

2 Literature Review

2.1 Introduction

Cheese is one of the most consumed dairy products in the world and is considered to be relatively nutritious due to its high protein and calcium contents (Reid & Yan, 2004). Approximately a third of the milk produced in the world is used in the manufacture of cheese (Farkye, 2004). It presents a method of preserving milk for a longer period of time than the fresh liquid (Everett, 2007).

Mozzarella cheese is one of the most consumed cheeses worldwide (Francolino, Locci, Ghiglietti, Iezzi, & Mucchetti, 2010). In the US in 2008 Mozzarella was the most consumed cheese with a consumption of 10.7 pounds per capita (4.85 kg). Mozzarella plays a large role in a considerable number of world markets, in particular the North American market (Eliot, Vuillemard, & Emond, 1998). It is common for cheese plants to manufacture in excess of 100,000 kg of pizza cheese daily (Kindstedt, Caric, & Milanovic, 2004). This scale of manufacture has led to a need for precise control over the manufacturing process as well as an in depth understanding of how changes in the process impact on the structure, functionality and the composition of the cheese.

Originating in Italy, Mozzarella cheese has evolved from a regional cheese of southern Italy (Rankin, Chen, Sommer, & Esposito, 2005) to being one consumed internationally. Traditional Mozzarella cheese is made from buffalo milk (Kindstedt, 1993a). However, the majority of the Mozzarella produced today is manufactured from pasteurised, part skimmed bovine milk (Fox *et al.,* 2000). Mozzarella is a member of the Pasta filata, or stretched curd, family of cheeses. The stretching of the curd gives the cheese it a unique fibrous texture (Kinstedt, 1993; Fox *et al.,* 2000; Ribero, Rubiolo, & Zorrilla, 2009).

Prior to World War II Mozzarella was only consumed in small amounts outside of Italy, however, after the war there was an explosion in the popularity of Mozzarella due to the

demand for Italian style products like pizza (Kindstedt, 1993b). More than 70% of all Mozzarella consumed in the US is used on pizzas (Ferris & Palmiter, 1987; Kindstedt, 1993a). The Mozzarella used as a pizza topping differs significantly to the traditional fresh Mozzarella cheese in both functionality and appearance.

The US has classified Mozzarella into four different categories based on composition: Mozzarella, low moisture Mozzarella, part skim Mozzarella and low moisture part skim Mozzarella (McMahon, Oberg, & McManus, 1993). The Low Moisture Part Skim (LMPS) Mozzarella is the most common variety due to its prominence on foods such as pizzas where it is used for its functional properties.

Table 2-1: Compositional standards for Mozzarella cheese in the US (Kindstedt, 1993a; USFDA, 1989)

Mozzarella Type	*Moisture Range (%)*	*Fat in dry matter (%)*
Mozzarella	>52 to ≤60	≥45
Low moisture Mozzarella	>45 to ≤52	≥45
Part skim Mozzarella	>>52 to ≤60	≥30 to <45
Low moisture part skim Mozzarella	45 to ≤52	≥30 to <45

The most common variation of Mozzarella cheese is that used for cooking purposes, in particular as a topping for pizzas (Walstra, Wouters, & Geurts, 2006). This pizza cheese is often referred to as a low-moisture part-skimmed (LMPS) Mozzarella due to its lower fat and moisture content (Kindstedt, 1993b). The cheese is produced from pasteurised cows' milk with a fat content of about 1.8% and generally a combination of thermophilic cultures such as *Lactobacillus* ssp. and *S. thermophilus* (Fox, Cogan, & Guinee, 2000). Although the majority of the Mozzarella produced worldwide use thermophilic cultures, there are a few exceptions where mesophilic starters are used (Kindstedt et al., 2004). The choice of

starter cultures depend on the acidification rate required in the curd through the cheese making process (Rankin et al., 2005). LMPS Mozzarella differs from table Mozzarella due to its lower fat content and the addition of *Lactobacillus* which is generally not added to table Mozzarella (Fox et al., 2000). The addition of *Lactobacillus* is due to the higher acidification rate needed in pizza Mozzarella to achieve the required moisture content (Kindstedt et al., 2004).

LMPS Mozzarella composition consists of between 30-40% fat to dry weight and a moisture content ranging from 45 to 52% (McMahon et al., 1993). Due to the allowable range in both the fat and moisture contents for LMPS Mozzarella, there is a degree of variation in the functional properties within the classification. Other types of Mozzarella can be used on pizzas, however, there are issues regarding the functional properties for this use. The food service industry rarely uses Mozzarella with moisture contents higher than 52% due to issues regarding shredding, matting and shorter shelf life (Bertola, Califano, Bevilacqua, & Zaritzky, 1996a).

Traditional Italian Mozzarella is a fresh unripened cheese that has a milky taste and is high in moisture (Francolino et al., 2010). However, Mozzarella used as a pizza cheese generally undergoes a period of ripening to attain a desired level of functionality (Kindstedt, 1993b). During the initial period after manufacture, Mozzarella undergoes a complex set of physiochemical changes that affects the structure and functionality of the cheese (Kindstedt, 2004). It differs from most cheeses in the fact that it is consumed in a molten state the majority of the time. Due to this fact, the melting properties of Mozzarella are critical to product performance as well as consumer acceptance (Kindstedt, Rippe, & Duthie, 1989).

2.2 Mozzarella

2.2.1 Processing steps and subsequent structural changes during Mozzarella manufacture

The manufacturing procedure used to produce Mozzarella cheese plays an important role in determining the structure of the product. The processes that the cheese undergoes during manufacture have evolved from relatively crude procedures to incorporating cutting edge technical processes.

The structure of a food product is one of its most important attributes due to it being a major factor in the behaviour and functional properties of the food. Foods have a hierarchical structure from the macro level down to the micro structural level. Each processing step impacts on the structure, and hence functionality, of a product to some extent (Auty, 2002).

2.2.1.1 *Milk*

Milk is a colloidal dispersion of proteins existing in a dynamic equilibrium with fat and lactose distributed in water (Auty, 2002). Bovine milk comprises between 30 and 35 g of protein per litre (Swaisgood, 2003). Bovine milk contains two distinct groups of proteins, the caseins and the whey proteins (Varnam & Sutherland, 2001). The caseins comprise approximately 80% of the protein content in bovine milk and are made up of four principal proteins: α_{s1}, α_{s2}, β and κ casein, in a ratio approximately 40:10:35:12 respectively, along with a number of minor proteins (Fox & McSweeney, 1997). Casein can bind to calcium due to phosphoserine residues present in their structure (Horne, 2002). Each of the different caseins has a different number of phosphoserine residues capable of binding calcium with α_{s1} containing 8, α_{s2} containing 10-13, β casein containing 5 and κ casein containing 1 phosphoserine residue per mole (Guinee & O'Brien, 2010).

Approximately 95% of the casein in bovine milk is present in the form of micelles, colloidal particles (Fox, 1984). These micelles are roughly spherical in shape and range in diameters from 50 to 500 nm (Horne, 2008). Horne (2008) states, casein micelles have a highly hydrated, open structure with levels of water between 2-3 g per gram of protein. The reason that micelles contain more water than they do casein, due to the spaces that exist between the casein particles (Walstra & Jenness, 1984). On a dry basis the micelles are approximately 94% protein with the other 6% being colloidal calcium phosphate (CCP), a collection of small ions mainly comprising calcium and phosphate (Fox, 1984). The amorphous calcium phosphate link to the casein at the caseins ester phosphate groups (van Vliet & Walstra, 1994). α_{s1}, α_{s2} and β casein are all insoluble at the calcium concentration present in milk, thus they arrange in such a way that the calcium soluble κ casein stabilises the micelle (Fox, 1984; Lucey, 2008). This is done in such a way that the micelles have a surface layer rich in κ casein that protrudes into the surrounding medium.

Casein is far more stable in the form of micelles than if they were not organised into this form (Holt & Horne, 1996). This micellar arrangement means that milk is able to withstand physiological changes to a greater extent than many other biological systems. The micelles contain roughly 65% of the 30 mM of calcium present in milk and only 10% is present as free calcium ions (Holt, Davies, & Law, 1986).

There has been much debate surrounding the internal structure of the casein micelles (McMahon & McManus, 1998; Walstra, 1999). A number of different models have been proposed over the last 40 years to explain the structure of the micelles (McMahon & McManus, 1998). The models fall into three main models: the sub-micelle model (Slattery & Evard, 1973; Walstra, 1990), the dual binding model (Horne, 1998, 2002) and the nanocluster model (Dalgleish, 2010; de Kruif & Holt, 2003; Holt, 1992). These models have evolved over time due to the advancement in techniques capable of measuring the structure & compositional makeup of the micelles (McMahon & McManus, 1998). The oldest of these models, and most frequently cited, is the submicelle model (de Kruif,

Huppertz, Urban, & Petukhov, 2012). This model proposed that the micelles were composed of smaller proteaceous subunits, or submicelles (Horne, 2008). The permutations of the submicelle model have included models where the submicelles are variable or constant size, composition and internal structure (Holt, de Kruif, Tuinier, & Timmins, 2003). The proposed models suggest that the submicelles have a hydrophobic interior and hydrophilic exterior and are held together to form casein micelles by calcium phosphate cross bridges or protein interactions (McMahon & McManus, 1998). The dual binding model suggests that the casein molecules are a block of copolymers that form micelles through polymerisation reactions through hydrophobic interactions or across calcium phosphate (Horne, 1998). It considers that the κ casein is a polymer chain terminator, stating this as the reason for its presence on the surface of the micelle (Horne, 2003). The nanocluster model describes the interior as a flexible array of casein molecules that are interlinked with calcium phosphate 'nanoclusters' (Auty, 2002; Holt & Horne, 1996). This model is derived from observations that the phosphopeptides in casein bind to and stabilise small domains of calcium phosphate (Dalgleish & Corredig, 2012). The formation of a micellar structure could occur due to crosslinking of these calcium phosphate nanoclusters by the highly phosphorylated α_{s1} & α_{s2} casein (de Kruif & Holt, 2003).

Currently the nanocluster model seems to be the most plausible based on the current research; however, further research needs to be conducted to gain a conclusive answer. With the nanocluster model becoming the most favoured model a number of proposed versions of the model have been suggested including an interlocking lattice (McMahon & Oommen, 2008) and the existence of water channels within the structure (Dalgleish, 2010), schematics of these models are displayed below.

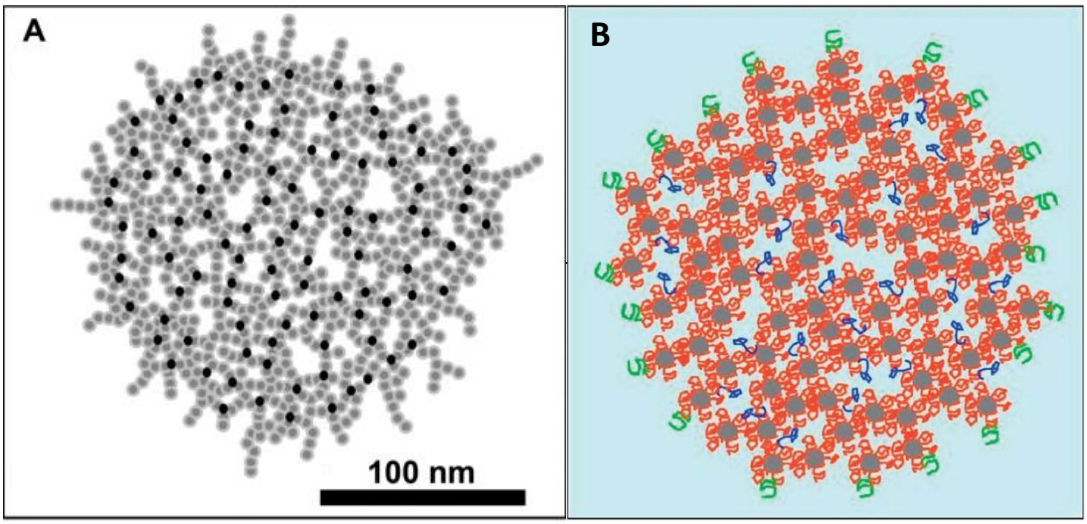


Figure 2.1 :A) Schematic diagram of the internal structure of the casein micelle with black calcium phosphate nanoclusters and interlocking chains of grey casein molecules (McMahon & Oommen, 2008). B) Schematic diagram of the casein micelle with grey calcium phosphate nanoclusters, blue β casein, green κ casein and red α casein with water channels through the micelle (Dalgleish, 2010)

During the cheese making process the micelles undergo a number of changes. However, after cheese manufacture there is still some level of the substructure that remains (Everett, 2007).

The fat globules that are dispersed in raw milk vary in size between 0.1-10 µm with approximately 90% existing between the size of 1 and 8 µm (Auty, 2002). These dispersed globules of fat are surrounded by a lipoprotein membrane, milk fat globule membrane (MFGM) (Keenan & Mather, 2006). The MFGM stabilises the fat globules from coalescing and also protects them from lipases present in milk (Ward, German, & Corredig, 2006).

2.2.1.2 *Pre-Treatment of Milk*

The pre-treatment of milk for Mozzarella making is a relatively new phenomenon coinciding with the occurrence of large scale manufacture. There are still numerous

manufacturers who do not pre-treat their milk; however, the majority have a pre-treatment step for reasons related to safety and consistency.

Pasteurisation has become a step in the pre-treatment of milk for cheese manufacture due to a number of countries and governing bodies having restrictions on the use of raw milk (Fox & Cogan, 2004). Pasteurisation is the heat treatment of raw milk to minimise the health hazards associated with pathogens (Ryser, 2002).

Pasteurisation is generally done by passing milk through a plate heat exchanger so that it is exposed to a high temperature for a relatively short time; 72°C for 15 seconds is standard practice to kill pathogens present in the milk (Bennett & Johnston, 2004). There are a number of different chemical and other changes that occur during heating; the extent of the changes depends on both the temperature and duration (Walstra & Jenness, 1984). Low pasteurisation (e.g. 15 s at 74°C) kills most microorganisms and inactivates some enzymes, high pasteurisation (e.g. 15 s at 90°C) kills all vegetative microorganisms, most enzymes are inactivated, whey proteins denature (in particular beta lactoglobulin) and –SH groups become exposed which promotes disulphide linkages. The process of pasteurisation alters the indigenous microflora of milk which enables the quality of cheese produced to be more uniform (Fox & McSweeney, 2004). However, care needs to be taken when pasteurising milk as it can damage the coagulability and curd forming ability of the milk.

One of the most important attributes in the manufacture of quality products is consistency (Solorza & Bell, 1995). Standardisation acts as a means of ensuring the starting point of the cheesemaking process is consistent between batches. This compositional adjustment of the milk is done to attain a desired composition of the cheese (Bennett & Johnston, 2004). Generally this is achieved by centrifugal separation of the raw milk into skim milk a cream. The skim milk stream is then combined with whole milk or the cream stream to obtain the desired level of casein and fat (Walstra & Jenness,

1984). Another method that is utilised in standardisation is the addition of non-fat dried (NFD) milk to the cheese milk prior to processing (Kindstedt, Hillier, & Mayes, 2010). Recently ultrafiltration has also been utilised to adjust the protein content of the milk (Bennett & Johnston, 2004). The concentration of protein in the milk is achieved as low molecular weight molecules such as salts, water and lactose pass through the membrane resulting in the concentration of the remaining components (Mistry, 2011). This permeate can then be added to milk prior to cheese making to control the protein content. Standardisation of milk is used to try and maintain compositional uniformity between batches of cheese manufactured.

Variation in milk composition can occur for a number of reasons including the breed of cow that the milk comes from as well as the season (Hill, 1995). This is due to different breeds of cow producing milk that differs in composition as well as the natural variation that occurs in composition throughout the year.

2.2.1.3 *Acidification*

The acidification of the milk has a large impact in most of the key steps in the manufacture of cheese (Fox & McSweeney, 2006). Acidification can be done by the use of starter cultures, direct acidification or a mixture of both (Kindstedt, 2004).

In acidification using starter cultures, the milk is inoculated with a thermophilic starter culture, which is generally composed of a mixture of both rod and cocci bacteria (Kindstedt, 1993a). Thermophilic starters are used to a greater extent globally in the production of pizza cheese than mesophilic starters as it makes it easier to obtain the desired moisture content of between 48 and 52% (Kindstedt, 2002). Starter selection is determined by the desired rate of acidification in the later stages of the cheese making process (Rankin et al., 2005). Most manufacturers utilise *Streptococcus salivarius* spp. *thermophilus,* cocci, alone or in combination with *Lactobacillus delbrueckii* ssp. *bulgaricus,* rods, *(Rankin et al., 2005)* or *L. helveticus* (Kindstedt, 2002). *S. thermophilus* is weakly

proteolytic and therefore is unable to produce enough free amino acids and small peptides from casein to sustain optimal growth and acidification in milk alone (Kindstedt, 1993a). The use of *L. bulgaricus*, which is more proteolytic, thus producing more free amino acids and peptides which stimulate the growth of *S. thermophilus.* The ratio of rod to cocci in the manufacture of Mozzarella influences the functional properties of the cheese due to its influence on structure (Yun, Barbano, Kiely, & Kindstedt, 1995). This is due to the early stages of acid production being dominated by *S. thermophilus,* while *L. bulgaricus* is more dominant during the latter stages of Mozzarella production. Changes to the rod:cocci ratio will change the rate of acid production which will influence the structure of the curd and thus the functional properties of the cheese. The role of the starter is to convert lactose to lactic acid and by doing so reduce the pH of the milk.

In the mid-sixties a breakthrough was made in the form of using direct acidification in place of lactic fermenters in the production of Mozzarella (Kindstedt, 2004). There are a number of advantages of using a direct acidification including reduced processing time, lower costs as well as providing a better means of standardising the characteristics of the cheese (Faccia, Trani, & Di Luccia, 2009). Another factor noted regarding the initial work done on direct acidification was that it produced Mozzarella that was suitable for use on pizzas immediately after manufacture (Kindstedt, 2004). However, if direct acidification is used completely in place of starter cultures there are implications in regards to the browning of the cheese when baked (Oberg, Wang, Moyes, Brown, & Richardson, 1991). This is due to starter cultures producing small peptides and amino acids that are able to react with residual sugars in the system when subjected to heat. Therefore the directly acidified Mozzarella will undergo browning to a lesser extent than cheese made with starter cultures.

One of the key purposes of acidifying milk prior to cheesemaking is to reduce the calcium content of the cheese (Metzger, Barbano, Rudan, & Kindstedt, 2000). This occurs due to the disassociation of colloidal calcium phosphate as the pH reduces (Auty, 2002). During the acidification process, calcium phosphate solubilises and proteins disassociate from the micelles. The release of proteins from the casein micelle was found to be temperature dependent with lower levels of protein released at higher temperatures (Dalgleish & Law, 1988). The maximum protein dissociation occurs at a pH of approximately 5.5 (McMahon, Du, McManus, & Larsen, 2009).

During the initial stages of acidification when the pH of the milk is lowered from its initial pH to a pH of 5.9, there is a progressive decrease in the casein particles hydration (Le Feunteun & Mariette, 2008). This is coupled with a decrease in the apparent particle radius of the casein micelle (Alexander, Corredig, & Dalgleish, 2006). However, as the pH is dropped further, the apparent particle radius of the micelle increases back to its original size by about a pH of 5.2 (Alexander et al., 2006). This return to initial size occurs as the hydration of the micelle returns to approximately its initial value (Le Feunteun & Mariette, 2008). This is a result of the casein micelles undergoing structural reorganisation during the acidification (McMahon et al., 2009). This is due to less calcium binding and ion pairing taking place with the reduction in the number of negatively charged amino acid side groups as the pH drops.

The stability of the external κ casein layer of the casein micelle remains relatively stable down to a pH of 5 (de Kruif & Holt, 2003). After this point, the κ casein layer collapses allowing the micelles to aggregate. However, cheese making utilises coagulants such as chymosin to cleave off the κ casein to promote aggregation at a desired pH.

Different gel properties have been observed when acidification is performed at different temperatures (Lucey & Singh, 1997)

The use of chemical acidifiers over starter cultures is widely used as it reduces production costs as well as providing a method of standardising the characteristics of the cheese (Faccia et al., 2009). Direct acidification also allows for lower calcium and higher moisture contents to be achieved (Kindstedt, 2007). The lower calcium content causes the formation of hydrated and swollen paracasein fibres on stretching causing the cheese to be drier, softer and gummy in texture as a young cheese. The type of acid used in direct acidification can influence the calcium content and rheology of the cheese (Keller, Olson, & Richardson, 1974). NMR spectroscopy identified that the mobility of the casein micelle does not change with pH (Rollema & Brinkhuis, 1989).

2.2.1.4 *Coagulation*

A number of different coagulants are now used to make cheese (Kindstedt, 1993a). The coagulant that has been traditionally used in cheese manufacture is rennet which is derived from the abomasum of young milk fed calves (Bennett & Johnston, 2004). Rennet is primarily made up of the enzyme chymosin (Fox, O'Connor, McSweeney, Guinee, & O'Brien, 1996) but also contains other enzymes such as pepsin in smaller quantities. Due to the increasing demand for clotting enzymes in the 1960's and the limited supply of calf stomachs for rennet, several substitutes were identified (Jacob, Jaros, & Rohm, 2010), including pepsin (Fox & Stepaniak, 1993), proteinases from different fungi (The most common from *Rhizomucor miehei* (Fox & McSweeney, 1996)), chymosin (Birkkjaer & Jhnk, 1985), as well as recombinant or genetically engineered chymosin (Jacob et al., 2010). The use of pepsin as a rennet substitute has several defects attributed to its use such as extensive proteolysis (Garg & Johri, 1994) and producing a coagulum with a more open structure than chymosin, allowing a greater amount of fat loss, as well as producing a cheese with a much softer body (Garg & Johri, 1994).

The addition of coagulants to milk has a dramatic effect on the structure of the system. The conversion that milk undergoes during coagulation can be classified as a two stage process (Fox et al., 1996). The primary stage of the rennet action is the production of para-κ-casein and soluble glycol macropeptides. This reaction takes place when the coagulation enzyme hydrolyses the κ casein at the Phe_{105}-Met_{106} site which is several times more susceptible to acid proteinases than any other peptide bond in the milk system (Fox, 1987). Although some coagulants do not attack the Phe_{105}-Met_{106} site, the majority used commercially in cheese making do attack this site. The majority of the glyco macropeptides are lost into the whey whilst the para-κ-casein remains attached to the exterior of the micelle (Fox & McSweeney, 1996). The α_{s1}, α_{s2} and β caseins are not hydrolysed during coagulation but may be hydrolysed during the ripening of Mozzarella.

There are a number of factors that have been found to affect the hydrolysis of κ casein by rennet. These include the pH, ionic strength, temperature, degree of glycosylation and the heat treatment of milk (Fox & McSweeney, 1997).

Heating milk above 60°C has been proven to have an adverse effect on the coagulation if the milk is exposed to the heat for a sufficient duration (Singh, Shalabi, Fox, Flynn, & Barry, 1988). If the heat treatment is severe, over 90°C for 10 minutes, milk does not coagulate. The heat treatment changes β-lactoglobulin so that it interferes with the casein micelles hindering the rennet action (Kannan & Jenness, 1961). This interference occurs due to the formation of a complex between β-lactoglobulin and κ casein (Sawyer, 1969).

Altering the pH of the milk affects the hydrolysis of the κ casein (Janhøj & Qvist, 2010). The optimal pH for chymosin activity in milk is pH 6.0, so reducing the pH from the natural pH in milk increases the rate of hydrolysis (Hooydonk, Boerrigter, & Hagedoorn, 1986). The charge on the micelles also decreases as the pH is dropped from the natural pH of milk (Janhøj & Qvist, 2010). The net effect of reducing the pH of milk on the hydrolysis of κ

casein is that the coagulation time decreases (Daviau, Famelart, Pierre, Goudedranche, & Maubois, 2000).

The ionic strength of the milk has an effect on the coagulation time (Daviau et al., 2000). As the ionic strength is reduced so is the coagulation time (Daviau et al., 2000; Famelart, Lepesant, Gaucheron, Le Graet, & Schuck, 1996). This is a result of higher ionic strengths causing a screening of the positive charge on the κ casein and the negative charge of the chymosin, restricting enzyme-substrate attraction (Daviau et al., 2000; Visser, Rooijen, & Slangen, 1980).

The secondary or non-enzymic phase of coagulation involves the aggregation of the casein micelles (Dalgleish, 1979). The stability of the micelle is due to the net negative charge as well as steric repulsions of the κ casein (Lucey, 2008). The hydrolysis of the κ casein destabilises the casein micelle due to a reduction in the zeta potential as well as reducing the intermicellular repulsions caused by the removal of protruding peptides (Fox, 1987). The reduction of the zeta potential is due to the release of the glyco macropeptides which diffuse away from the micelle (Lucey, 2008). The reduction in the steric hindrance allows the paracasein micelles to aggregate together (Walstra, Vandijk, & Guerts, 1985). This aggregation of the micelles occurs when approximately 85% of the κ casein has been hydrolysed (Fox & McSweeney, 1997). However, individual micelles do not participate in gelation until approximately 97% of the κ casein has been hydrolysed. In order to promote coagulation at a lower degree of κ hydrolysis, a lower pH can be used as well as increasing the temperature of the system (Fox, 1987). Micelles stay discrete until approximately 60% of the visual coagulation time, at which point they begin to aggregate into chain like structures. These chain-like structures continue to aggregate forming clumps, clusters and eventually a gel like network (Lucey, 2008). The strength of the gel, curd tension, is an important factor in terms of the cheese yield (Fox et al., 1996). The curd tension is affected by the same variables as the coagulation.

The coagulation of the rennet altered micelles is dependent on the concentration of calcium ions (Fox & McSweeney, 1997). These calcium ions may act by crosslinking micelles with serine phosphate residues or charge neutralisation (Fox et al., 1996). Colloidal calcium phosphate (CCP) is also important in the coagulation process as if the level of CCP falls below 20%, coagulation will not occur (Fox, 1987). This is due to the removal of CCP causing micelle dissociation and may cause the charge on the micelles to increase. The reduction in CCP can be offset by increasing the concentration of calcium ions (Fox & McSweeney, 1997). If there are no free calcium ions the destabilised micelles will not aggregate (Lucey, 2008).

Coagulation of renneted micelles is also dependent of the temperature of the milk, with normal bovine milk not coagulating at temperature below 18°C unless the calcium content is increased (Fox et al., 1996). The type of coagulant used has an impact on the rate of micelle aggregation as well as the development of gel strength (Fox, 1987).

The subsequent changes to the proteins in Mozzarella after renneting can be considered to be due to the change in free energy that occurs when the macropeptide portion of κ-casein is cleaved off (McMahon & Oberg, 1998).

2.2.1.5 *Dehydration*

The dehydration process can be divided into a number of key steps: cutting, cooking, draining, Cheddaring and milling.

Once the curd has reached a desired degree of firmness the gel is usually cut or broken (Fox et al., 1996). The cutting of the gel enhances syneresis (Kindstedt, 1993a; Walstra & Vandijk, 1983). The gap between the blades used to cut the gel is instrumental in controlling the syneresis related to cutting, with smaller gaps cutting the gel to a greater extent which enhances syneresis. The amount of cutting is dependent on the type of cheese that is being manufactured, with low moisture cheeses cut to a greater degree

than high moisture cheeses. As the Mozzarella used on pizzas has a moisture content generally in the bounds of between 48 and 52%, it is generally cut with blades that are widely spaced, leading to a lower degree of cutting in Mozzarella than lower moisture cheeses. Structurally cutting breaks apart the gel allowing the fluid entrapped within to flow.

Due to the majority of Mozzarella being produced using thermophilic starter cultures, the cooking temperature of the cheese is about 41°C (Kindstedt et al., 2004). This is done as it provides a desirable temperature for the starter culture to convert lactose into lactic acid. The cooking of the curd and whey mixture plays a fundamental role in the control of syneresis by influencing curd shrinkage and acid development (Bennett & Johnston, 2004). The cooking temperature used in the manufacture of the Mozzarella curd is an easily adjustable parameter that has an effect on a number of variables (Yun, Kiely, Barbano, & Kindstedt, 1993). When the cooking temperature is increased there is a subsequent loss in moisture, reduced rate of proteolysis and an increase in the apparent viscosity. The impact that the cooking of the curd has on the functional properties of the Mozzarella depends on the heat stability of the coagulant as well as the amount and activity of the starter. Cooking causes the fat globules to aggregate; however, the milk fat globule membranes that surround the globule remain relatively intact.

The draining step in the manufacture of Mozzarella cheese essentially is the process that separates the curd from the whey (Akkerman, Buijsse, Schenk, & Walstra, 1996). This simply separates the liquid fraction from the solid gel structure. The pH at drainage is the key factor affecting the demineralisation of the curd (Kiely et al., 1992). The effect of this on calcium is that the majority of the non-micellar calcium is lost in the whey while the micellular calcium is retained within the curd (Metzger, Barbano, Rudan, & Kindstedt, 2000). The altering of the pH at which the whey is drained impacts the level of calcium lost in the whey. Hence, the pH at drainage determines the functional stability of Mozzarella curd for the stretching process as well as the properties of the finished cheese (Ak &

Gunasekaran, 2003; Keller et al., 1974). The draining occurs once a desired temperature has been reached, referred to as the draw pH (Kiely et al., 1992). This pH depends on the type of cheese being produced and the desired composition of the cheese. There are four major steps in the drainage: a) additional whey is expelled from the curd grains b) the curd grains deform c) curd grains partially fuse together c) whey flows out of the curd bed (Akkerman et al., 1996; Akkerman, 1992).

The Cheddaring process allows the curd to knit together to form curd granules (Auty, 2002; Bennett & Johnston, 2004). This occurs when the para-casein micelles fuse together to form a more continuous protein phase (Auty, 2002). The fusion of these curd grains into a coherent mass is an essential part of the formation of most cheeses (Akkerman, Lewis, & Walstra, 1993).

2.2.1.6 *Salting*

Salting of the cheese curd is a critical step in the cheese making process (Bennett & Johnston, 2004). The amount of salt and the method of addition of salt to the cheese play an important role in key characteristics of the cheese (Sutherland, 2002). The salting of Mozzarella can occur either in the form of dry salting or brine salting (Kindstedt, 2002). Dry salting is where salted is added directly to the curd prior to the stretching in Mozzarella production. Prior to dry salting, the curd is generally milled to maximise the surface area exposed to the salt. The dry salting of the curd is a means of controlling the moisture content as it promotes syneresis. The Mellow is the process that occurs once the salt has been added to the curd. It involves the mixing and uptake of salt as well as the subsequent moisture loss (Bennett & Johnston, 2004).

The other method of salting, brine salting, occurs after the Mozzarella has been stretched, moulded into the desired shape and has been cooled in water (Kindstedt, 1993a). Brine salted Mozzarella has a salt gradient that is highest at the surface and lowest in the centre of blocks of cheese (Farkye, Kiely, Allshouse, & Kindstedt, 1991). The brine that the cheese

in soaked in needs to have the pH and the calcium content adjusted so as to prevent leaching of calcium and lactic acid out of the cheese which result in defects to the cheese (Sutherland, 2002). The salt in the cheese is mainly present in the aqueous phase (Sutherland, 2002).

2.2.1.7 *Stretching*

Stretching refers to the process in which the Mozzarella curd is subjected to thermo-mechanical treatment involving the application of shear stress to the plasticized curd (Kindstedt et al., 2004; Kindstedt, 2007). The stretching process gives Mozzarella its unique structure and fibrous texture (Kinstedt, 1993; Fox *et al.,* 2000; Ribero, Rubiolo, & Zorrilla, 2009). Industrially the curd is generally plasticized and kneaded in hot water or a dilute brine solution (Kindstedt, 2007). This is generally performed using mechanical mixers with single or multiple screws to knead the curd in the hot water which is controlled by steam injection (Kindstedt et al., 2004).

The plasticisation and stretching is governed by the level of casein associated calcium at the time of stretching, which is in turn governed by the total calcium content and the pH of the curd (Kindstedt, 2007). The ability to plasticise is determined by the amount of casein associated calcium phosphate that is available to crosslink the amorphous para-casein matrix when heat is applied to the curd (Kimura, Sagara, Fukushima, & Taneya, 1992; Kindstedt et al., 2004; Kosikowski & Mistry, 1997; Lawrence, Creamer, & Gilles, 1987; Lucey & Fox, 1993).

There are two key conditions that are needed to be controlled for optimal stretching of the curd (Kindstedt, 2007). The first is that the curd needs to be sufficiently acidified and demineralised. The second condition for optimal stretching is the heat transfer during the stretching process.

Stretching can be considered a two stage process (Kindstedt et al., 2004). The first stage involves the milled curd entering the hot water, settling at the bottom and increasing in temperature so that it becomes a plastic workable consistency. The second stage of the process refers to the kneading and stretching that occurs as the plasticised curd is worked by the augers.

During the stretching process the amorphous para-casein matrix of the curd is aligned into fibres that are roughly parallel with channels of fat globules and free serum between them (Kindstedt, 2007). The serum channels contain water, residual proteins, minerals, fat globules and bacterial cells (Everett, 2007). The heating that occurs during the stretching reduces the activity of residual rennet in the curd which reduces the extent of primary proteolysis during ripening (Lucey, 2007).

A problem that can occur with the use of screws stretching the curd is that at high screw speeds tearing of the curd can occur (Renda, Barbano, Yun, Kindstedt, & Mulvaney, 1997). The tearing is caused by the curd not being fully plasticised as it doesn't have time to get up to an adequate temperature. The heterogeneous quasi-laminar structure created during the stretching process is instrumental in a number of the key functional properties of Mozzarella (Kindstedt, 2007).

Free serum has been identified following the stretching process of Mozzarella cheese curd, however, whether this is caused by the heating of the curd, the shearing of the curd or a combination of the two is unknown. This will be explored as part of this thesis.

2.2.1.8 *Effect of Storage*

Although it is considered an unripened cheese, Mozzarella does undergo significant structural changes during storage that have a dramatic effect on the functionality of the cheese (Kindstedt, 1993b). Immediately after manufacture, low moisture Mozzarella cheese does not possess the desired functional properties for its application (Kindstedt et

al., 2004). This fresh Mozzarella has a tough, fibrous texture and does not stretch or flow well when melted. This is due to the thick paracasein fibres that are formed during stretching being initially hydrophobic, favouring strong protein-protein interactions which resist flow and stretching (Kindstedt, 2007).

Mozzarella undergoes significant structural and functional changes during the first few weeks of aging whereby it becomes softer and becomes more stretchable (Kindstedt, 1993a). During this short period of aging, some of the β-casein partially disassociates from the casein matrix and becomes the main intact casein in the serum phase (Everett, 2007). This is due to lessening in the hydrophobic forces in the matrix.

Proteins become less aggregated and become more hydrated during maturation (McMahon & Oberg, 1998). This protein swelling is enhanced by high salt and low ionic calcium concentrations which lead to an increase in the tendency for the cheese to melt (Everett, 2007). This is likely to be caused by the reduction in protein-protein interactions increasing the ease of which the protein aggregates flow (Joshi, Muthukumarappan, & Dave, 2004b; McMahon, Fife, & Oberg, 1999). The increase in hydration of proteins causes the amount of expressible serum to decrease dramatically over the first few weeks of aging (Guo & Kindstedt, 1995). Salt promotes structural swelling of the protein matrix and casein solubilisation through peptizing (Guo, Gilmore, & Kindstedt, 1997). Lowering the level of calcium present in the cheese will increase the peptising action of salt (Paulson, McMahon, & Oberg, 1998).

Although short term aging of Mozzarella is needed due to it improving the shreddability and allowing the water binding capacity to increase, aging for a period beyond 2-3 weeks generally results in a progressive deterioration in the shredding characteristics (Kindstedt, 1995). This is due to the texture progressively becoming soft and gummy making shredding difficult.

How the Mozzarella is stored after processing has an effect on the functionality of the cheese (Oberg, Merrill, Brown, & Richardson, 1992). The length of refrigerated and frozen storage has an effect on the textural properties of the Mozzarella (Tunick, Mackey, Smith, & Holsinger, 1991).

Another significant factor affecting the structure of Mozzarella is the proteolytic breakdown that occurs during aging (Costabel, Pauletti, & Hynes, 2007; Farkye et al., 1991; Fox & McSweeney, 1996). Proteolysis occurs as residual enzymes present in the cheese hydrolyse the casein causing a breakdown in the protein matrix (Fox, 1989). The amount of residual coagulant impacts on the proteolytic breakdown that occurs during aging (Kindstedt, 1993a). This is influenced by the stretching process where the curd is exposed to heat and shear. The additional heat treatment largely inactivates the chymosin present in the curd, while the more heat stable plasmin remains active (Lawrence et al., 1987). Plasmin predominantly hydrolyses β casein (Farkye & Fox, 1991) resulting in the breakdown of β casein in most Mozzarella's being greater than that of α_{s1}-casein (Farkye et al., 1991). This preferential breakdown of β-casein is due in part to the complex protease-protease inhibitory system of plasmin in milk (Ismail & Nielsen, 2010). Plasmin in milk exists in its zymogen form, plasminogen (Grufferty & Fox, 1988), and its conversion and activity governed by a number of activators and inhibitors (Ismail & Nielsen, 2010). Both the plasminogen activator inhibitor and the plasmin inhibitor are affected by heat (Ismail & Nielsen, 2010) while the plasminogen activator is heat stable (Lu & Nielsen, 1993). This allows the conversion of plasmin from plasminogen to occur and in the absence of an inhibitor the plasmin can hydrolyse the casein in the cheese. Plasmin is the main enzyme responsible for the breakdown of β-casein in cheese (Feeney, Fox, & Guinee, 2001).

Using electrophoresis it was found that Mozzarella had a greater percentage of intact casein than either Cheddar or Gouda cheeses (Creamer, 1976). This is likely to be due to the temperature treatment that occurs to the curd during the manufacture process.

Freezing Mozzarella is of commercial interest due to the ability to arrest physiochemical changes during the ripening process as well as a means of extending shelf-life (Kuo & Gunasekaran, 2009). There have been a number of defects associated with thawed cheese after it has been subjected to conventional freezing (Reid & Yan, 2004). For Mozzarella these defects include poor cohesiveness, discolouration, fat leakage, watery surface and an acid flavour (Johnston, 2000). When cheese is subjected to conventional freezing where heat is removed progressively, the nucleation of water begins at the surface of the cheese and an ice front forms, progressing towards the centre (Reid & Yan, 2004). As the ice front advances, the solute concentration in the remaining liquid becomes more concentrated as they are excluded from the ice front. Due to this there are a number of changes that can occur in the cheese as a result of freezing. The expansion of water as it changes to ice crystals weakens the protein matrix leading to the cheese having a more porous structure and being softer (Graiver, Zaritzky, & Califano, 2004). However, this effect is only significant if sufficient bulk water is present, as in fresh Mozzarella prior to water absorption into the protein phase (Everett, 2007).

The temperature and speed used in freezing Mozzarella is an important factor in the melting properties of the cheese (Oberg et al., 1992). If the freezing rate is sufficiently fast, large crystals will not form and the protein matrix may not be disrupted enough to impact on texture (Cervantes, Lund, & Olson, 1983). A slower rate of freezing has been shown to increase the meltability of Mozzarella, likely due to large ice crystals damaging the protein matrix (Oberg et al., 1992). The form in which Mozzarella is frozen, in terms of being in block form or shredded, also has an effect on the stretch and melting properties. The stretching properties of the Mozzarella were greatest in shredded cheese due to the rapid freezing limiting the size of the ice crystals.

In most applications Mozzarella is used in a shredded form therefore many manufacturers shred the product prior to distribution. There are a number of problems concerning shredding Mozzarella related to the texture of the cheese (Bertola, Califano, Bevilacqua, &

Zaritzky, 1996b). If the cheese is too soft there are issues regarding the clogging of the cutting blades as well as the shredded cheese matting into sticky aggregates. If the cheese is too firm, shredding will result in the shattering of particles due to the brittleness of the cheese.

2.2.1.9 *Advances in processing & future developments*

The mechanisation of the cheese process has resulted in a number of processing tools that aid in the manufacture of products. Vats are used in commercial cheese manufacture to control the processing of cheese from milk to a cut coagulum (Bennett & Johnston, 2004). Commonly used vats include TetraPak's OST, APV's Curdmaster as well as Damrow and Scherping vats (Johnston, Barclay, & Honore, 2010). These enclosed vats are mechanised and automated to cope with large volumes of milk and hygiene requirements of modern processing facilities (Bennett & Johnston, 2004). These vats allow a number of unit operations to be conducted in one piece of equipment and allow a greater level of control over variables associated with processing. The majority of these vat systems contain revolving knife panels, automated rennet addition, a heating jacket, a whey removal system and CIP systems (Bennett & Johnston, 2004).

A number of machines have been developed to process cheese curd following the production of a cut coagulum in vats. Two such machines used for the processing of cheese curd are the Cheddarmaster, made by AVP, and the Alfomatic, made by Tetra Tebel (Law, 2001). These machines have the cut coagulum pumped into them and drain it, pass it along a set of Cheddaring belts, mill the curd and salt it. The salted curd is then generally passed through an auger to stretch the curd prior to being pressed and packed.

Extrusion has been identified as a method of producing Mozzarella continuously in an efficient and economical way (Muliawan & Hatzikiriakos, 2008). Extrusion is the process in which a material is subjected to high shear, high temperature, over a short time period. As extrusion is a thermo-mechanical process it is similar in nature to the stretching process

that Mozzarella curd traditionally is subjected to. Extrusion has the advantage of increasing productivity while reducing the production costs associated with processing (Wiedman & Strobel, 1987).

The use of rolling in the production of Mozzarella has been identified as a potential processing tool (Mitsoulis & Hatzikiriakos, 2009; Muliawan & Hatzikiriakos, 2008). Rolling is the process in which a material is passed between counter rotating rollers and in the food industry is generally used as a forming process (Levine & Drew, 1990; Xiao, Charalambides, & Williams, 2007)

2.2.1.10 *Low Fat Mozzarella*

Cheese contains enough milk fat to make it a reasonably large source of dietary fat (Zhou & Mulvaney, 1998). A major challenge in the manufacture of reduced fat Mozzarella is achieving sensory properties similar to the full fat variety (Bhaskaracharya & Shah, 2001) as well as maintaining the desired functionality.

The most important way of improving the functionality of lower fat cheese is to increase the moisture content to achieve moisture to protein ratio that is similar to full fat cheeses (McMahon & Oberg, 1998). The main textural problems associated with reduced fat cheeses are crumbliness, excessive hardness, reduced meltability and poor stretching properties (Bhaskaracharya & Shah, 2001). It has been identified that lower fat Mozzarella is firmer than the full fat variety due to more cohesiveness and springiness (Tunick et al., 1991). This is due to the rheological properties of cheese being strongly influenced by the lipid fraction (Zhou & Mulvaney, 1998). The amount of calcium present in low fat Mozzarella is higher than the full fat variety; this contributes to the hardness of the cheese (Zisu & Shah, 2005).

2.2.2 Components within Mozzarella

The compositions of the components used to produce Mozzarella have a large influence on the end composition of the cheese, in particular the milk. The use of standardisation limits any variance in the cheese composition due to variability in the raw products (Francolino et al., 2010).

Cheese has three main components, fat, water and protein (Green, 1997). However, there are a number of other minor components that are important to the structural and functionality of the cheese. The salt and minerals, such as calcium, in cheese play an important role in cheeses such as Mozzarella.

There are different variations on Mozzarella from around the world, many with differing composition. In terms of the Mozzarella made from bovine milk, the composition differs depending on the intended application for the Mozzarella. In the US, Mozzarella is categorised into 4 different groups based on their composition, in particular the moisture and fat in dry matter quantities, as shown in Table 2-1 previously (Kindstedt, 1993a; McMahon et al., 1993). Each of the different compositions gives rise to a number of differing functional properties.

2.2.2.1 *Casein*

Caseins are a group of proteins that make up approximately 80% of the protein content of bovine milk (Fox & McSweeney, 1997) and are precipitated from milk at a pH of 4.6 (Hill, 1995). Casein plays an essential role in cheese as it provides the structural network holding the other components together. The casein acts as an emulsifying agent stabilising the oil in water emulsion (Shimp, 1985). Shimp (1985) states this is due to the ability of the surface active proteins binding to both the oil and water phases. This ability arises due to most caseins having a polar calcium phosphate group at one end while the other end is

non-polar. The casein plays a role in almost all of the structural changes occurring throughout the cheese making process (Aguilera, 2004).

The coagulation of the casein micelles in milk is responsible for the formation of curd in the cheese making process (Hill, 1995). The integrity of the protein structure is influenced not only by the total amount of protein, but also the amount of intact protein present (Yun, Hsieh, Barbano, & Rohn, 1994). During the commercial manufacture of Mozzarella where the curd is stretched, heated and moulded, the proteins form continuous, interconnected, smooth-walled fibres (Ribero, Rubiolo, & Zorrilla, 2009). These fibres are separated by large open channels containing the fat globules and water (Kuo, Gunasekaran, Johnson, & Chen, 2001). Over time the protein network adsorbs the water from these channels, causing the protein network to swell. During storage, the casein in the Mozzarella continues to be broken down into smaller fragments (Tunick et al., 1991), most notably α_{s1} and β casein depending on residual enzyme activity.

2.2.2.2 *Fat*

The fat in Mozzarella is a very important attribute, contributing to the functional properties including texture, taste, appearance and melt (Rudan, Barbano, Yun, & Kindstedt, 1999). Due to the stretching of the curd, the microstructure of Mozzarella is different to most other cheeses. Due to this structure the fat globules, instead of being distributed evenly throughout the casein matrix, exist in channels which they initially occupy with some water (Ribero et al., 2009). This is due to the fat interrupting the fusion of the protein matrix, resulting in spaces in which excess serum can be retained (McMahon et al., 1999).

The size of the fat globules has a large influence on the texture of the cheese (Everett, Ding, Olson, & Gunasekaran, 1995b). During refrigerated storage, the fat globules increase in size (Joshi, Muthukumarappan, & Dave, 2004a).

Fat has a significant effect on the hardness, gumminess, meltability and chewiness of Mozzarella (Tunick et al., 1991). This effect is more pronounced as the moisture content of the cheese is decreased. An important function that fat in Mozzarella plays in its use as a pizza topping is the formation of free oil during melting. If there is an inadequate amount of free oil the cheese will become dehydrated and burn when cooked (Yun, Kiely, Barbano, et al., 1993). However, if excessive quantities of free oil forms it has a detrimental effect of the appearance of the cheese (Kindstedt & Rippe, 1990; Tunick, 1994).

2.2.2.3 *Water*

Moisture is one of the key components in most cheeses (Gunasekaran & Ak, 2003). Mozzarella is often considered to be a high moisture cheese (with a moisture range between 45 and 60%), even though the majority manufactured is referred to as a low moisture variety, and thus the structure and functionality are significantly influenced by the water in the system.

The physical state of water in food has an important role in the structural and functional properties of the food material (Godefroy, Korb, Creamer, Watkinson, & Callaghan, 2003). In a food system, water is generally grouped into two general categories; bulk water and bound water. The bound water in a food such as Mozzarella can be viewed as being either expressible or entrapped water (McMahon et al., 1999).

In Mozzarella the bound water is the fraction that is constrained by the proteins. This bound fraction does not have a solvent function and is also unfreezable (Rowney, Roupas,

Hickey, & Everett, 2004). Entrapped water relates to the fraction of water that is impeded by the protein structure and cannot be separated out using centrifugation (McMahon et al., 1999). The expressible fraction of water in the cheese relates to the fraction that is free to move within the cheese structure and can be separated out using centrifugation. The term bound water has been used less frequently in recent publications as it has been replaced with statements relating to the level of casein association, as the water is not strictly bound to the protein. Therefore the water present within Mozzarella can be either in a free state or associated with the protein. Within each of these groups there are varying levels of freedom and association with the proteins.

The distinct microstructure caused by the stretching of Mozzarella means that the distribution of water is quite different to most other cheese types (Godefroy, Korb, et al., 2003; Kuo et al., 2001). In Mozzarella, water is present as a continuous phase dispersed in a porous casein matrix (Guo & Kindstedt, 1995). However, immediately following manufacture some of the water is present in a free state within the channels. After manufacture there is a dynamic relationship between the casein matrix and the serum phase in the Mozzarella (Gunasekaran & Ak, 2003). During the initial period of storage of about 10 days, the expressible fraction of water in the Mozzarella is absorbed into the protein matrix (Kuo et al., 2001). This water is still in the bulk phase; however, it became entrapped in the protein matrix (McMahon & Oberg, 1998).

2.2.2.4 *Salt*

The key reason for the addition of salt to cheese curd is to arrest the further growth of lactic acid bacteria and control undesirable microbial growth (Rowney et al., 2004). The preservation effect caused by adding salt to curd is due to its effect on water activity (Guinee & Fox, 2004a). The salt causes an increase in the osmotic pressure of the aqueous phase which results in dehydration of bacterial cells, killing or preventing their growth. The salt also acts to drive out moisture from the curd.

The salt content of Mozzarella has a direct effect on the functionality of the product (Pastorino, Hansen, & McMahon, 2003; Rowney et al., 2004). The salting of cheese causes the proteins in the curd to interact more strongly with the water in the serum phase (McMahon & Oberg, 1999). This causes the cheese to have less expressible serum as it is absorbed into the protein matrix. This is due to the addition of the salt causing an increase in the ionic strength of the cheese curd, which causes an increase in the solvation of the proteins (Pastorino et al., 2003). The salt, especially at low calcium concentrations, enhances protein to water interactions and reduces interactions between protein molecules (McMahon & Oberg, 1998). Therefore the salt alters the free energy state of the proteins which causes changes in the arrangement of the protein matrix.

The inhibition of cultures in the cheese due to salting also has the effect of leaving unfermented sugars which can be involved in browning reactions (McMahon et al., 1993). These unfermented sugars can undergo Mallard browning reactions with the amino acids (Johnson & Olson, 1985). Salt provides an additional function in cheese, contributing to the flavour (Guinee & Fox, 2004a).

2.2.2.5 *Calcium*

Calcium is one of the key minerals in dairy products due to its prevalence in milk. It is one of the key components that play an important role in the microstructure of cheese (Everett, 2007). Calcium is present in a number of forms within cheese including being associated with anions such as phosphate, citrate and chloride. The quantity of calcium in cheese is one of the main parameters in controlling protein functionality (McMahon & Oberg, 1999). The calcium content of Mozzarella can be manipulated by altering the manufacturing of the cheese (Metzger, Barbano, Rudan, & Kindstedt, 2000).

Lowering the calcium content causes the protein matrix to become more hydrated and smoother (Joshi, Muthukumarappan, et al., 2004a). This lowering of the calcium increases the meltability of the cheese, however, it does cause the firmness to decrease (McMahon & Oberg, 1999). The ionic calcium concentration increases with both a decrease in the pH of the serum as well as an increase in the salt content (Lawrence et al., 1987)

Calcium exists in two states in cheese: soluble calcium and insoluble colloidal calcium within the casein micelles (Lucey & Fox, 1993). It is the colloidal calcium that influences the structure of the cheese. The amount of casein-associated calcium is determined by the total calcium content as well as the distribution between the soluble and colloidal calcium (Ge, Almena-Aliste, & Kindstedt, 2002). The loss of calcium phosphate from the casein micelle during the cheese making process determines the extent of micelle disruption, thus determining the basic structure of cheese (Lawrence, Heap, & Gilles, 1984).

Casein micelles provide a means of delivering high concentration of calcium to young mammals without causing pathological calcification of the mammary glands (Holt & Carver, 2012). The locking up of calcium phosphate into their internal structure is one of the most important biological functions that the casein micelles have (Horne, 2009).

2.2.2.6 *Acidulants*

pH plays a major role in the texture of the cheese, as changes in pH are directly related to chemical changes occurring in the cheese curd (Ramkumar, Campanella, Watkinson, Bennett, & Creamer, 1998). The pH plays a key role in gelation and through the subsequent stages of cheese making (Mishra, Govindasamy-Lucey, & Lucey, 2005).

The pH of cheese curd is mainly dependent on the amount of starter or acid added, the temperature of cook and the rate of salting. The final pH of the cheese is largely dependent on the pH that the whey is drained at (Ramkumar et al., 1998).

Starter cultures in cheese manufacture perform two key roles; dropping the pH of the milk by converting lactose into lactic acid and also having a role in the structural and biochemical changes that occur during ripening (Bennett & Johnston, 2004). The starter cultures used to convert lactose into lactic acid are generally comprised of both rod and cocci bacteria (Kindstedt, 1993a). The ratio of rod to cocci in the starter culture used in the manufacture of Mozzarella was identified as having no influence on the general composition of the cheese (Yun, Barbano, Kiely, et al., 1995). However, there was some impact on the on the functional properties of the cheese when this ratio was varied.

The other method of modifying the pH is directly adding acid into the milk. The type of acid used and the pH influence the functional properties of the cheese (Keller et al., 1974). The melting properties of direct acidified Mozzarella are dependent on the pH of the milk at coagulation (Ak & Gunasekaran, 2003).

2.2.2.7 *Whey*

Cheese is generally made with the casein fraction of the milk proteins. However, it is financially advantageous to incorporate the whey into the cheese rather than separating it out. The use of whey in cheese increases the nutritional content as well as increasing the yield of the cheese (Lelievre, 1995). The development of new technology, such as ultrafiltration) has enabled whey to be incorporated in to a cheese matrix (Hinrichs, 2001). However, it is uncommon for cheese to incorporate whey protein as it can negatively impact on a number of functional properties, such as melt for Mozzarella cheese.

2.2.3 Functional Properties of Mozzarella

The microstructure of cheese has a large influence of its functional properties (Joshi, Muthukumarappan, et al., 2004a). The functionality of Mozzarella cheese refers to the key attributes such as meltability, free oil formation and stretchability that make it suitable for use as a pizza topping (Rowney et al., 2004). Functionality has found to be strongly influenced by the pH, calcium to casein ratio as well as the extent of casein solvation (Kindstedt, 2004).

2.2.3.1 *Unmelted Mozzarella*

In order to facilitate even distribution on products as well as uniform melting, Mozzarella is generally shredded or diced (Yun, Kiely, Kindstedt, & Barbano, 1993). Therefore a key functional property of unmelted Mozzarella is in regards to its ability to be shredded. Shreddability is a term used to describe a number of characteristics including the ease with which the cheese is shredded, the integrity of the shreds, whether even shredding occurs or fines are formed, and whether the shreds remain free formed or matt together after being shredded (Kindstedt, 1995). Shreddability is of importance due to the major applications, such as its use on pizzas, requiring the cheese to be in a shredded state. If the cheese is too soft and wet, there are problems such as clogging and matting during shredding. However, problems also exist when the cheese is too dry and hard, with the shreds shattering due to brittleness of the cheese (Bertola et al., 1996b). The firmness of Mozzarella can be controlled by manipulating the density and the structure of the casein matrix (Rankin et al., 2005). This is generally done by altering the calcium content and the pH of the curd. Manipulating these two parameters, especially during the draining step, can have a significant influence on the firmness of the finished cheese (Lawrence et al., 1984).

Unlike many cheeses, flavour is not a very important attribute for Mozzarella for pizzas; rather a lack of flavour is desired (Kindstedt, 1993a).

2.2.3.2 *Melted Mozzarella*

Due to Mozzarella generally being consumed in a molten state (Bertola et al., 1996a), many of the key functional properties relate to how it behaves when melted.

The use of Mozzarella as a pizza topping is the most important application that the cheese is put to due to the sheer volume used for this purpose. Many large pizza chain restaurants have very specific quality parameters that they give their Mozzarella suppliers. These generally relates to properties such as browning, melting, blistering and free oil formation.

2.2.3.2.1 Meltability

Meltability can be defined as the ease with which cheese can flow when heated (Muthukumarappan, Wang, & Gunasekaran, 1999). The meltability of Mozzarella is due to the combined effect of fat and the balance between protein to protein and protein to water interactions (McMahon & Oberg, 1998). It relates to the cheese's ability to form a continuous melt with no individual particles present (McMahon et al., 1993). The melting process is a progression from the firm solid individual shreds of cheese to a semi-solid mass. When subjected to heating, at first the cheese does not change shape but does rapidly increase in temperature (Rankin et al., 2005). The cheese then reaches the softening point, which is the temperature the cheese begins to flow. The cheese matrix collapses and the individual shreds matt together to form a semi-solid mass. Once the cheese has completely melted the change in height is minimal and the temperature of the cheese approaches that of the heated environment. Meltability is generally expressed in

terms of the decrease in the height of a sample or the increase in the sample area (Kindstedt, 1993a).

Initially after manufacture Mozzarella has unacceptable melting properties as it forms a tough, elastic, nonhomogeneous, semi-solid mass that has a granular appearance (Kindstedt, 1995). As the cheese ages it give a more desirable melt as the water holding capacity has increased and there is no separation of water, it has greater stretching and elastic properties. However, Mozzarella becomes excessively soft and fluid after extended aging of over 4 or so weeks, depending on the composition and process.

There is a trade-off between meltability and the firmness of Mozzarella. This can be controlled by the manipulation of the calcium content of the cheese (McMahon & Oberg, 1999). At higher calcium contents Mozzarella is firm but becomes less firm and increasingly more meltable as the calcium content decreases. Meltability is affected by casein solvation, temperature, age, level of proteolysis, fat and moisture levels (Everett & Auty, 2008). Any factor that reduces the casein-casein interactions will cause an increase in the meltability of Mozzarella (Everett & Auty, 2008).

2.2.3.2.2 Free Oil Formation

The release of oil during cooking is a key functional property related to the use of Mozzarella on pizzas. If an insignificant amount of free oil is released the pizza will lack a characteristic sheen and the cheese becomes dehydrated causing excessive browning and burning to occur (Yun, Kiely, Kindstedt, et al., 1993). This is due to the oil creating a hydrophobic film that prevents evaporating occurring from the cheese (Kindstedt et al., 2004). However, if there is an excessive release of free oil it gives the pizza an undesirable appearance and is considered a defect (Kindstedt & Fox, 1991). The release of free oil when Mozzarella is heated is a property that needs to be carefully controlled, with insufficient amounts and excessive amounts being undesirable (Kindstedt et al., 2004). The formation of free oil when Mozzarella is heated is governed by the coalescence of fat

globules within the paracasein channels and the disassociation of the paracasein fibres, which causes them to collapse and flow (Kindstedt, 2007). The collapse of the paracasein fibres allows the pooled liquid fat in the channels to flow and merge together.

The tendency of the fat globules to coalesce and form free oil when the cheese is melted is related to the strength of the emulsion (Kindstedt, 1995). The casein present in the cheese is the main emulsifying agent, and is thus a key factor affecting the formation of free oil.

The fat in dry matter of the Mozzarella influences the amount of free oil that is formed (Kindstedt, 2007). The higher fat content means that there is a greater interruption of protein-protein interactions which causes the paracasein fibres to collapse and flow more readily. The storage and aging of Mozzarella has an effect on the amount of free oil that is released from the melted product. The amount of free oil present in Mozzarella increases from about 36% 3 days post manufacture to 50% by day 21 (Kindstedt & Fox, 1991). This increase indicates the extent of change occurring during the aging process.

The salt content of the cheese also has a significant effect on the quantity of free oil released (Kindstedt, 2007). Increasing the salt content of Mozzarella reduces the free oil formation. Another factor that influences the release of free oil in Mozzarella is the screw speed during the stretching process (Renda et al., 1997). This is due to its role in the dispersion of fat throughout the cheese.

2.2.3.2.3 Stretchability

One of the key functional properties of Mozzarella, that made it the cheese of choice for pizzas, is the ability to stretch when melted (McMahon & Oberg, 1998). Although having no distinct rheological definition, the stretch can be referred to as the ability to form

fibrous strands which extend under tension. This ability of Mozzarella to form strings when stretched is its most distinguishing property (Ak & Gunasekaran, 2003).

Consumers of pizza products, whether they are from a restaurant or from a supermarket freezer, expect the Mozzarella on the topping to have a reasonable stretchability and elasticity whilst being in a semisolid form. This is due to the image advertising products containing Mozzarella generally depicting strands of melted cheese on the packaging (Ak & Gunasekaran, 2003).

The stretchability of Mozzarella is governed by the casein associated calcium present in the cheese. If the calcium to casein ratio is too high the curd will tear and fracture during stretching, while too little results in a complete loss of structure and stretch (Kindstedt, 2002). The amount of calcium present in the curd is dependent on how much is lost at whey drainage; however, the distribution of the calcium within the curd is dependent on the pH at the time of stretching.

2.2.4.2.4 Browning & Blistering

The appearance of Mozzarella changes dramatically from application to a pizza, through baking and cooling (Metzger, Barbano, Rudan, Kindstedt, & Guo, 2000).

Excessive browning is of concern due to many pizza manufacturers using temperatures of over 260°C (Matzdorf, Cuppett, Keeler, & Hutkins, 1994). The browning of Mozzarella when heated is a property that is of specific interest to pizza manufacturers. The browning occurs due to residual galactose and lactose in the cheese that undergo Maillard browning when subjected to heat (Johnson & Olson, 1985). Small peptides and amino acids serve as reactants for the reducing sugars to undergo the browning process (Kindstedt & Guo, 1997b). These small peptides and amino acids are produced by the starter culture in the milk. If Mozzarella is made using direct acidification without the presence of starters, the

cheese does not brown to the same extent as when starter cultures are used (Oberg et al., 1991).

The blistering of Mozzarella on pizzas is another functional property related to the appearance of a baked product that needs to be controlled. Blisters occur when water vapour bubbles under the cheese surface (Kindstedt, 2007). The extent of blistering depends on the tensile strength and viscoelastic properties of the melted cheese.

2.3 Imitation Mozzarella & Model Systems

Imitation cheeses or analogues are cheese-like products that are made by blending together a number of individual components to make a cohesive cheese-like mass (Bachmann, 2001; Noronha, O'Riordan, & O'Sullivan, 2008). The use of imitation cheese blend products allows manufacturers to manipulate individual components to influence texture, nutrition as well as potential economic benefits (Bachmann, 2001; Pereira, Bennett, McMath, & Luckman, 2002). The processing parameters used in the manufacture of these cheese products can also be manipulated to achieve desirable functional properties (Noronha, Duggan, Ziegler, O'Riordan, & O'Sullivan, 2008). Imitation cheeses, as well as providing a product that can be made to meet particular customer requirements (Noronha, O'Riordan, et al., 2008), also provide an ideal system for analysing the effects of different factors on the cheese product. These cheeses are labelled as 'imitation', 'analogue', 'artificial', 'substitute', 'filler', 'extruded' or 'synthetic' (Muir, Tamime, Shenana, & Dawood, 1999).

Imitation Mozzarella or Pizza cheese analogues (PCA) (Sherkat & Walker, 2002) are terms used to describe the wide range of cheese products used as alternative pizza topping to traditional Mozzarella. Imitation or analogue Mozzarella has become of increasing importance due to the demand for convenience foods, such as pizzas, as well as the desire for manufacturers to reduce costs (Ennis, O'Sullivan, & Mulvihill, 1998; Sherkat & Walker, 2002). These pizza cheese analogues are one of the biggest uses of functional caseins (Fox & Kelly, 2004).

The majority of casein-based imitation cheeses have been specifically manufactured for use on frozen pizzas and have thus been manipulated so that the textural and melt properties meet the requirements for this use (Noronha, O'Riordan, et al., 2008). The use of rennet casein in the manufacture of imitation and processed cheese is common practice as it produces products with a ridged structure that also have good melting and

stretching properties (Aimutis, 1995). Rennet casein is insoluble in water so in order to hydrate it calcium chelating salts are added to disrupt the calcium mediated cross bridges between the casein (Ennis et al., 1998). This gives the proteins a greater degree of mobility and freedom which enhances the casein to form networks, increasing the viscosity of the dispersion. These cheese analogues produced with rennet casein need to be manufactured so that the hydration properties of the casein remain constant through its shelf life (O'Sullivan & Mulvihill, 2001). The paracasein derived from solubilising rennet casein is desirable in the manufacture of imitation Mozzarella as it produces a product with good functional properties (Aimutis, 1995). These properties can be controlled by the manufacturer by varying the composition of the product. To adjust the stretching properties of the imitation Mozzarella the sodium to calcium ratio can be changed.

Although these cheeses are made in alternative ways to traditional Mozzarella, these cheeses are commonly referred to as Mozzarella and are becoming increasingly more common.

2.3.1 Model Systems

It is difficult to use real cheese to investigate the relationship between composition and texture due to a number of factors (Marshall, 1990). One of the main reasons is that natural cheeses are not homogeneous or isotropic and therefore their rheological behaviour is nonlinear outside specific limits (Tunick, 2000). Thus model systems using cheese analogues offer a convenient medium to investigate these relationships. Cheese analogues provide a good model system for research purposes due to the composition uniformity that can be obtained over time and different batches (Pereira, Bennett, Hemar, & Campanella, 2001). Another reason as to why model systems are advantageous to work with over real cheese is that they generally have more simplistic processing requirements (Watkinson et al., 2001).

Previous uses of model systems include the development of a system to determine the effect of varying the amount of colloidal calcium phosphate on the rheological properties of Cheddar cheese (O'Mahony, McSweeney, & Lucey, 2006). The effect of different caseins on microstructure and meltability has been investigated using a model processed cheese system (Savello, Ernstrom, & Kalab, 1989).

The protein within the cheese analogue structure reduces the surface tension at the oil and water interface, which increases the stability of the emulsion (Ennis & Mulvihill, 1999; Shimp, 1985). If a dry protein is used in the model system it needs to be properly hydrated in order to perform this function properly (Ennis & Mulvihill, 1999). The majority of cheese analogues are manufactured using rennet casein (Guinee, 2002a). The hydration of rennet casein requires the addition of calcium-sequestering salts to disrupt calcium mediated cross-bridges amongst the proteins (Aimutis, 1995; Ennis & Mulvihill, 1999).

Mozzarella/pizza cheese analogues have been used as model systems to investigate the effects of altering the composition and processing parameters on the functional properties of the product (Ennis & Mulvihill, 1999; O'Sullivan & Mulvihill, 2001; Sherkat & Walker, 2002).The majority of Mozzarella analogues use rennet casein as a protein source due to its ability to give a product with good melting and stretching properties (Aimutis, 1995). Sherkat & Walker (2002) found that the aging of the analogue cheese was not necessary due to bacteria and natural milk enzymes not being present in the product. However, they did find that the product did benefit from a short storage time. Work has also been done on the effect of rennet casein used in Mozzarella analogues (Ennis & Mulvihill, 1999; Ennis et al., 1998; O'Sullivan & Mulvihill, 2001). This work has looked at effect of hydration (Ennis et al., 1998), effect of different types of rennet casein (Ennis & Mulvihill, 1999) as well as the physio-chemical characteristics of the rennet casein (O'Sullivan & Mulvihill, 2001) in Mozzarella analogues.

2.4 Methods for measuring structure & functional properties

There are many different techniques that are commonly used to characterise different cheese products. Most food systems, including cheese, are soft condensed matter existing over a range of hierarchical nano and microstructures (Fischer & Windhab, 2011).

The microstructure of foods can be analysed directly using visual techniques, such as microscopy, or the use of indirect techniques such as instrumental measurements (Auty, 2002). There has been a huge advancement in instrumentation to gain a greater insight into the structure and texture of cheese since the 1940s (Everett & Auty, 2008).

However, the results gained by an individual technique are relatively useless in isolation. Thus in order to maximise the usefulness of a measurement technique it is advantageous to combine its results with those from a number of different measurement systems.

2.4.1 Physical Properties

2.4.1.1 *Rheological analysis*

Rheology is the study of the deformation and flow of matter (Laurati et al., 2009; Rao, 2007b; Tunick, 2000). Rheological responses occur at a macroscopic level but are influenced by the properties and changes occurring at a microscopic and molecular level (Genovese, Lozano, & Rao, 2007). The difficulty in rheology lies in linking the macroscopic rheological properties to the changes and properties at the microscopic and molecular level (Rao, 2007a). The microstructure of a material is related to its physical properties such as viscosity, texture, firmness and elasticity (Velez-Ruiz & Canovas, 1997).

The rheological properties of cheese refer to how it behaves relative to applied stress and strain such as during compression (O'Callaghan & Guinee, 2004). Cheese is classified as a viscoelastic material, in rheological terms, because it behaves with liquid and solid

characteristics when subjected to stress or strain (Guinee, 2002b). The rheological properties of cheese are determined by its microstructure, composition, macrostructure and the physiochemical state of its components (O'Callaghan & Guinee, 2004). Being related to the structure and composition, the rheological properties also undergo significant changes during ripening (Rosenberg, Wang, Chuang, & Shoemaker, 1995). This is because the rheological properties are related to the structure, composition and strength of the bonds within the cheese network (O'Callaghan & Guinee, 2004) with a number of these parameters changing during storage.

The rheological properties of cheese are important due to their influence on the handling, texture and eating quality, the use of cheese as an ingredient, its ability to retain its shape as well is the ability of the cheese to retain gas if required (O'Callaghan & Guinee, 2004). The textural and functional properties of Mozzarella strongly influence consumer acceptability (Ak & Gunasekaran, 1997). These functional attributes are related to the rheological properties of the cheese.

2.4.1.1.1 Large strain

Large strain deformation tests are useful for food gels that hold their shape (Rao, 2007a). These tests include shear/torsion, cutting, compression tests (O'Callaghan & Guinee, 2004). These types of tests break down the gel network allowing comparisons to be drawn with sensory properties (Bowland & Foegeding, 1999). This type of testing has been used to assess the viscoelastic properties of cheeses (Holsinger, Smith, & Tunick, 1995).

Uniaxial compression

The most common type of rheological assessment of cheese involves the application of a linear uniaxial displacement (O'Callaghan, O'Donnell, & Payne, 2002). This typically involves subjecting a cube or cylindrical sample of cheese to large strains between two

parallel plates and dynamically measuring both the displacement and force (Guinee, 2002c). Compression tests of cheese utilise equipment such as an Instron (Creamer & Olson, 1982) or TA XT texture analysers (Truong, Daubert, Drake, & Baxter, 2002). These devises allow a sample to be compressed at a fixed rate to a predetermined level while recording the force as a function of the displacement (Guinee, 2002c).

Uniaxial compression measurements yield information about the mechanical and fracture properties of a material (Wium, Qvist, & Gross, 2007a), with the maximum force recorded being related to the hardness at the level of compression applied and the fracture determined by the initial peak force. A sample that undergoes uniaxial compression is distorted in various directions simultaneously, with any fracture that occurs in the sample more likely to be the result of the shear force caused by the distortion of the sample (O'Callaghan et al., 2002).

The advantages of using uniaxial compression as a measurement technique for assessing the texture of cheese include: being simple, rapid, and can be applied to almost any cheese. One disadvantage of uniaxial compression is that to achieve reproducible results samples need to be cut to precisely the same size and dimensions (Guinee, 2002c), which can be a difficult task in cheese. The samples must also all be at the same temperature for comparisons to be made.

Uniaxial compression has been used to assess the hardness and fracture of a number of different cheeses including: Feta (Wium, Qvist, & Gross, 2007b), Cheddar (Ak & Gunasekaran, 1992), Gouda (Bertola, Califano, Bevilacqua, & Zaritzky, 2001), Swiss (Rohm & Lederer, 1992) and Mozzarella (Casiraghi, Bagley, & Christianson, 1985).

Uniaxial compression has been used to evaluate how different levers affect the textural properties of Mozzarella including: the effect of draw pH and storage (Yun, Hsieh, Barbano, & Kindstedt, 1994), the effect of the inclusion of whey deplete retentate (Brandsma & Rizvi, 2001).

Texture profile analysis

Texture profile analysis (TPA) involves measurements using a double bite uniaxial compression (Guinee, 2002c). This method was developed to imitate the compressive action of molar teeth during mastication (Bourne, 1978).

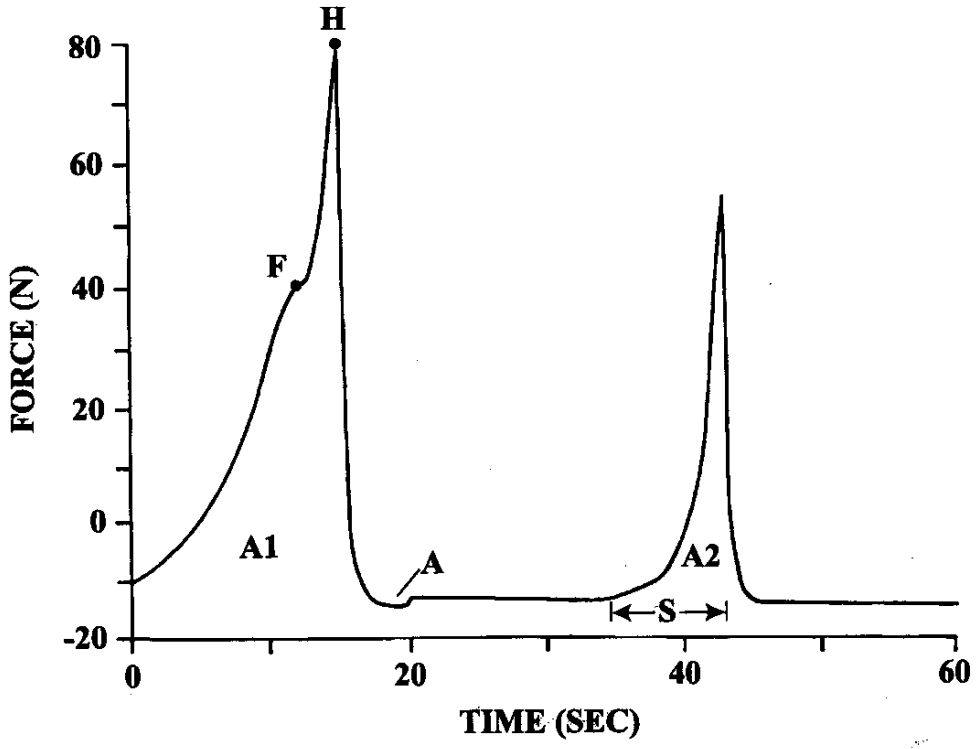


Figure 2.2: An example of a TPA curve obtained for Mozzarella taken from Tunick (2000).

Figure 2.2 is an example of the texture profile analysis on Mozzarella, With a number of features identified including the fracture point (F) and the hardness (H) of the sample (Tunick, 2000). Beyond these two parameters, texture profile analysis can be used to calculate a number of other parameters including: the cohesiveness (A2/A1), springiness (S), adhesiveness (A) and gumminess (hardness x cohesiveness) (Bourne, 1978). One of the

advantages of TPA is the possibility of correlating textural properties to sensory properties of a food (Agulheiro-Santos & Roseiro, 2012).

Texture profile analysis has been applied as a measurement technique to assess a number of different dairy gel systems including: whey protein gels (Tang, McCarthy, & Munro, 1995), yogurt (Sandoval-Castilla, Lobato-Calleros, Aguirre-Mandujano, & Vernon-Carter, 2004), processed cheese (Joshi, Jhala, Muthukumarappan, Acharya, & Mistry, 2004; Piska & Štětina, 2004), and Cheddar cheese (Mistry & Kasperson, 1998).

TPA has been used as a tool for evaluating how a number of factors affect the texture of Mozzarella including: assessing the impact of coagulant type (Yun, Barbano, & Kindstedt, 1993), the effect of coagulant concentration (Kindstedt, Yun, Barbano, & Larose, 1995), the effect of reducing fat particle size by homogenisation in reduced fat Mozzarella (Rudan, Barbano, Gu, & Kindstedt, 1998), the effect of milk pre-acidification (Metzger, Barbano, Kindstedt, & Guo, 2001) and the assessment of fat reduction (Rudan et al., 1999).

2.4.1.1.2 Small strain rheology

Small amplitude oscillatory rheology (SAOR) is a rheological technique also referred to as a dynamic rheological experiment (Rao, 2007a). This technique involves a sinusoidal oscillating stress or strain applied to a material with a frequency, ω (Aguilera, 1995). The measurements taken are the stress, strain and amplitude ratio during the oscillations. This type of dynamic rheological testing is useful for investigating characteristics of gels as well as gelation and melting properties (Rao, 2007b). This allows for the determination of the viscous and elastic component of a food product (Lucey, 2008). There are three types of dynamic tests that give useful information about gel systems: 1) frequency sweep at a fixed temperature;2) temperature sweep at a fixed temperature; and 3) shear rate sweep

at fixed frequency and temperature (sometimes referred to as a time sweep). Based on these tests information can be gained to describe the organisation of the gel network (Bowland & Foegeding, 1999). The parameters that can be determined from these tests include the storage or elastic modulus, G', the viscous or loss modulus, G", as well as the loss tangent, tan δ (Lucey, 2008). The elastic modulus relates to the energy stored per oscillation cycle, the viscous modulus relates to the energy lost per cycle and the loss tangent relates to bond relaxation as a gel is deformed (Lucey, 2002). The use of rheological data along with information about the structure and properties of foods can lead to a greater understanding of the relationship between them (Genovese et al., 2007).

The application of SAO to cheese is done so that the strain is within the linear viscoelastic region so that the structural breakdown that occurs is largely reversible (Everett & Auty, 2008). SAO has been applied to a wide range of cheese systems as a means of assessing their physical properties. These applications have included: assessing how the fat content of Cheddar cheese affects physical properties (Guinee, Auty, & Fenelon, 2000); the effect of the change in the calcium equilibrium in Cheddar (Lucey, Mishra, Hassan, & Johnson, 2005); the characterisation of the melt properties of an imitation cheese (Mounsey & O'Riordan, 1999); examination the role of moisture content of cheese analogues (Pereira et al., 2001); and the effect of different curd washing methods in Colby cheese (Lee, Johnson, Govindasamy-Lucey, Jaeggi, & Lucey, 2011).

The technique has been applied to Mozzarella to gain information about how the cheese changes with storage (Joshi, Muthukumarappan, & Dave, 2004d), temperature (Muliawan & Hatzikiriakos, 2007; Tunick, 2010) and composition (Joshi, Muthukumarappan, & Dave, 2004c; Sheehan & Guinee, 2004; Van Hekken, Tunick, Malin, & Holsinger, 2007).

Behaviour of Mozzarella during heating is of particular interest due to the majority of the cheese being used as a pizza topping. The rheological properties of Mozzarella are highly temperature dependent (Ak & Gunasekaran, 1996). Below temperatures of 60°C,

Mozzarella behaves as a viscoelastic-plastic as it maintains its structure up until this point (Muliawan & Hatzikiriakos, 2007). After 60°C has been reached, the structure is completely broken and Mozzarella behaves as a viscoelastic fluid.

Rheology has been proven to be a useful tool for assessing the physical properties of cheese systems. Therefore the use of rheological methods to assess Mozzarella in this investigation is advantageous.

2.4.1.2 *Melt*

As LMPS Mozzarella is predominantly consumed in a molten state (Bertola et al., 1996a); assessing the meltability of the cheese is of great importance. The melting properties are generally assessed by measuring the change in the dimensions of a fixed size sample after exposure to heating. The most common tests for evaluating the meltability of cheese are the Schreiber and the Arnott melt tests (Kuo, Wang, & Gunasekaran, 2000; Park, Rosenau, & Peleg, 1984). The Schreiber test involves measuring the maximum diameter that a sample of cheese spreads during heating (Muthukumarappan et al., 1999). It involves placing a plug of cheese on a Petri dish and heating at 232°C for a 5 minute period (Park et al., 1984). Once the melted cheese has been allowed to cool for 30 minute, the maximum diameter of the melted sample can be measured.

The Arnott melt test assesses the reduction in the height of a cylinder of cheese following heating (Ustunol, Kawachi, & Steffe, 1994). It involves exposing a cylinder of cheese to a temperature of 100°C for a period of 15 minutes and measuring the height before and after the heat treatment (Arnott, Morris, & Combs, 1957).

A modification proposed to the Schreiber test involves carrying out the test at a lower temperature of 90°C to prevent charring and to conduct the test on an aluminium plate, as it was found to have less variation than a Petri dish (Muthukumarappan et al., 1999).

A number of other methods to evaluate the meltability of cheese have been used including UW Meltmeter (Kuo et al., 2000) and capillary rheometers (Smith, Rosenau, & Peleg, 1980).

Due to the end use of the majority of LMPS Mozzarella produced being as a pizza topping, an evaluation of the melt of samples in this project would be advantageous.

2.4.2 Microstructural Analysis

2.4.2.1 *Microscopy*

Microscopes are used as a visual technique to analyse the microstructure of food products (Auty, 2002). They provide a powerful tool in assisting in the understanding of the relationship between physico-chemical changes in a system and the texture of the product (Everett & Auty, 2008). The main microscope techniques applied to the study of dairy products are compound light, stereo, confocal, scanning electron and transmission electron microscopes (Auty, 2002).

2.4.2.1.1 Confocal Microscopy

Confocal laser scanning microscopy (CLSM) is a microscopy technique that offers a method of observing a sample without disturbing its internal structure (Everett, Ding, Olson, & Gunasekaran, 1995a; Joshi, Muthukumarappan, et al., 2004a). The confocal microscope was invented in 1955 by Minsky (Minsky, 1988). Confocal refers to the common focal point of the image of the illumination pinhole and the back projection of the detection pin-hole (Everett et al., 1995b). The laser scanning allows the penetration of the surface of a sample and the visualisation of thin optical sections (Joshi, Muthukumarappan, et al., 2004a; Lopez, Camier, & Gassi, 2007). It has the ability to make selective observations of the optical sections without being affected by the image of out-of-focus regions above and below the plane (Everett et al., 1995a). Due to this optical sectioning it allows the accurate assessment of fat globules and the protein network in its 3-dimensional structure (Hassan, Frank, & Corredig, 2002). This is done by building up a number of adjacent planes to reconstruct a 3-D image of the sample (Everett et al., 1995b).

Other advantages of the CLSM, other than its ability to observe the internal structure without altering it (Joshi, Muthukumarappan, et al., 2004a), include: reduced preparation time due to samples not needing fixation or dehydration; the ability to continuously monitor a sample; and the ability to reconstruct the 3-D microstructure of the sample (Auty, Fenelon, Guinee, Mullins, & Mulvihill, 1999). Another advantage is the ability to fluorescently stain specific components within a sample (Heertje, Vandervlist, Blonk, Hendrickx, & Brakenhoff, 1987).

CLSM has been used extensively to study the microstructure of dairy products including milk protein gelation (Auty et al., 1999; de Kruif et al., 1995), sodium caseinate emulsions (Dickinson, Radford, & Golding, 2003; Ye & Singh, 2001), model cheese systems (Floury et al., 2009; Smith, Carr, Golding, Reid, & Zhang, 2011; Trivedi et al., 2008), feta cheese (Hassan et al., 2002), cream cheese (Fenoul, Denmat, Hamdi, Cuvelier, & Michon, 2008), Emmental cheese (Lopez, Camier, et al., 2007) and many others.

CLSM have been shown to be a useful technique in studying the microstructure of Mozzarella during the processing and ripening as it enables the imaging of the distribution of fat and protein (Auty, Twomey, Guinee, & Mulvihill, 2001; Hassan et al., 2002). It has also been used in numerous studies to assess the microstructure of Mozzarella including: work illustrating the effect of calcium (Guinee, Feeney, Auty, & Fox, 2002; Joshi, Muthukumarappan, et al., 2004a), pH (Guinee et al., 2002), the addition of phospholipase (Lilbæk et al., 2006) and the effect of storage (Auty et al., 2001).

2.4.2.1.2 Scanning Electron Microscopy

Electron microscopes offer a high resolution technique for studying the microstructure of a material (Everett et al., 1995b; Joshi, Muthukumarappan, & Dave, 2003a). Electron microscopy consists primarily of an electron gun encased in a high vacuum (Auty, 2011).

The electron beam is focused on the sample with electromagnets (Auty, 2002). In scanning electron microscopy (SEM) the image is created by electrons impinging on the surface of the sample and emitting secondary electrons (Auty, 2011). These secondary electrons are collected to form a topographic image of the sample (Aguilera & Bouchon, 2008; Auty, 2002). The first SEM was developed in 1937 by Von Ardenne (1937) following the work by Knoll in 1935.

One of the key disadvantages of electron microscopy is the sample preparation that is involved, including dehydration, which can lead to artefacts (Everett et al., 1995b; Kalab, 1984; Liboff, Goff, Haque, Jordan, & Kinsella, 1988). The dehydration of samples is needed as high vacuums are used to ensure a clear path for electrons (Aguilera & Bouchon, 2008). Another limitation of microscopy methods is the limited field of veiw that can be observed.

There are a number of different types of SEM, most notably conventional high vacuum SEM where a dried sample is examined and cryo-SEM where a sample is examined when frozen to below -80°C (Kaláb, Allan-Wojtas, & Miller, 1995). However, this project will focus on the use of conventional high vacuum SEM due to the access to equipment.

SEM has been used to illustrate the structure of many different dairy products including: casein micelles (Auty, 2011; Horne, 2002), yogurt (Auty, 2011; Lucey, 2007), spray dried milk powder (Auty, 2002), Cheddar cheese (Hall & Creamer, 1972), and Emmental cheese (Lopez, Camier, et al., 2007).

SEM has been used to study the microstructure of Mozzarella cheese during manufacture (Kiely et al., 1992; Oberg, McManus, & McMahon, 1993). It has also been used as a tool to assess changes within the protein structure during maturation (McMahon et al., 1999) and the effect of freezing on the structure of the cheese (Kuo & Gunasekaran, 2003). SEM has also been used to evaluate the effect of a number of processing and compositional levers on the structure of Mozzarella including: the effect of high pressure microfluidization

(Tunick, Van Hekken, Cooke, Smith, & Malin, 2000), the effect of calcium (Joshi, Muthukumarappan, et al., 2004a), how low fat Mozzarella compares to LMPS (Tunick, Mackey, et al., 1993), and the investigation into the role of salt in the structure of non-fat Mozzarella (Paulson et al., 1998). This is due to its ability to produce a detailed image of the protein and channel structure within the cheese.

Both confocal and scanning electron microscopy allow different detailed examination of the internal structure Mozzarella, indicating that both techniques would be beneficial in this study in conjunction with other tools.

2.4.3 Component Mobility

2.4.3.1 *Dielectric Analysis*

The impetus for an investigation into dielectric properties was based on the suggestions from the body funding this project. Interest in the dielectric properties of agricultural and food products dates back to around 1900 (Nelson, 1991). This initial work was in regards to the conductivity of grains to determine their moisture content. One of the main interest in dielectric properties of food products is in relation to predicting heating rates as well as describing the behaviour of the product when subjected to high frequencies (Venkatesh & Raghavan, 2005). Dielectric spectroscopy is also of interest in terms of the make-up of food material as the dielectric properties are closely related to the composition and structure of food (Hall, Zhuo, & Gabriel, 1994).

A dielectric probe works by emitting electromagnetic energy in the form of micro and radio waves. When the electromagnetic wave hits an object, part of it is reflected while part of it is transmitted, including the part that is absorbed (Venkatesh & Raghavan, 2005). The absorption of the electromagnetic energy has two primary mechanisms, dipolar relaxation and ionic conduction (Datta, Sumnu, & Raghavan, 2005). This means that the dielectric properties of a substance are dependent on their moisture and salt content, with water being the primarily contributor to dipolar rotation and salt being an ionic compound. These two parameters, moisture and salt are very important in a wide range of food products.

The fundamental electrical property that describes the interaction between electromagnetic energy and material is the relative complex permittivity. The relative complex permittivity, ε^*, is the permittivity of the material, ε, divided by the permittivity of free space, ε_0 (Nelson, 2006). The complex permittivity can be broken down into the following equation:

$$\varepsilon^* = \varepsilon' - j\varepsilon''$$

Equation 2-1: Equation for the complex permittivity as calculated by the dielectric constant (ε') and dielectric loss factor (ε").

The real part of this equation is the dielectric constant, ε'. The dielectric constant relates to the ability of the material to store electromagnetic energy (Datta et al., 2005; Icier & Baysal, 2004) also known as the capacitance of the material (Laaksonen & Roos, 2000). The imaginary part of the complex permittivity, ε" or loss factor refers to a materials ability to dissipate electromagnetic energy (Datta et al., 2005; Icier & Baysal, 2004) or the conductance of the material (Laaksonen & Roos, 2000). Another important parameter is the loss tangent, tan δ, which can be calculated from dividing the loss factor by the dielectric constant (Hall et al., 1994). The loss tangent is also known as the dissipation factor and is often used as a descriptive dielectric parameter (Datta et al., 2005; Nelson, 2006). In food systems it has been widely reported that the dielectric constant, ε', is related to the moisture content (Berbert et al., 2001; Everard, Fagan, O'Donnell, O'Callaghan, & Lyng, 2006; Kudra, Raghavan, Akyel, Bosisio, & van de Voort, 1992), while the dielectric loss factor, ε", has been related to the salt content of foods (Green, 1997; Kudra et al., 1992).

The dielectric constant, ε', and the loss factor, ε", can aid in estimating the penetration depth (δ_p) of the microwaves as well as establishing a temperature profile for products (Herve, Tang, Luedecke, & Feng, 1998). This power penetration depth relates to the distance over which 63% of the power is dissipated (Datta et al., 2005). This is related to the material properties of the sample being analysed. The penetration depth is an important parameter due to its ability to characterise the temperature distribution of microwave heated foods (Ahmed, Ramaswamy, & Raghavan, 2008).

There are many factors that have an effect on the measured dielectric properties of a food product. These include factors related to both the structure and composition of the material being analysed.

There are a number of different ways of assessing the dielectric properties of a material. The selection of a technique is dependent on the nature of the material being assessed, the frequency of interest, the degree of accuracy required as well as the test conditions (Datta et al., 2005). Contact probes have provided a means of non-destructive measurements for food analysis and quality control (Hall et al., 1994).

Another key parameter of dielectric testing is the frequencies used to measure the sample. This is because the dielectric properties of a material can vary with frequency (Datta et al., 2005). The frequencies used are important due to different interactions within the sample occurring at differing frequencies. The main interactions affected by differing frequencies are those of dipolar rotation and ionic mobility (Fagan, Everard, O'Donnell, Downey, & O'Callaghan, 2005; Herve et al., 1998; Wang, Tang, Rasco, Kong, & Wang, 2008). Ionic loss is typically predominant at frequencies below 1 GHz (Nyfors & Vainikainen, 1989). Whereas dipolar polarisation is typically more significant above 1 GHz, although it still influences the dielectric response of a material at lower frequencies as well (Ryynänen, 1995). The frequencies used are also of particular interest in regards to gaining an understanding of microwave heating. This is due to specific frequencies being set aside for use in microwave ovens & industrial microwave heating (Herve et al., 1998).

The measurement of dielectric properties offers a low cost, accurate and near instantaneous method of monitoring many of the critical control parameters in a process (Fagan et al., 2005). Dielectric spectroscopy has been used to assess the material properties of a number of food systems. This includes the assessment of the moisture content of foods. The traditional methods of measuring the moisture content of a product are time consuming and generally destructive (Trabelsi, Krazsewski, & Nelson, 1998). Therefore a method such as dielectric analysis is advantageous due to the fact it offers a non-destructive quick measurement option.

The assessment of the dielectric properties of cheese is a relatively recent endeavour. The first notable assessment was in 1997 in which the dielectric properties of Cheddar cheese were examined (Green, 1997). This investigation identified relationships between the composition, namely moisture content, and the dielectric properties of the cheese. The technique was also used to assess the relationship between the chemical, dielectric and sensory properties of Edam cheese during maturation (Kubis, Krivanek, & Gajdusek, 2001).

Dielectric spectroscopy was used to predict the inorganic salt content and moisture content in process cheese (Fagan et al., 2005). This identified that the moisture of the process cheese dominated the dielectric constant. They also noted that the increase in inorganic salt content of the process cheese resulted in an increase in the dielectric loss factor with moisture also having an underlying effect. From their results they developed a chemometric model to predict the moisture and inorganic salt content in process cheese.

Dielectric spectroscopy was also utilised to assess 16 different process cheese samples over a frequency range from 0.3 to 3 GHz (Everard et al., 2006). In this investigation they also assessed the effect of temperature on the dielectric properties of the process cheese samples. Both the dielectric constant and loss factor decreased over the frequency range used to assess the samples. They also identified that the composition of the process cheeses samples affected the dielectric response to temperature.

Mozzarella represents a more dynamic system than process cheese as it experiences significant structural changes after manufacture. Due to these changes being linked to the functionality of the cheese, an assessment of the dielectric properties during storage may provide insight into these changes.

2.4.3.2 *Magnetic Resonance*

Nuclear Magnetic Resonance (NMR) is based on nuclear magnetism which is a property held by a number of atomic nuclei that contain an odd number of protons, neutrons or both (Chary & Govil, 2008). It has become a relatively popular technique in a number of different industries as it is allows non-invasive probing of a material down to the molecular level (Dykstra, 2006). NMR has been proven to have numerous useful applications in the dairy industry as it can be used to study heterogeneous samples (Duce, Amin, Horsfield, Tyszka, & Hall, 1995).

The nuclei of certain atoms possess an intrinsic spin with angular momentum (L) that gives it a magnetic dipole moment (μ) (Dykstra, 2006). These two properties are related by gyromagnetic ratio, γ, which is nucleus dependent. Placing the nuclei into a magnetic field will cause the nuclear magnetic moments to align either partially parallel (α spin state) or antiparallel (β spin state) with the direction of the magnetic field (Simpson, 2008). This is caused by the magnetic dipole experiencing a torque when placed in the magnetic field which causes the alignment (Dykstra, 2006). The two spin orientations, α and β, have corresponding energies of $\pm \frac{1}{2}\gamma\hbar B_0$ with the higher energy state, β, having the + spin. The tilted momenta precess around the magnetic field, B_0, with a frequency, called the Larmor frequency (ω_0), and a random phase described by equation below (Capozzi & Cremonini, 2008)

$$\omega_{0=}\gamma B_0$$

Equation 2-2: Equation for the Larmor frequency as determined by the gyromagnetic ratio and magnetic field.

Due to the nature of NMR it is a relatively insensitive technique (Chary & Govil, 2008; Rovnyak et al., 2003). The sensitivity of a nucleus is directly proportional to the cube of the nuclear magnetic moment of the nuclei. ^{1}H has the highest magnetic moment (Barba, Jaimez-Auguets, Rodriguez-Sinovas, & Garcia-Dorado, 2007) making it the most sensitive

nuclei which is useful due to it being found in high quantities in biological systems (Chary & Govil, 2008). The relative insensitivity means that it can only be utilised to identify information relating to abundant components in a food system (Gianferri, Maioli, Delfini, & Brosio, 2007).

There are a large number of different NMR imaging sequences that have been used that differ in the parameters regarding image contrast and the speed at which the data is acquired (Duce et al., 1995). The contrast of the images is dependent on the concentration of protons, their ^{1}H longitudinal (T_1) and transverse (T_2) relaxation times, as well as the presence of mass transport processes such as diffusion (Duce et al., 1995).

The application of NMR in the food industry began in the 70's when bench-top NMR systems became commercially available (Mariette, 2009). Until recently this work has focused on time domain (TD) techniques that focus on bulk properties such as water and fat (Charlton, 2009). These measurements rely on the intrinsic properties of the proton nucleus when a sample in a magnetic field is subjected to a pulse of radio frequency. A major break-through in NMR occurred in 1966 with the development of Fourier transform (FT) spectroscopy (Chary & Govil, 2008). When this was applied to the signal resulting from a pulse of TD radiofrequency on a sample, the results were dramatically improved.

Low-resolution NMR relaxometry deals with the abundant components in a system by assessing the amplitude and decay rates in a NMR signal (Gianferri, Maioli, et al., 2007). This form of NMR has the advantage of not requiring any sample preparation as well as its ability to take rapid measurements.

The use of high-resolution (HR) NMR has gained an important role in food characterisation due to its ability to identify and quantify all major low-medium molecular weight components in a sample without the need for destructive processes such as separation (Baroni, Bubici, Ferrante, & Aime, 2009). This method utilises higher strength magnetic

fields and has allowed the measurement of specific resonance frequencies corresponding with specific components in the food system (Charlton, 2009).

Recent advances have also led to NMR becoming a powerful tool for determining 3D structures (Chary & Govil, 2008). Nuclear magnetic resonance imaging (MRI) and magnetic resonance microscopy (MRM) are techniques used to image samples at various scales. A magnetic resonance image is constructed using a combination of pulsed magnetic field gradients with radiofrequency pulsing to encode spatial position in the phase and frequency of the received signal (Tyszka, Fraser, & Jacobs, 2005).

It offers a powerful technique for assessing structural and compositional changes within a system (Mariette, 2009). NMR has been used as a technique to study a number of different factors in cheese. NMR provides a non-destructive and rapid technique to assess the quantity, structure and dynamic characteristic of water (Duce & Hall, 1995; Kuo et al., 2001), solid fat content (McClements & Povey, 1988; Singh, McClements, & Marangoni, 2002) as well as identifying the effect of pH, temperature and calcium on the structure of casein micelles (Rollema & Brinkhuis, 1989).

2.4.3.2.1 T2 (spin-spin) Relaxation

Spin relaxation measurements can be used to study molecular interactions as well as gaining information regarding molecular tumbling (Godefroy, Creamer, Watkinson, & Callaghan, 2003). Relaxation is the process whereby nuclear spins come to thermal equilibrium among themselves (Callaghan, 1993). T2 or spin-spin relaxation is the measurement of the transverse relaxation of nuclei, which varies as a function of molecular motion (Mariette, 2011). It is essentially the destructive interference in the precessing magnetisation due to each spin precessing at a slightly different rate (Cowan, 1997).

T2 relaxation measurements were used to monitor the changes in the water mobility during aging of Mozzarella (Kuo et al., 2001). This study showed that NMR was able to identify that water became less mobile in Mozzarella during the first 10 days of aging. It also identified that water was held in at least two different states, that in the bulk phase and that which is associated with the casein matrix. NMR has also been used to identify the effects of freezing Mozzarella (Kuo, Anderson, & Gunasekaran, 2003). The study by Kuo et al. (2003) identified changes caused due to the formation of ice crystals and their subsequent damage to the protein matrix.

2.4.3.2.2 Diffusion

The translational motion of molecules within a system can be assessed using Pulsed Gradient Spin Echo (PGSE) techniques to investigate the diffusion properties of molecules (Callaghan, 1991; Mair et al., 2001). This technique is based on well-defined linear gradient pulses that change the magnetic field strength probed locally by the protons of a molecule (Mariette, 2011). If a molecule diffuses spatially within the magnetic field gradient there is a reduction in the NMR signal; a faster diffusion rate results in a greater reduction in the NMR signal (Mariette, 2009).

The diffusion of solvent molecules is impeded by macromolecules due to both the required diversion to diffuse around the macromolecule and the momentary inhibition caused by interaction with the macromolecule (Gottwald, Creamer, Hubbard, & Callaghan, 2005). The diffusion properties of solvent molecules in porous media are an area that is gaining increasing attention. At very short time scales the solvent molecules can freely diffuse, however, at long time scales the molecules are restricted by the gel network (Fridjonsson, Bernin, Seymour, Nyden, & Codd, 2011). There are two aspects associated with porous media having reduced diffusion movement of molecules (Song, 2009). The first, at short time scales, is due to solvent molecules near the surface of the pores having

their movement restricted by the surface to volume ratio (S/V_p) of the pores (Latour, Kleinberg, Mitra, & Sotak, 1995; Mitra, Sen, Schwartz, & Le Doussal, 1992). The second, at long time scales, is limited by the tortuosity (α) of the network (Song, 2009). The tortuosity of a porous media is governed by the connectivity of the pores (Latour et al., 1995), and is the ratio of the path length that a molecule travels to the geometric length (Brown et al., 2012).

A Pade approximate can be used to estimate the surface to volume ratio and the tortuosity, as In Equation 2-3 below (Mair et al., 2001).

$$\frac{D(t)}{D_0} = 1 - (1 - \frac{1}{\alpha}) \frac{\left(4\sqrt{\frac{D_0 t}{9}}\sqrt{\pi}\right)\left(\frac{S}{V_p}\right) + \left(1 - \frac{1}{\alpha}\right)(\frac{D_0 t}{D_0 \theta})}{\left(1 - \frac{1}{\alpha}\right) + \left(4\sqrt{\frac{D_0 t}{9}}\sqrt{\pi}\right)\left(\frac{S}{V_p}\right) + \left(1 - \frac{1}{\alpha}\right)(\frac{D_0 t}{D_0 \theta})}$$

Equation 2-3: Pade approximate for diffusion measurements

The diffusion (D) at time t was normalized by the free diffusion of water (D_0) and includes a fitting parameter θ that represents the time for a particle to diffuse the distance required to reach the tortuosity limit (Brown et al., 2012).

The majority of work related to diffusion in cheese has involved the use of relaxation diffusion correlations to investigate structural dynamics of cheese. These studies include: the comparison of various dairy products (Hurlimann, Burcaw, & Song, 2006), investigating the effect of salt contents in Mozzarella with aging (Hubbard, Watkinson, Creamer, Gottwald, & Callaghan, 2005) and examination of the effect of age on the dynamics of Mozzarella and Gouda at 5 and 40°C (Godefroy, Korb, et al., 2003). These correlation techniques utilize 2D inverse Laplace inversion NMR to probe the intricate nature of heterogeneous systems, assessing the complex multi-exponential behaviour of their diffusion and relaxation rates (Hubbard et al., 2005).

Magnetic resonance techniques are a powerful tool for assessing component mobility in complex media such as cheese.

2.4.3.2.3 Solid-state ^{31}P NMR

^{31}P magic angle spinning solid state NMR is a technique that can be used to investigate phosphorus (Rondeau-Mouro, Gobet, Mietton, Buchin, & Moreau, 2009a). This method used with a combination of a ^{31}P single pulse, cross polarisation and dipolar dephasing enables the resonances of phosphates with different levels of mobility and proximity to protons to be distinguished (Bak, Rasmussen, Petersen, & Nielsen, 2001). This allows the signals to be discriminated in regards to mobility, with immobile phosphorus in the insoluble phase and mobile phosphorus in the soluble phase (Gobet et al., 2010).

Phosphorus NMR has been used to investigate colloidal calcium phosphate in casein micelles (Bak et al., 2001) and the distribution of phosphorus in native casein micelles (Thomsen, Jakobsen, Nielsen, Petersen, & Rasmussen, 1995). More recently the technique has been applied to cheese to evaluate the distribution and mobility of phosphates (Gobet et al., 2010; Rondeau-Mouro et al., 2009a). However, apart from the initial investigation into phosphate groups in cheese by Rondeau-Mouro et al. (2009a) and Gobet et al. (2010), very little work has been conducted using ^{31}P NMR in cheese. This raises the question as to whether it would be a useful technique to evaluate the changes occurring within the structure of cheese during storage.

Nuclear magnetic techniques have been proven to be powerful tools to elicit information about the structure and component mobility of cheese. The ability of these techniques to gain a detailed understanding of structure and structural change suggest that it would be useful to study Mozzarella.

3 Specific Research Objectives and Structure of Thesis

One of the main objectives behind the studies in this thesis was the elucidation of the structure of Mozzarella cheese with a particular focus on component mobility. It was hypothesised that the structure of Mozzarella would be determined in part by the ionic environment surrounding the protein matrix and thus it would be desirable to utilise techniques that could quantify the state of both ions and water. Dielectric spectroscopy (DS) was identified as a non-destructive method for studying the state of water and ions that had previously been used for this purpose in static systems. It was hypothesised that DS could be used to study the mobility or state of ions and water in Mozzarella. A model analogue Mozzarella system was chosen so that the composition could be systematically varied (in particular, the calcium content) and the dielectric response measured. The outcomes of this first study are described in Chapter 4.

With the newly developed DS method in hand but not yet proven, attention turned to natural Mozzarella. DS plus conventional techniques such as microscopy and NMR were used to study water movement in Mozzarella during the first 20 days of storage (Chapter 5) and then on to 120 days (Chapter 6). The objective of these studies was to continue the evaluation of DS as a tool and then to compare it to established techniques for monitoring water movement such as NMR.

For the longer maturation, Cheddar cheese was added to the study so it could be used as a reference point for changes in the Mozzarella structure. In addition, gel electrophoresis was used to monitor break down of caseins. The outcome of Chapter 5 highlighted the need to understand where the free water evident in the first few days of the Mozzarella storage was coming from. As part of the subsequent study, the opportunity was taken to investigate the structure of Mozzarella during manufacture. The objective of this work was to pinpoint the stage at which the free water appears. It was determined that the

stretching step was the point at which the moisture moves from inside the protein network into the channels containing the fat.

Few studies have been published that investigate Mozzarella structure during heating. Another key objective for this study was to elucidate structural change and component mobility during heating. To this end, the commercial Mozzarella from the previous study was monitored during the 120 day shelf-life study by carrying out temperature sweeps (4-90^{o}C) using DS and small amplitude oscillatory rheology (SAOR). The SAOR data was used to construct Arrhenius plots of complex viscosity – these were then differentiated to highlight sometimes subtle changes. This is reported in Chapter 7.

While the DS and SAOR study of Mozzarella was novel, the study highlighted the need for a better technique for determining water movement in Mozzarella during heating. Conventional NMR can be used to measure water movement during storage but a Magnetic Resonance Imaging (MRI) facility in Montana offered the opportunity to study materials over a range of temperatures. Six weeks were spent at Montana State University (MSU) with the objective being to evaluate MRI as a tool to study cheese during heating. Significant method development work was carried out and then the technique was applied to commercially available store bought samples Mozzarella and Cheddar. Two Cheddar samples of different maturity were studied. From diffusion measurements, the Pade approximate was used to measure diffusion of water and infer porosity and tortuosity. This work is described in Chapter 8. Having established the new method and carried out an initial look at shop-bought cheeses, the technique was used to study a sample of one day old Mozzarella during the first 18 days of storage. This is described in Chapter 9.

Time limited the studies at MSU so the new techniques were transferred to the Massey University NMR Facility. It was hypothesised that Mozzarella made with different levels of

calcium should have different structures and therefore differences in porosity and tortuosity should be detectable by measuring the diffusion of water using the new technique. Mozzarella cheeses with 4 different calcium concentrations were prepared in the Fonterra Research and Development Centre's pilot plant. These cheeses were matured for 40 days and tested regularly during storage using DS, microscopy, PAGE and water diffusion. The detail and outcomes of this final study are described in Chapters 10.

The ultimate objective of this overall study was to find new techniques that would elucidate the structure of Mozzarella and help understand how this structure changes with composition, storage and application. The learning's from each of the individual studies were used to describe a conceptual model of the structure of Mozzarella and how it changes during storage and upon heating.

4 Assessing Dielectric Spectroscopy as a Tool for Studying Composition and Structure of a Model Cheese System

4.1 Introduction

The structure of food influences an array of attributes associated with the product. Cheese is a good example of this as its microstructure governs its functional properties, including meltability and shredability. The structure of a cheese system is affected by its components including protein, fat, moisture, pH and calcium. Calcium affects both the structural and functional properties of cheese (McMahon et al., 1993). One of the reasons for this is the large impact that calcium has over protein to protein interactions within the cheese matrix (Pastorino et al., 2003; Paulson et al., 1998). More details of the importance of calcium are given in Section 2.2.2.5. Mozzarella is one such cheese that calcium plays a vital role in structure and functionality (McMahon & Oberg, 1999).

There are many different techniques used, individually and in combination, to assess the microstructure of cheese products from visual means such as microscopy to indirect instrumental methods (Auty, 2002). Dielectric spectroscopy is a tool that has found increasing use in the food industry as it provides a means of assessing the structure and composition of a material (Hall et al., 1994). It works by emitting a spectrum of high frequency electromagnetic waves into the sample and measuring how the material interacts with the energy. This interaction is broken down into two components:

- how the material stores energy, the dielectric constant (ε')
- how it dissipates energy, the dielectric loss factor (ε") (Venkatesh & Raghavan, 2004).

The dielectric properties are dominated by dipolar rotation and ionic interactions within the sample being examined. In foods, water is the primary component responsible for dipolar rotation while salts are responsible for the ionic interactions (Datta et al., 2005). Dielectric spectroscopy has been used to: study the dielectric properties of Cheddar

cheese (Green, 1997) and processed cheese (El-Bakry, Duggan, O'Riordan, & O'Sullivan, 2010); assess changes during the ripening of Edam (Kubis et al., 2001); and for prediction of moisture and salt in processed cheese (Fagan et al., 2005).

Analogue cheeses provide a useful model system for this type of study due to the compositional uniformity and controllability that can be maintained over time and different batches (Pereira et al., 2001). Due to these products having simpler processing conditions than traditional cheeses, it makes them cheaper to produce and also allows a certain amount of tailoring to meet specific compositional and functional requirements (El-Bakry et al., 2010). This is generally accomplished by producing the analogue cheese using simplified processing procedures and ingredients (Watkinson et al., 2001). Although these systems are generally uniform in nature, care needs to be taken to make sure that there is full hydration of the protein source to ensure this is the case.

Model systems have been used extensively in the past to investigate varying properties of cheese including investigating the effect of colloidal calcium phosphate in Cheddar (O'Mahony et al., 2006) and assessing the effect of different caseins on a model processed cheese (Savello et al., 1989).

The objectives of this investigation were to:

- Adapt a methodology to study cheese using dielectric spectroscopy
- Develop a model cheese system and manipulate the calcium content to study the role calcium plays in the structure of the cheese using well established techniques including confocal microscopy and compression testing.
- Analyse the model system using dielectric spectroscopy and evaluate its usefulness as a tool for studying cheese

4.2 Materials and Methods

4.2.1 Materials

Table 4-1: Material for model system

<table>
<tr><th>Material</th><th>Supplier</th><th>Product information</th></tr>
<tr><td>Milk Protein Concentrate (MPC 4864)</td><td>Fonterra Co-operative Group Limited, NZ</td><td>Product ID: 1576 BU03 A2336
Make date: 03/09/2010
<table><tr><th>Protein (%)</th><th>Fat (%)</th><th>Ash (%)</th><th>Moisture (%)</th><th>Carbohydrates (%)</th></tr><tr><td>81.5</td><td>3.5</td><td>7.0</td><td>5.8</td><td>2.2</td></tr></table></td></tr>
<tr><td>Fresh Frozen Milk Fat for recombination</td><td>Fonterra Co-operative Group Limited, NZ</td><td>Product ID: 4172 DQ30 E6579
99.9% Fat, <1% moisture</td></tr>
<tr><td>Glucono-delta lactone</td><td>Hawkins Watts, Auckland, NZ</td><td>Batch: 5009454-R401
Best Before: 01/07/2012</td></tr>
<tr><td>Sodium Chloride</td><td>Dominion Salt, Mount Maunganui, NZ</td><td>Food grade</td></tr>
<tr><td>Calcium Chloride</td><td>Hawkins Watts, Auckland, NZ</td><td>Lot: 2009190298-R117
Best Before: 01/03/2014</td></tr>
</table>

4.2.2 Confocal Microscopy

Thin sections of the refrigerated cheese samples were cut using a scalpel blade and the protein stained with 1% Fast Green and the fat stained with 0.5% Nile Red. A small quantity of each of the dyes was mixed together in a ratio of 1:1. The staining of the sample was done by placing 10 µl of the mixed dyes on a cover slip and immersing the thin section of cheese in the dye. The sample was left to sit in the dye for a period of 20

minutes. A concave slide was then placed onto the cover slip so that the sample of cheese and dye were in the concave section of the slide. Pressure was applied to the slide so that the excess dye on the cover slip created a seal that keeps the cover slip attached to the slide. The slide was then placed on the microscope stage for examination. The Illumination was provided by an Argon laser at 488 nm and a Helium/Neon laser at 633 nm. Scanning was done sequentially to excite the two dyes with a zoom factor of 1. The samples were examined using a Leica SP5 Confocal Laser Scanning Microscope (CLSM) (Leica Microsystems, Wetzlar, Germany) with a 10x and 20x dry objective as well as a 40x and a 62x oil immersion objective. This was done to observe the microstructure of the model system at room temperature.

At least three images were collected for each model system at magnifications of 20x, 40x and 62x. The images were collected and viewed using Lecia LAS AF Lite software (Leica Microsystems, Wetzlar, Germany).

4.2.3 Texture Profile Analysis

The cylindrical samples of cheese, 32 mm diameter and 20.5 mm in height, were analysed using a TA-XT Plus Texture Analyser (Stable Micro Systems, Godalming, England) performing a double compression texture profile analysis (TPA).

The cheese samples were removed from the refrigerator, cut with a cork borer, wrapped in plastic wrap and left to reach equilibrium in the temperature controlled laboratory set at 20°C for a period of 4 hours. A thermocouple was used to ensure that the samples had reached 20°C prior to testing. This was done by inserting the thermocouple into the centre of a sample of cheese identical to the samples for texture analysis. Samples were then placed on the centre of the platform and compressed in two successive cycles recording the force and time parameters. Samples were compressed to 50% of their original height (Cortez, Furtado, Gigante, & Kindstedt, 2008) using a 35 mm diameter Teflon probe with a 50 kg load cell. From this data the parameters of hardness, springiness and cohesiveness

were calculated. Three batches of eight samples were tested for each of the various systems.

4.2.4 Uniaxial compression

Samples were analysed using a TA-XT Plus Texture Analyser (Stable Micro Systems, Godalming, England) performing a uniaxial compression test. Cheese samples were cut into cylinders of 25 mm height and a diameter of 20 mm. Samples were compressed to 80% of its original height using a 60 mm diameter Teflon probe. The compression speed was 0.83 mm/s (ISO, 2006).

The cheese samples were removed from the refrigerator, cut with a cork bore, wrapped in plastic wrap and left to reach equilibrium in the temperature controlled laboratory set at 20°C for a period of 4 hours. As with the TPA analysis, a thermocouple was used to assess the temperature of a non-tested sample of the same geometry to ensure the samples had reached the required temperature prior to testing. Samples were then placed on the centre of the platform and compressed, recording the force. From this data the parameters of hardness and force required to fracture the samples were obtained. The compression was replicated at least 20 times per sample.

4.2.5 Dielectric Analysis

Dielectric measurements were made using an Agilent 85070E high temperature dielectric probe connected to an Agilent 8712ET network analyser (Agilent Technologies, California, USA). The system was calibrated using three known standards air, a shorting block and Milli-Q water at 25°C.

A cylindrical cheese sample, 28.5 mm in diameter and 31 mm in height, was taken directly from the refrigerator and placed in the jacketed sample holder and raised up to the dielectric probe using a laboratory jack until the sample was in constant contact with the

probe. An initial 100 point frequency sweep from 200 MHz to 1.3 GHz was taken at 4°C, to assess the dielectric properties of the samples at the temperature of refrigeration. The water bath attached to the heating jacket was set to 25°C and the cheese was left for a period of 45 minutes when the next 100 point frequency sweep was conducted and the dielectric data was recorded. The water bath was increased incrementally by 10°C every half an hour with the dielectric data recorded at the end of the half hour period. Measurements were taken in sets of four at each of the temperature steps recording both the dielectric constant and the dielectric loss factor. The dielectric constant and the dielectric loss factor were determined using Agilent 85070E dielectric probe kit software (Agilent Technologies, California, USA). In order to ensure the cheese sample had reached the required temperature prior to sampling a thermocouple was inserted into the sample. Two replicates were taken from each of the three batches manufactured for each of the different calcium variations.

4.2.6 Model System

4.2.6.1 *Model system formulations*

Table 4-2: Table of formulations (quantities in grams)

	VC1	VC2	VC3	VC4	Na1	Na2	Na3	Na4
MPC	148.5	148.5	148.5	148.5	148.5	148.5	148.5	148.5
Fat	102.5	102.5	102.5	102.5	102.5	102.5	102.5	102.5
Deionised Water	234.5	234.5	234.5	234.5	234.5	234.5	234.5	234.5
Salt	10	10	10	10	10	10.73	11.560	13.19
GDL	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5
$CaCl_2$	0	1.38	3.03	6.06	0	0	0	0

4.2.6.2 *Varying the calcium content*

The model system was produced with four differing ratios of calcium to protein: 1% (VC1), 1.4% (VC2), 1.9% (VC3) and 2.8% (VC4) (w/w) to gain a range of levels between that of the base powder and a commercial Mozzarella product, Mozzarella T Cheese (Fonterra Co-operative Group Limited). Batches (~500 g) were manufactured on a Brabender Sigma mixer S300, a twin screw z blade mixer, attached to a variable speed drive. The temperature for the mixer was controlled using a water bath attached to the jacket of the mixer.

The fat was placed in the cooker and left to melt at 35°C. The MPC, salt, GDL and calcium chloride were added to the melted fat and the mixer set to 135 rpm. The water was slowly added to the other components over a 10 minute period. The water bath was increased to a temperature of 70°C over a period of 15 minutes. Once the 15 minute period had elapsed, the speed was reduced to 54 rpm. After 10 minutes of mixing at this lower speed the cheese was removed and placed into moulds. The moulds were wrapped in plastic wrap to avoid moisture loss and drying. They were then placed on a metal bench and allowed to cool to room temperature for a period of 2 hours before being transferred into a refrigerator. The pH and moisture content were measured for each of the different samples with the moisture measurements made by drying the samples in an air oven at 108°C for 16 hours.

4.2.6.3 *Varying sodium content*

The model system was manufactured as above; however, the four calcium levels that were used in the initial experiment were replaced by the molar equivalent of sodium. These samples, as shown in the formulation table (Table 4-2), had additional salt added to the model system in quantities of 0 g (Na1), 0.727 g (Na2), 1.596 g (Na3), 3.191 g (Na4).

The dielectric properties of these samples were assessed and compared to the results of the calcium experiment.

4.2.6.4 *Critical Calcium level*

It was evident from the initial experiment that a calcium to protein ratio in the range of 1 to 2.8% has a significant impact on structure (4.2.6.2). To characterise the relationship of calcium to structure over this critical range a second set of experiments was conducted. The model system was manufactured, as stated above, with progressively increasing calcium to protein ratios from 1% to 2.8% (w/w), with emphasis on the range between VC3 and VC4. The effect of the added calcium was assessed in regards to the effect it had on the hardness of the resulting cheese.

4.2.6.5 *Effect of processing*

To investigate the role that calcium was playing in the 2.8% sample (VC4), the point at which the calcium was added during processing was varied. This calcium level was chosen as it is the same as a commercial Mozzarella cheese. Addition points were chosen at the point of water addition (A), at the end of the 10 minute mixing step (B), mid-way through the temperature ramp (C) and at the beginning of the kneading step (D), as seen in Figure 4.1 below.

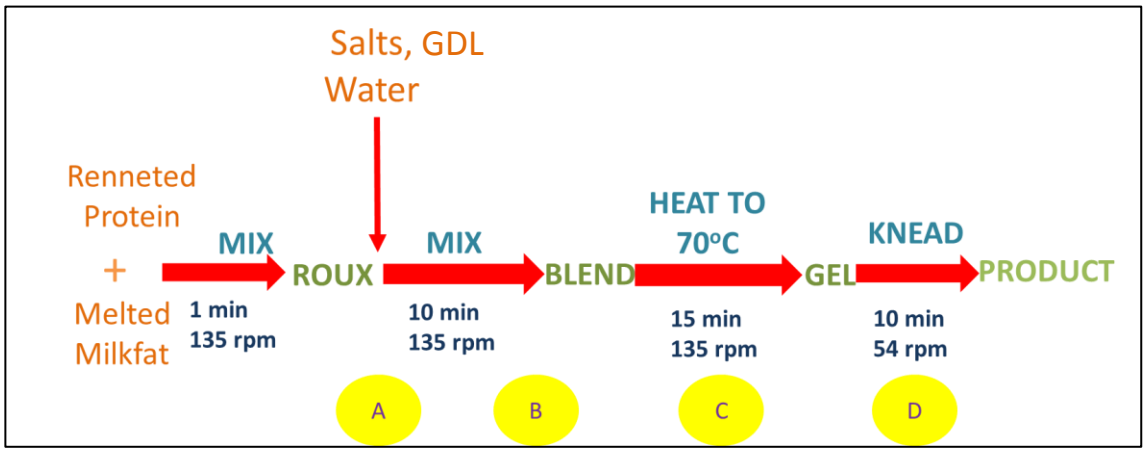


Figure 4.1: Model system manufacturing process

An additional system was manufactured where the calcium was added at point A but the mixing time prior to heating was extended from 10 to 30 minutes. The calcium chloride was dissolved in 10 ml of deionised water prior to being added to the system. An Omega TQ513 rotary torque sensor (Omega Engineering Inc, USA) was fitted in line to the Brabender unit. This was done as it enabled the torque to be monitored during processing and related to the viscosity of the system.

4.3 Results and Discussion

4.3.1 Model system development

A model system was required that would allow various components to be manipulated – particularly calcium. A composition close to Low Moisture Part Skim (LMPS) Mozzarella was desirable, preferably with similar levels of calcium and other minerals. This precluded rennet casein-based analogue Mozzarella that contains emulsifying salts due to their higher mineral content. A functional milk protein concentrate (MPC) from Fonterra Cooperative Group was selected as the protein source. There is precedent for its use in model cheese (Carr, Coker, Kells, Elston, & Ferreira, 2010). This MPC is a calcium-reduced MPC that has excellent solubility and dispersion characteristics. These characteristics are essential for making a fully hydrated, homogeneous model cheese. Unlike with processed cheese manufacture, emulsifying salts are not required to form a block of model analogue cheese. Therefore a model system could be manufactured using this functional MPC with a calcium concentration equal to and lower than LMPS Mozzarella.

It should be noted however, that the MPC contains whey protein (~20% of the total protein). Whey protein is not usually present in Mozzarella and has the potential to change the functionality of the model cheese, particularly the melt characteristics (as discussed in 2.2.2.7). However, the focus of this piece of work was on assessing dielectric spectroscopy as a tool for studying cheese structure rather than functionality.

The acid source chosen for the model system was glucono-delta lactone (GDL). GDL was chosen as it slowly hydrolyses to form gluconic acid (Salles et al., 1995). Slow acidification was more desirable for the model system as the use of GDL results in a more uniform pH drop rather than a localised one that can occur when some stronger acids are used, thereby avoiding inhomogeneous precipitation of the protein.

4.3.2 Observations during Manufacture

During the initial mixing VC1 formed small pellets of protein visibly coated with fat while the other three samples formed a dough-like mass. As the temperature of the samples increased, VC1 started to knit together as the temperature approached 50°C, VC2 and VC3 increased in viscosity and slowly approached a texture similar to VC1. However, following its initial increase in viscosity, during the initial mixing step, VC4 started to lose viscosity as the temperature was increased above 35°C. When the samples reached a temperature of approximately 55°C they started to form a cohesive mass. In the case of VC1 the cohesive mass seemed relatively uniform; however, it was coated with a thin yellow layer of fat on the surface. Both VC2 and VC3 formed what appeared to be a homogeneous, semi-solid mass at this temperature. VC4, however, lost all structural rigidity by 55°C and became a low viscosity, water-like liquid.

After manufacture VC1, VC2 and VC3 formed a slightly rubbery cheese-like mass with VC1 appearing slightly yellower in comparison to the other samples. VC4 was poured into its moulds and set to form a soft paste that was white in colour. With the increase of calcium chloride in the system there was a corresponding decrease in pH.

Table 4-3: Analysis of model systems

	Moisture (%)	pH
VC1	45.9	5.50
VC2	45.7	5.47
VC3	45.2	5.44
VC4	45.1	5.29

In order to determine the impact of pH on the formation of the different structures, each sample was manufactured at a pH of 5.3. This was achieved by manipulating the quantity of GDL added to the model system. All four samples were observed to follow the same trends as those observed in the samples manufactured at different pHs. When the moisture content of the samples was analysed it was found that they were all relatively similar, ranging from 45.9 to 45.1%.

4.3.3 Confocal Imaging

The confocal laser scanning microscope (CLSM) allowed the dispersion of protein and fat within the system to be observed. When comparing the microstructure of the four different samples using CLSM, significant differences were observed as shown in Figure 4.2.

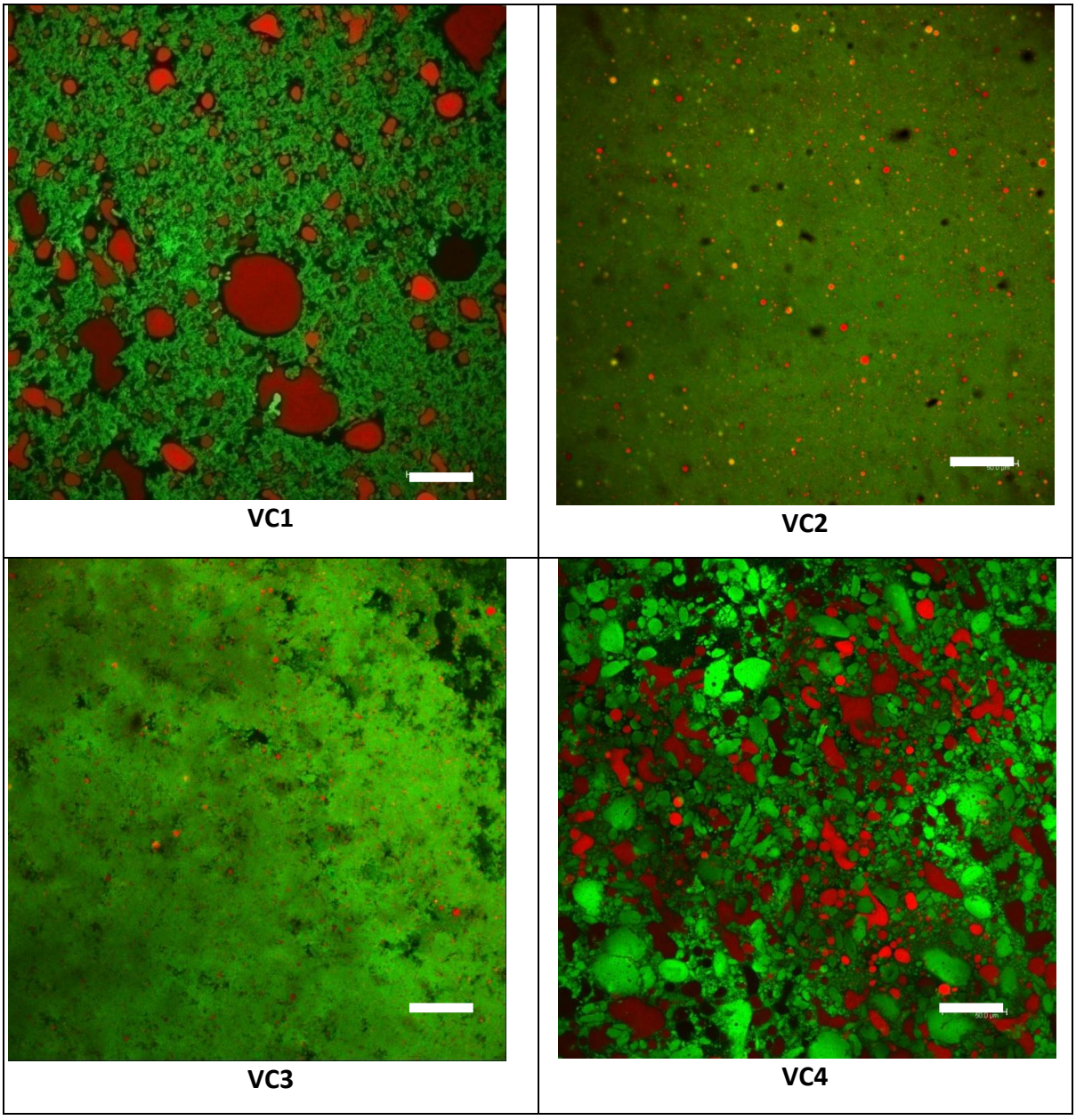


Figure 4.2: Confocal scanning laser microscope images from 1% (VC1), 1.4% (VC2), 1.9% (VC3) and 2.8% (VC4) calcium to protein [40 x objective, scale bar = 50 µm]

The yellower colour observed visually by eye in the VC1 samples is likely due to the large fat globules shown in the confocal image of the sample or small quantities of free fat. The formation of small protein clusters in VC4 indicates that the continuous protein network present in the other samples is not present in this sample. The absence of this three

dimensional protein network would explain why the sample remained pasty when cooled to room temperature. VC2 and VC3 exhibited a fine dispersement of fat globules within the protein matrix. This difference in the observed protein structure is likely due to the influence of the calcium on the emulsifying ability of the casein present in the system (Caric & Kaláb, 1987; Ye & Singh, 2001). In the work of Ye and Singh (2001) the key mechanism involved in the role of calcium in the emulsifying ability of casein is related to the calcium binding to phosphoserine, reducing electrostatic repulsions between the molecules. They reported that low concentrations of calcium initially assisted in the formation of small aggregates that adsorb to the surface of the fat droplets and the calcium reducing charge repulsion aiding in the closer packing at the interface. However, at high calcium-to-protein ratios protein bridging flocculation results in higher levels of casein aggregation, combined with a decrease in the flexibility of casein may limit the coverage of the fat droplets (Ye & Singh, 2001). This is a similar trend to that observed in acidic caseins and sodium caseinate where lower concentrations of calcium chloride increased the emulsifying ability of casein, while higher concentrations had the opposite effect (Mohanty, Mulvihill, & Fox, 1988).

4.3.4 Texture Profile Analysis

The mean results of the texture profile analysis (TPA) are displayed in Table 4-4 below. Three parameters from the TPA testing were compared: the hardness, springiness and cohesiveness. These parameters were determined in accordance with Bourne (1978). The results indicate distinct differences between the samples.

Table 4-4 – Texture profile analysis of samples [20°C]

Sample	Hardness (N)	Springiness	Cohesiveness
1% (VC1)	124.6	0.79	0.60
1.4% (VC2)	237.6	0.89	0.71
1.9% (VC3)	206.1	0.85	0.64
2.8% (VC4)	27.4	0.21	0.24

As the confocal images of the 2.8% sample (VC4) showed, the protein is present as discrete ball-like structures rather than the continuous protein structure that is present in the other samples. This explains why the hardness value obtained for this sample was substantially lower than that of the other samples. The lack of springiness and cohesiveness in the sample is likely to be due to the same reason; that is there is no rigid or elastic network keeping the structure intact. Although VC1 has a hardness that is between that of VC4 and VC3, both the values for springiness and cohesiveness are relatively close to those of VC2 and VC3. The lower hardness of this sample is likely due to the low calcium content limiting the number of protein to protein interactions in the final product (Metzger, Barbano, Kindstedt, et al., 2001). This looser network also results in the formation of larger pockets of fat globules which may deform under pressure during testing. However, the continuous protein matrix present in this sample is likely to be enabling it to deform and reform. This gives VC1 a much higher springiness and cohesiveness in comparison to VC4 which only has dispersed bundles of protein rather than a continuous network. An analysis of variance was conducted on the TPA results to identify whether the difference between the samples was significant. Significant differences were found to exist between the samples (P=0.000). A Tukey's test revealed that VC2 and VC3 were grouped together with both being separate groups. A further ANOVA comparing VC2 and VC3 indicated that no significant difference existed between

the two samples at a 95% confidence interval (P=0.058). A maximum standard deviation in the hardness results was recorded at 20.3.

Both VC2 and VC3 exhibited high values for hardness, springiness and cohesiveness. This is likely to be caused by the increase in the calcium mediated protein to protein interactions throughout the protein matrix. A greater number of calcium-mediated protein-to-protein interactions will result in a firmer gel structure (Joshi, Muthukumarappan, et al., 2004a). This is due to the increased level of crosslinking leading to a more connected rigid casein matrix. Also the finer dispersion of fat within both VC2 and VC3 will reduce the contribution of fat to the microstructure of the cheeses, with the protein dominating the material properties of the systems. A finer disbursement of fat has been observed to result in an increase in the apparent viscosity of cheese (Rowney, Hickey, Roupas, & Everett, 2003). VC2 exhibited a higher level of all three attributes. The texture profile analysis on the samples indicated that VC4 had relatively low levels of structural integrity in comparison with the other samples. At the highest calcium ratio, the product is very soft and pasty with little cohesion. This is consistent with the low viscosity observed during manufacture and the discontinuous protein structure evident in the micrograph. The texture of the model system follows a similar trend to previous studies where the calcium initially aided in emulsification but at higher concentrations lead to a disruption of the formation of structure (Caric & Kaláb, 1987; Mohanty et al., 1988).

4.3.5 Dielectric Spectroscopy

4.3.5.1 *Dielectric method development*

An assessment was made of the method developed by Fonterra for using dielectric spectroscopy to study cheese (Kong & Zhang, 2010). This was done by analysing a commercial sample of Mainland Edam cheese (Fonterra Cooperative Group, New Zealand) and conducting multiple replicate analyses to identify the repeatability of the method.

This identified that the pre-existing method had a large degree of variation in the results between samples. A number of factors were identified as potential causes of the sample to sample variation including: the removal of the sample between measurements leading to compression between measurements and the temperature being based on a thermocouple reading from the side of the sample rather than from where the dielectric was placed. Adjustments were made to the method to reduce errors. In addition to these factors a number of other variables were also assessed to identify the effect they had on the dielectric readings of the samples. These included investigating the effect of the compression of the sample by the probe, the heating rate of the sample, temperature gradients within the sample during heating as well as whether there was a difference between a grated or plug sample of cheese.

Thermocouples were placed into cheese samples inside the jacketed dielectric sample holder. The sample was subjected to a number of different heating regimes including step changes and heating ramps. The time taken to obtain a desired change in temperature was also measured. From this it was observed that temperature steps, with adequate thermal equilibration between steps, were the most reliable way of obtaining a desired temperature. Based on the observed results from this experiment, adjustments were made to the heating rate used for dielectric testing and a stepwise heating regime was implemented. The resulting heating profile used in subsequent experiments was similar to that of Everard *et al* (2006), however, a longer equilibration period was used in this experiment as the dielectric properties were observed to take close to 25 minutes to stabilise, indicating the place where the dielectric measurements were taken on the sample required this time to reach thermal equilibrium.

To assess the role of compression of the dielectric probe on the cheese, samples were placed in the sample holder and incrementally compressed while taking dielectric readings. As the cheese sample was progressively compressed, no change was observed in the dielectric properties until the sample was compressed to approximately two thirds of

its original height. However, compression of grated samples was found to impact on the dielectric properties of the cheese. The dielectric properties were found to increase as the grated sample was compressed up until the point where they were the same as the plug of cheese. This indicated that the air pockets present between the individual grated pieces of cheese were depressing the dielectric response of the cheese. This is due to dielectric spectroscopy being influenced by the density of samples (Venkatesh & Raghavan, 2005). However, the experiment did suggest that if a plug of cheese was used a small amount of compression, sufficient to eliminate entrapped air between the probe and cheese surface, will not affect the results obtained by the dielectric probe.

Following the analysis of the method and examination of factors that could influence the results, modifications to the method were proposed and validated using a sample of commercial cheese. The validation involved performing a frequency sweep from 200 MHz to 1.3 GHz on the commercial Edam cheese and replicating it six times. This validation was carried out at four temperatures: 8, 21, 37 and 57°C. The refined method was found to produce repeatable results with a much smaller error than the previous method. The maximum standard deviation for the six replicates of the validation experiment at each of the four temperatures was found to be 0.86.

4.3.5.2 *Dielectric analysis of the model system*

Both the dielectric constant (ε'), Figure 4.3 below, and dielectric loss factor (ε"), increase with temperature and decrease with frequency.

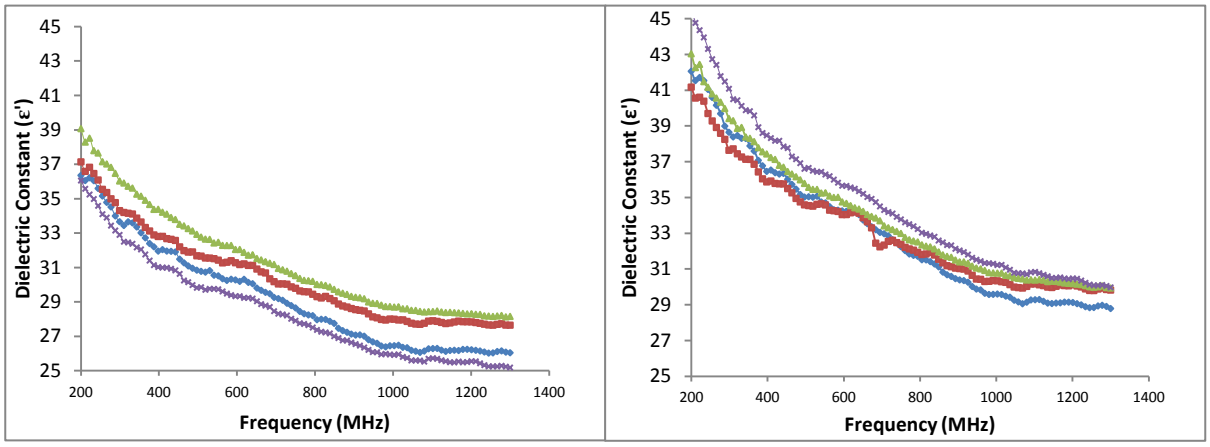


Figure 4.3- Dielectric constant of samples at 25°C (left) and 85°C (right). [△1% (VC1), ● 1.4% (VC2), ◆ 1.9% (VC3) and X 2.8% (VC4)]

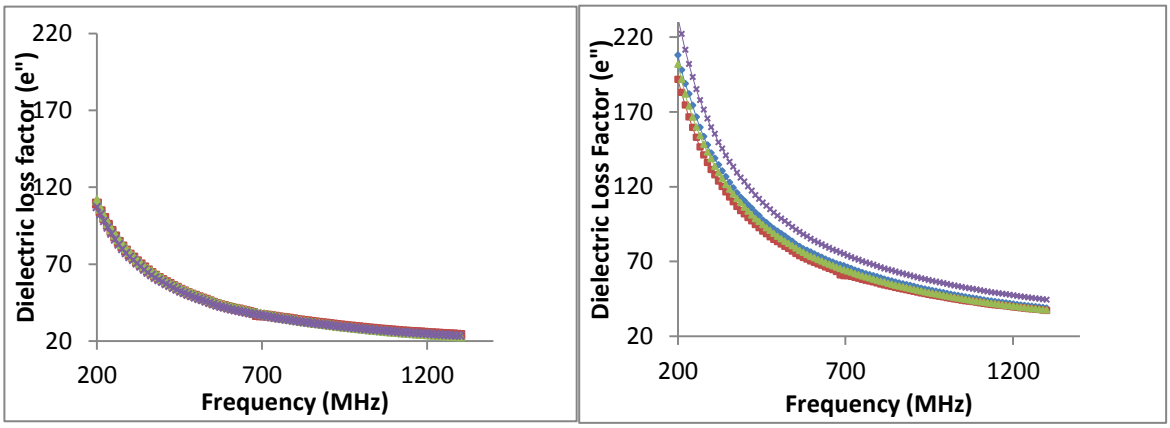


Figure 4.4: Dielectric loss factor of samples at 25°C (left) and 85°C (right). [△ 1% (VC1), ● 1.4% (VC2), ◆ 1.9% (VC3) and X 2.8% (VC4)]

By inspection of Figure 4.3 it is evident that the dielectric constant can be used to detect distinct differences between the samples held at the same temperature and between temperatures. The differences observed with temperature between samples are possibly due to the greater amount of energy that the components have in the system, resulting in an increase in molecular mobility. In particular the dielectric constant and loss factor of the 2.8% calcium to protein sample (VC4) shifted substantially more than the other

samples. This is likely due to the loose structure in the VC4 sample allowing a greater amount of component mobility at elevated temperatures. The overall differences between the dielectric constant profiles of the different samples are likely due to the ability of components, namely water, to move through the system.

The changes in the dielectric constant and dielectric loss factor with temperature are displayed below at calcium concentrations of 1% (VC1), 1.4% (VC2), 1.9% (VC3) and 2.8% (VC4).

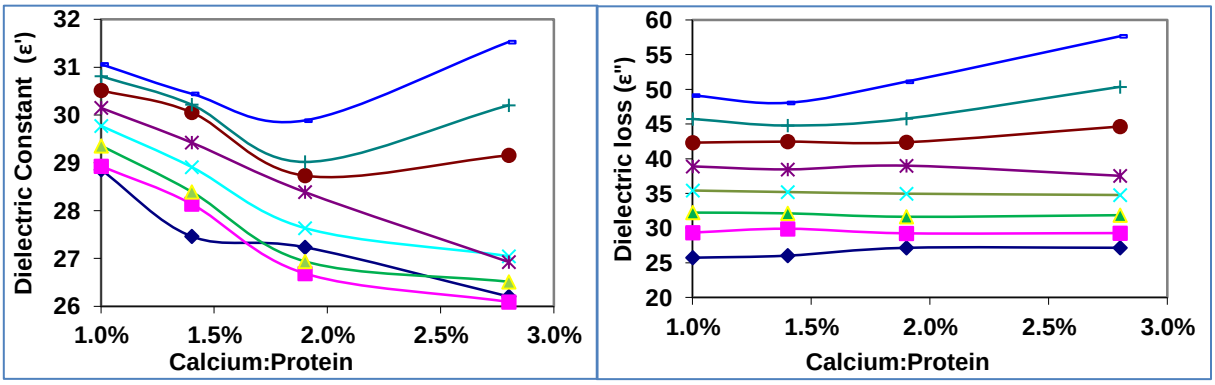


Figure 4.5: Dielectric constant and dielectric loss of the samples at 948 MHz. [♦ 4°C, ■ 25°C, ▲35°C , x 45°C, * 55°C, ● 65°C , + 75°C , — 85°C]

The shift in the dielectric constant and dielectric loss factor with temperature were both found to be greatest at lower frequencies. The dielectric loss factor indicated that there was a very slight change in the ionic concentration between the different samples. As a greater number of ions were added to the system, it would have been expected that the dielectric loss factor would increase with the concentration of calcium. The increase in the addition of calcium chloride will cause the ionic strength to increase in accordance with Equation 4-1 below. Where I is the ionic strength, c_i is the molarity ($molL^{-1}$) and z_i is the valance of each ion.

$$I = \frac{1}{2}\sum C_i Z_i^2$$

Equation 4-1: Ionic strength

Using Equation 4-1 the increase in ionic strength was calculated as 0.699. This assumes that all of the added salt species are in an ionic form. However, the slight increase in dielectric loss factor over this range suggests that some of the added calcium may not be in an ionic form, rather precipitating from the aqueous phase. The increase in the dielectric loss factor with temperature indicates that there was a greater level of ionic interactions occurring at 85°C than at 25°C, which may be facilitated by the increase in the mobility within the aqueous phase. Overall the dielectric properties identified a slight change in composition that had an effect on the movement of components within the system at different temperatures. A similar decreasing trend in ε' and ε'' with frequency was observed in process cheese over the frequency range of 0.3 to 3 GHz (Everard et al., 2006) and in a macaroni and cheese product (Nelson & Bartley Jr, 2000). In their results, it was also noted that the decrease in the dielectric properties is less at higher frequency than at lower frequency which is in agreement with our findings. At 948 MHz very little difference in the dielectric loss was identified between the varying calcium concentrations at temperatures of 55°C and below. Above 55°C the dielectric loss factor was found to increase with the increasing calcium content of the model system. The dielectric constant showed a decreasing trend with increasing calcium apart from at the 2.8% calcium concentration at temperatures between 65 and 85°C. This is likely to be related to the mobility of the components within the looser structure enabling a greater degree of movement of water in particular, as it is not constrained by a continuous protein network.

There have been many reports of foodstuffs that have dielectric properties that increase with temperature such as meat (Zhang, Lyng, Brunton, Morgan, & McKenna, 2004), cheese (Everard et al., 2006) and whey protein gels (Wang, Wig, Tang, & Hallberg, 2003). In the case of model cheese, as the temperature rises, it receives more and more energy and its ionic and dipolar movements become more active. The change in the calcium content produced systems with a range of structures (Figure 4.2). The increase in calcium

content enables greater protein-protein interactions which are reflected in the increase in firmness (Table 4-4).

At 25°C the dielectric loss was found not to change significantly when the calcium content was varied. Previous studies have indicated that the dielectric loss factor can be used to assess changes in the ionic concentration of a material (Fagan et al., 2005; Kubis et al., 2001). It would therefore be expected that an increase in the concentration of calcium in the system would result in an increase in the dielectric loss. However, this trend was not exhibited when the calcium concentration was increased. One possible explanation for this response is the calcium is binding to the casein in the system. This would mean that even though the concentration of ions added to the system was increasing, the ionic mobility was not affected as the calcium was not present in a free state.

To identify whether this was an effect caused by the calcium binding to the protein, the model systems were remade with sodium replacing calcium on a molar basis. The dielectric loss factor of the samples were measured at 25°C and plotted in Figure 4.6.

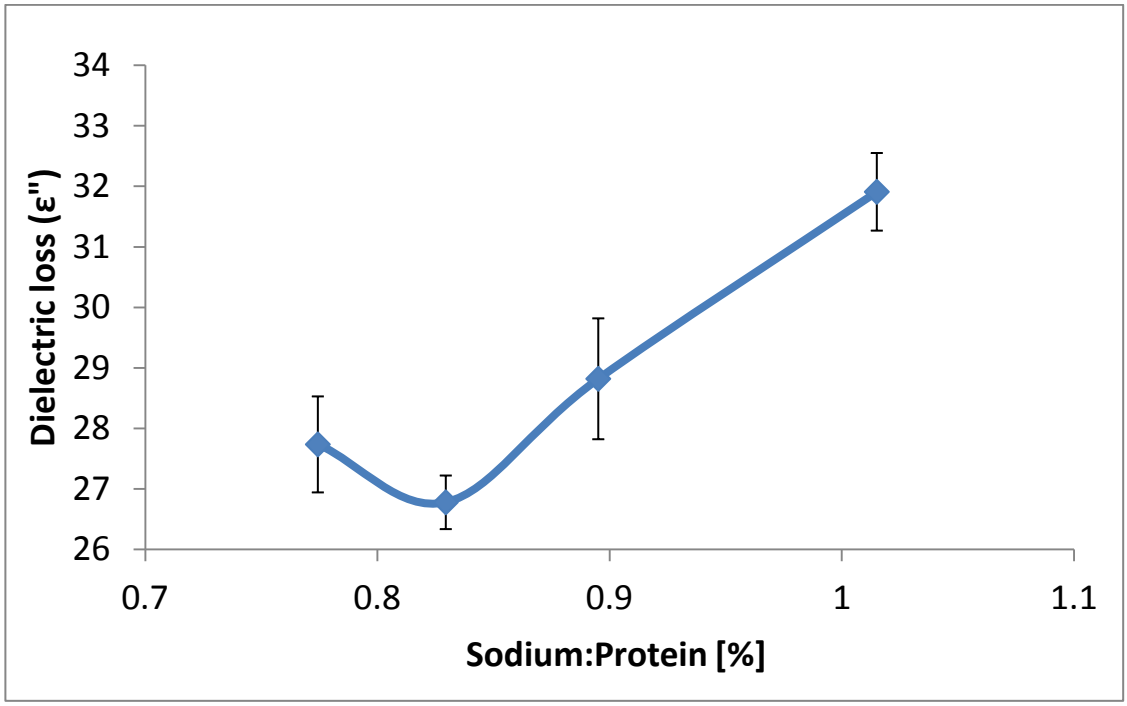


Figure 4.6: Effect of replacing calcium with the molar equivalent of sodium on the dielectric loss at 25°C and 948 MHz

This indicates that when the number of ions of sodium in the model system was increased, the dielectric loss also increased. This differs from the response observed when the calcium concentration was increased at 25°C. As sodium is a monovalent cation it has a lower affinity to bind to the casein than the divalent calcium (Guo, Campbell, Chen, Lenhoff, & Velev, 2003), therefore the addition of sodium will result in a greater number of free ions in the system. As the concentration is increased, the quantity of free ions also increases resulting in a greater level of ionic mobility present in the system and leading to an increase in the dielectric loss factor. The sodium study therefore lends credence to the theory that added calcium in the model system is precipitating, presumably onto existing nanoclusters, within the cheese system.

Based on this investigation it was difficult to ascertain differences among samples of the model system with various calcium concentrations using dielectric spectroscopy. This was despite distinct differences existing in the structures of the samples. This indicates that

dielectric spectroscopy alone was not suitable for predicting structure in this model system nor detecting gross differences in the calcium content of this model system. It is evident however from Figure 4.5 that dielectric spectroscopy can potentially be useful in detecting and characterising subtleties in the distribution of colloidal and ionic calcium. In particular it is interesting to note the change in behaviour of the dielectric constant with increased calcium as a function of temperature. The data indicates that at calcium contents above 2% and elevated temperatures, there is an increase in the dielectric constant above 55°C which is in contrast to the observations at cooler temperatures wherein there is a linear decrease in the dielectric constant with calcium concentration regardless of the temperature. One further issue with working with this model system is the difference in the state that added calcium exists in compared to in its native form in cheese.

The calcium present in natural cheese exists in two states: soluble calcium and insoluble colloidal calcium (Lucey & Fox, 1993). The amount of calcium associated with casein is determined by the total calcium content as well as the distribution between the soluble and colloidal calcium (Ge et al., 2002). The colloidal calcium has been identified as playing a more critical role in the structure and functional properties of cheese than the soluble calcium (Joshi, Muthukumarappan, & Dave, 2002; Lucey & Fox, 1993). The model system differs in structure to natural cheese as the protein source is a calcium depleted system and has ionic calcium added. This means that it may behave differently to natural cheese. Additional experimental work could be conducted in order to gain a more detailed understanding of how the state of calcium affects the structure of the model system.

Therefore in order to truly identify the utility of dielectric spectroscopy in measuring differences in cheese samples, a study needs to be conducted using a natural cheese. This would allow an assessment to be undertaken where changes in the ionic and dipolar nature of the samples could be related to the ions and water as they exist in their native state within cheese.

4.3.6 Critical calcium level

Following the initial investigation into calcium levels in the model cheese system, additional experiments were conducted to investigate the changes in the system dynamics. The system produced with a calcium to protein ratio of 2.8% (VC4) became a liquid during manufacture and formed a weak gel upon cooling rather than a solid mass with a continuous protein matrix. To investigate this change in structure, the calcium content of the system was increased incrementally to identify whether the transition was gradual or occurred at a specific concentration. The samples were analysed using texture profile analysis so that properties including hardness and springiness could be compared between the samples.

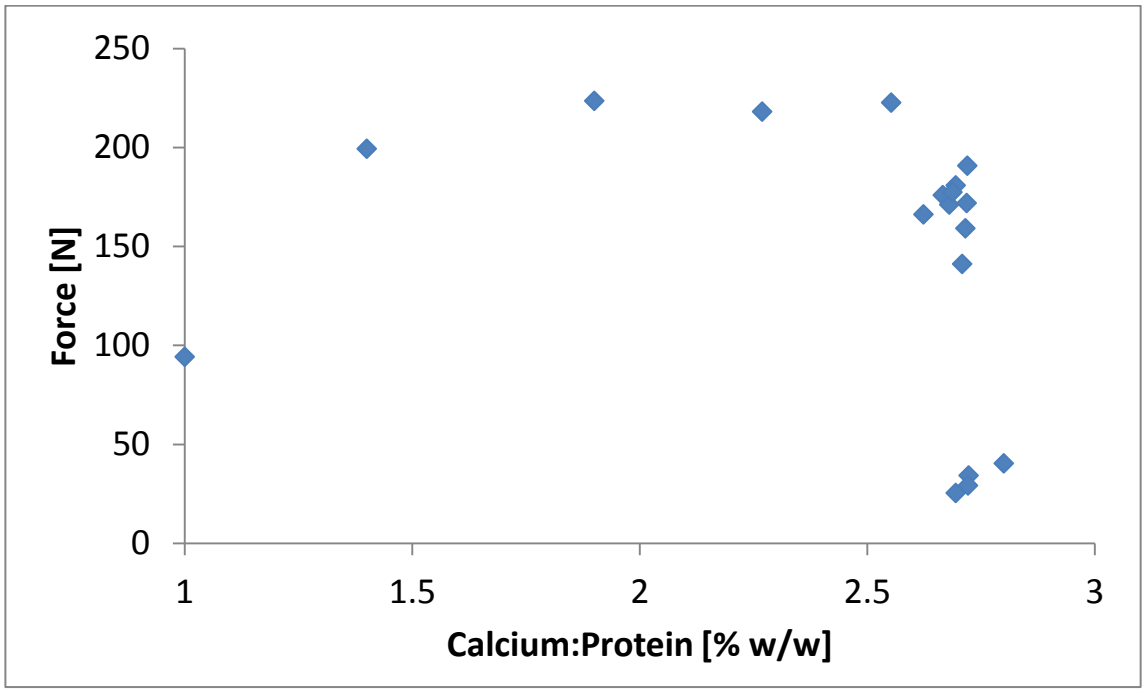


Figure 4.7: Effect of calcium on the hardness of the model system

The results shown in Figure 4.7 indicate that the hardness of the resulting cheese increased initially with an increase in the calcium content. This initial increase in hardness

is likely to be due to the increase in the number of calcium mediated protein to protein interactions resulting in the formation of a stronger gel network (Joshi, Muthukumarappan, et al., 2004a). Another factor that could impact the hardness of the samples is the change in the size of the fat globules. This is possibly due to small fat globules deforming to a lesser extent than large globules (Rudan et al., 1998). Also smaller fat globules disrupt the protein network to a lesser extent than large fat globules; potentially resulting in a firmer more elastic gel structure. The hardness was then identified as decreasing slightly between 2.5 and 2.7%. This suggests that even though the samples are still forming a rigid solid, the calcium is interfering with the caseins ability to form a network. The point that the system ceases to form a solid cheese mass was a very specific calcium to protein ratio between 2.720 and 2.721%. This is reflected in the rapid drop off in the hardness recorded during texture profile analysis. This change was very noticeable during manufacture with the higher calcium concentration samples forming a liquid at high temperatures; that formed a gel when cooled. This differed from all of the samples with lower calcium concentrations as they formed cohesive solid masses during manufacture.

This systems differs from a rennet casein-based system, the most popular choice for analogue cheeses (Ennis et al., 1998), as it starts at a lower calcium concentration and has calcium added. Rennet casein based systems require the addition of chelating salts to disrupt the calcium-mediated protein-to-protein cross bridges (O'Sullivan, Singh, Munro, & Mulvihill, 2002). This enables the rennet casein to become partially hydrated so it can emulsify the oil phase. For caseinate systems, calcium has been shown to initially improve the emulsifying ability of the casein, however, high concentrations of calcium lead to a disruption to this ability (Caric & Kaláb, 1987; Mohanty et al., 1988), due to precipitation. Although not a caseinate system, this model system displayed a similar trend.

One possible mechanism for the model systems decrease in emulsification and the eventual inability to form a solid is that the added calcium is causing the system to behave

in a similar fashion to rennet casein. As a large proportion of the calcium in the system is added in the form of calcium chloride, most of it is present in an ionic form. This leads to a large number of calcium mediated protein to protein cross bridges to form, reducing the ability of the protein phase to become hydrated before formation of a 3 dimensional gel structure (Joshi, Muthukumarappan, et al., 2004c).

4.3.7 Effect of Processing

The point of calcium addition during processing was identified as having a significant effect on the samples produced. The samples where calcium was added before the heating step (A and B), including the extended mixing time sample, were all observed to form a soft paste, as in the previous part of the experiment. The sample where the calcium was added midway through the heating step (C) was observed to form a cohesive cheese like mass. However, this sample did not incorporate all of the fat into the structure of the sample, with a pool of molten fat left in the bottom of the Brabender after processing. The sample produced when the calcium was added at the start of the kneading step (D) was observed to form a cohesive cheese-like mass. This product was softer than samples produced at lower calcium levels. However, unlike sample C, this sample had the fat incorporated into the structure.

4.3.7.1 *Inline torque measurements*

In order to investigate the role of calcium and heat on the structure of the model system during processing, an inline torque system was used. The inline torque measurements indicated that each of the samples had a distinct torque profile related to the development of its structure during processing. The torque required by the motor to maintain a constant mixing speed can be related to the viscosity of the system. The greater the torque required by the motor, the more viscous the sample.

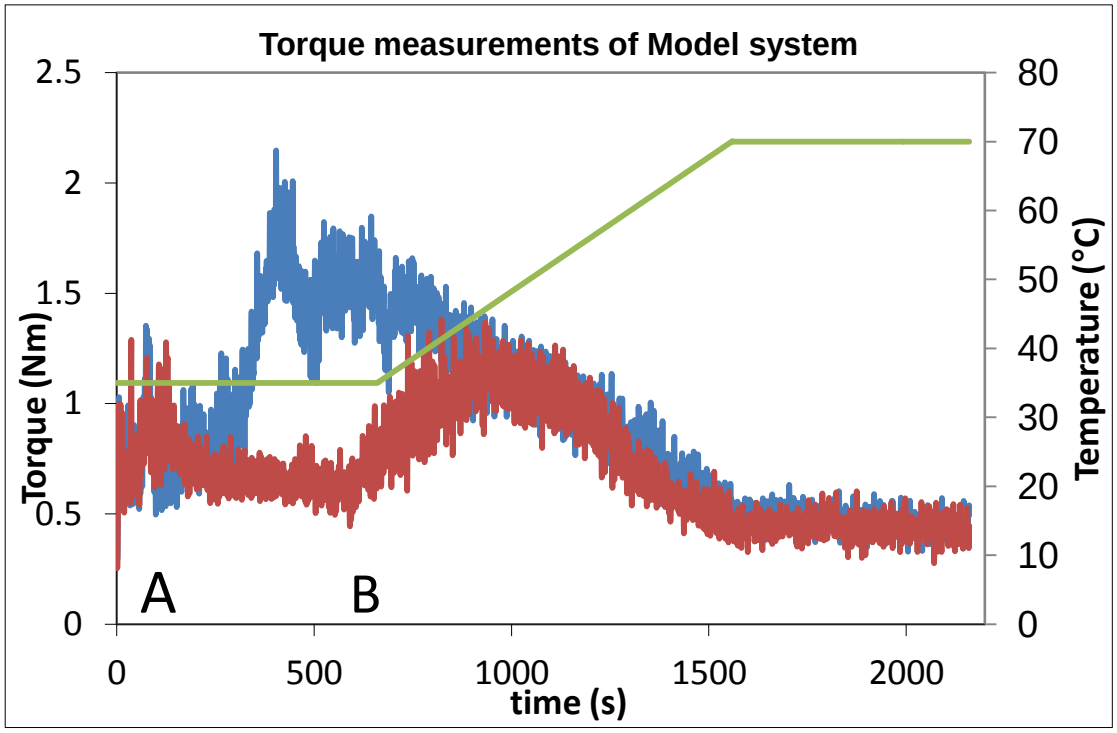


Figure 4.8: A comparative torque profile of model systems produced with calcium added at points A ( ) and B ( ) [Temperature].

As shown in Figure 4.8, initially both samples underwent a period of variable torque during which the components were first mixed together. Following this, sample A was observed to have a brief period where little change was evident in the torque profile. This was followed by a rapid increase in the viscosity of the system, indicated by the rise in the torque. The viscosity of the system was then found to drop off in an approximately linear fashion. The initial part of this decrease occurred independent of temperature, as it occurred prior to the temperature ramp. As the temperature was increased, the viscosity of the sample was found to continue to decrease. Once the temperature stabilised at 70°C, so did the viscosity of the system.

After the initial variation, sample B maintained a relatively stable viscosity until the calcium was added to the system. Following the addition of calcium, the viscosity was observed to increase. However the maximum torque was found to be lower for sample B

than sample A. Sample B then mirrors sample A in the decrease in viscosity up to 70°C followed by a stabilisation when the system has reached a constant temperature.

The lower maximum torque in sample B may be the result of the action of calcium during the hydration of the protein powder. Sample A has the calcium added with the water at the beginning of processing. The calcium can cause large aggregates as the powder particles do not have sufficient time to break apart prior to the bridging effect of calcium. Whereas, in sample B, the protein powder is allowed to hydrate prior to the addition of calcium. This allows the powder can breakdown into micelles and the addition of the calcium forms smaller aggregates resulting in a lower viscosity.

The inline monitoring of the samples during processing indicated that the addition of calcium initially assists in the formation of structure. This is due to calcium assisting in the formation of protein-to-protein cross bridges (Ennis et al., 1998). However, as the concentration of calcium exceeds the critical calcium concentration, the system ceases to form a solid at high temperatures.

4.3.7.2 *Processing effect on uniaxial compression*

In order to determine the effect that the point of calcium addition had on the resulting texture of the model cheeses, uniaxial compression was carried out to study the hardness of the samples.

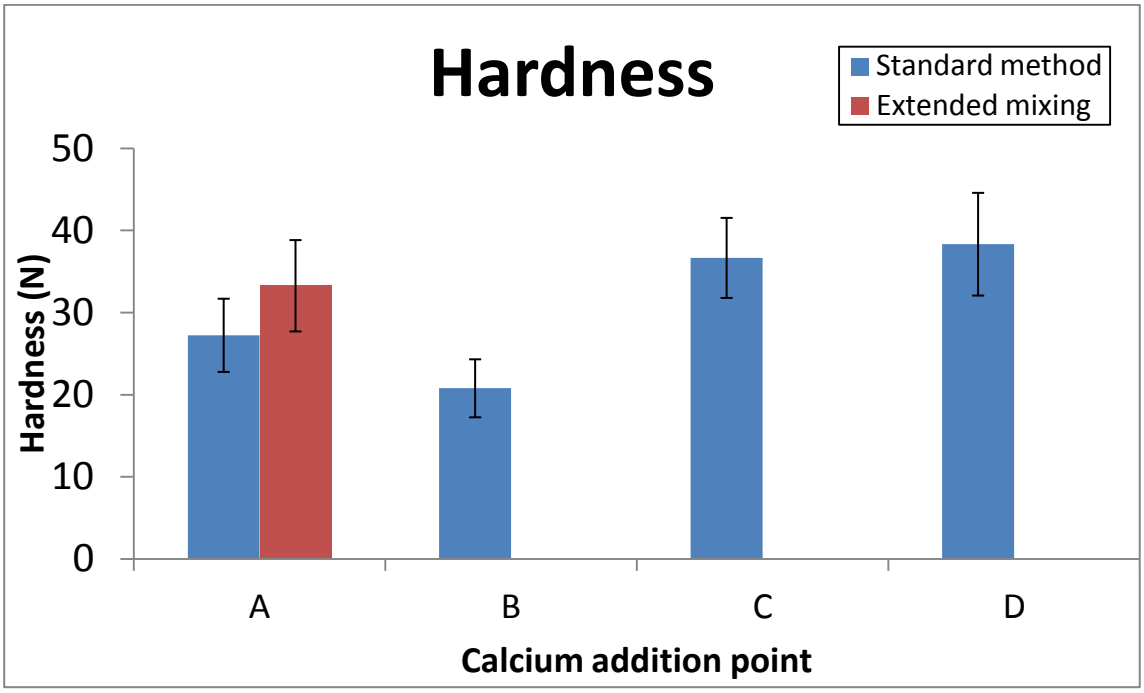


Figure 4.9: Hardness of model systems with different points of calcium addition [Calcium:Protein = 2.8%]

A one way ANOVA was conducted based on the hardness results to identify whether there were any significant differences between the samples. This analysis produced a p value of 0.00, indicating that not all of the sample population means are the same.

Based on this finding a Tukey's analysis was carried out on the data to identify where the differences occurred. This found that there were three distinct groups that were significantly different from one another. The first, where no significant difference was identified between the samples, included sample C, D and the samples with the extended mixing time. Although no difference was found between the measured hardness of these three samples, they were all identified as having different observable bulk structures. However, it is likely that differences in the hardness between these samples would be evident if the experiment was carried out at different temperatures. As the structures of the samples differ, the contribution that the fat imparts to the hardness would differ in

the samples, especially at temperatures where the fat would be in a molten state. However, due to the time constraints of this study this work was not conducted.

The other two groups were sample A and sample B, each found to be significantly different from one another and the previous group. Sample B was identified as having the lowest hardness out of the three distinct groups while sample A had a hardness between the other two groups.

The potential mechanisms that could be responsible for this difference in hardness could be an ion migration effect, as mentioned earlier. Another possibility is that the additional mixing time results in a homogenisation effect. This would be the result of the extra mixing breaking down the size of the fat particles in the system. Smaller fat particles deform to a lesser extent than large pockets of fat (Rudan et al., 1998), therefore the hardness of the resulting system is likely to be higher.

4.3.7.3 *Processing effects on dielectric profile*

Dielectric spectroscopy was used to identify whether there were any differences in component mobility between the different model cheese samples. The samples produced with the calcium added at point C and D both formed a solid during processing, however, sample D formed a porous, foam-like structure and sample C was observed to have large pockets of fat dispersed within the structure. This made these two samples unsuitable for dielectric spectroscopy due to the large inhomogeneities in the structure interfering with the signal obtained from the sample.

The dielectric constant was analysed over a frequency range from 200 MHz to 1.3 GHz. The results are displayed below at a frequency of 1250 MHz. This frequency was chosen as the dielectric constant is dominated by dipolar rotation, which is the most dominant mechanism above a frequency of 1 GHz (Ryynänen, 1995).

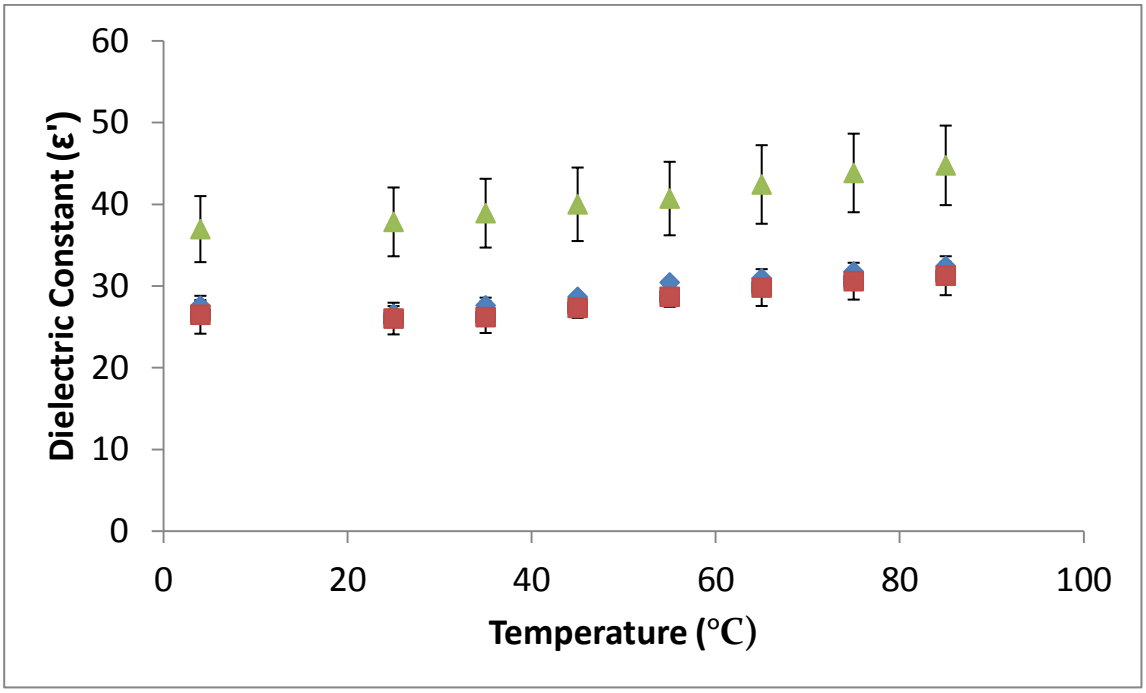


Figure 4.10: Change in the dielectric constant at 1250 MHz with temperature (Sample: ■ A, △ B and ◆ Extended)

All three samples exhibited a trend where the dielectric constant increased with temperature. Sample B was found to have a much higher dielectric constant at each temperature than the other two samples analysed. A higher dielectric constant is indicative of a greater level of dipolar mobility, dominated by water in food systems (Datta et al., 2005; Kudra et al., 1992). As all of the samples have similar moisture contents, the difference in dielectric constant could be the result of free water within the system. This is due to the dielectric constant not only being sensitive to the moisture content, it can also be used to interpret the level of water mobility within a system (Tsoubeli, Davis, & Gordon, 1995).

The dielectric loss factor was also used to assess the three different systems. Full frequency sweeps were conducted on the samples and a frequency of 255 MHz was chosen to display the relationship between the dielectric loss factor and temperature. This

frequency was chosen due to the dominant effect of ionic conduction at lower frequencies (Fagan et al., 2005).

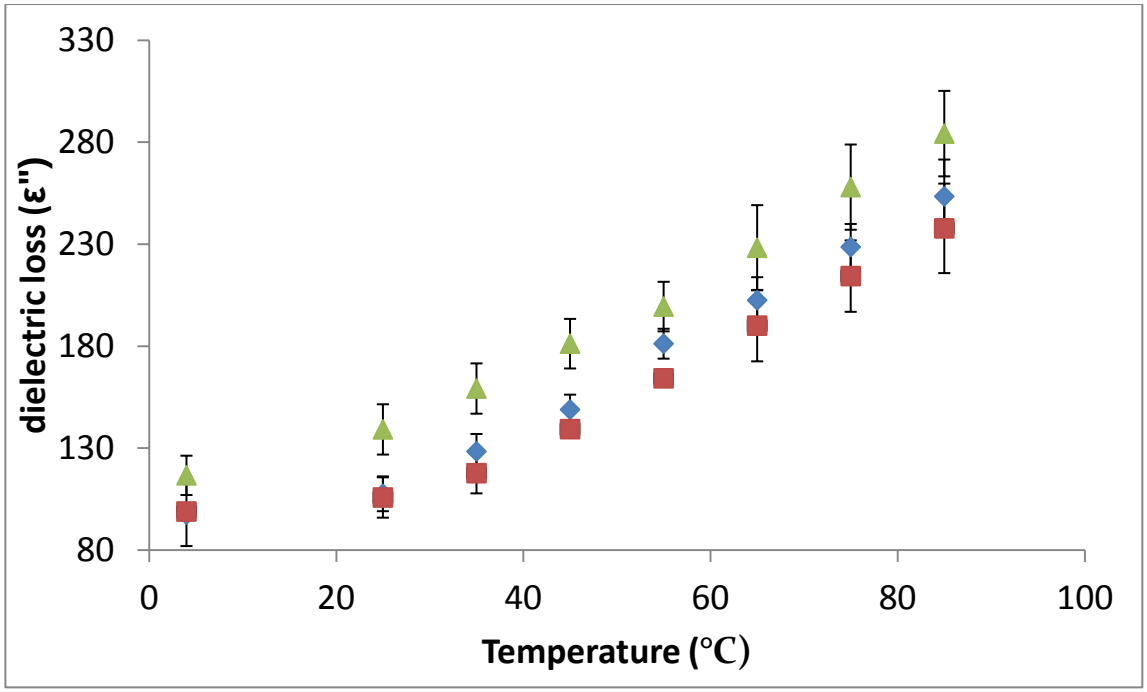


Figure 4.11: Effect of temperature on the dielectric loss factor at 255 MHz (Sample: ■ A, △ B and ◆ Extended)

The dielectric loss factor was found to increase with temperature in all three samples. As with the dielectric constant, the dielectric loss factor was found to be higher in sample B than the other two samples at each temperature studied. This indicates that the ions within sample B are more mobile than the other two samples. This suggests that there is a greater quantity of free ions present in the sample as the composition is identical to the other samples.

No significant difference was found to exist between the sample with the extended mixing time and sample A. This suggests that if migration of calcium is occurring during processing, the extended mixing time does not result in further migration than the standard mixing time of 10 minutes. The effect of the homogenisation due to additional mixing could result in the difference in the hardness in between system A and the

extended mixing time. The additional mixing would likely result in the reduction in the size of the fat globules within the system, modifying the contribution that the fat imparts to the hardness of the cheese.

4.3.8 General discussion

A model system was developed and the calcium content manipulated to produce a number of cheese systems with different structures. The difference in structure was observed using confocal microscopy and was found to impact on the texture of the various model systems, as identified by TPA. A repeatable method for utilising dielectric spectroscopy was developed; however, it produced an unexpected result when assessing the model system with various calcium levels. The response from the dielectric loss factor was found to stay relatively constant over the range of calcium contents used despite previous studies linking the dielectric loss with salts (Fagan et al., 2005; Kubis et al., 2001). This difference suggested that the system created for this work behaved differently from those in literature. One possible cause of this behaviour is in relation to the protein based used for the creation of the model system. Unlike other proteins used for model cheese systems used in literature, this protein has a much lower calcium content. This difference in calcium contents may be responsible for the behaviour in this model cheese system when calcium was added back. One possible mechanism responsible for this lack of dielectric response is a consequence of the casein having free sites where calcium can bind, limiting its molecular mobility. This potential lack of calcium mobility in the model system would result in little change in the dielectric loss constant. Differences were identified between the samples when they were heated. One possible reason for this is the disassociation of calcium ions from the casein occurring with temperature. In order to identify whether the lack of a response in the dielectric loss factor at 25°C was due to a lack of sensitivity or other factors, the samples of the model system were recreated with the molar equivalent of sodium. These samples with increasing sodium levels were found

to influence the dielectric loss factor, unlike the calcium adjusted samples. This suggests that there is an effect caused by the calcium binding to the casein in the system. As sodium is a monovalent cation and has a lower affinity to bind to casein than calcium (Guo et al., 2003), therefore there are likely to be a greater number of free sodium ions than the system with the molar equivalent of added calcium. These results indicated that the state of calcium within the model system was affecting the results of the investigation.

The importance of calcium equilibria between the ionic and colloidal states was highlighted in the critical calcium experiment as the model system ceased to form a three dimensional protein matrix at calcium concentrations in the vicinity of commercial Mozzarella. This identified an initial increase in the hardness of the model system as calcium was added to the model system. This is likely to be due to the increase in the number of calcium-mediated protein-to-protein interactions that creates a stronger gel structure (Joshi, Muthukumarappan, et al., 2004a). As the calcium content was further increased the hardness dropped off slightly followed by a sudden decrease in the hardness of the model system. This is similar to studies that have shown that the addition of calcium to casein initially aids in emulsification, however, at high concentrations it can disrupt this ability (Caric & Kaláb, 1987). This suggests that the mechanism responsible for the change in the hardness of the model system is dominated by how the calcium interacts with the casein present in the system.

The minerals and casein in milk exist in a dynamic equilibrium between the soluble and colloidal phases (Walstra, Geurts, Noomen, Jellema, & van Boekel, 1999). The model system used for this study utilised a calcium depleted protein source and has ionic calcium added to it in the form of calcium chloride. One possibility is that the calcium added to the calcium depleted system can assist in the shielding of the renneted micelles or migrate into the micelles. In order for the calcium to do the latter, it must first exist outside of the micelles, shielding the surface charge. As the calcium can neutralise the surface charge of renneted casein micelles (Dalgleish, 1983), the particles can come together and the

viscosity of the system increases. If the calcium was then to migrate into the micelles, the shielding of the surface charge of the particles would reduce leading to a reduction in the viscosity of the system. This is similar to observations made during the processing of sample A, with the initial increase in viscosity followed by a decrease, independent of temperature. However, the trend in viscosity may also be the result of the hydration of the protein powder. Initially the dry powder, when in contact with the water, will become sticky and result in the formation of asymmetrical particles during mixing leading to a higher viscosity. As the powder becomes fully hydrated the particles fall apart becoming micelles, resulting in a reduction in viscosity.

In sodium caseinate solutions the addition of calcium causes aggregation leading to precipitation (Carr, Southward, & Creamer, 2003). This is due to the calcium binding to the phosphoserine residues of the casein molecules, reducing electrostatic repulsions (Ye & Singh, 2001) and thus allowing hydrophobic attractive forces to dominate. The extent of the aggregation is highly sensitive to the concentration of calcium (Dickinson & Golding, 1998). Initially as the calcium concentration is increased, the viscosity of the system also increases (Carr, Munro, & Campanella, 2002). However, a peak viscosity is reached and the appearance of the solution changes from being translucent to being opaque (Carr et al., 2003). This change in the appearance of the solution indicates that the casein is forming colloidal particles. The 'micellerization' of casein involves dramatic changes in the conformation and association of the molecules (Mulvihill & Fox, 1989). As the 'micelles' or aggregates are tightly bound particles they exert less shear on the other particles, causing a subsequent decrease in viscosity (Carr et al., 2002). The continued addition of calcium to the system causes a decrease in viscosity (Carr et al., 2003) until precipitation occurs (Carr et al., 2002). This continued decrease in viscosity with an increasing calcium concentration indicates that the calcium is involved in additional cross-linking within the micelle causing a tighter network of casein molecules. One could postulate that a similar mechanism is at play within the model system, whereby calcium added to a calcium depleted micellar

protein source can initially migrate into the micelle and aid in crosslinking. However, at high concentrations of calcium the protein precipitates, due to the aggregation kinetics occurring at a fast rate so aggregates form rather than a continuous protein matrix. The research performed by Carr *et al,* (Carr et al., 2002; 2003) demonstrating this phenomenon, was carried out at a pH of 6.7. This differs from the model systems; however, similar trends are conceivable at a lower pH, as lower pH would be expected to merely alter where the equilibrium point would exist, as it is still above the isoelectric point of the casein.

The point during processing that the calcium was added to the model system was found to impact on the structure of the model system. As with the previous results, this experiment indicated that the state of calcium added to the calcium-depleted protein source was having an impact on the formation of the structure of the model cheese system. There are a number of potential mechanisms that could be responsible for the changes occurring in the model system. Ion migration due to the state of calcium in the model system may influence how the samples were formed and how they behaved when tested. Another factor that could influence the structure and texture of the model system is the changes in the extent of mixing leading to a homogenisation effect. Small fat globules deform to a lesser extent than large pockets of fat in cheese which impacts on the measured hardness of samples (Rudan et al., 1998).

Based on the experiments conducted on the model system it was difficult to relate changes in the structure to that of a natural Mozzarella system. The properties of the model system were also likely to have impacted on the dielectric results obtained in the experiment making an evaluation of its use as a technique to analyse cheese problematic. The state that components (e.g. calcium) exist within the model cheese system may differ from a natural cheese product making comparisons between the systems difficult.

4.4 Conclusion

A method was developed to assess the dielectric properties of a cheese sample. This was based on a refinement of a pre-existing method, where potential errors were assessed and the method refined. The application of the technique to a model system indicated that the dielectric properties of a model cheese system are independent of calcium levels at room temperature. This was despite significant differences observed in the structure, as determined by confocal microscopy, and texture of the four samples produced. It can therefore be concluded that the role of calcium in structure formation is via colloidal interactions (which cannot be measured by dielectric spectroscopy) rather than ionic interactions (which can be measured by dielectric spectroscopy).

The state that the calcium exists in the model system was likely to differ to that in the native calcium in cheese. This difference in the state of calcium was potentially responsible for the differences in the behaviour of the model system in comparison to a real cheese system. It is also likely to be the reason for issues relating to the dielectric measurements of the model system. Although useful for establishing the various testing methodologies, the model system, due to its differences in comparison to natural Mozzarella cheese, was difficult as a medium to ascertain information about a natural cheese system. Therefore future studies as part of this project should focus on a natural Mozzarella product.

Based on the results of this investigation it was difficult to evaluate whether or not dielectric spectroscopy was a useful tool for assessing changes in a cheese system. However, it did indicate the importance of the colloidal interactions within the cheese system. Limited information was gained from the change in the ionic nature of the model cheese systems. In literature, dielectric spectroscopy has also been used to study changes in dipolar rotation, which in food is linked to water molecules. Therefore the progression

to natural Mozzarella may give insights into the changes occurring during various stages in the maturation process.

5 The assessment of water mobility in Mozzarella cheese during the initial stages of storage

5.1 Introduction

Cheese has a bi-continuous structure consisting of a casein matrix interrupted by fat (de Kruif et al., 1995). The water in the cheese structure is entrapped within the protein phase. Low moisture part skim Mozzarella cheese is one of the most consumed cheeses in the world (Francolino et al., 2010). Mozzarella is commonly referred to as a fresh cheese; however, it often undergoes a period of ripening to attain a desired level of functionality (Kindstedt, 1993b). Mozzarella differs in structure to most other cheeses due to the thermo-mechanical stretching step that the cheese undergoes (Kindstedt et al., 2004; McMahon et al., 1999). This process leads to the formation of a fibrous texture where the continuous protein fibres are interrupted by channels of fat and serum (Kindstedt, 2007). Over time the composition of these channels changes as free water within the channels is absorbed into the protein matrix (Kuo et al., 2001).

The state that water exists in influences both the structural and functional properties of a food (e.g. melt in Mozzarella) (Godefroy, Korb, et al., 2003). As the distribution of water changes within Mozzarella over this initial storage period, the functional properties of the cheese change (Kindstedt, 1995). Therefore gaining an understanding of the progressive change in the state of water within Mozzarella is of importance.

The majority of techniques used to assess the moisture content or molecular mobility of water in foods are time consuming and/or destructive (Trabelsi et al., 1998). Dielectric spectroscopy offers a rapid non-destructive technique to characterise the moisture in a material (Lizhi, Toyoda, & Ihara, 2008). Dielectric spectroscopy works by emitting a spectrum of electromagnetic waves and measuring how it interacts with a sample (Smith et al., 2011). The interaction is measured by two parameters, the dielectric constant and

the dielectric loss factor. These parameters relate to how the sample stores and dissipates energy, respectively (Venkatesh & Raghavan, 2004). The dielectric properties are dominated by dipolar rotation and ionic conduction (Ryynänen, 1995). Water is the primary component in food responsible for dipolar rotation while the salts are the dominant factor associated with ionic conduction (Datta et al., 2005). Dipolar polarisation is perhaps the most dominant loss mechanism above a frequency of 1 GHz; however, it still influences the dielectric response of a material at lower frequencies (Ryynänen, 1995). A number of studies have related the dielectric constant to the moisture content of a material over a wide range of frequencies including: apples at 915 and 2450 MHz (Feng, Tang, & Cavalieri, 2002); soils between 0.3 and 1.3 GHz (Peplinski, Ulaby, & Dobson, 1995); a model meat emulsion at 900 and 2800 MHz (Ohlsson, Henriques, & Bengtsson, 1974); and onions between 200 MHz and 20 GHz (McKeown, Trabelsi, Tollner, & Nelson, 2012).

Dielectric spectroscopy has been applied to a number of cheese systems including Cheddar (Green, 1997), Edam (Kubis et al., 2001), process (Fagan et al., 2005) and analogue cheeses (Smith et al., 2011). These studies have looked at the moisture content, ion concentration and how changes in structure influence component mobility. A positive linear relationship was identified between the dielectric constant and the moisture content of process cheese (Fagan et al., 2005). It is unknown whether such a linear relationship also exists in Mozzarella as the water distribution in Mozzarella differs from process cheese due to water existing in a free state immediately after manufacture: the changes in the state of water may impact on the dielectric response.

The previous chapter identified dielectric spectroscopy as a potential tool for monitoring changes in the ionic composition of a model cheese system. This chapter will explore its use in a real cheese system undergoing maturation changes involving structure and component mobility. In particular, its ability to measure water mobility within Mozzarella.

The objectives of this work were to:

- Monitor the changes occurring in structure and water mobility within Mozzarella during the first three weeks of storage.
- Identify whether dielectric spectroscopy could be utilised as a tool for assessing water mobility within Mozzarella cheese.

5.2 Material and Methods

5.2.1 Material

A commercial 10 kg block of Mozzarella cheese was obtained immediately after a 24 hour rapid cool cycle following manufacture (Fonterra Cooperative Group, New Zealand). The 10 kg block was then cut into 20 equal sized blocks, vacuum packed and stored at 4°C. The blocks were randomly selected on each day of the trial. The moisture content of each sample on each day of analysis was determined by drying 2 g of each sample for 16 hours in an air oven at 106°C in triplicate (NZTM 3.12.6).

5.2.2 Confocal Microscopy

Confocal microscopy was used to monitor visual changes in the distribution of protein, fat and free water within the Mozzarella samples on day 1, 8 and 15. Confocal microscopy was carried out as stated in Section 4.2.2.

5.2.3 Scanning Electron Microscopy

Cheese samples were examined using a FEI Quanta 200 Scanning Electron Microscope (FEI Electron Optics, Eindhoven, Netherlands).

Cheese samples were defatted by washing in a series of acetone solutions increasing in concentration from 50%, 75%, 95% and 100% for half an hour per wash. The sample was then soaked in an additional 100% acetone solution for an hour. The samples were then fractured prompted with a razor blade after critical point drying, with liquid CO_2 as the critical point fluid. The fractured samples were selected based on suitability of the fracture (based on the presence of a flat fracture plane). The samples were mounted on aluminium specimen stubs and sputter coated in gold using a Bal-tec SCD 050 sputter coater (Bal-tec, Balzer, Germany). The samples were analysed using the high vacuum mode on the microscope. The samples were fractured in two directions: parallel and perpendicular to the protein fibres. Samples were prepared in triplicate and at magnifications of 120, 500, 1000 and 2000x on the quadrant of the SEM screen..

5.2.4 NMR Spectroscopy

Samples of cheese (4 mm depth, 4 mm wide and 12 mm in height) were cut using a razor blade. The samples were placed in a 5 mm diameter ceramic NMR tube. The samples were then placed in a Bruker AMX 200 MHz horizontal wide-bore magnet (Bruker, Rheinstetten, Germany).

T2 relaxations were performed using the Carr Purcell Meiboom Gill (CPMG) spin echo pulse sequence (Carr & Purcell, 1954; Meiboom & Gill, 1958). The relaxation of the water present in the cheese was fitted to a bi-exponential model based on the equation (Chaland, Mariette, Marchal, & De Certaines, 2000; Kuo et al., 2001):

$$I = Ae^{\left(\frac{-t}{T2a}\right)} + Be^{\left(\frac{-t}{T2b}\right)}$$

Equation 5-1

The T2 component relates to the molecular mobility of the water with the T2a component relating to the fraction that has the shortest relaxation time due to being associated with

the protein (Altan, Oztop, McCarthy, & McCarthy, 2011), while the T2b relates to the water in a free state which has a longer relaxation time. I represents the intensity, A and B represent the proton intensity proportional to the different populations of water in the sample, and t represents time. The NMR measurements were conducted in triplicate at 20°C

5.2.5 Expressible Serum

Cheese samples were finely grated (approximately 1 mm diameter particles) and approximately 15 grams weighed into a centrifuge tube. The sample was then spun at 12,500 x g at 20°C for a period of 1 hour (Kindstedt & Guo, 1997a). The liquid in the tube was then decanted into a container. The moisture content of the serum was determined by placing the samples in a vacuum oven at 70°C at 76 mm Hg for 5 hours and measuring the mass lost. This method to determine the moisture content was used due to the serum being decanted into a plastic container and to avoid losses due to transfer, the entire container was dried.

5.2.6 Dielectric Spectroscopy

Dielectric spectroscopy was carried out on the samples three times a week over a three week period. The analysis was carried out at 20°C using the method developed in Section 4.2.5. The dielectric constant was measured in triplicate over the storage trial.

5.2.7 Composition

The composition of the cheese was assessed immediately following manufacture. The methods used were New Zealand test methods (NZTM). The moisture content was

determined by drying in an air oven at for a 16 hour period (NZTM 3.12.6). The fat content of the different cheese samples was identified using the Schmid-Bondzynski-Ratzloff method (NZTM 3.6.4). The protein content was calculated based on the analysis of total nitrogen within the cheese samples using the Kjeldahl method (NZTM 3.15.8). The ash content was determined by ashing the cheese at 550°C (NZTM 3: 4.2). The salt content was determined using an autotitrator (NZTM 3.9.6). The calcium concentration in the cheese was calculated using inductive coupled plasma (ICP) mass spectrometry (NZTM3: 9.21). The compositional testing was carried out at the Fonterra Research and Development Centre by the Analytical Services team.

5.3 Results and Discussion

5.3.1 Composition

Table 5-1: Composition of Mozzarella

	Moisture (%)	Fat (%)	Protein (%)	Ash (%)	Calcium (%)	Salt (%)
Mozzarella	49.1	21.2	26.1	2.46	0.64	0.90

5.3.2 Confocal Microscopy

During storage, significant changes in the structure of cheese occur. In order to visualise these changes in the cheese structure, microscopy methods have been used. Confocal microscopy allowed the dispersion of fat, protein and free water to be observed, as shown in Figure 5.1 below. The dark areas observed in the images correspond to the free water present in the cheese. The images displayed below were taken at a magnification of 62x and are representative of what was observed between replicates and at the various magnifications.

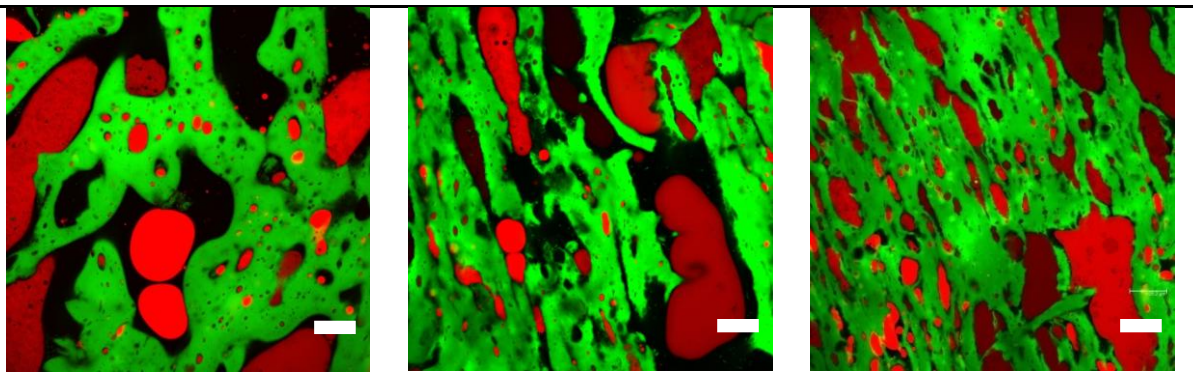


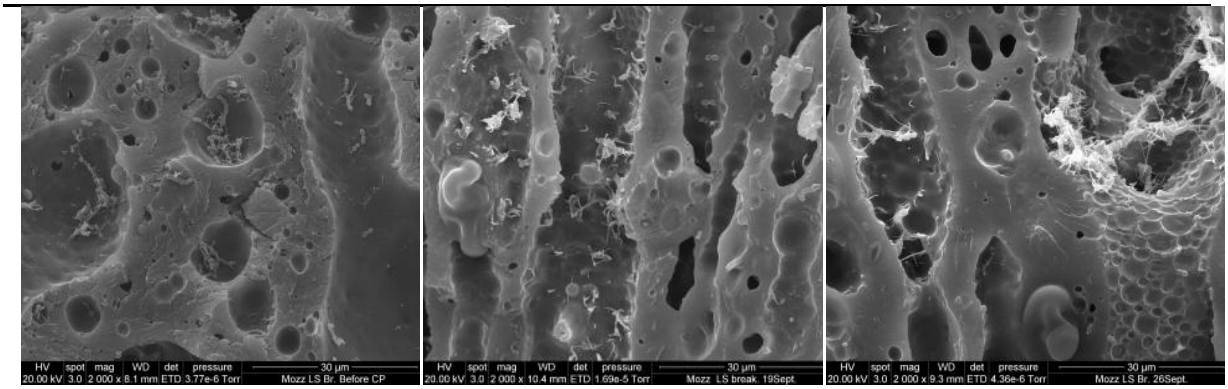
Figure 5.1: Confocal micrographs showing the change in structure over a 15 day period 62x magnification – scale bar is 25 µm (green = protein, red = fat & black = free water)

The confocal image shows the change in structure that occurred over the first 15 days following manufacture. On day 1 a large amount of free water was evident in the images, surrounding the fat entrapped within the casein matrix. Over time the quantity of free water was observed to decrease as it was absorbed into the protein. This absorption of the water causes the protein to swell. The swelling of the proteins puts pressure on the fat in the channels causing a deformation of the fat within the channels. This gradual swelling of the casein following manufacture was noted by Auty *et al* (2001). This causes a progressive change in the morphology of the fat from existing in a roughly spherical state to filling the long thin channels. This progressive change in free water, which is related to the physical state of water, will influence the functional properties of the cheese via modification to the hydration of casein which is the dominant structural element (Kuo et al., 2001). The subsequent change in the morphology of the fat within the cheese will also affect the functional properties of the Mozzarella.

5.3.3 Scanning Electron Microscopy

The images obtained from confocal microscopy showed that there was a significant change in the structure of the Mozzarella. In order to gain a greater level of detail, scanning electron microscopy (SEM) was identified as a method for elucidating the

progressive structural changes of the protein matrix and channels. The use of SEM allowed the structure of the protein network of the Mozzarella to be observed as well as the space left by the removal of free water and fat. SEM was able to show the structure at a much smaller scale to that obtained by confocal microscopy.



Day 1	Day 8	Day 15

Figure 5.2: SEM micrographs at 2000x magnification showing the progressive change in the structure of protein channels over 15 days

The change in the surface of the protein channels from smooth walls to a rough dimpled appearance has been documented in previous SEM studies of Mozzarella (Kiely et al., 1993; McMahon et al., 1999; McMahon & Oberg, 1999; Oberg et al., 1993). In a number of these past studies the presence of these indentations has been attributed to a transition in the state of fat within the cheese. These past investigations have described the fat within the channels as being in a liquid state immediately following manufacture and deform leaving a smooth wall. As the fat solidifies during storage, the solid fat provides resistance to the protein resulting in the observed indentations in the protein wall (McMahon & Oberg, 1999).

The current work offers additional insights through the incorporation of confocal microscopy into the experimental design, allowing the free water to be spatially mapped. While it may be true that the fat globules are in a molten state during the early stages of storage, the confocal micrographs suggest that the ‘softness’ of fat is unlikely to be the

cause of the absence of dimples immediately following manufacture. The appearance of the indentations on the surface of the protein channel walls coincides with the decrease in free water seen in the confocal images. Therefore, contrary to earlier reports discussing the dimpling effect during storage, the lack of indentations during the first few days of storage is likely to be caused by the free water in the system surrounding the fat and preventing the protein from pushing against it, thus not leaving indentations. As the quantity of free water in the fat/serum channels decreases over the first 15 days of storage, the indentations caused by the fat globules become evident. As seen in the confocal micrographs, this absorption of free water leads to the swelling of the protein matrix which applies pressure to the fat. This progressive swelling causes a deformation of the fat forcing it to form long thin clusters within the channels. As the last of the free water is absorbed into the protein swollen protein pushes against the fat, leaving indentations. Additionally, the size of the indentations (between approximately 1 and 7 µm) indicates that the native fat globules have survived processing and are either fully intact or partially agglomerated, as native milk fat globules range in size from less than 0.2 to 15 µm (Walstra, 1995).

5.3.4 NMR Spectroscopy

A change in free water present within the structure of Mozzarella was observed using confocal microscopy. In order to quantify this water movement within the structure over the maturation period nuclear magnetic resonance (NMR) was utilised. It is a technique capable of isolating both the relative level of water entrapment in cheese as well as the mobility of the molecules in each state (Kuo et al., 2001). The fraction of water that was identified as having a longer T2 time was classed as the free fraction of water, whereas the fraction with the shorter T2 time was described as being associated with the protein phase (Altan et al., 2011).

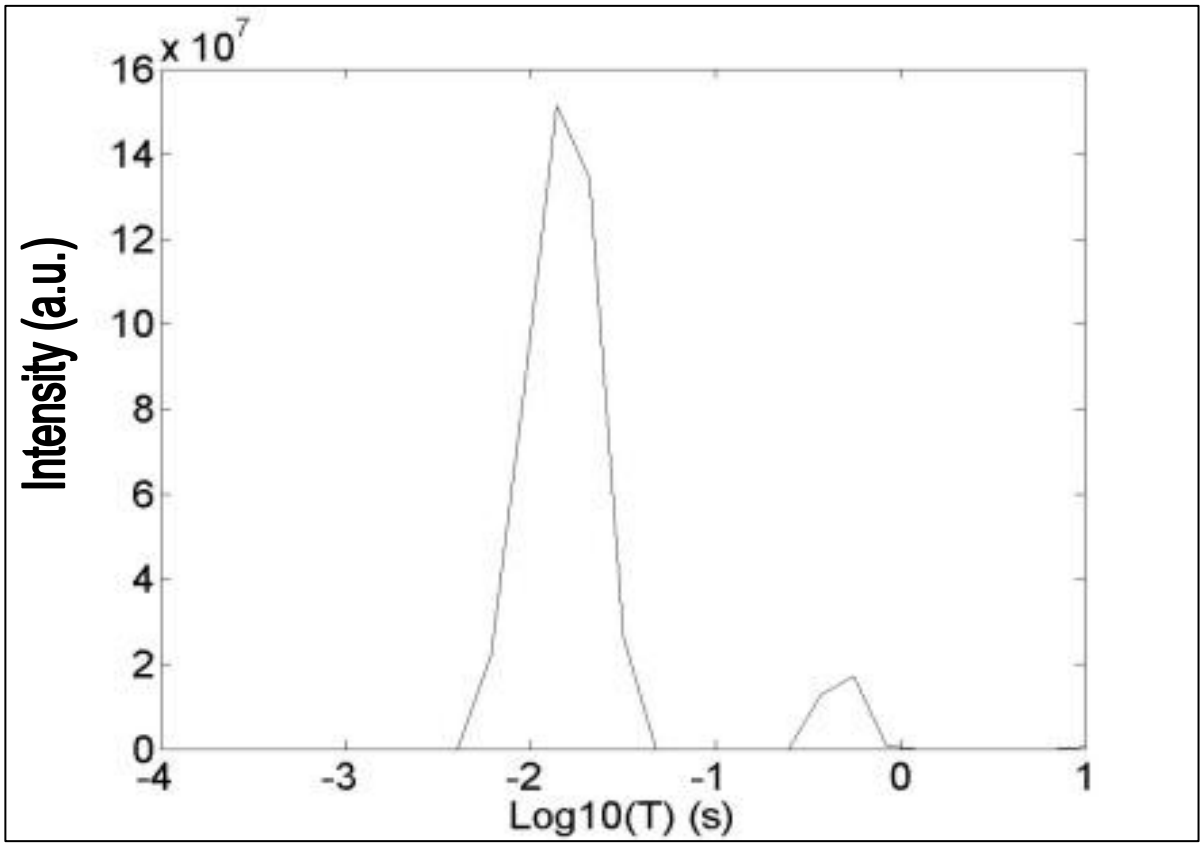


Figure 5.3: An example of the distribution of T2 relaxation times for water in Mozzarella

The relative proton intensity of water in each group of relaxation times can be used to identify the proportion of water that was associated with the protein and the proportion that was free. By monitoring the change in the relative quantities of each state of water the change in the amount of free water present in the Mozzarella can thus be monitored, as shown in Figure 5.4 below.

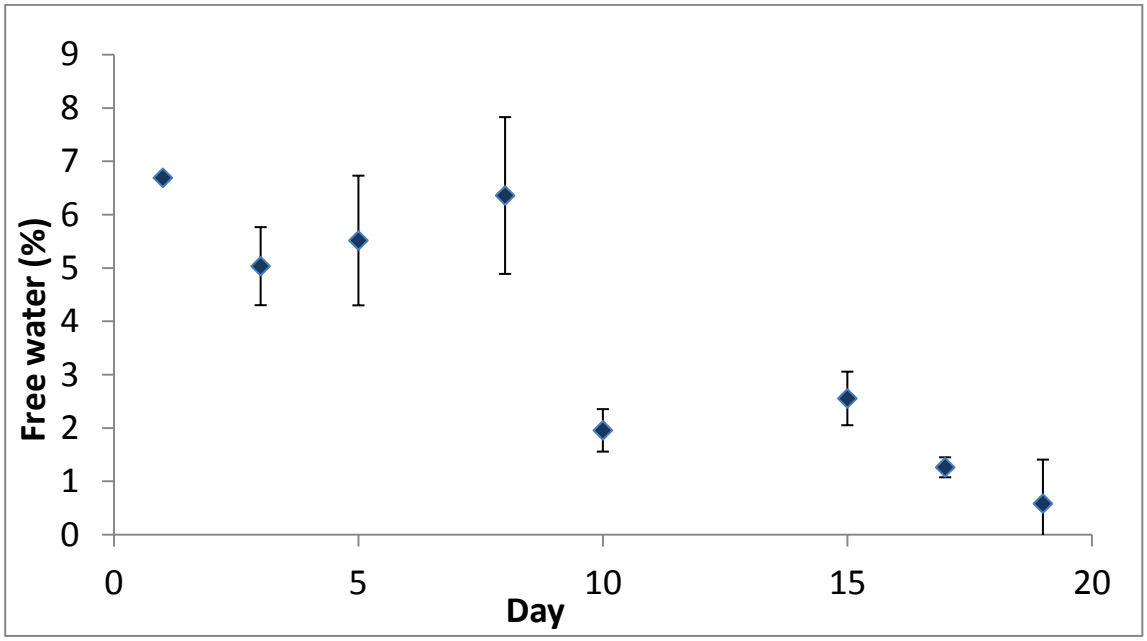


Figure 5.4: Change in free water in Mozzarella over the first 20 days of storage

The amount of free water in Mozzarella was found to decrease over the first 20 days of the trial. This difference in the quantity of free water within the Mozzarella sample over the maturation trial was identified as being significant, $p=3.14^{-4}$. This decrease corresponded with an increase in water associated with the protein in the cheese. This change in the free water in Mozzarella is similar to that observed in pasta filata and non-pasta filata Mozzarella by Kuo *et al* (2001). This follows a similar trend as the confocal micrographs which illustrate the apparent decrease in free water with time.

The error identified in the T2 NMR results is possibly due to the size of the samples analysed, natural inhomogeneity within the cheese and the fact that measurements were mostly conducted in only duplicates. The size of the samples analysed in conjunction with the natural variation that exists within Mozzarella could result in sample to sample variation. With the limited number of replicates that were able to be conducted, this sample to sample variation resulted in error in the T2 results depicted by the error bars in Figure 5.4 representing ±1 standard deviation.

5.3.5 Expressible Serum

Both confocal microscopy and NMR identified free water during the initial stages of maturation. A method for extraction of this free water from the cheese that has been used in previous studies, is to separate it by centrifugation (Kindstedt & Guo, 1997b; McMahon et al., 1999). This method uses centrifugal force to move liquid that is not physically entrapped or bound to the protein out of the solid cheese mass. This liquid includes some fat as well as free water present in the cheese. In light of the morphology of the indentations observed in the SEM micrographs, the existence of fat in the serum phase would be expected as a portion of fat globules are non-interacting.

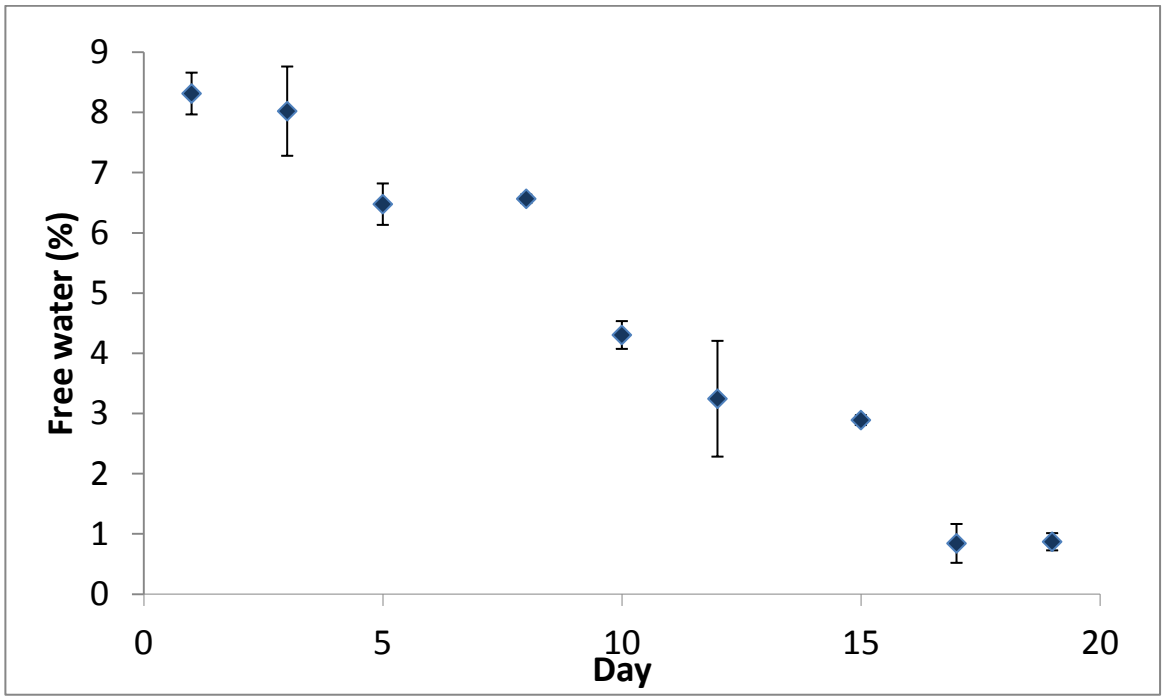


Figure 5.5: Change in expressible moisture over the 20 days following manufacture.

The percentage moisture expressed as a result of centrifugation was found to decrease in a linear manner over the first few weeks following the manufacture of the cheese. This change in expressed moisture was found to be significant, $p=3.22x10^{-6}$. This is similar to the trend identified in previous studies monitoring the change in expressible moisture in Mozzarella (McMahon et al., 1999). It also mirrors the trend identified in the NMR data

with the quantity of free water present in the Mozzarella decreasing with time. The linear decrease indicates that the mechanism for water movement within Mozzarella occurs at the same rate throughout the early stages of storage.

5.3.6 Dielectric Spectroscopy

Water movement within the cheese structure was identified using microscopy as well as by monitoring the NMR response and expressible moisture. Dielectric spectroscopy has been used as a tool for assessing the water mobility in a number of food products. This is due to the dielectric constant being dominated by the rotation of dipolar molecules, which in food systems is primarily water (Datta et al., 2005).

The dielectric constant of the cheese was measured at 20°C over the 20 day storage period. There were no identifiable peaks or trends in the raw data and thus to facilitate analysis, three frequencies from the 100 point frequency sweep were isolated and displayed on Figure 5.6 below. These arbitrary frequencies were chosen as they represented the lower, middle and upper limits of the network analyser used in the experiment.

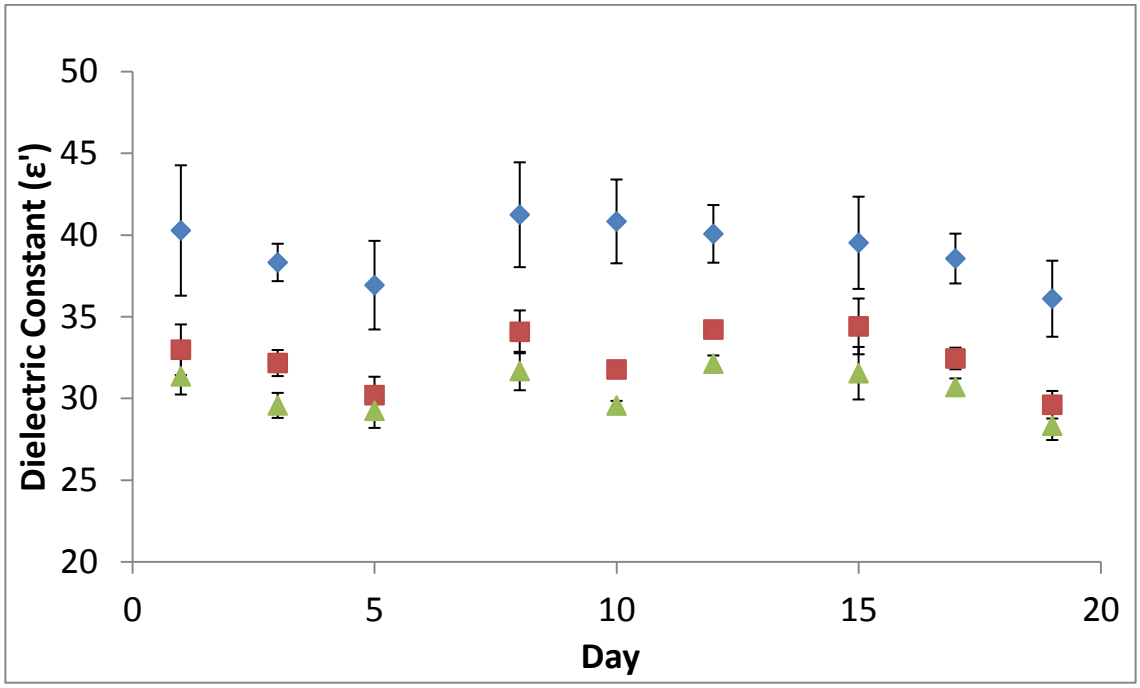


Figure 5.6: Change in the measured dielectric constant over a 20 day period (● 244, ■ 750 & ▲ 1260 MHz)

The moisture content of the blocks was recorded on each day of sampling to monitor block to block variation. This was done as previous work by Fagan *et al* (2005) identified a strong correlation between the dielectric properties and moisture content for process cheese. However, no obvious trend was identified when the dielectric constant was compared to the moisture content of the Mozzarella samples, as seen in Figure 5.7 below.

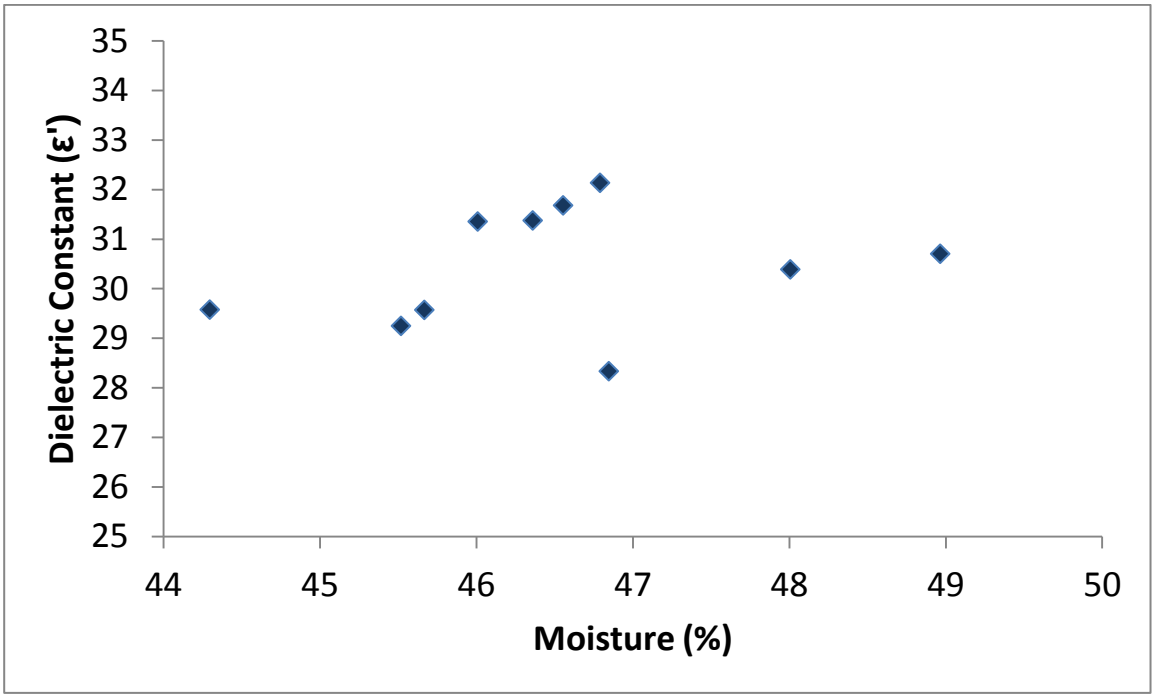


Figure 5.7: Relationship between the moisture content and dielectric constant measured at 1.26 GHz in Mozzarella

No trend was evident between the measured dielectric constant and the moisture content of the samples displayed in Figure 5.7 above. This differs from the strong positive linear relationship between these two parameters found by Fagan *et al* (2005) for process cheese, shown in Figure 5.8 below.

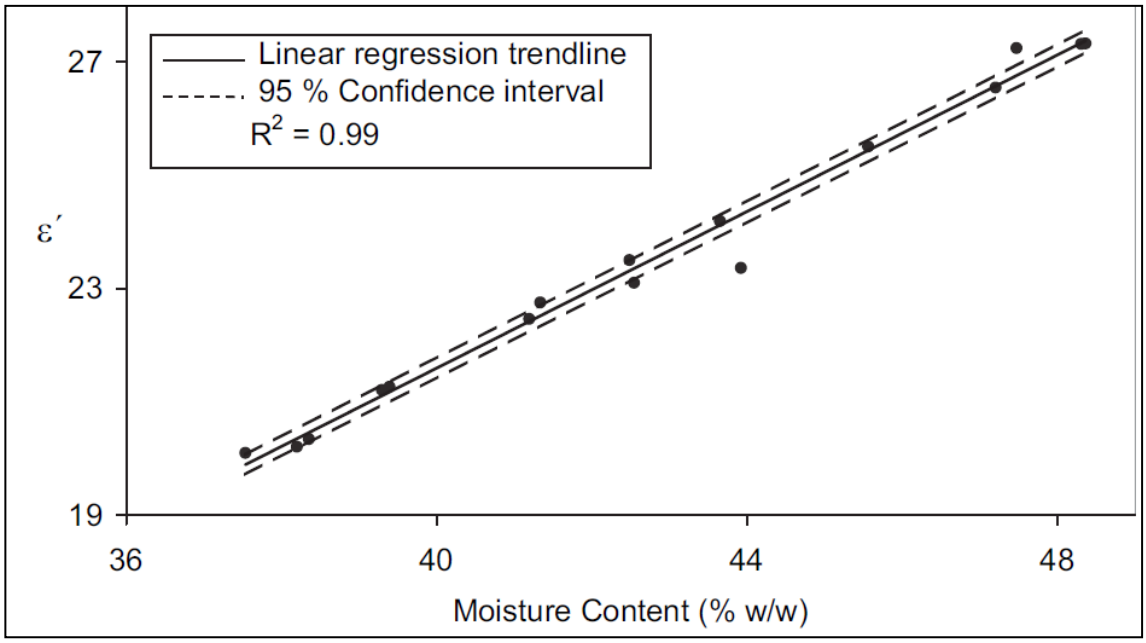


Figure 5.8: Relationship between moisture content and the dielectric constant (ε') of process cheese at 2.46 GHz (Fagan et al., 2005).

The points from the study conducted by Fagan et al. (2005) were replotted to look at the relationship that they identified. The equation from the resulting linear relationship is displayed below, where ε'_p is the predicted dielectric constant and M is the moisture content (%).

Equation 5-2

$$\varepsilon'_p = 0.694M - 6.1629$$

Assuming that water in both process cheese and Mozzarella is structured in a similar way via casein hydration then it is reasonable to assume the same relationship (Equation 5-2) between e' and moisture holds for Mozzarella. The use of this equation allowed a predicted dielectric constant for the Mozzarella samples at each of their respective moisture contents to be calculated. An examination of the residuals from this analysis showed that the difference between the actual dielectric constant measured and that

predicted using Equation 5-2 decreased with the age of the cheese, as shown in Figure 5.9 below.

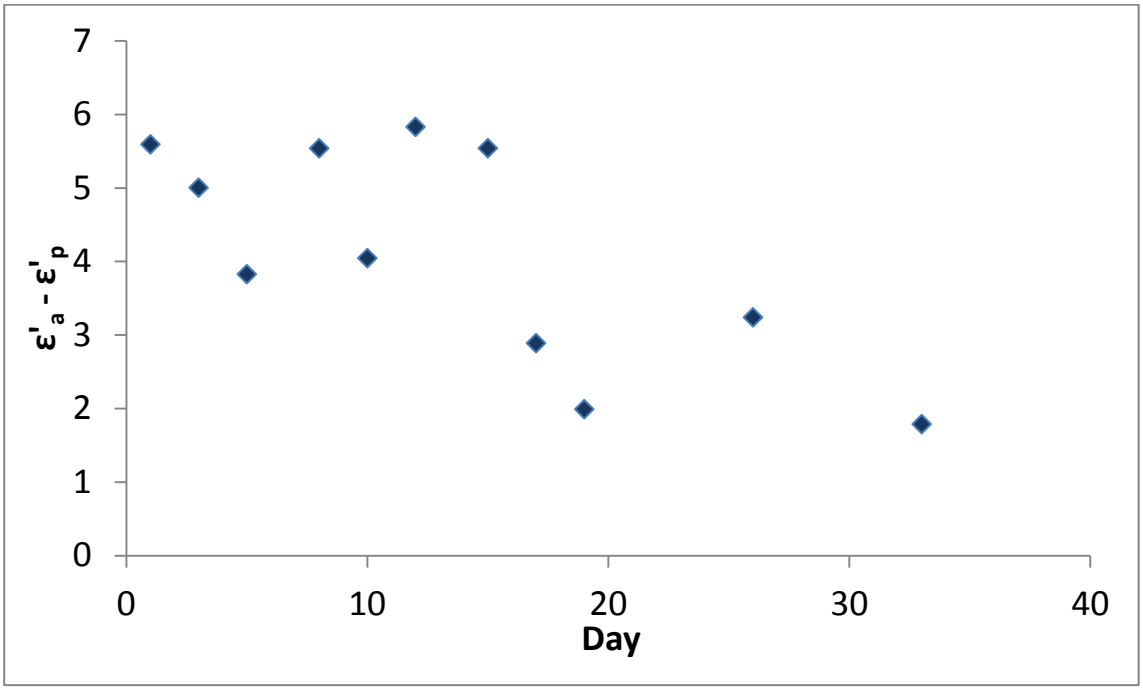


Figure 5.9: Change between the actual (ε'_a) and predicted (ε'_p) dielectric constant with time

A negative linear relationship (r^2=0.54) was identified in the difference in actual and predicted dielectric constant with time, as displayed in Figure 5.9: Change between the actual (ε'_a) and predicted (ε'_p) dielectric constant with time, Figure 5.9. This trend indicated that the mobility of the water within Mozzarella decreased with time. As the predictive model was developed for a process cheese system, which is stable in comparison to natural cheese (Kapoor & Metzger, 2008) with little change occurring in the structure during storage in comparison with Mozzarella, the difference in the state that water exists in, within the cheese, needs to be considered. Mozzarella differs from process cheese as it has free water present in its structure during the first few weeks following manufacture, whereas process cheese does not. It is possible that the variation contributed by variation in free water content with time is obscuring the observance of a linear relationship between ε' and moisture. Therefore the difference in actual dielectric

constant and that predicted via the linear regression model (Equation 5-2) was plotted against the percentage free water in the cheese, as determined by NMR, in Figure 5.10 below.

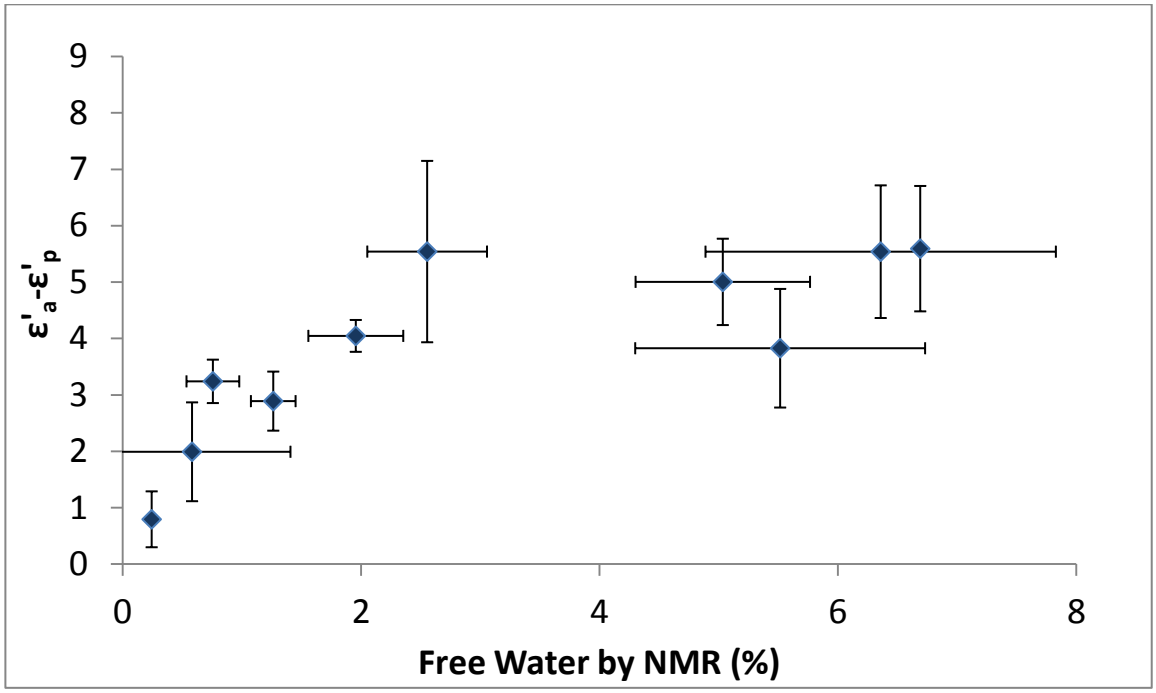


Figure 5.10: Difference in the actual and predicted dielectric constant versus free water determined by NMR [error bars = ±1 SD]

Figure 5.10 illustrates the weak relationship between the free water in Mozzarella and the difference between the actual dielectric constant and that predicted by Equation 5-2. This indicates that the dielectric constant is not just sensitive to the change in the total quantity of water present in the system; it is also related to the state that the water is in. The difference between the predicted and actual dielectric constant was largest when there was a greater quantity of free water present in the system. As the quantity of free water in the system decreases, so does the difference in dielectric constants. This is as expected as the dielectric constant is related to the molecular mobility of the water molecules in the cheese system. As the level of association between the water and the protein phase increases the water molecules become more restricted in their movement.

Based on the relationship between the dielectric constant and free water by NMR, a predictive model was developed. This enabled the quantity of free water in Mozzarella to be calculated as a function of the moisture content and the dielectric constant. By fitting an exponential function to the data the equation below was created; where M is the moisture content (%) and ε'_a is the measured dielectric constant.

$$\%\ \text{free water} = e^{\frac{\epsilon' a - 0.694M - 9.1297}{1.2871}}$$

Equation 5-3

This equation allows the percentage free water in Mozzarella to be predicted based on the dielectric constant and the moisture content.

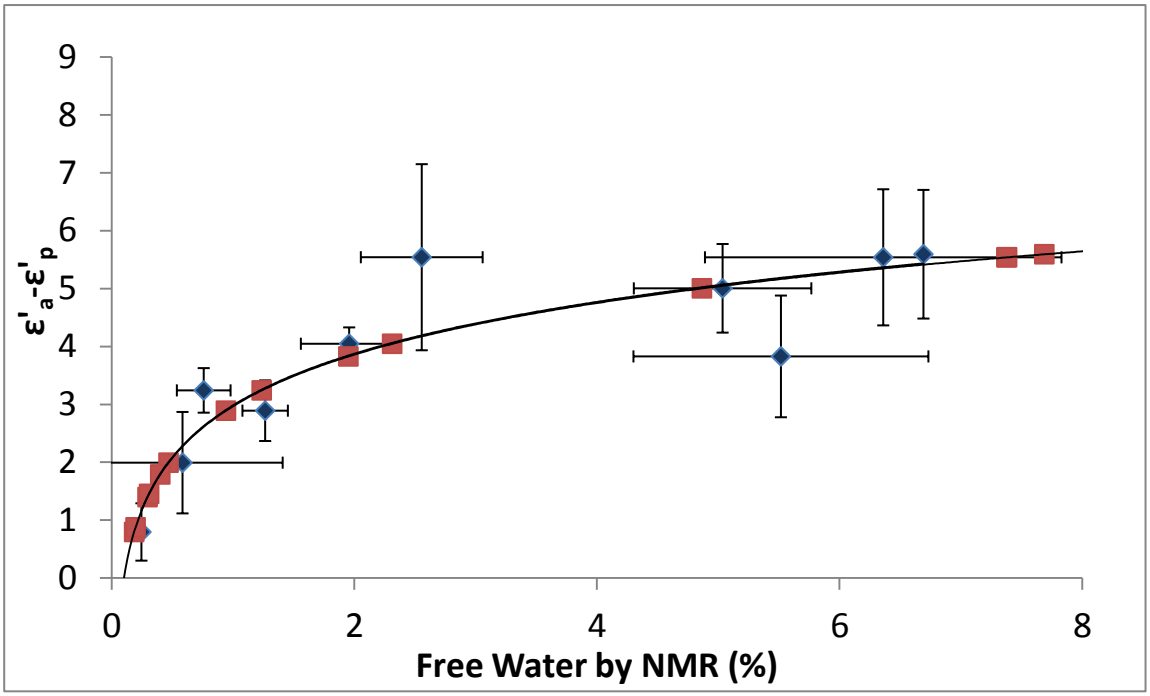


Figure 5.11: The predicted free water calculated by Equation 5-3 (● = measured data at 1.26 GHz & ■ = predicted data)

An analysis of the ability to predict free water content via dielectric spectroscopy and moisture content is shown in Figure 5.11, which plots free water predicted by the new model (Equation 5-3) against free water as measured by the accepted technique of NMR.

A regression fit demonstrates a reasonable fit, producing an R^2 of 0.81. A limitation in the experiment including the number of samples that could be assessed on each day of the trial impacted on the variation in the data. However, these preliminary results indicate that the dielectric constant may be useful in measuring the progressive change in free water in Mozzarella samples of a known moisture content.

The predictive model for process cheese based on the findings of Fagan *et al* (2005) was based on dielectric measurements at 2460 MHz. Although fitted to data obtained at 1260 MHz in this experiment it is unlikely to have a large impact on the trends observed in the data. To investigate this, the model developed was fitted to the other two frequencies isolated in Figure 5.6, 244 and 750 MHz. These lower frequencies exhibited an identical trend to the data at 1260 MHz; however, the predicted quantity of free water was higher than that predicted at 1260 MHz, most notably for the 244 MHz. A similar analysis was carried out on soils by Peplinski et al. (1995), where a model based on data obtained over a frequency range of 1.4 to 18 GHz was applied to data collected between 0.3 and 1.3 GHz and only required a small linear adjustment to be made for a perfect fit.

This experimental work indicated that dielectric spectroscopy could be used as a possible tool for assessing the change in water mobility in Mozzarella cheese during aging. The implication of this is that for a known moisture content, the dielectric constant could provide a rapid, non-destructive testing method for determining the quantity of free water present in Mozzarella. Although this initial study showed some promise in the use of dielectric spectroscopy, further work is needed to validate the proposed model. In particular it is necessary to replicate these results before the new dielectric method is utilised in industry. Additional experiments assessing the validity of the predictive model are discussed in 10.3.13.

5.3.7 General discussion

Significant changes were noted in the structure of Mozzarella during the first three weeks following manufacture. The distribution of water in Mozzarella was observed to change using confocal microscopy, with water migrating from pools of apparent free water into the progressively swelling casein network. This coincided with a change in the appearance in the surface of the channels within the cheese observed using SEM. These images identified a transition from smoothed walled channels to dimpled walls similar to those observed in previous studies (McMahon et al., 1999). In contrast to earlier studies the comparison of the confocal images and those obtained from SEM suggests that initially the fat in Mozzarella is surrounded by water. Over time the water is absorbed into the protein (Kuo et al., 2001) causing the casein matrix to swell so that it is in contact with the fat globules in the channels and thus create an imprint or dimple.

Both NMR and the expressible serum experiment indicated that the quantity of free water within Mozzarella decreased over the initial storage period. This is similar to trends identified in past studies of Mozzarella with NMR (Kuo et al., 2001) and expressible serum (Kindstedt & Guo, 1997b; McMahon et al., 1999). It also confirms the observations made using microscopy in regards to water migration. The implication of the NMR and expressible serum measurements in regards to free water is that there is a transition that occurs over the first few weeks following manufacture. This change in the mobility of the water will have repercussions in the physical properties of the cheese as the protein phase becomes more hydrated. It is also possible that the change in the mobility of the water mobility within Mozzarella will impact on the ions present in the cheese. As a result the ionic strength and pH in localised areas within the casein matrix may vary as the level of water associated with the protein phase increases. The change in the distribution of water within Mozzarella is likely to have implications not only related to other components within the cheese, such as ions, but also to the physical properties of the cheese, which is explored throughout the remainder of the thesis.

Unlike the other measurement techniques, no obvious trends were identified in the results from dielectric spectroscopy over the first 20 days of storage. Despite a linear relationship being identified between the dielectric constant and moisture content in process cheese by Fagan *et al* (2005), no such trend was identified in the Mozzarella data. This suggested that other factors were influencing the dielectric constant other than just the moisture content of the Mozzarella samples. One factor that has been identified to affect the dielectric properties in other material is the state that the water exists in (Feng et al., 2002; Mashimo, Kuwabara, Yagihara, & Higasi, 1987). The ability to structure water is dependent on the components that are in contact with the water. Protein is a key component in the cheese system that has the ability to structure water. In the study of Fagan and co-workers the protein content was constant over all the process cheese samples tested while the moisture content was varied by substitution with fat. In the current work while it was noted that moisture content varied among samples it is not known what the protein content for each sample was. Therefore it is possible to have two samples with identical moisture contents but with differing moisture to protein ratios.

A decreasing trend was identified in the difference between the actual dielectric constant and that predicted by applying the process cheese equation proposed by Fagan *et al* (2005) to Mozzarella. One key difference between Mozzarella and a process cheese is the presence of free water during these initial stages of storage. Over this period of storage the quantity of free water in Mozzarella decreases (Kuo et al., 2001; McMahon et al., 1999), in a similar manner to the trend identified in the difference between measured dielectric constant and the predicted dielectric constant based on the process cheese model. Modifying the predictive model of moisture content in process cheese (Fagan et al., 2005) enabled a model be to be constructed to predict free water in the Mozzarella sample.

Due to this investigation being carried out on a single sample of commercial cheese, validation of the proposed dielectric model over the compositional range typically found

in cheeses will need to be conducted in future work. However, based on these initial results dielectric spectroscopy could possibly be used as a tool for studying free water within Mozzarella cheese. There are a number of advantages of a predictive model for free water based on dielectric spectroscopy, including being a rapid non-destructive technique (Trabelsi et al., 1998). Dielectric measurements are near instantaneous and relatively inexpensive in comparison with methods such as NMR.

As with the other measurement techniques, dielectric spectroscopy was also identified as being influenced by the free water within Mozzarella and the subsequent change in water mobility during storage. One possibility for this change in free water within Mozzarella is due to weakening hydrophobic interactions within the cheese. Mozzarella differs from most other cheeses, such as Cheddar, due to an additional processing step where the curd is stretched (McMahon & Oberg, 1998). In this processing step Mozzarella curd is thermo-mechanically stretched by augers (Kindstedt et al., 2004). As part of this the curd is worked in hot water or brine and attains a temperature generally between 55 and 65°C (McMahon & Oberg, 2011). In this temperature range hydrophobic interactions are at their maximum strength (Nemethy & Scheraga, 1962). Strong hydrophobic interactions could lead to the casein matrix contracting, syneresing some of the water entrapped within. The cheese is then chilled and stored at refrigerated temperatures (Kindstedt, 2002). At the lower temperature of storage the hydrophobic interactions weaken, which may allow the free water to migrate back to within the protein matrix (McMahon & Oberg, 2011).

Other structural changes that are occurring in Mozzarella during this initial period of storage include a redistribution of the calcium within the cheese. This involves a slight increase in the proportion of soluble calcium in the Mozzarella during storage (McMahon & Oberg, 2011). This corresponds to a decrease in the number of calcium-mediated protein-to-protein interactions resulting in a looser gel (Joshi, Muthukumarappan, et al., 2004a). This loosening of the gel matrix may allow water to migrate in to the casein

network. This change in calcium distribution could also impact on the ionic strength of the system as there would be a greater number of free calcium ions in the system. This change in the proportion of soluble calcium and the subsequent change in protein-to-protein interactions is also likely to influence the diffusion properties of water within the cheese, namely the tortuosity. This effect will be investigated in detail in Chapter 10.

Another factor affecting structural changes occurring during storage is proteolysis (Costabel et al., 2007), although limited over the time frame investigated. Proteolysis is the process where residual enzymes in the cheese hydrolyse the casein molecules (Coker, Crawford, Johnston, Singh, & Creamer, 2005; Fox, 1989). Liberated carboxyl and amino groups due to the cleaving of casein molecules are able to structure water, reducing the water activity of cheese (Fox & McSweeney, 1996). The extent of proteolysis is very limited over the storage period for this experiment so is unlikely to have a large effect on free water. However, further research will be done to assess proteolysis in future work.

This work builds on past research characterising changes occurring in Mozzarella during the initial stages following manufacture, by bringing together a number of techniques previously used to monitor cheese to gain a more holistic view of changes in the cheese over this stage of storage. It also applied dielectric spectroscopy to identify its usefulness in studying Mozzarella. All of the measurement techniques to study the changes in the structure and component mobility were influenced by the free water present immediately following manufacture and the subsequent change in water mobility.

5.4 Conclusions

The distribution of water within Mozzarella was observed to change over the first few weeks following manufacture. Both NMR and the expressible moisture measurements identified a decreasing trend in free water similar to that seen in the confocal micrographs. By taking into account the sample to sample variation in the moisture content, the dielectric constant has the potential to predict the free water present in

cheese of a known moisture content.

Water migration was identified as having a significant inpact on the microstructure of Mozzarella. However, water migration is not the only mechanism that affects the structure of cheese during storage. Therefore a more detailed study, monitoring changes occuring over a longer maturation period using a variety of tools to assess the structure of Mozzarella, needs to be undertaken to identify what other drivers are responsible for structural change during the maturation process.

6 An investigation into the mechanisms driving structural change in Mozzarella and Cheddar cheese during maturation

6.1 Introduction

Cheese undergoes significant structural rearrangement during maturation. These changes in structure have a direct effect on the functionality of the cheese.

Mozzarella and Cheddar cheese are two of the most consumed cheeses in the world. Mozzarella differs from Cheddar in manufacturing due to a processing step where the curd is heated and stretched, giving the curd its fibrous structure (McMahon & Oberg, 1999). This additional processing step leads to, among other changes, the distribution of water within Mozzarella being quite different to Cheddar (Guo & Kindstedt, 1995). The stretching process can be considered to be a two stage process involving the curd entering the hot brine and increasing in temperature followed by the kneading of the curd (Kindstedt et al., 2004). Although a great deal of work has dealt with the structure before and after stretching, there is a lack of structural studies carried out to identify the drivers of change during the stretching process.

Although commonly referred to as a fresh cheese, commercial Mozzarella generally undergoes a period of ripening to attain a desired level of functionality (Kindstedt, 1993b). Immediately following manufacture, Mozzarella has been identified to have free water present within its structure (Kuo et al., 2001). Over time the water is absorbed into the protein matrix, reducing the amount of free water that can be measured in the cheese. This change in water mobility in Mozzarella was highlighted in the previous chapter where a number of different techniques were used to characterise the progressive decrease in free water.

Nuclear magnetic resonance (NMR) has been used as a tool to monitor the change in the association of water with the protein within the cheese structure (Chaland et al., 2000;

Gianferri, D'Aiuto, Curini, Delfini, & Brosio, 2007; Godefroy, Korb, et al., 2003; Hinrichs et al., 2004; Kuo et al., 2001) and this technique was successfully applied. This was observed in the previous chapter to assess water migration in a fresh Mozzarella cheese sample.

Proteolysis, which is an important mechanism in the development of cheese flavour, is also one of the major drivers of structural change in many cheeses. This breakdown of caseins in different cheeses has been extensively studied and reviewed (Creamer & Olson, 1982; Creamer, 1976; Fenelon & Guinee, 2000; Fox, 1984; Fox & McSweeney, 1996). The type and rate of proteolytic breakdown varies between cheeses depending on their processing conditions, storage conditions and composition. Gel electrophoresis is a method that has been utilised to assess the breakdown of protein fractions within cheeses during maturation.

This study aims to bring together a variety of techniques for characterising cheese to determine how the thermally treated Mozzarella compares to Cheddar.

Therefore the objectives of this study were:

- Characterise changes during the evolution of the structure and component mobility of Mozzarella and Cheddar cheese over a 120 day storage period.
- Determine potential drivers for structural change and associated mechanisms in both Mozzarella and Cheddar. In particular, the drivers associated with the movement of water within Mozzarella following manufacture.

6.2 Materials and Methods

6.2.1 Material

Commercial samples of Mozzarella and Cheddar cheese were obtained immediately after a 24 hour rapid cool cycle following manufacture (Fonterra Cooperative Group, New Zealand). The blocks, 1x 20 kg block of Cheddar and 2x 10 kg blocks of Mozzarella, were

cut into 40 equal sized blocks and were vacuum packed. The blocks were stored at 4°C in a refrigerator for the duration of the trial. The blocks were randomly selected on each day of the trial.

The composition of both cheeses was determined in accordance with the methods described in Section 5.2.7 by the Fonterra Research and Development Centre's Analytical Services team.

The moisture content of each cheese was monitored on each day of testing to assess the natural variation within each block of cheese. This was done by drying a sample of each cheese in an air oven for 16 hours at 108°C.

6.2.2 Confocal Microscopy

Confocal microscopy was used to assess the changes occurring in the structure of both the Mozzarella and Cheddar samples. Images of Mozzarella were collected both parallel and perpendicular to the protein fibres. Imaging was carried out at regular intervals over the course of the 120 day trial. The method used to assess the samples using confocal microscopy is stated in Section 4.2.2.

6.2.3 Urea-polyacrylamide gel electrophoresis

Significant changes in the structure of the protein matrix were observed using microscopy over the course of the maturation trial. In order for change to the protein matrix to occur there must be a mechanism allowing mobility of the protein. A possible mechanism could be a gradual breakdown of caseins which could not only weaken the existing structure but additionally render the existing structure thermodynamically unstable and thus create a driving force for protein movement toward a more stable conformation. To assess this theory PAGE was used to investigate the extent of breakdown of both α_{s1} and β casein.

Samples of cheese were frozen at -30°C on days 1, 15, 30, 60, 90 and 120 of the maturation trial. The samples were then thawed at 4°C and shredded. The samples were then processed in accordance with Creamer (Creamer, 1991) method for alkaline urea gels. Duplicate samples were analysed to assess the change in both α_{s1}- and β- caseins.

6.2.4 NMR Spectroscopy

The quantity of free water within both Mozzarella and Cheddar was assessed over the course of the 120 day storage trial using NMR. The method used to analyse the samples is stated in the previous Section 5.2.4.

6.2.5 Uniaxial Compression

Uniaxial compression was used to monitor the changes occurring in the hardness of both the Mozzarella and Cheddar samples over the 120 day period. The method used is described in Section 4.2.4.

6.2.6 Meltability

The meltability of the cheese samples was assessed using the Modified Schreiber method disclosed by Muthukumarappan et al. (1999). Cylinders of cheese 25 mm in diameter and 20 mm in height were cut and left to reach equilibrium in the temperature controlled laboratory set at 20°C for a period of 4 hours. The cylinders were placed on a 2.2 mm thick aluminium plate and placed in a laboratory oven at 90°C for a period of 5 minutes. The samples were removed from the oven and allowed to cool. The diameters of the samples were then measured and the percentage increase in diameter calculated.

6.2.7 Scanning Electron Microscopy

SEM was used to monitor changes in the protein matrix of both Mozzarella and Cheddar over the maturation trial. Samples of Mozzarella were fractured both in the direction parallel and perpendicular to the protein fibres. Images were collected on four samples of each cheese at the 4 magnifications used previously. The method used to assess the samples is stated in Section 5.2.3.

6.2.8 Analysis of key processing steps for Mozzarella

In order to identify the stage during processing that free water becomes evident in the structure of Mozzarella, a pilot plant trial was conducted. This allowed samples of Mozzarella to be obtained at specific points during the manufacturing process. This experiment was conducted on a pilot scale as it was not possible to collect samples at the desired points during commercial manufacture. The pilot plant trial was conducted in 400 L vats. An assumption made was that that the commercial manufacturing conditions were similar to those done on the pilot scale as they were manufactured in the same way. It is recognised that there may be some variation with regards to time and temperature profiles through key steps and the composition of the initial cheese milk. However, it is believed that any variation that may exist will be of minor importance compared to the effect of the cook temperature and stretching operations. Therefore correlations between the commercial sample and the pilot scale production of Mozzarella are likely to be valid.

Samples were collected after dry salting, after the curd was immersed in the hot brine of the cooker stretcher and after the curd was stretched. The samples were sealed in plastic bags and immersed in -4°C brine to try and minimise the changes occurring in the samples. The samples were removed from the brine solution and then confocal and scanning electron microscopy were used to view the structure and distribution of components within the cheese. These analyses were conducted within 24 hours of obtaining the samples.

6.3 Results and Discussion

6.3.1 Composition

Table 6-1: Compositional analysis of commercial samples of LMPS Mozzarella and Cheddar.

	Moisture (%)	Protein (%)	Fat (%)	Ash (%)	Calcium (%)	Salt (%)
Mozzarella	49.1	26.1	21.2	2.46	0.64	0.90
Cheddar	37.9	22.5	33.6	4.19	0.74	2.18

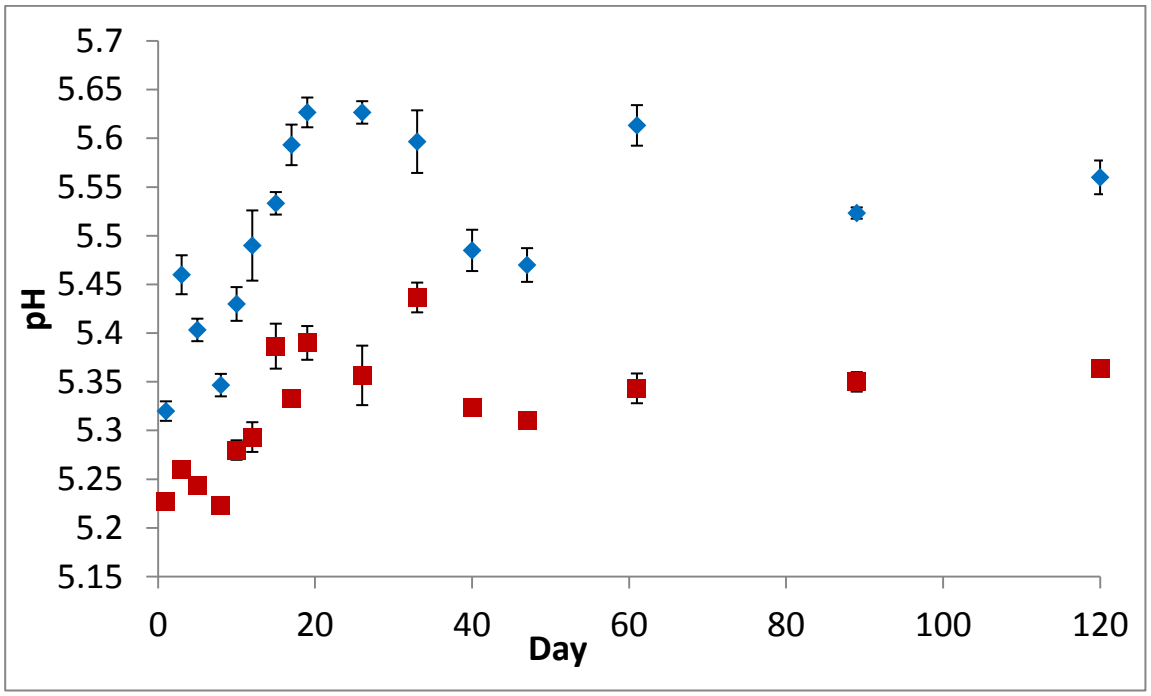


Figure 6.1: Change in the pH of Mozzarella (■) and Cheddar (▲) over a 120 day storage period

The pH of both Mozzarella and Cheddar increased following manufacture up until approximately day 20. This increase in pH in each of the cheeses following manufacture may be the result of the solubilisation of colloidal calcium phosphate (Hassan, Johnson, & Lucey, 2004), which has been reported previously (Lee, Johnson, Govindasamy-Lucey,

Jaeggi, & Lucey, 2010). The consequence of this is that there is a progressive increase in calcium and phosphate ions within the aqueous phase. The increase in the quantity of free phosphate ions in the aqueous phase may result in an increase in the buffering capacity of the cheese (Hassan et al., 2004). The mechanism of the buffering that may occur in cheese could be the result of the most stable form of phosphate being dihydrogenphosphate ($H_2PO_4^-$) (Ferreira, Oliveira, & Rocha, 2003). Therefore as the CCP solubilises the phosphate ions form $H_2PO_4^-$, removing hydrogen ions and as a result causing an increase in the pH of the system.

Another factor that may influence the pH of cheese during the extended storage is proteolysis of the casein. As the protein is hydrolysed, protons are liberated into the surrounding media as the peptide bonds are cleaved (Spellman, McEvoy, O'Cuinn, & FitzGerald, 2003). The increase in the number of protons within the aqueous phase will result in a decrease in the pH of the cheese. Lipolysis, although likely to be limited in the cheese samples in this study, may also have an effect on pH. This is due to liberation of free fatty acids (FFA's) (Collins, McSweeney, & Wilkinson, 2003), which may reduce pH. Therefore, if proteolysis and lipolysis affect the pH during the storage trial it would be in the form of a reduction in pH.

6.3.2 Confocal Microscopy

During the maturation process, significant changes in the bulk structure of cheese occur. In order to observe these changes occurring in the cheese structure, microscopy methods were employed. Confocal microscopy was used to observe the dispersion of fat, protein and free water. Mozzarella was cut in two different directions, longitudinally sectioned and transverse sectioned (parallel and perpendicular to the fibre structure respectively).

Images were collected at 20x, 40x and 63x magnification. The images displayed below are those taken at a magnification of 40x. The dark area observed in the images corresponds to the free water present in the cheese.

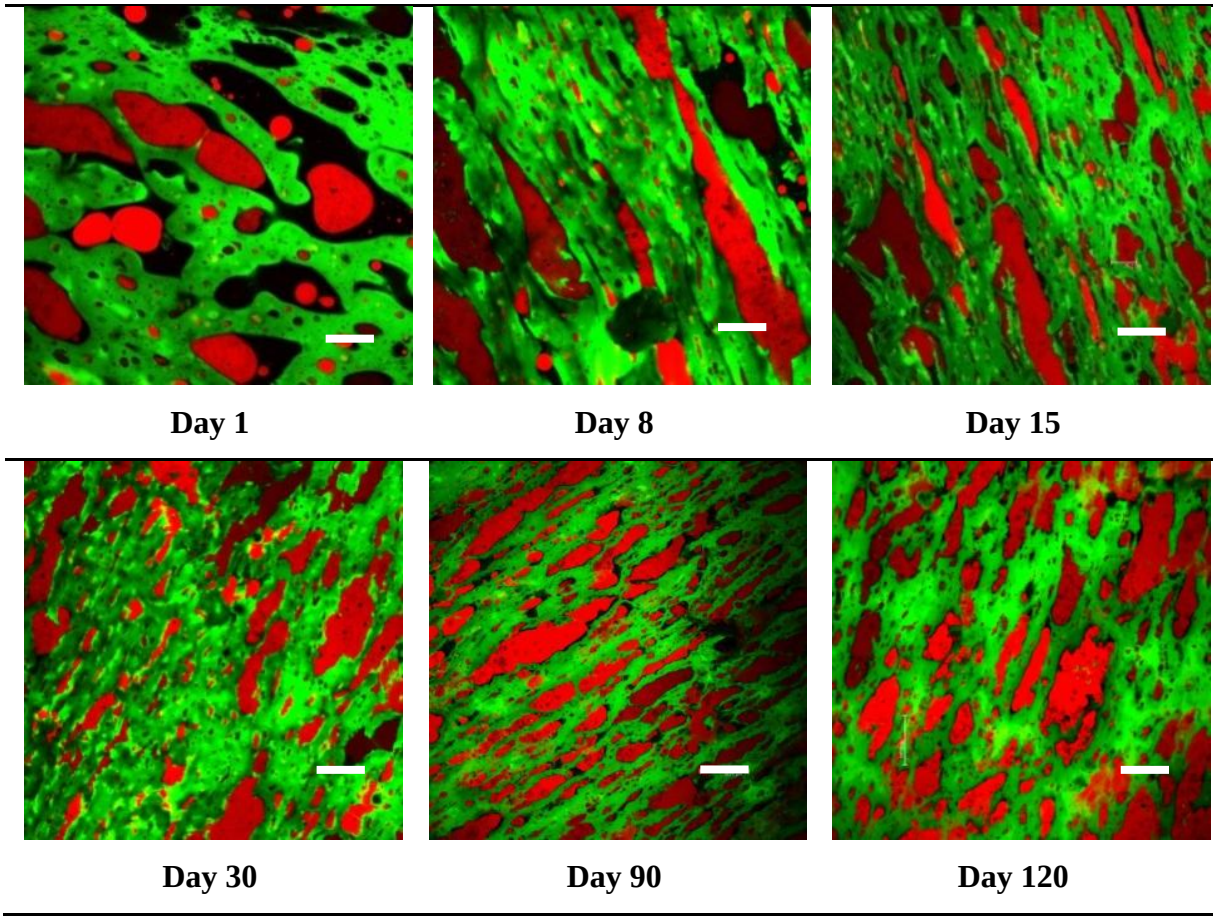


Figure 6.2: Confocal micrographs of mozzarella at different ages – samples taken parallel to protein fibres (Scale bar = 50 µm).[Green = protein+water, red = fat, black = water]

The images of Mozzarella cut parallel to the protein fibres showed the channels of protein and fat in the cheese structure. Over the 120 days of this study, significant changes in the structure of the channels were observed. The free serum in Mozzarella was seen to decrease from day 1 through to 15, at which point the water was no longer visible in the confocal images. The observed reduction in visible free serum is likely to be due to absorption of water by the protein phase. This explanation is supported by the

concomitant appearance of long fat channels which would be expected through the swelling of the fibril protein matrix and the resulting pressure compressing the fat.

After this initial adjustment in structure, the distribution of fat within the cheese continues to change. The cheese is subjected to proteolysis whereby the protein is gradually broken down into fragments (Coker et al., 2005). This causes a relaxation of the protein matrix (Tunick, Malin, et al., 1993) causes deformation of the channelled fat structure causing them to become more spherical with time. During storage the protein may revert to a more energetically favourable conformation (the fibres developed during stretching likely to be a high energy state). However, the change in the protein structure is more likely to be driven by the weakening of the matrix due to proteolysis This change in the distribution of fat during storage is similar to observations made by Auty *et al* (2001).

The trends in the distribution of the fat within the protein channels were confirmed by assessing the average axial ratio of the fat pockets (length divided by the width). This indicated that the axial ratio increased from 1.51 to 5.09 between day 1 and day 15, indicating that the fat was becoming more elongated. This decreased to 2.05 on day 120 indicating that the fat within the cheese was becoming more spherical over this time interval, backing up the observations in the images. On day 1 of the storage trial free serum was identified in the confocal images surrounding the fat within the channel. The average axial ratio of the entire channel was identified as being 2.53 in comparison to that of the fat at 1.51. This indicates that the shape of the channels differs from that of the fat within the channels, with the fat being more spherical in nature to the elongated channels. In addition to a difference in the average axial ratio, differences were identified in the standard deviation of the axial and longitudinal dimensions, with the entire channel on day 1 having a standard deviation in the axial direction of 22.6 µm and 58.7 µm in the longitudinal dimension in contrast to 13 µm and 21 µm respectively for the fat. This indicates that the pockets of fat within the cheese were more uniform in both dimensions in comparison to the entire channels on day 1. However, to quantify the morphology of

the fat within the cheese more accurately a greater number of confocal micrographs would need to be assessed to gain a representative picture of the fat, particularly due to the anisotropic nature of Mozzarella.

The confocal images of Mozzarella cut across the protein fibres, in Figure 6.3 below, showed a similar trend to the images cut in a parallel direction. The free serum present in the cheese structure was absorbed over the first 15 days following manufacture and the fat within the system forms larger clusters during maturation.

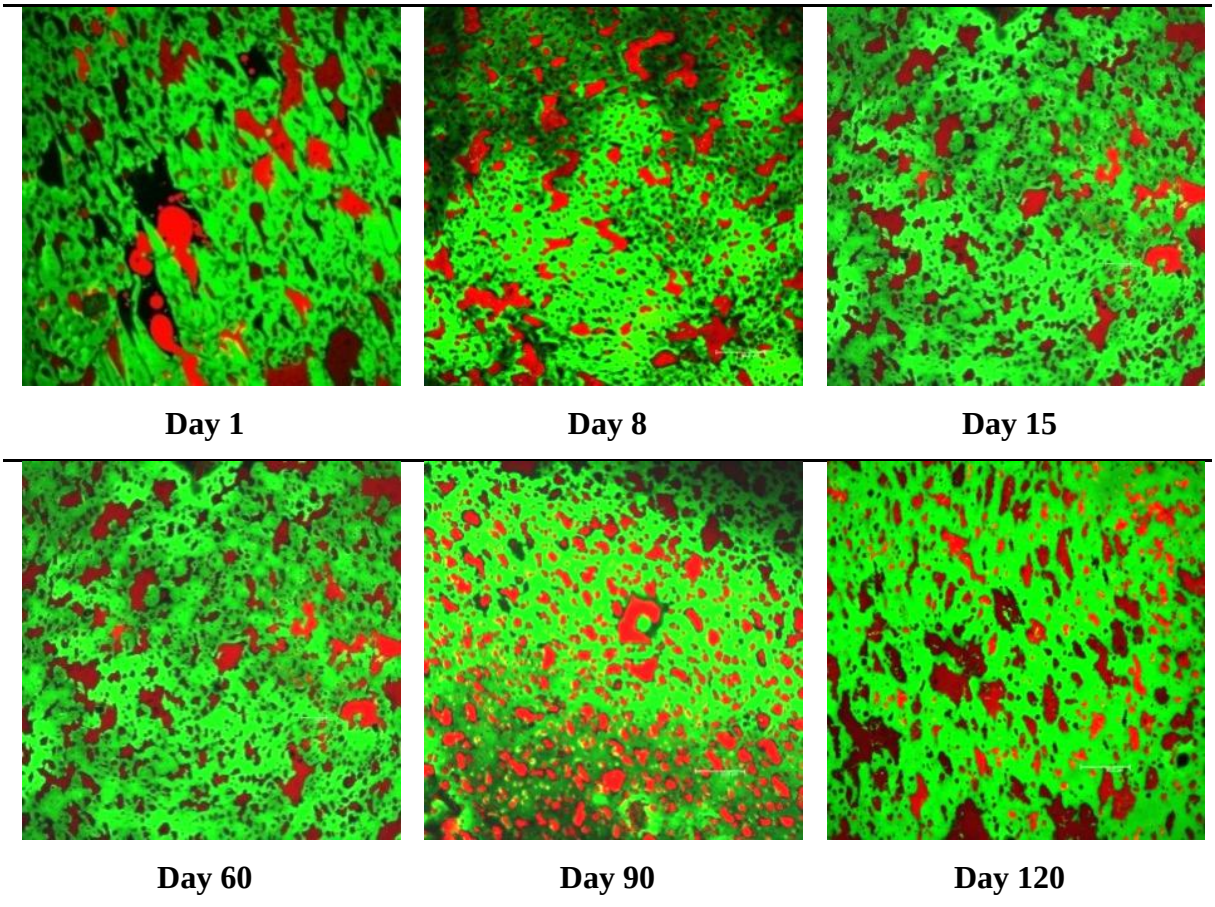


Figure 6.3: Confocal micrographs of mozzarella at different ages – samples taken across the protein fibres

The functionality window for the Mozzarella sample, as described by the Fonterra Research and Development Centre as the timeframe where the cheese is at it optimum functionality from a commercial standpoint, studied is 8 to 12 weeks following manufacture, when the structure is between the measurements and images taken on day 60 and 90.

The analysis of the fat within the Mozzarella over the storage trial, based on the confocal micrographs, indicated changes in the average axial ratio, as in those images collected parallel to the protein fibres. However, the trend exhibited in the images collected perpendicular to the channels was the reverse of that identified in the previous micrographs. Over the storage trial the average axial ratio changed from 2.8 on day 1, decreasing to 2.4 on day 15 and then increasing to 2.7 on day 120. The change in the axial ratio in the micrographs collected perpendicular to the protein fibres in Mozzarella were found to be smaller than the change observed in those collected parallel to the fibres. A progressive decrease was identified in the axial and longitudinal dimensions over the storage trial indicating that the distribution of the fat in the image was becoming more uniform with time. This change was from a standard deviation of 9.6 µm in the axial dimension and 17.2 µm in the longitudinal dimension on day 1 of the storage trial to 5.1 µm and 15.8 µm respectively on day 120 of the storage trial. This conforms with the trends that can be visually observed in the micrographs from the beginning of the storage trial through until day 120.

Over the 120 day trial, changes were observed in the distribution of fat and protein within the structure of Cheddar cheese (Figure 6.4). By inspection of the confocal images the pockets of fat within the cheese initially decreased in size over the first month following manufacture, followed by a subsequent increase in size over the remainder of the trial.

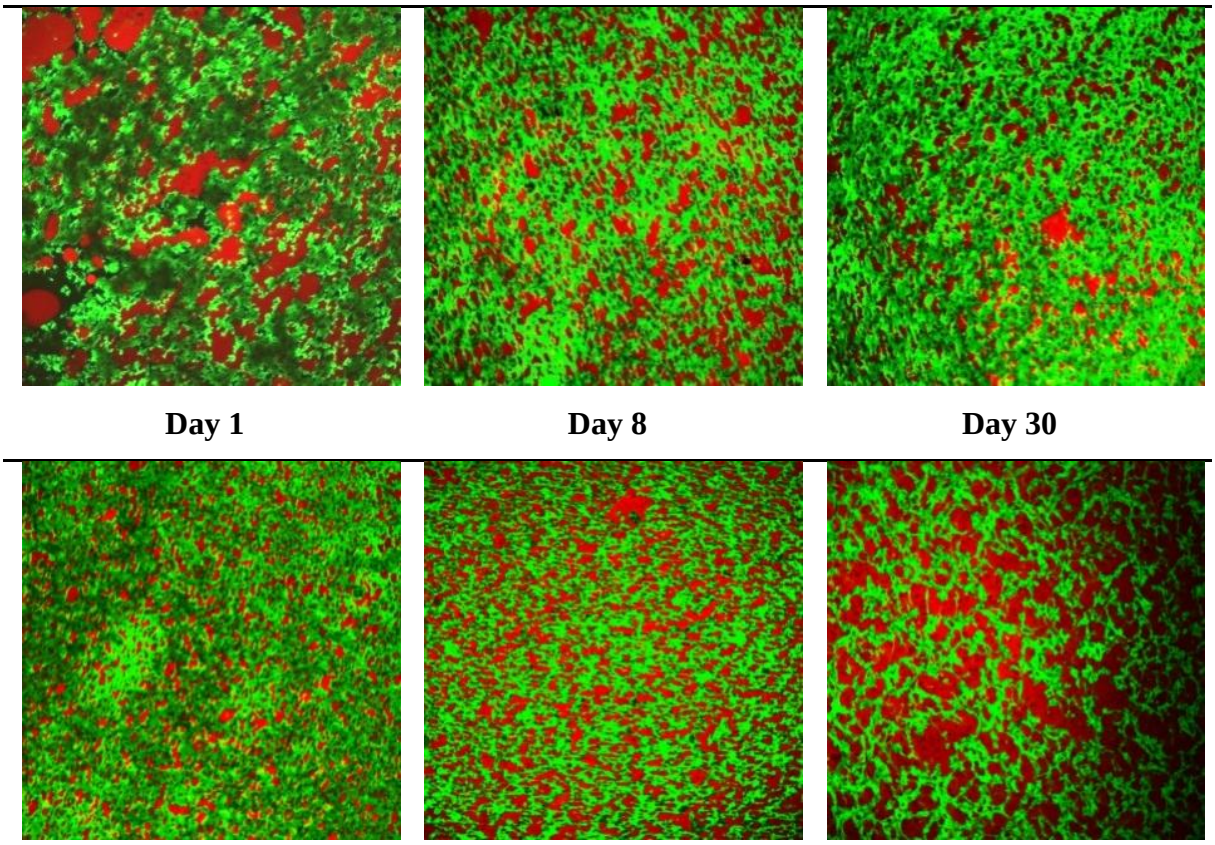


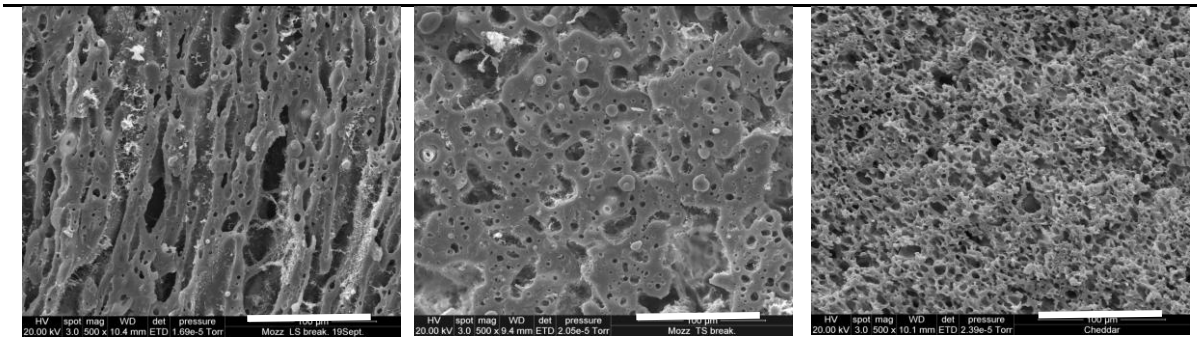
Figure 6.4: Confocal micrographs of Cheddar at different ages

Figure 6.4 indicates the changes in the distribution of fat and protein within Cheddar over the 120 day trial. Unlike the early confocal micrographs of Mozzarella, no free water was identified upon inspection of the images obtained for Cheddar. As in Mozzarella, the protein in Cheddar is subjected to proteolysis (Fox & McSweeney, 1996). This will result in a gradual weakening in the protein matrix (Tunick, Malin, et al., 1993) which may result in the observed change in the distribution of the fat within the cheese. As the casein matrix becomes looser the fat can cluster together into a more energetically favourable state. It was evident in the SEM images of Mozzarella in the previous chapter that fat exists either in its native state or partially coalesced state as a consequence of high localised phase volume.

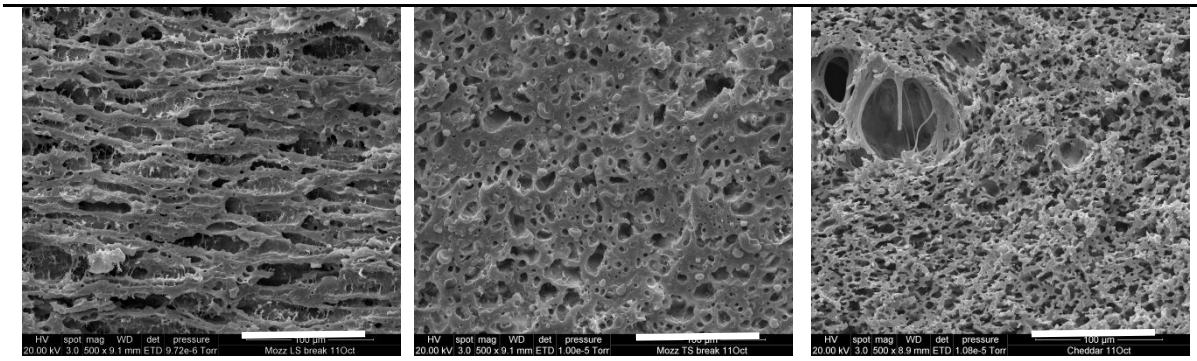
As with the Mozzarella images the average axial ratio of the fat pockets within the Cheddar samples was assessed over the storage trial. This showed that the average axial ratio decreased from day 1 to day 30 from a value of 2.6 to 2.08. This indicates that over this initial storage period the fat within the Cheddar became more spherical in nature. Following this, the mean axial ratio increased slightly to 2.14 on day 120. The standard deviation in both the axial and longitudinal dimensions decreased from day 1 to day 30, from 6.7 µm and 25.3 µm to 3.1 µm and 5.8 µm respectively, indicating the fat pockets within the cheese were becoming more uniform. An increase in the standard deviation in both dimensions was observed up to the end of the storage trial where on day 120 they were 5.3 µm in the axial dimension and 12.5 µm in the longitudinal dimension. This behaviour is evident by examination of the confocal micrographs over the 120 day storage trial.

6.3.3 Scanning Electron Microscopy

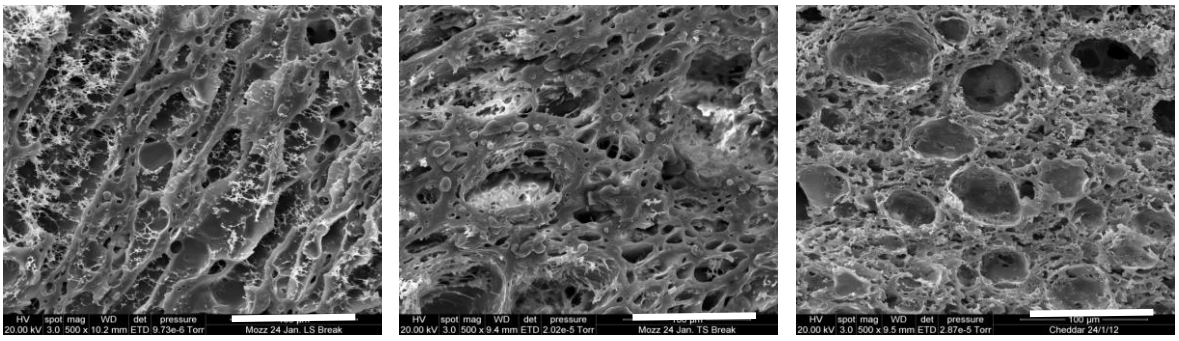
The confocal images of both Mozzarella and Cheddar indicated that substantial changes were occurring in the structure of the cheeses over the 120 day period. Scanning Electron Microscopy (SEM) allows a detailed view of the protein matrix of the cheeses to be obtained. The samples are defatted and dried leaving the protein matrix and voids previously occupied by fat and serum.



Day 8	Day 8	Day 8



Day 30	Day 30	Day 30



Day 120 Mozzarella – Parallel to protein fibres	Day 120 Mozzarella – perpendicular to fibres	Day 120 Cheddar

Figure 6.5: SEM micrographs following the progressive change in the structure of Mozzarella and Cheddar (scale bar = 100 µm).

The SEM micrographs of the Mozzarella samples cut parallel to the protein fibres allowed a detailed view of the channel structure within the cheese. Initially the channels between the protein fibres were observed to be long and thin. However, with extensive aging the

channels become wider and shorter confirming the observations made in the confocal images.

In addition to the changes in the morphology of the channels, strands of protein become evident within the channels. As the cheese is stored at low temperatures β-casein is able to migrate out of the protein network (Everett, 2007). This is due to the hydrophobic interactions within the cheese weakening at the temperature of storage.

In the Mozzarella samples taken perpendicular to the protein fibres, an end on view of the channels could be gained to assess changes with time. These micrographs depicted the change from the initial structure to one with much larger openings. This, along with the confocal images and the SEM micrographs cut parallel to the protein fibres, indicates that following the initial swelling of the protein fibres, the fat channels within Mozzarella become wider with extensive aging.

The confocal images of Cheddar indicated that large pockets of fat had formed by day 120. This is confirmed by the SEM images that show large voids in the protein matrix on day 120. As the protein matrix weakens during proteolysis (Karami, Ehsani, Mousavi, Rezaei, & Safari, 2009; Tunick, Malin, et al., 1993), the fat is able to cluster together to form large pools, as indicated in the confocal images and the voids in the SEM micrographs.

6.3.4 Urea PAGE

The level of α_{s1}-casein and β-casein present in the Cheddar and Mozzarella cheeses were monitored over the 120 day maturation process.

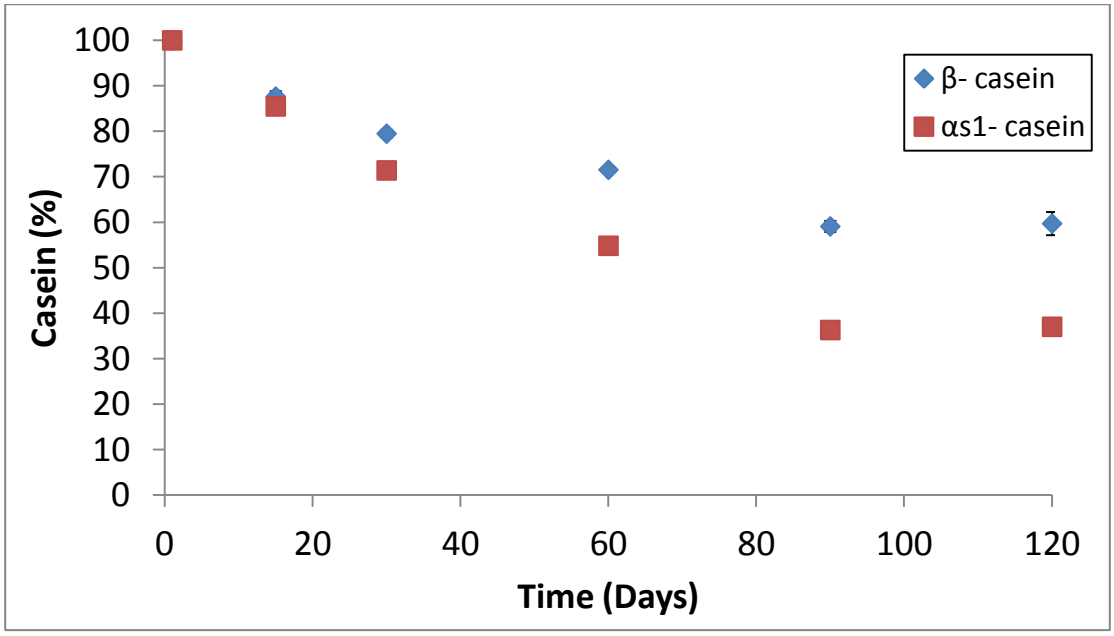


Figure 6.6: Breakdown of casein in Mozzarella over 120 days.

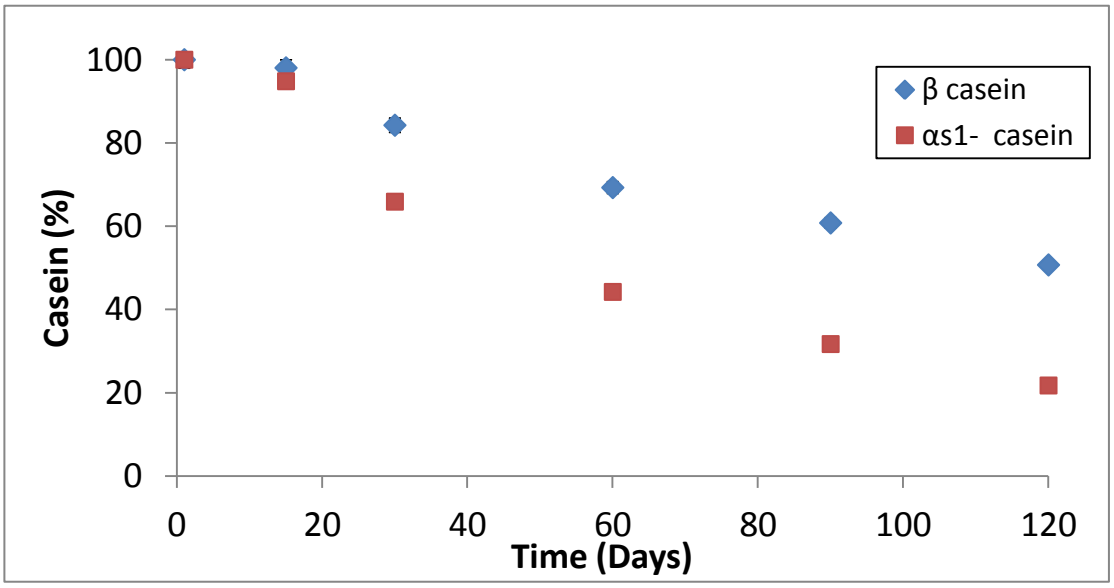


Figure 6.7: Breakdown of casein in Cheddar over 120 days.

The level of both α_{s1}-casein and β-casein were found to decrease during storage in both the Mozzarella and the Cheddar samples. Both the Cheddar and Mozzarella samples were observed to have a greater rate of breakdown of α_{s1}-casein than β-casein over the storage trial. This trend in the breakdown profile in the Mozzarella sample was unexpected, as the preferential breakdown of β-casein was expected in the cheese.

A greater breakdown of β-casein was expected due to the additional heat treatment that Mozzarella is subjected to during the stretching process. This additional heat treatment acts by largely inactivating the chymosin present in the curd, while the more heat stable plasmin remains active (Lawrence et al., 1987). The mechanism for this, as discussed in Section 2.2.1.8, is part of the complex protease-protease inhibitory system of plasmin in milk (Ismail & Nielsen, 2010). Plasmin in milk exists in its zymogen form, plasminogen (Grufferty & Fox, 1988), and its conversion and activity governed by a number of activators and inhibitors (Ismail & Nielsen, 2010). Both the plasminogen activator inhibitor and the plasmin inhibitor are affected by heat (Ismail & Nielsen, 2010) while the plasminogen activator is heat stable (Lu & Nielsen, 1993). This allows the conversion of plasmin from plasminogen to occur and in the absence of an inhibitor the plasmin can hydrolyse the casein in the cheese. Plasmin is the main enzyme responsible for the breakdown of β-casein in cheese (Feeney et al., 2001). Proteolytic breakdown of casein in New Zealand Mozzarella typically is driven predominantly by plasmin, while chymosin in combination with plasmin is responsible for the majority of the breakdown in Cheddar.

However, this was not the case in this study, with Mozzarella having a greater level of β-casein than α_{s1}-casein in the cheese sample. This suggests that the heat treatment of the Mozzarella curd was not sufficient to fully inactivate the rennet. Therefore the residual chymosin present within the cheese during storage hydrolyses the casein within the Mozzarella in a similar manner to the Cheddar sample. However, the percentage decrease in intact α_{s1}-casein was lesser in the Mozzarella than the Cheddar cheese indicating that some of the chymosin may have been inactivated by the stretching step. Both of the

cheeses showed a significant level of protein breakdown over the trial period which results in a weakening of the gel structure (Karami et al., 2009; Tunick, Malin, et al., 1993). The progressive weakening in the gel structure coincides with the progressive pooling together of fat globules to form localised pockets within the cheese. The weakening of the gel structure will impact on the material properties including the hardness and the ability for the cheese to flow when heated, which will be explored in more detail in sections 6.3.6 and 6.3.7.

6.3.5 NMR

The movement of water within the cheese structure was observed using confocal microscopy. In order to quantify this water and the rate of movement within the structure over the maturation period, we used Nuclear magnetic resonance (NMR) as done in the Section 5.3.4 previously.

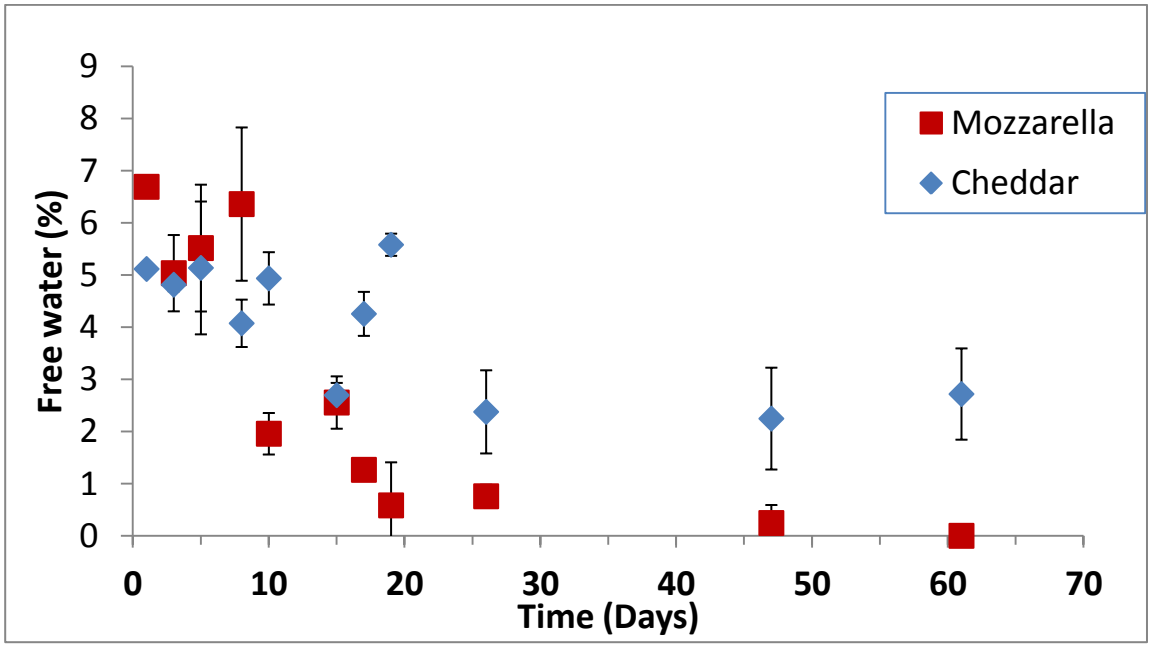


Figure 6.8: Change in the percentage water in a free state as determined by NMR in Mozzarella and Cheddar cheese over a 60 day period

The percentage of water in a free state in both Mozzarella and Cheddar was found to decrease over the first 20 days of the trial, as illustrated in Figure 6.8. This decrease corresponded with an increase in the quantity of water associated with the protein within the cheese, as determined by NMR. The level of free water present in the Mozzarella was identified as being significantly different over the maturation trial, $p=3.8x10^{-6}$. The changes observed in Mozzarella were similar to those observed by Kuo *et al,* (2001). Mozzarella has a higher moisture content than Cheddar (Table 6-1) so the decrease in free water had a much greater effect on the structure of the cheese. Comparatively, as a percentage of the entire composition of the cheese samples, the initial free water content for Mozzarella was 3.34% and 1.93% in Cheddar. In Mozzarella this dropped to zero, with the cheese going from having 3.34% free water to no free water. The Cheddar sample had an overall change in the quantity of free water within the entire cheese of 0.98%, as it decreases from comprising of 1.93% to 0.95%. Therefore, not only was Mozzarella identified as having a higher quantity of free water making up the cheese initially, but the change in free water during storage was greater than the Cheddar. However, as with the Mozzarella, the variation in the free water in the Cheddar samples was identified as being significant, p=0.00146.

Unlike Mozzarella, the confocal micrographs did not indicate the presence of free water within the structure of Cheddar. In the previous chapter, Section 5.3.5, an expressible serum method was used to monitor free water in Mozzarella, as done in previous studies (Guo et al., 1997; McMahon et al., 1999). Over this period the method was also applied to Cheddar (unreported). This identified the quantity of expressed moisture was an order of magnitude lower than Mozzarella, with the maximum extracted on day 1 at a level of 0.5% dropping to less than 0.1% by day 5. However, the NMR data indicate the free water does reside within Cheddar cheese following manufacture. Two factors that are likely to influence these measurements are the lower moisture content of Cheddar in comparison with Mozzarella and the distribution of free water differing from Mozzarella due to the

absence of channels in Cheddar. As with Mozzarella the free water within Cheddar will probably occupy the space between the protein and the fat. However, unlike Mozzarella, the fat within Cheddar is dispersed in globules throughout the gel network (Rogers, McMahon, Daubert, Berry, & Foegeding, 2010) resulting in small quantities of free water possibly surrounding these pockets of fat. This difference in the distribution of the free water would make it more difficult to extract using centrifugation and more difficult to observe in confocal microscopy.

In the previous chapter (Section 5.3.7), the change in the distribution of free water over the initial few weeks following manufacture was explained as being possibly due to the weakening of hydrophobic interactions during storage. The temperature that both cheeses are made are higher than the temperature of storage, leading to a subsequent weakening of hydrophobic interactions (McMahon & Oberg, 2011). As Mozzarella reaches a higher temperature during manufacture, where a greater quantity of free water will be expressed, a greater decrease in the quantity of free water within the cheese following manufacture during storage would be expected. This was confirmed by the NMR analysis of the two cheeses during the storage trial.

The change in the distribution of water was identified as being similar to the trend observed in pH during the first 20 days of the storage trial. This suggests that water migration may influence the pH, possibly as a result of an interaction with the CCP. However, as CCP was not monitored in this investigation no conclusions can be drawn about the change in the distribution of calcium. This will be monitored in future work (Chapter **Error! Reference source not found.**) to aid in the interpretation of the results.

6.3.6 Uniaxial Compression

Previous techniques used in this study identified significant changes to the structure over the course of the maturation trial. These structural changes will impact on the functional

properties of the two cheeses. Uniaxial compression was used to evaluate the changes to the hardness and fracture properties of the cheeses.

Uniaxial compression involves compressing the cheese sample to a specific percentage of its original height, recording the force required to do so as well as the force at which the sample fractures. From these recorded forces an evaluation of the hardness of the cheese samples can be gained along with the fracture point.

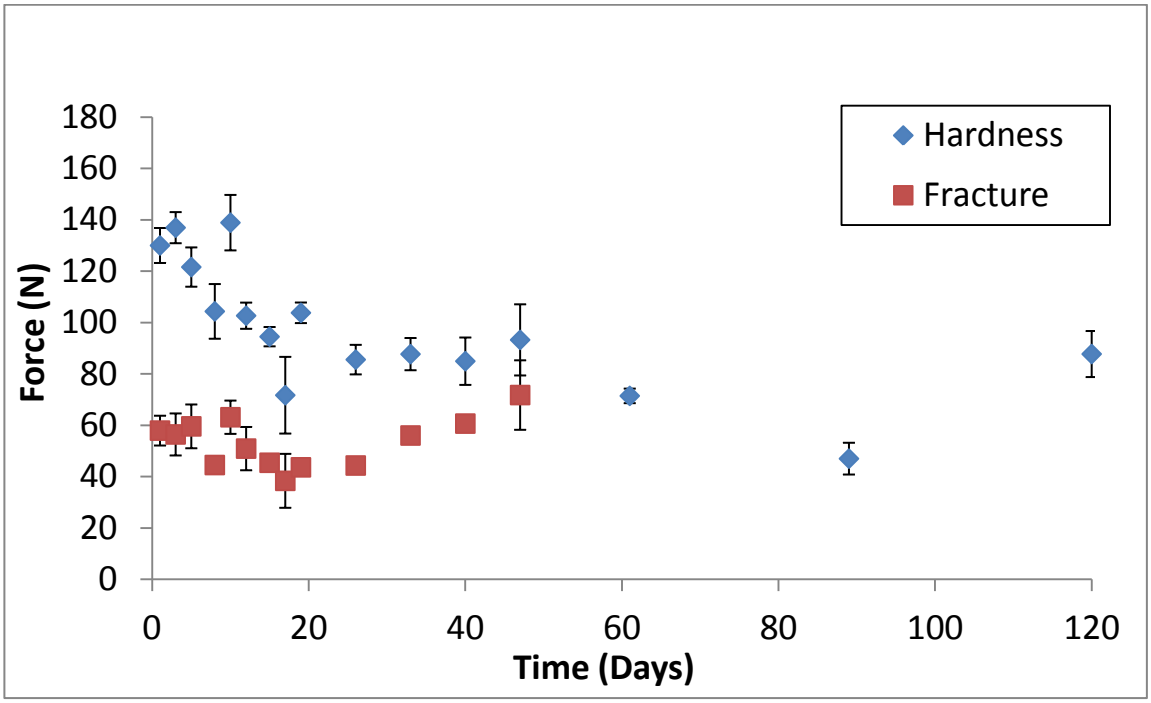


Figure 6.9: Hardness and fracture data of Mozzarella stored for 120 days

The hardness of the Mozzarella was found to decrease over the course of the first 20 days following maturation, as identified in Figure 6.9. This decrease in hardness coincides with the water migration occurring within the cheese structure. The force at which the samples of Mozzarella fractured was found to stay stable over the first 20 days and then increase until day 47. After this point, the force required to fracture the samples became greater than the force required to compress the samples to the specified height. Because of this, no fracture of the Mozzarella samples was observed after day 47.

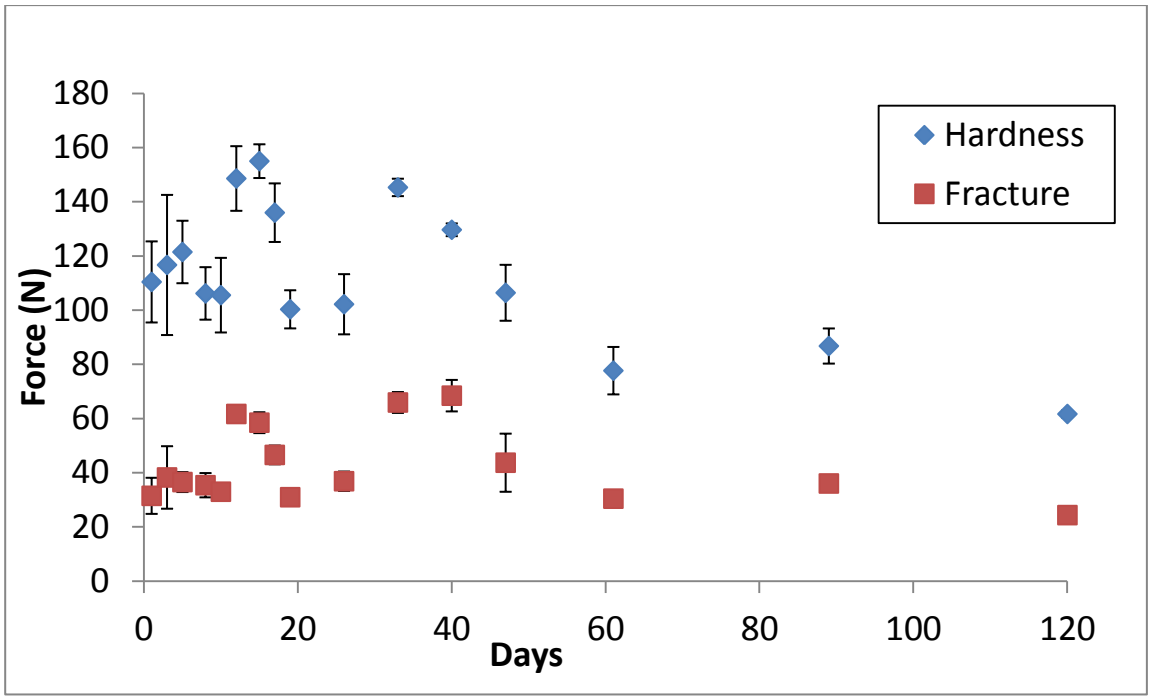


Figure 6.10: Hardness and compression data for Cheddar stored for 120 days

The hardness of Cheddar was found to decrease over the 120 day storage trial, as shown in Figure 6.10. The force required to fracture the Cheddar at followed a similar pattern to the hardness of the samples, when subjected to a 80% compression.

The proteolytic breakdown of the protein within both cheeses is likely to strongly affect the resulting hardness of the cheeses, with a decrease in intact casein leading to a weaker structure (Creamer & Olson, 1982). The weakening of the gel structure of both Mozzarella and Cheddar cheese results in a gradual softening of the cheeses as proteolysis occurs (Tunick, Malin, et al., 1993). This weakening of the gel structure during the later stages of the maturation profile coincides with the change in the distribution of fat within the cheese structure.

As the fat within the cheese structure is mostly solid at 20°C, it has a significant contribution to mechanical and textural properties such as hardness and elasticity (Bryant,

Ustunol, & Steffe, 1995). The changes observed in the distribution of the fat globules in Mozzarella cheese are likely to have a significant impact on the hardness of the cheese (and melt properties). The initial change in morphology of the fat occurs as a result of the pressure from protein as it hydrates due to migration of free water within the system over the first 20 days. This coincides with the decrease in the hardness of the Mozzarella over this period. The changes in the fat distribution within the channels over the latter parts of the maturation of Mozzarella are likely to influence the changes in the hardness over the rest of the trial.

The pockets of fat globules within Cheddar were also observed to change over the course of the 120 day maturation period. As the pockets of fat were observed to get progressively larger, the cheese was identified as being less hard. In addition to the weakening of the gel structure due to proteolysis (Tunick, Malin, et al., 1993), larger pockets of fat can deform to a greater extent than small fat pockets (Rudan et al., 1998) which could also contribute to the overall decrease hardness.

6.3.7 Melt

The melt properties of the cheeses were assessed using the Modified Schreiber Test. The meltability of both Mozzarella and Cheddar cheeses was found to increase over the 120 day period studied.

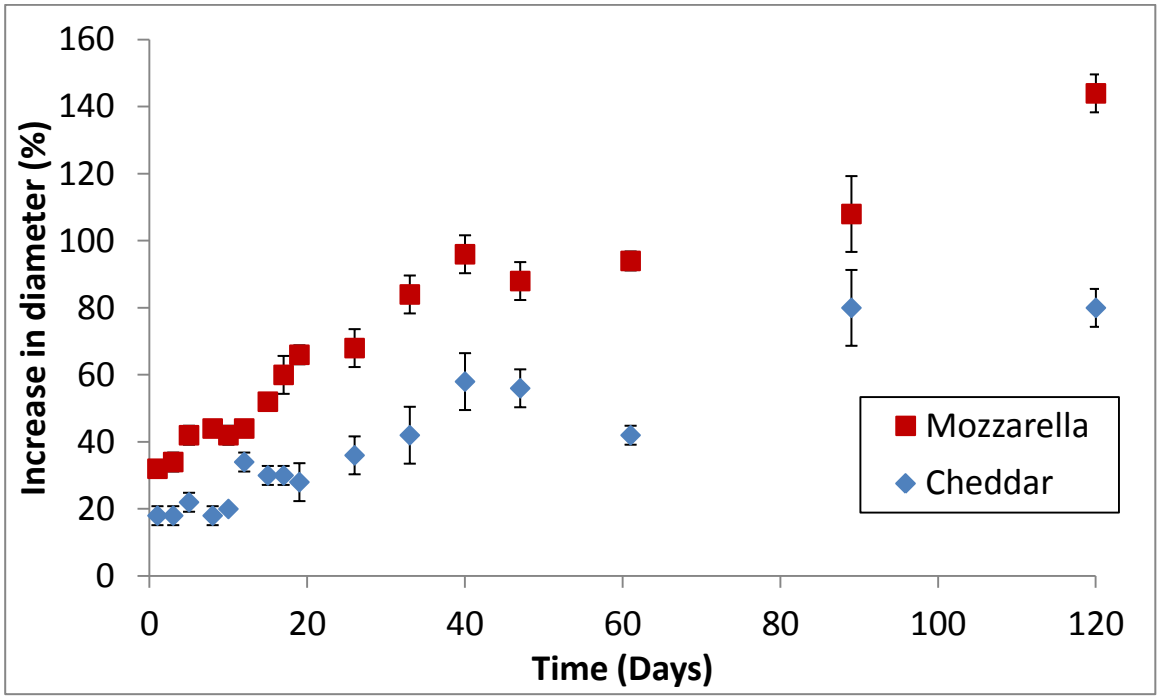


Figure 6.11: Change in the meltability of the samples over a 120 day period

The meltability of both Mozzarella and Cheddar cheese was found to increase over the 120 day storage trial, as shown in Figure 6.11. As expected the meltability of Mozzarella was found to be significantly higher than that of Cheddar on each corresponding day. This is due to the fibrous structure of Mozzarella enhancing flow, with protein fibres able to move past each other (Paulson et al., 1998). The increased melt and decreased hardness mirror the breakdown in protein observed in the urea PAGE gels. This correlation is likely causal and related to the proteolytic breakdown of casein producing a weaker gel structure (Karami et al., 2009; Tunick, Malin, et al., 1993) resulting in a softer cheese that can flow more readily when heated.

6.3.8 Structural differences during manufacturing

Mozzarella was identified initially as having a greater quantity of free water present in its structure in comparison with Cheddar. Apart from a higher moisture content and higher moisture-to-protein ratio, one of the key differences between the two cheeses is the

stretching step that Mozzarella undergoes. This thermo-mechanical treatment involves the heating and shearing of the curd (Kindstedt et al., 2004). In order to identify when, during this processing, the free water becomes evident within the structure of Mozzarella, a pilot plant trial was conducted. This allowed samples of Mozzarella to be taken during processing so that microscopy could be carried out on the structure. The images of the curd, heated curd and stretched curd are shown in Figure 6.12 below.

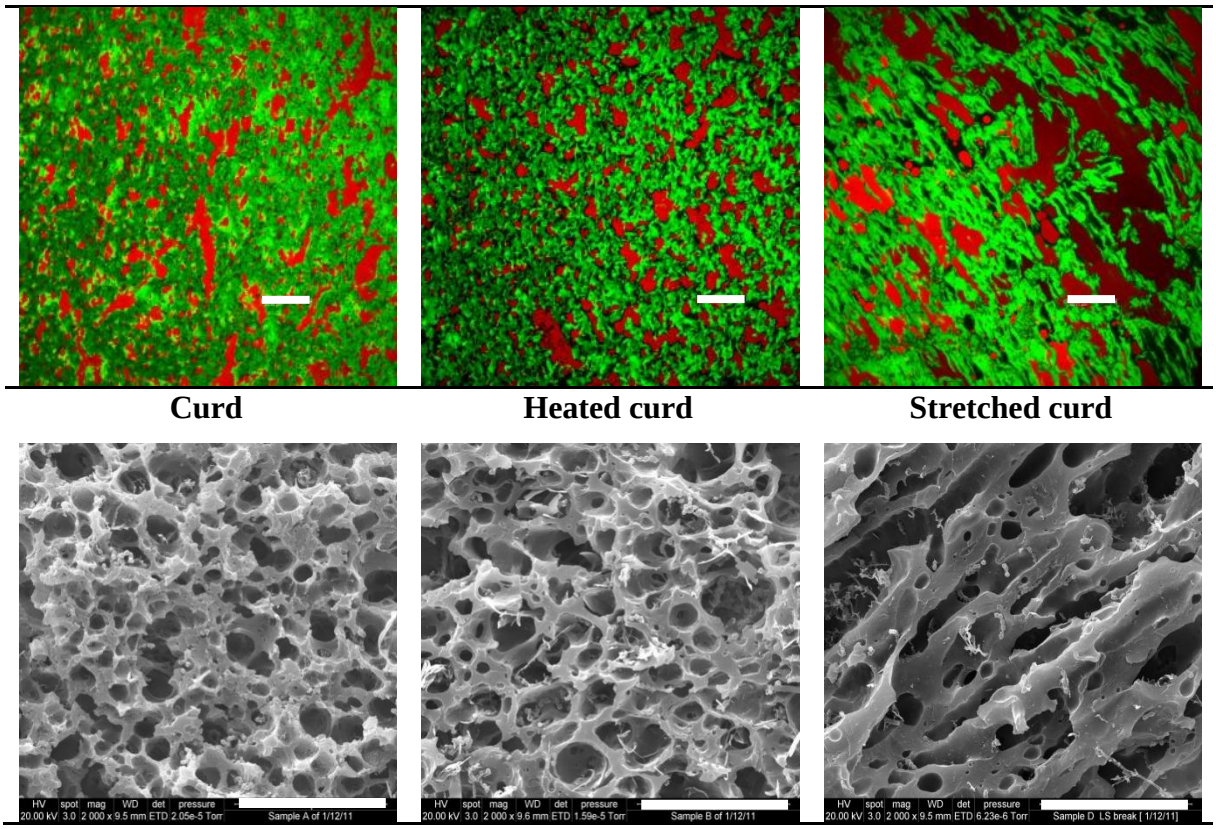


Figure 6.12: Confocal using a 40x objective (Scale bar = 50 µm) and SEM (scale bar = 30 µm) micrographs during processing.

The confocal images identified the presence of free water when the Mozzarella curd was heated in the cooker stretcher (as indicated by the black regions). This is coupled with an increase in the pore size observed in the SEM images when the curd is heated. This indicates that the free water present in the structure of the Mozzarella is caused by the additional heat treatment step during the stretching of the curd. This is potentially driven

by the strengthening of the hydrophobic interactions during the heating of the curd (Bryant & McClements, 1998) which leads to the protein matrix contracting and forcing entrapped water out into a free state. The curd reaches temperatures between 50-60°C in the cooker stretcher which is in the temperature range where hydrophobic interactions are at their strongest (Nemethy & Scheraga, 1962).The stretching of the curd results in the redistribution of the free water within the curd structure so that it occupies the channels between the protein fibres (Oberg et al., 1993). When the cheese cools, the hydrophobic interactions weaken allowing the water to migrate back to within the protein (McMahon & Oberg, 2011). As the cheese is stored at low temperatures, the rate of migration of the water into the protein is slow and occurs over a number of days. The migration of the water from the channels, where it surrounds the fat, into the protein is energetically favourable.

6.3.9 General Discussion

Significant changes were identified in the structure of both Mozzarella and Cheddar cheese over the 120 day storage trial. These changes in structure influenced the functional properties of both cheeses, with decreases in hardness and increases in meltability observed. One of the key drivers in the structural changes occurring in both cheeses was proteolysis. As discussed earlier, the proteolysis in both cheeses arises as a consequence of the hydrolysis of casein by the residual rennet and plasmin. As the protein matrix weakens during proteolysis (Karami et al., 2009; Tunick, Malin, et al., 1993), the fat is able to cluster together to form large pools, as observed in both the confocal and SEM images. This change in fat structure in conjunction with the weakening of the gel structure results in a reduction in the hardness of the cheeses during maturation. The weakening of the casein matrix due to intact casein being broken down into fragments (Coker et al., 2005) will result in a reduction in the structural rigidity of the cheese enabling the cheeses to flow to a greater extent as the cheese matures. This weakening of the protein matrix due

to proteolysis (Tunick, Malin, et al., 1993) will also impact on the hardness of the cheeses. The change in fat cluster size also may influence the hardness of the cheese, as large fat pockets are able to deform to a greater extent than smaller pockets resulting in a softer cheese when compressed (Rudan et al., 1998).

Mozzarella was consistently found to melt to a greater extent than Cheddar over the course of the maturation trial. This is likely to be due to the fibrous structure enhancing flow (Paulson et al., 1998), Mozzarella's higher moisture-to-protein ratio and the lower calcium-to-protein ratio than Cheddar. A lower calcium to protein ratio reduces the number of calcium mediated protein-to-protein interactions (Metzger, Barbano, Kindstedt, et al., 2001). This in turn leads to a weaker gel structure in the Mozzarella in comparison to the Cheddar cheese, which can flow more readily when heated. As proteolysis occurs the protein structure of both cheeses weakens (Tunick, Malin, et al., 1993) allowing the cheeses to flow more readily when heated.

The water migration in Mozzarella, as discussed in the previous chapter, is one of the main structural changes occurring during the first few weeks of storage. One mechanism that has been linked to this water migration, as stated in Section 5.3.7, is the solubilisation of calcium during storage (McMahon & Oberg, 2011). This phenomenon of an increasing proportion of soluble calcium in cheese is not limited to Mozzarella as it has been reported in a number of other cheeses including Cheddar (Hassan et al., 2004) and Colby (Lee et al., 2010). The solubilisation of calcium has also been linked to the softening of cheese during maturation (O'Mahony, Lucey, & McSweeney, 2005). This increase in soluble calcium occurs despite the pH of these cheeses rising initially following manufacture. It is possible that this change in calcium is due to water migration, as the water in these cheeses becomes more associated with the casein in the system. As water migrates into the protein matrix following heating, the CCP nanoclusters may find themselves in 'excess' water for the localised ionic environment, changing the equilibrium that exists between the soluble and insoluble calcium. This possible relationship between

water migration and calcium equilibrium will be explored in Chapter **Error! Reference source not found.**. NMR suggests that as a cheese matures the distribution of relaxation times for casein associated water decreases, indicating that the water has become associated with the casein to a greater extent. Proteolysis results in the liberation of carboxyl and amino groups due to the cleaving of casein molecules which are able to bind water, reducing the water activity of cheese (Fox & McSweeney, 1996). It is possible that this water migration modifies the solubility constant of calcium resulting in a progressive solubilisation of insoluble calcium during storage.

Other possible causes for the measured increase in soluble calcium in these studies (Hassan et al., 2004; Lee et al., 2010; Lucey et al., 2005; O'Mahony et al., 2005) include the release of small phosphopeptides during proteolysis that have calcium bound to them, influencing the measured level of soluble calcium. However, since the soluble calcium was not assessed in this study, judgement based on its effect cannot be made. In hindsight the level of soluble calcium should have been monitored over the course of this trial. It would therefore be advantageous to monitor it in future work as done in Chapter **Error! Reference source not found.**.

The stretching process that Mozzarella curd is subjected to during processing has a large effect on the cheese, impacting on both the structure and structural changes during storage (Kindstedt, 1993b). The microscopy study of this process indicated that the heating of the curd may be responsible for the free water present in the structure of the cheese. This was based on free water being visible in the confocal images and corresponding SEM images indicating an increase in the size of pores within the structure of the cheese. The presence of free water during heating is likely to be due to hydrophobic interactions within the protein being at their maximum strength at temperatures in the region of 50 to 60°C (Nemethy & Scheraga, 1962). The effect of heating on the release of free water within cheese will be explored using additional techniques in Chapters 8, 9 and **Error! Reference source not found.**. This free water was observed to decrease during the

storage of the cheese. This is likely to be due to these hydrophobic interactions weakening at the temperature of storage (McMahon & Oberg, 2011), allowing the water to migrate back to within the protein. The heating of the curd is not only responsible for the presence of free water within the cheese structure it also influences the proteolytic breakdown of the casein. This is due to the increasing temperatures during processing leading to a greater degree of inactivation of chymosin (Lawrence et al., 1987), plasmin plasminogen activator inhibitor and the plasmin inhibitor (Ismail & Nielsen, 2010). However, in the commercial Mozzarella study, the temperature reached by the curd during processing did not result in proteolysis being dominated by plasmin, as the breakdown of α_{s1}-casein was preferential to β-casein.

The stretching of Mozzarella curd is not only important due to the development of the cheese's fibrous texture (Kindstedt, 1993a) but the heating that occurs as part of the process also governs free water (Kindstedt et al., 2004) and influences subsequent proteolysis (Lawrence et al., 1987).

6.4 Conclusion

The structure of both Mozzarella and Cheddar cheese undergo significant structural changes following maturation. Mozzarella differs from Cheddar as it has a greater quantity of free water present in its structure immediately following manufacture. Previous research has shown that the stretching process gives Mozzarella cheese its fibrous texture and also produces the free water present in the structure. This research adds to the current knowledge by taking an in-depth look at the changes in the structure of Mozzarella during the stretching process. This exposed the heating of the curd in the cooker stretcher as the driver primarily responsible for the release of free water into the cheese structure. The subsequent mechanical stretching of the curd acts to orient the protein fibres and distributes the free water into the newly created fat channels. The

absorption of the free water into the protein matrix over the first few weeks following manufacture has significant impact on the functional properties measured (melt and hardness) of the cheese along with changes in fat distribution. Both cheeses exhibited proteolytic breakdown over the maturation period which strongly influenced the structural changes occurring. The changes that were observed in the structure of both cheeses significantly impacted their functional properties.

The majority of low moisture part skim Mozzarella is consumed in a molten state, primarily due to its use as a pizza topping. Therefore an investigation into the effect of the structural changes identified during storage in this study on the cheese during heating needs to be carried out.

7 An investigation into the effect of maturation on the dielectric and rheological properties of Mozzarella cheese during end-use heating

7.1 Introduction

Low moisture part skim Mozzarella is one of the most consumed cheeses in the world (Francolino et al., 2010). This is primarily due to its use as a pizza topping (Kindstedt, 1993b). In this application Mozzarella is melted during the pizza baking process. Therefore, the majority of Mozzarella is consumed when melted (Bertola et al., 1996a).

The structure of Mozzarella differs from most other cheeses (Kindstedt & Guo, 1997b), which is linked to its unique behaviour when melted. Primarily the structure of Mozzarella is governed by the thermo-mechanical processing step; whereby the curd is stretched by augers, aligning the amorphous paracasein matrix into roughly parallel channels interrupted by fat and serum (Kindstedt, 2007; McMahon & Oberg, 2011). This heterogeneous quasi-laminar structure is instrumental in a number of the key functional properties of Mozzarella (Kindstedt, 2007), including those related to the melted cheese.

During end-use heating a number of changes occur within the structure of Mozzarella. As the temperature of cheese is increased, the fat present within the structure will progressively transition into a liquid state until it is all liquid at approximately 40°C (Tunick, 1994). The heating also results in the number and strength of the bonds/interactions within the cheese matrix decreasing (Guinee, Auty, & Mullins, 1999; Horne, Banks, Leaver, & Law, 1994; Taneya, Izutsu, & Sone, 1979). Chief amongst these are casein to casein interactions which play a pivotal role in the melting properties of cheese (Lucey, Johnson, & Horne, 2003; Park et al., 1984). Other interactions increase in strength during heating including hydrophobic interactions and electrostatic repulsions (Bryant & McClements, 1998). In the previous chapter the strengthening hydrophobic

interactions at the temperature of stretching was identified as one of the potential drivers for the free water present within the cheese structure following manufacture.

Although Mozzarella is generally described as an unripened cheese, it does undergo significant structural rearrangement after it is manufactured (Kindstedt, 1993b). These structural changes are linked to the functional properties of the cheese (Joshi, Muthukumarappan, et al., 2004a). The previous chapter identified significant changes in the structure of Mozzarella following manufacture. The structural changes included water migration and proteolysis. These observed changes have a direct effect on how the cheese behaves during end-use heating.

The objectives of this chapter were to:

- Assess the changes occurring in the rheology and component mobility of Mozzarella during heating and the effect that storage has on these changes.
- Evaluate the use of dielectric spectroscopy to study the changes occurring with temperature.

7.2 Materials and Methods

7.2.1 Material

A commercial sample of low moisture part skim (LMPS) Mozzarella was obtained immediately after a 24 hour rapid cool process following manufacture (Fonterra Cooperative Group). The 10 kg block was cut into 20 roughly 500 g blocks, individually vacuumed packed and stored at 4°C. On each day of analysis a block was removed from the refrigerator at random and samples were cut in the appropriate size for rheology & dielectric spectroscopy.

The samples of cheese used for this study were taken from the same block of Mozzarella as used in the previous chapter.

7.2.2 Rheology

Small amplitude oscillatory rheology was conducted on two MCR301 controlled stress and strain rheometers (Anton-Paar, Germany). The first was used from day 1 to day 11 while the second rheometer, a replacement to the first which malfunctioned, was from day 26 onwards.

Disks of cheese 2.2 mm in height (Lucey et al., 2005) and 25 mm in diameter were cut. The disks were wrapped in plastic wrap and placed in a temperature controlled lab set at 20°C for a period of 1 hour. The cheese samples were placed on a serrated plate and had a 25 mm serrated top plate lowered onto it until a normal force of 1 N was applied. Serrated plates were used to prevent slippage from occurring (Rosenberg et al., 1995; Yun, Hsieh, Barbano, & Rohn, 1994). In order to prevent the cheese samples drying out over the course of the temperature sweep a moist tissue was placed around the outside of the bottom plate and a cover placed over the sample.

An amplitude sweep was conducted to identify the linear viscoelastic region (Mounsey & O'Riordan, 2006; Sutheerawattananonda & Bastian, 2007) for the Mozzarella. This was done at 20°C and 1 Hz at 40 points between 0.01 and 100% amplitude.

A temperature sweep was carried out on the Mozzarella samples during the maturation period. The cheese was heated from 20 to 90°C at a rate of 4°C a minute with 120 points collected over the sweep. The samples were subjected to an angular frequency of 10 s^{-1} at a strain rate of 0.03%. The temperature sweeps were replicated at least three times.
Data was assessed using RheoPlus software (Anton-Parr, Germany) and used to gain information about the storage modulus (G'), the loss modulus (G") and the complex viscosity (η*).

7.2.3 Dielectric Spectroscopy

Temperature sweeps were carried out using dielectric spectroscopy as stated in Section 4.2.5. Initial readings were taken at 4°C immediately following the removal of the sample from the refrigerator. The water bath attached to the heating jacket was then set to 20°C and left for a period of 45 minutes before measurements were carried out. The temperature was incrementally increased by 10°C every half hour until a temperature of 90°C was obtained. The measurements were conducted in duplicate on each day of the trial.

7.3 Results and Discussion

The previous chapter discussed the changes occurring in the structure and functionality of Mozzarella and Cheddar cheese over a 120 day maturation period. As Mozzarella is predominantly consumed in a molten state (Bertola et al., 1996a), its behaviour during heating is of importance. This aspect is discussed below.

7.3.1 Rheological Temperature Sweep

The storage (G') and loss (G") moduli were both found to decrease over the course of the temperature sweep. G' is related to the elastic or solid like properties of a material whereas G" is related to the viscous or liquid like properties (Biswas, Muthukumarappan, & Metzger, 2008). The rate of decrease was found to be greatest in the storage modulus, leading to the two parameters intersecting during the course of the temperature sweeps. The loss tangent (tanδ) is a parameter that indicates the relationship between the storage & loss moduli (Rao, 2007a) and is calculated using Equation 7-1 below.

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

Equation 7-1: Calculation of the loss tangent

It is often used to assess the transition of a material from a predominantly elastic state to a viscous state (Tung & Dynes, 2003).

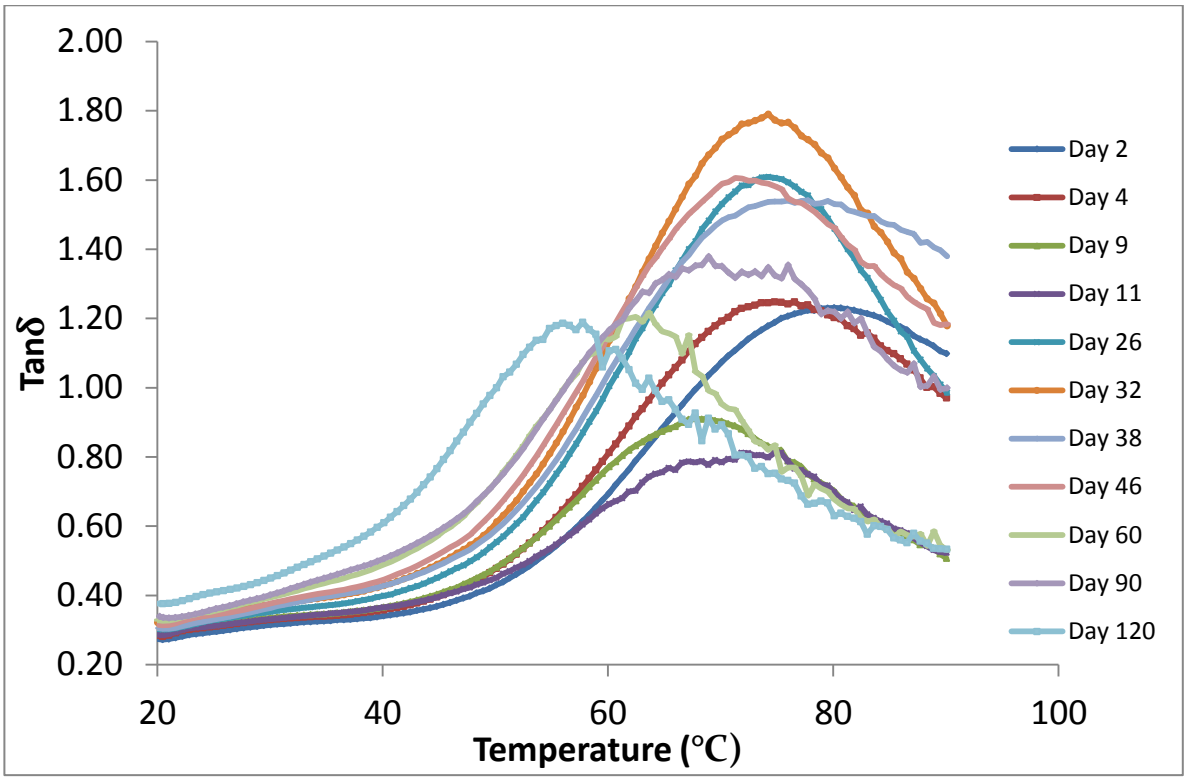


Figure 7.1: Temperature sweep monitoring the change in tanδ over the 120 day storage trial

Figure 7.1 indicates changes in the loss tangent over the temperature sweep at various days during the storage trial. All of the samples were observed to have an increasing tanδ with temperature up until the sample reaches a maximum between 50 and 80°C. Following this the tanδ decreased until the end of the temperature sweep. The shape of the curve produced from the temperature sweep was found to change over the course of the maturation period. The trends exhibited in Figure 7.1 were consistent with observations made by Govindasamy-Lucey, *et al* (2005b) Arimi, *et al* (2010) and Lucey, *et al* (2003).

The curves indicate that the loss tangent initially increased with temperature as the loss modulus became more dominant than the storage modulus. This is due to the viscous properties of the cheese becoming more dominant than the elastic properties over this temperature range. Factors that affect the progression of cheese from a semi-solid to behaving in a liquid-like manner, resulting in an increase in tanδ, include the transition of fat into a liquid state (Lucey et al., 2003), strengthening hydrophobic interactions and electrostatic repulsions (Bryant & McClements, 1998) resulting in flow. The melting of the fat, which occurs up until 40 °C (Tunick, 2010), within the cheese structure will cause a decrease in the elastic and increase in the viscous properties of the cheese.

Additionally, as the temperature increases, the hydrophobic interactions will increase in strength (Bryant & McClements, 1998) until they are at their maximum strength between 50 and 60°C (Nemethy & Scheraga, 1962). As a consequence of this, the proximity within the hydrophobic regions of the protein matrix will increase, forcing water previously entrapped within the matrix into a free state. This contraction of the protein fibres which, lubricated by the molten fat and free water, contributes to the flow of the cheese (Tunick, Malin, et al., 1993).

The solubilisation of calcium within the casein matrix over the course of the maturation trial is reported to have a significant effect on age related textural and rheological properties (Hassan et al., 2004; Lucey et al., 2005; O'Mahony et al., 2005). A change in the state of calcium within the cheese will result in a decrease in the number of calcium-mediated casein-to-casein interactions (Joshi, Muthukumarappan, et al., 2004a). This will cause the protein gel structure to weaken, resulting in a softening of the cheese (O'Mahony et al., 2005). This weakening of the protein matrix will influence the storage and loss moduli, particularly when the fat is in a molten state and the protein matrix dominates the rheological properties of the system.

The fat within the cheese may also be involved in changes occurring at high temperatures. This is due to the tendency of Mozzarella to express free oil during melting (Kindstedt & Rippe, 1990; Tunick, 1994). This exclusion of fat from the cheese system will cause the elastic properties of the remaining system to become more pronounced due to the reduction in liquid fat. This is possibly the cause for the decrease in the loss tangent at high temperatures.

The magnitude of the maximum loss tangent was found to initially increase from the start of the maturation trial up until day 32 (with the exception of days 8 & 11). Following day 32 the maximum loss tangent was found to progressively decrease. This trend is consistent with observations made by Govindasamy-Lucey, Jaeggi, Johnson, Wang, and Lucey (2005a) in a number of their pizza cheese samples. The age-related increase in the loss tangent has been attributed to changes in the insoluble calcium content and proteolysis (Lucey et al., 2003). The decrease in the maximum loss tangent after day 32 was due to an inflection in both the storage and loss moduli. An example of the difference in the inflection point of the dielectric constant is displayed in Figure 7.2 below.

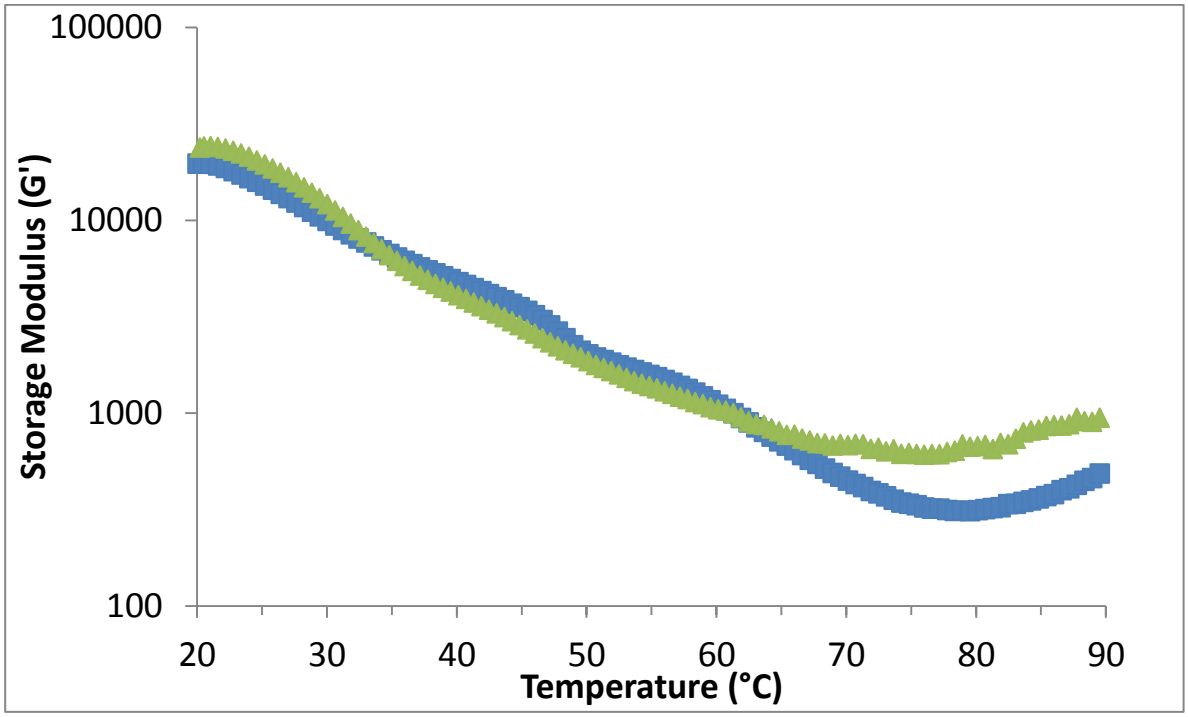


Figure 7.2: Example of the change in the storage modulus (G') with temperature during the storage trial (■ Day 32 and ▲ Day 90).

Figure 7.2 indicates that the inflection point in the storage modulus curve above 60°C occurred at a lower temperature in the more mature Mozzarella sample. This trend is consistent with the rest of the data collected past day 32 of the storage trial, with an inflection in both the storage and loss moduli occurring at a decreasing temperature over the trial. The storage modulus increased at a faster rate than the loss modulus leading to the overall decreasing trend in the loss tangent. The inflection in the two parameters occurred at a progressively lower temperature over the storage trial. The inflection in both the storage and loss moduli may be the result of fat and moisture loss during the melting of the cheese samples. As the cheese matures it melts at a progressively lower temperature, this enables the losses to occur at a lower temperature.

The temperature at which the storage and loss moduli crossed over ($\tan\delta=1$) was observed to occur at a progressively lower temperature over the course of the maturation trial. This cross-over temperature has been used to indicate the point at which the cheese

begins to flow/melt (Prow & Metzger, 2005; Sutheerawattananonda & Bastian, 2007; Zhou & Mulvaney, 1998). This indicates that over the storage trial, the temperature at which the Mozzarella began to flow decreased. During storage Mozzarella undergoes solubilisation of calcium (McMahon & Oberg, 2011) and proteolytic breakdown resulting in a weakening of the casein matrix (Costabel et al., 2007; Farkye et al., 1991). As the matrix weakens with time, the cheese flows more easily when heated.

The curves created as a result of the temperature sweeps on Day 9 & 11 were found to differ from the others obtained over the 120 day trial, as depicted in Figure 7.1. The loss tangent profile differed for these two days as they were observed not to exceed a tanδ value of 1, which is commonly used as an indicator of the point at which cheese melts. This indicates that the storage modulus exceeded the loss modulus during the entire temperature sweep. However, the shape of the loss tangent curve is consistent with the other days analysed. This suggests that although following a similar trend to the other days, the loss modulus does not exceed the storage modulus. These results imply the cheese sample stays in a state where it exhibits more solid-like behaviour than liquid-like behaviour and does not therefore melt. However, when observing the sample at elevated temperatures it was observed to flow. Alternatively, a potential cause of this difference is as a result of a malfunctioning rheometer. The rheometer initially used at the beginning of the trial malfunctioned and ceased working after the temperature sweep conducted on day 11. It is possible that the rheometer was experiencing problems prior to stopping.

In the previous chapter the melting properties of the cheeses were assessed using a simple melt test. This measured the spread of the sample after a fixed period of heating. This measurement technique is routinely carried out in industry to assess the meltability of Mozzarella samples as it is a relatively simple test that can be carried out on multiple samples at once. By comparing the results from the modified Schreiber melt test and the temperature at which tanδ=1 for each sample point of the maturation trial, a relationship between the temperature of flow and spread can be identified.

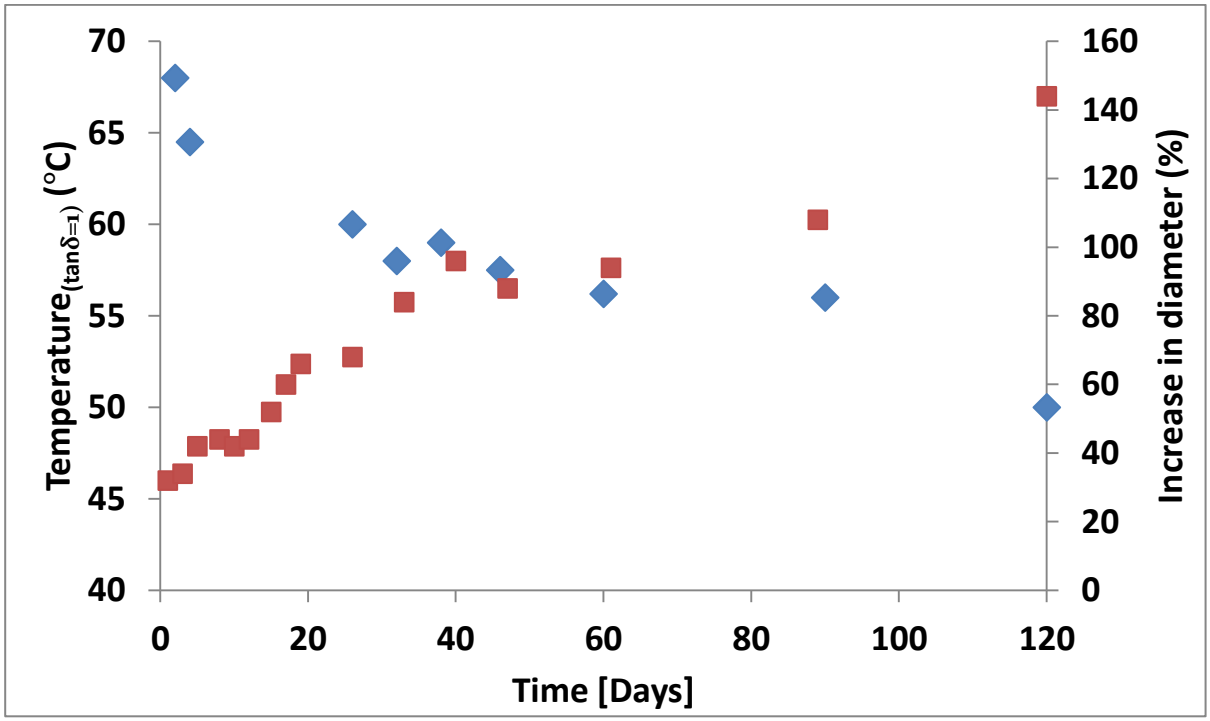


Figure 7.3: Comparison of tanδ=1 and the increase in diameter during storage (◆ temperature at which tanδ=1, ■ Schreiber melt test results)

The temperature that the storage and loss moduli were found to be equal (tanδ=1) over the course of the maturation trial was inversely proportional to the increase in diameter in the modified Schreiber melt test, as shown in Figure 7.3. This relationship can be explained in the following way: with age the cheese begins to flow at a lower temperature, thus the sample has a greater time to flow during testing. Another factor that could influence the degree of spread is a lower viscosity at high temperatures as the Mozzarella samples matured. In order to assess the trends between the two variables the results from each test were plotted against each other.

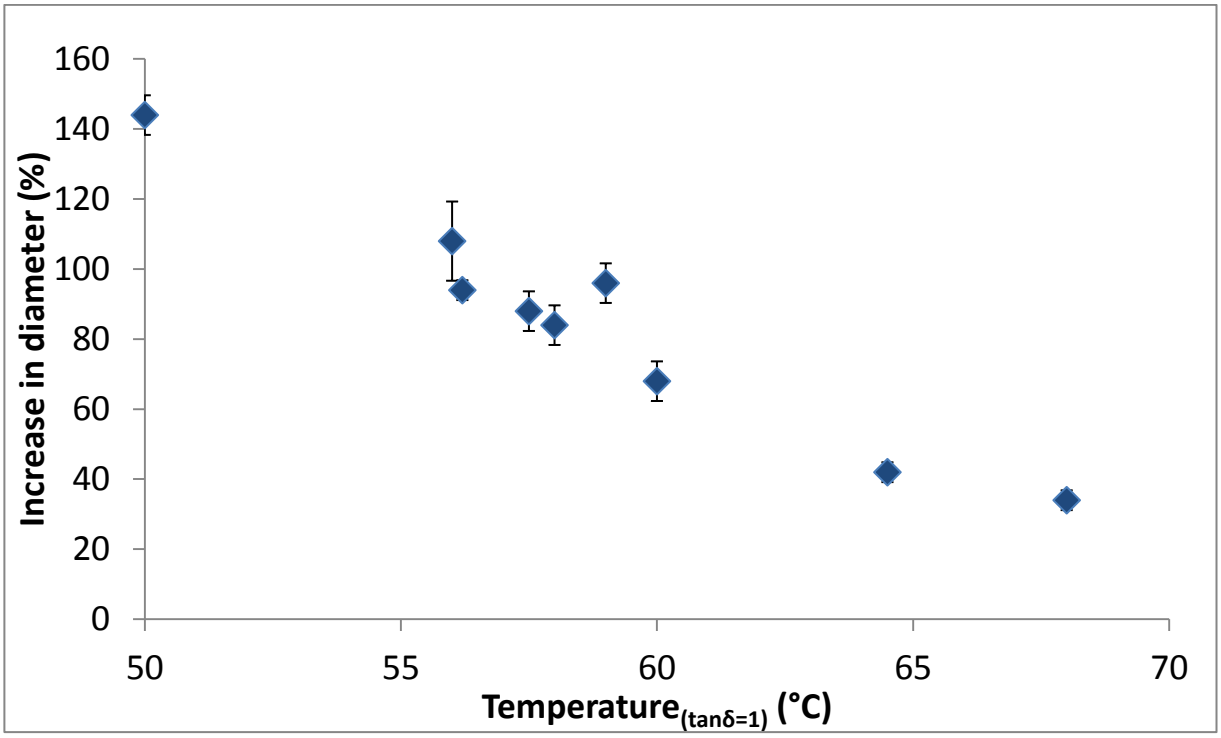


Figure 7.4: Relationship between the Schreiber melt test and crossover of the viscous and storage moduli

A linear relationship (R^2=0.945) was identified between the results obtained from the modified Schreiber melt test and the temperature at which G' and G" crossed over (tanδ=1). Figure 7.4 indicates that as the temperature that tanδ=1 progressively reduces for a Mozzarella sample, there is a corresponding increase in the spread in the modified Schreiber melt test.

The complex viscosity, η* (Pa.s), is the resistance to flow while under oscillatory shear conditions (Tunick & Nolan, 1992). By substituting the complex viscosity into the Arrhenius equation we can assess the change in the resistance to flow with temperature as well as the activation energy of flow, E_a ($Jmol^{-1}$) (Tunick, 2010).

$$\eta^* = A\, e^{\frac{-E_a}{RT}}$$

Equation 7-2: Arrhenius equation using complex viscosity

Where T is the absolute temperature of the sample (K), R is the gas constant (8.314 $Jmol^{-1}K^{-1}$) and A is a pre-exponential factor (Pa.s).

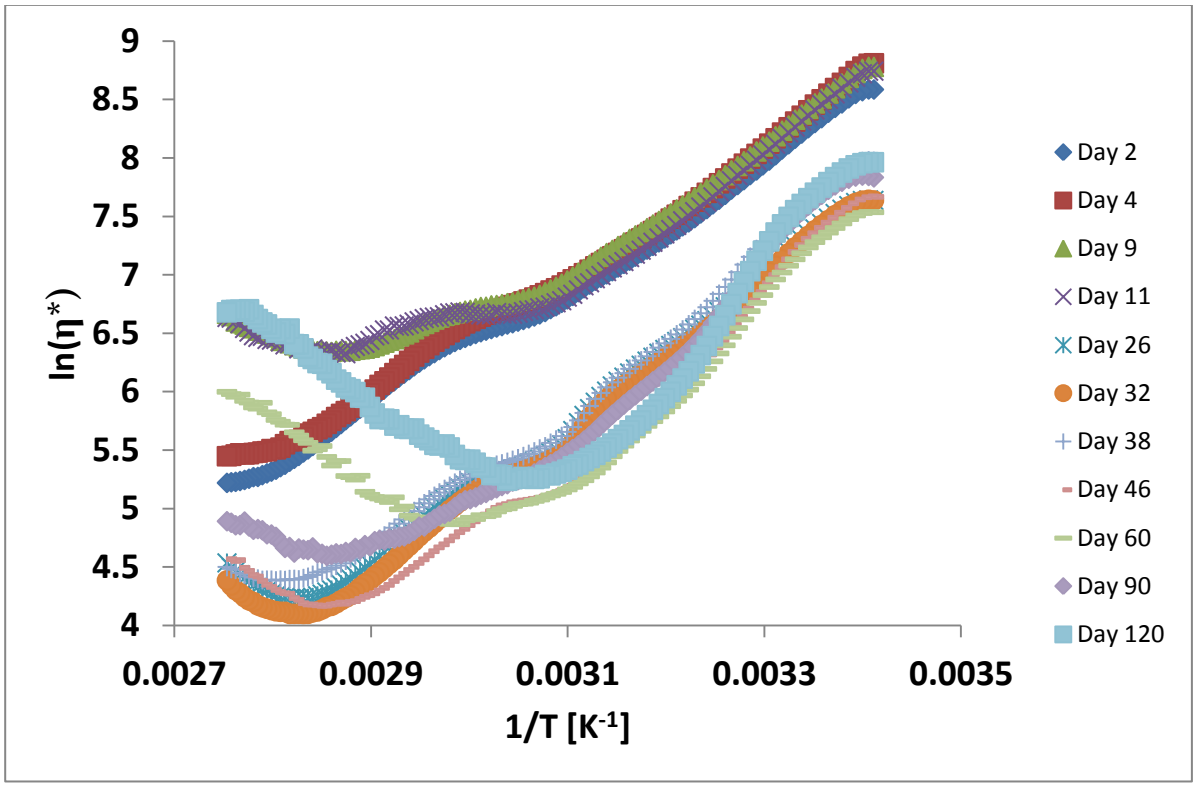


Figure 7.5: Arrhenius plot of the change in complex viscosity with temperature over the storage trial

A gap was identified between the data obtained on days 2 to 11 and 26 to 120 in Figure 7.5. This gap coincided with a malfunction with the original rheometer utilized for this study. Due to this malfunction a period existed between day 11 and 26 where no rheological data could be obtained. From day 26 to day 120 a different rheometer of the same make and model was utilized for the study. The data obtained from the original rheometer was found to be higher than the data from the second rheometer. Based on this, no absolute values were assessed as a means of comparing the temperature sweeps

conducted on the various days studied. However, the trends observed in the data were consistent between the data obtained on the first and second rheometers.

In order to inspect the changes in the Arrhenius plot the data was re-plotted to show the temperature in degrees Celsius, Figure 7.6 below. This was done so that changes in viscosity could be related back to temperatures more accurately.

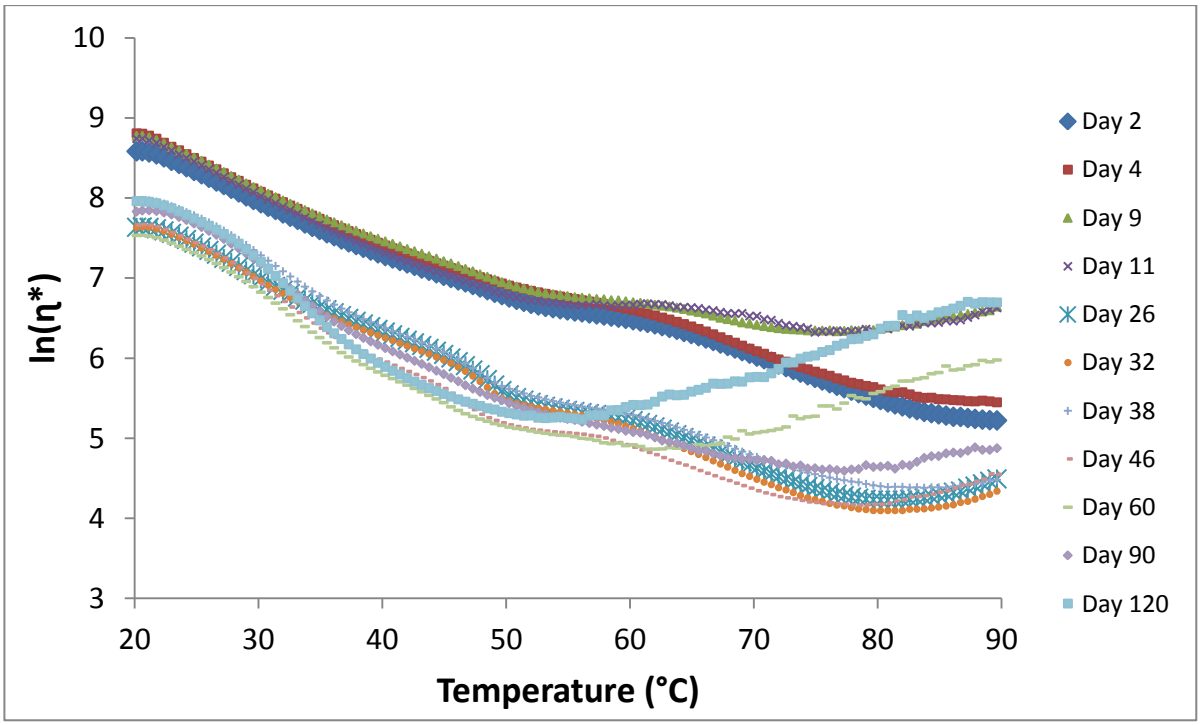


Figure 7.6: Arrhenius plot displayed as a function of temperature in degrees Celsius

All samples were observed to decrease in viscosity as the temperature increased. The trend in all of the data sets showed an initial curvature followed by a linear region and followed by an inflection at high temperatures. The curvature at the beginning and end of the temperature sweep were found to be more pronounced as the maturation trial progressed.

The initial curvature was observed at temperatures from 20°C to 35°C. This is likely to be caused by the transition of fat within the cheese structure to a liquid state over this

temperature range, consistent with previous rheological studies of cheese (Muliawan & Hatzikiriakos, 2007; Reparet & Noel, 2003). With the increasing temperature the fat globules become increasingly more deformable as the quantity of liquid fat increases (Kindstedt, 2007), resulting in a decrease in the viscosity of the cheese. This increase in slope was observed to become more prominent as the Mozzarella aged. As the cheese ages the protein matrix weakens due to proteolysis (Karami et al., 2009; Tunick, Malin, et al., 1993) allowing the fat to form larger, more spherical clusters, as seen in the previous chapter.

Following this initial curvature, the complex viscosity continues to decrease in a roughly linear manner with increasing temperature until reaching approximately the temperature where the cheese began to flow, where the complex viscosity deviated from linearity, similar to the trends illustrated by Tunick (2010). This curvature was observed to occur at progressively lower temperatures over the maturation trial. This mirrors the trend observed in the progressive change in the temperature that $\tan\delta = 1$ over the same period. The increasing viscosity as part of the inflection in the plot is possibly the result of water and fat losses following the cheese beginning to flow. When the cheese begins to flow some of the fat and water within the channel structure of the cheese is no longer held in place by the casein matrix and escapes. The resulting viscosity could be the result of the protein becoming more dominant as it loses the fat filling the structure and becomes slightly dehydrated as it expresses water. In this way the distance between protein strands would decrease and thus facilitate increased protein-protein interactions and hence an increase in apparent viscosity. It is interesting to note that the fresh Mozzarella cheese in Section 6.3.6 was harder than when aged and this phenomenon was also attributed to protein hydration.

To further characterise the Arrhenius plot, a differential analysis was conducted. This was done to extend the current analysis by delving deeper into understanding fluctuations in the Arrhenius plots. This allowed the rate of change in the slope to be assessed against

temperature. This allowed a more accurate assessment of the slope as well as identifying the points of inflection more clearly. The slope of the Arrhenius plot is proportional to the activation energy of flow, E_a (Tunick, 2010). The assessment of E_a has been applied to liquid foods as it identifies the energy required to overcome the resistance to flow (Rao, 1977) and more recently to examine the flow associated with cheese melt (Tunick, 2010). In the work of Tunick (2010) the activation energy of flow (E_a) was used as a means of quantifying the flow of cheese.

As the gas constant (R) does not vary, the change in the slope of the Arrhenius plot is due to fluctuations in the activation energy of flow. In order to assess this change, the slope of the Arrhenius plot was assessed over the storage trial (Figure 7.7).

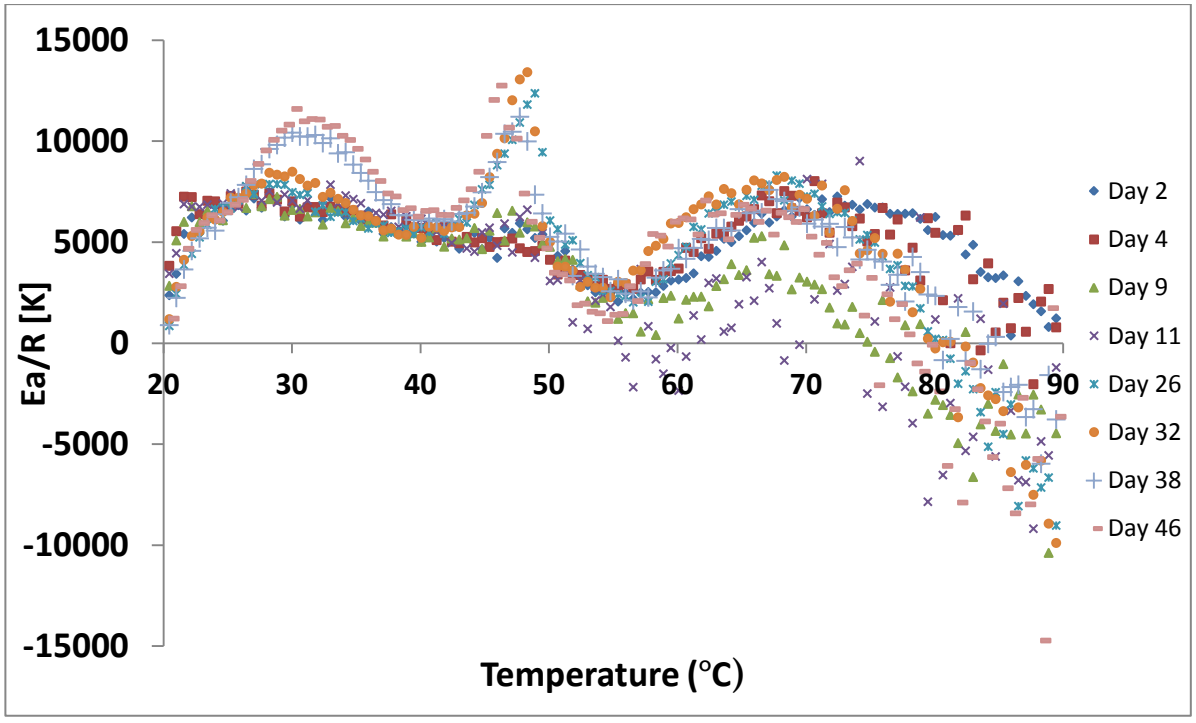


Figure 7.7: Change in the slope of the Arrhenius plot over the course of the temperature sweep

The fluctuations in Figure 7.7 are related to how the slope of the Arrhenius plot changes, with an increase indicating a greater rate of decrease in viscosity and a decrease indicating

a drop in the rate of decrease in viscosity. A peak in the data indicates where a particular event occurs resulting in a sharp decrease in viscosity. An initial increase in the slope was observed at the beginning of the temperature sweep. This is likely to be due to solid fat within the cheese structure transitioning into a liquid state. The change in the state of fat cause the cheese to become more liquid-like (Lucey et al., 2003), resulting in a decreased resistance to flow and a subsequent increase in the activation energy of flow. At approximately 30°C another increase in slope was observed. This was found to be more pronounced as the maturation trial progressed. A possible explanation for this increase is the melting of a polymorph of fat with a high melting point. This conforms with a storage trial of Emmental cheese that identified the formation of high melting point fat between 30-36°C during aging using a differential scanning calorimeter (Lopez, Briard-Bion, Camier, & Gassi, 2006). Milk fat is composed of a number of different melting point fat polymorphs, including α, β' and β polymorphs which melt by approximately 22, 30 and 35°C respectively (Walstra, van Vliet, & Kroek, 1995). The β polymorph is the most stable of these different fat crystals in milk fat (Lopez, Briard-Bion, Beaucher, & Ollivon, 2008). The fat may progressively transition into the more stable β polymorph of fat during the maturation process. As higher quantities of solid fat in cheese leads to a greater viscosity (Shukla, Bhaskar, Rizvi, & Mulvaney, 1994), having a greater amount of high melting point fat would result in a greater quantity of solid fat to exist at higher temperatures. This would result in a more pronounced drop in viscosity as the higher melting point fat melts. However, an argument against this theory is that the limited quantity of solid fat between 30 and 35°C in cheese may not have such a substantial effect on viscosity.

An additional change in slope was identified between 45 and 50°C for the samples. As with the previous increase in slope, this increase also increased in magnitude as the cheese matures up to day 46. However, beyond day 46 it diminishes substantially, as shown in Figure 7.8 below. At this temperature the fat within the system will be in a molten state (Tunick, 2010). This peak also occurs prior to the temperature at which the cheese was

identified as beginning to flow. This could be due to a structural reorganisation within the casein matrix at this temperature. As the temperature rises the strength of the hydrophobic interactions within the protein matrix will increase in strength (Bryant & McClements, 1998), which may lead to a progressive contraction of the network. A slight contraction may enable fat pockets to move within the channels and pool together into larger pockets. As the cheese matures, the solubilisation of calcium and proteolysis lead to the casein matrix becoming less rigid (O'Mahony et al., 2005). Therefore the fat within the channels may be able to move more freely and form into larger clusters during heating. However, further investigation is required to identify what is responsible for this peak.

The slope of the Arrhenius plot was observed to decrease prior to reaching 55°C indicating that the activation energy of flow decreased. One possible explanation for this is that as the temperature of the cheese increases the hydrophobic interactions within the casein matrix become stronger (Bryant & McClements, 1998). This increase in hydrophobic interactions may result in the casein matrix contracting slightly forcing water out of the matrix to surround the fat within the cheese structure. As a result of this, the casein matrix will become slightly tougher as is less hydrated.

This is followed by a final increase in the slope of the Arrhenius plot above 55°C. The start of this increase is consistent with the temperature at which the cheese begins to flow. As the cheese continues to melt the complex viscosity continues to decrease, indicated by the increase in slope up until approximately 70°C. After this point the slope of the Arrhenius plot decreased until the end of the temperature sweep. The decrease in slope could be a result of Mozzarella expressing free oil upon melting (Tunick, 1994) as well as potential moisture losses as the cheese begins to flow. The release of fat will result in the cheese structure becoming tougher causing a resulting increase in the resistance to flow.

At high temperatures (above 70°C) variability in the data became evident. This variability became progressively more pronounced as the maturation trial progressed, as can be

seen in Figure 7.8 below. The variability at these temperatures is likely to be a consequence of the proteolysis, whereby the casein matrix is broken down into fragments with time (Coker et al., 2005). As the matrix weakens, the cheese is able to flow more as the temperature increases as well as possibly resulting in a greater number of breakages of bonds as the sample is oscillated with temperature.

The data obtained for the temperature sweeps conducted on days 60, 90 & 120 were separated from the prior days studied and displayed on a separate chart, Figure 7.8 below. This was done due to high temperature variation resulting in poor data beyond 50°C.

The functionality window for the Mozzarella cheese studied is between 8 to 12 weeks following manufacture. This corresponds with the period of time between day 60 and 90 of the storage trial.

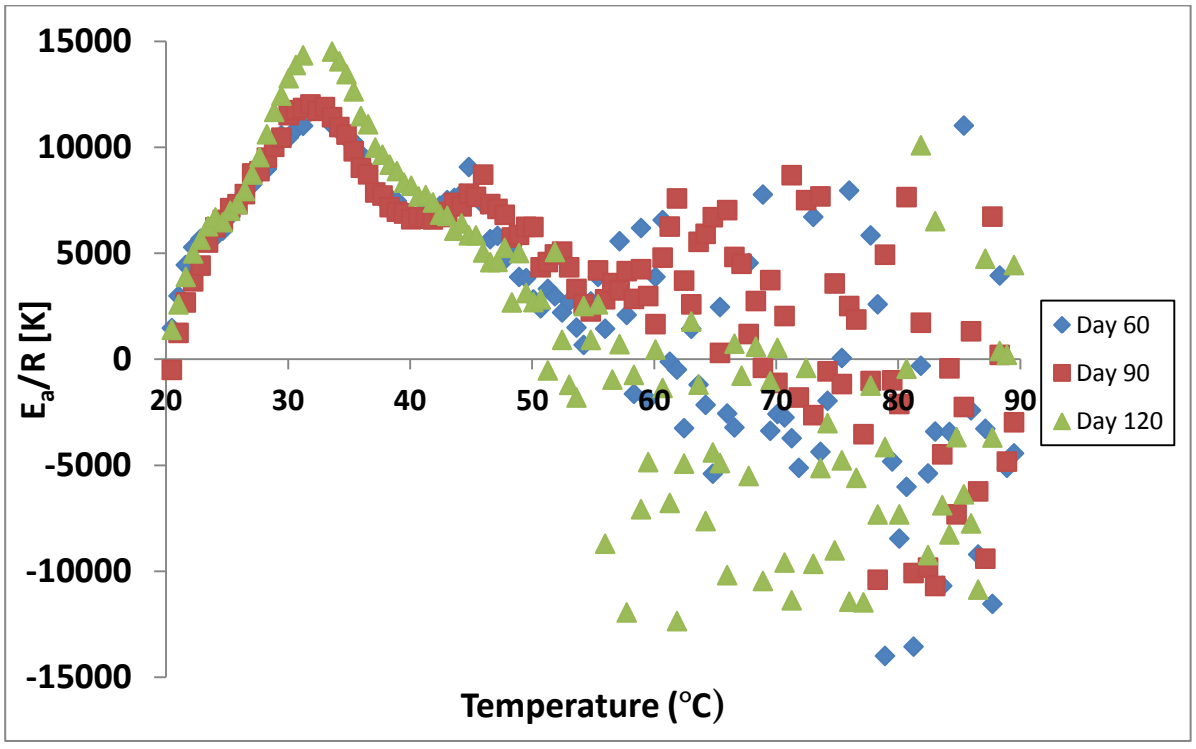


Figure 7.8: Change in the slope of the Arrhenius plot over the course of the temperature sweep on day 60, 90 & 120

The data obtained from day 60 to day 120 indicated a lot of variation in the slope of the Arrhenius plot above 50°C. This variation is consistent with the curvature in the Arrhenius plot (Figure 7.5) at high temperatures where the complex viscosity begins to increase. The variation in the data is likely to be a result of the proteolysis that the cheese has undergone by day 60 to day 120, as shown in Section 6.3.4. The proteolysis will cause a progressive weakening of the casein matrix as the protein is broken down with time (Coker et al., 2005). By day 60 the amount of intact α_{s1}-casein had reduced to 63.2% and β-casein to 54.8%, which by day 120 had reduced to levels of 48.4% and 37.0% respectively.

The peak identified between approximately 30 and 35°C was observed to continue to increase in size as the cheese matured. If, as mentioned earlier the polymorphic state of fat may be changing, with a greater quantity of fat transforming into a high melting point polymorphic form as the cheese continues to age.

The peak identified between 40 and 50°C, which increased in magnitude from day 1 to day 46, was found to diminish over the remainder of the trial. In order to identify what is responsible for this change in the complex viscosity over this temperature range further research is required. If, as suggested earlier, it is possibly due to the movement of fat within the channels of the cheese the decrease in the magnitude of the peak could be due to larger fat clusters developing with storage. The confocal and SEM images in the previous chapter indicated that as the cheese matures the fat formed larger, more spherical clusters within the casein matrix. As large clusters already exist in these samples, the movement of fat to form larger pockets during heating may be less pronounced. However, further work would need to be undertaken before any solid conclusions can be made regarding this peak including repeating the experiment using a number of different techniques to aid in the analysis of the cheese such as coupling the rheological measurements with differential scanning calorimentry and NMR.

7.3.2 Dielectric Temperature Sweep

Dielectric spectroscopy was used to monitor changes in component mobility over the 120 day trial from 4 to 90°C.

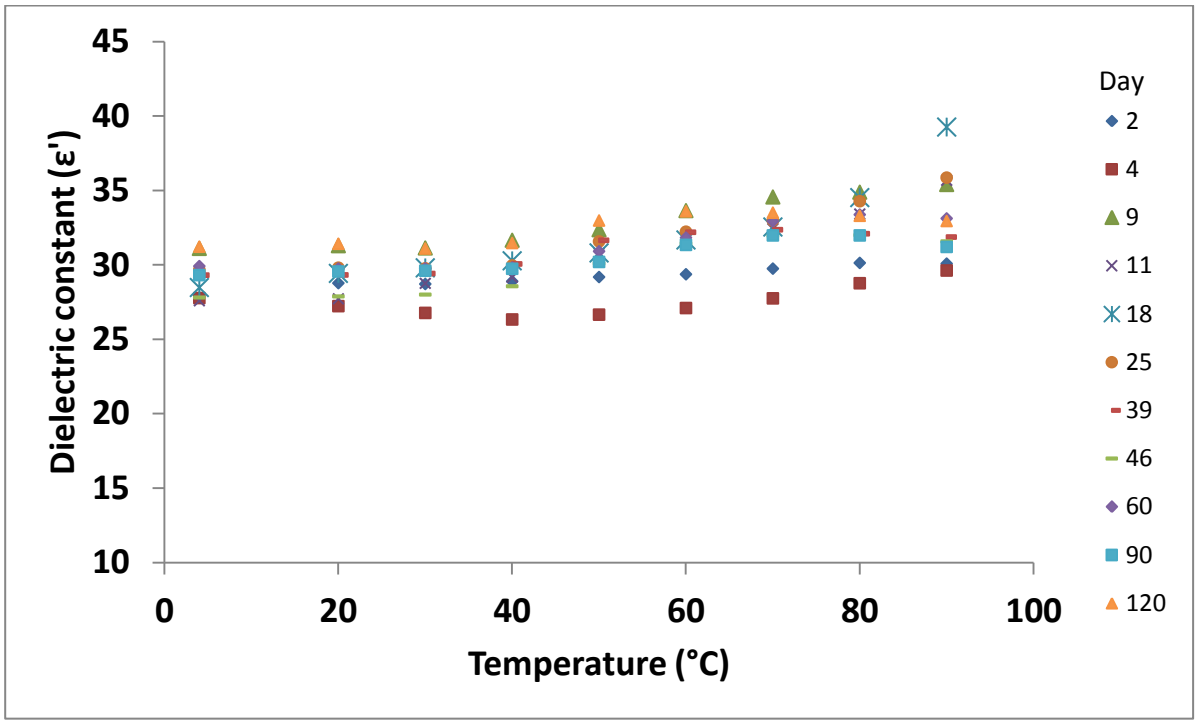


Figure 7.9: Dielectric constant versus temperature during the storage trial [1260 MHz].

The dielectric constant at 1260 MHz increased with temperature for all of the samples assessed over the duration of the maturation trial, as shown in Figure 7.9. Very little change was identified between 4 and 30°C on each day of the storage trial at the beginning of the temperature sweep. Above 30°C the dielectric constant was observed to increase in a linear manner until the sample reached 70°C where it deviated from linearity. Variation was exhibited on the different days of the storage trial at each temperature studied. This variation was smallest at the beginning of the temperature sweep (18% at 4°C) and increasing with temperature with 34% difference between the lowest and highest dielectric constant at 90°C. No ordered trends were identified between

the different days of the storage trial, suggesting the variation in the dielectric constant may be due to sample to sample variation rather than trends as a result of maturation. This was confirmed by a statistical analysis of the data which identified that there was a significant difference between the dielectric constant as a result of heating on each day of the trial, p=0.02, while no significant difference was identified between the different days at each temperature of the trial, p=0.50.

As discussed previously, the dielectric constant has been related to the moisture content in food systems (Berbert et al., 2001; Everard et al., 2006; Kudra et al., 1992) and water mobility (Tsoubeli et al., 1995). As dipolar rotation is typically the most significant mechanism at frequencies greater than 1 GHz (Datta et al., 2005; Ryynänen, 1995), the dielectric constant is displayed at a frequency of 1.26 GHz in Figure 7.9.

The increase in the dielectric constant with temperature suggests that the water molecules within the cheese structure are becoming more mobile. The previous chapter suggested that the heating of Mozzarella curd is the driver responsible for free water to be present within the structure of the cheese. As the temperature increases, an increase in the hydrophobic interactions within the casein matrix (Bryant & McClements, 1998) will likely result in the matrix progressively contracting forcing out a greater quantity of water that can exist in a free state. As free water is more mobile than the water molecules associated with the protein matrix (Altan et al., 2011), a progressive increase in water mobility could explain the increasing dielectric constant with temperature.

In order to better characterise the changes occurring over the storage trial in the dielectric constant, a plot was constructed to show how the dielectric constant changed with time at 20 and 90°C, Figure 7.10 below.

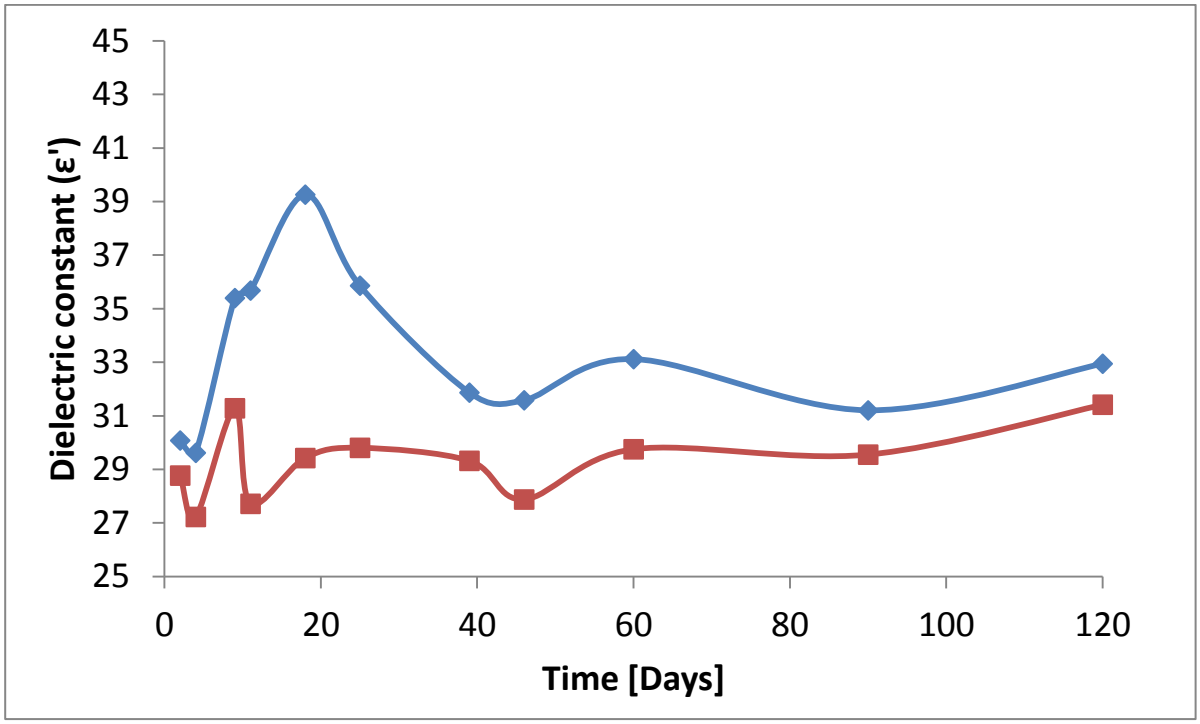


Figure 7.10: Change in the dielectric constant over the 120 day trial at ■ 20°C and ◆ 90°C [1240 MHz].

The dielectric constant at both 20 and 90°C was found to vary over the course of the 120 day storage trial. At 20°C the dielectric constant was observed to fluctuate between 27 and 31 over the course of the storage trial. The fluctuation in the data suggests, as mentioned previously, that sample to sample variation in composition is responsible for the variation. However, the fluctuations in the dielectric constant over the storage trial were not found to be significant at a 95% confidence interval, p=0.3.

The dielectric constant is influenced by the moisture content of a material (Berbert et al., 2001; Everard et al., 2006). Within a block of cheese the moisture content varies, which could influence the dielectric data. It was also found in Section 5.2.6 that the free water in Mozzarella influenced the dielectric constant. In Section 6.3.8 it was found using confocal microscopy that heating resulted in the presence of free water within the structure of Mozzarella curd. The variation in the dielectric constant at 90°C is possibly due to changes in the quantity of free water at elevated temperatures. Another factor that could

influence the dielectric constant is changes in the molecular mobility of both the free water and casein-associated water. A technique that could shed light on changes in water mobility at elevated temperatures is relaxation measurements using NMR. This could provide information pertaining to both the state and level of mobility of water within the cheese. Therefore this technique was applied in Chapters 8 and 9 to assess water mobility at elevated temperatures.

The dielectric loss factor, like the dielectric constant, was found to increase with temperature, as shown in Figure 7.11.

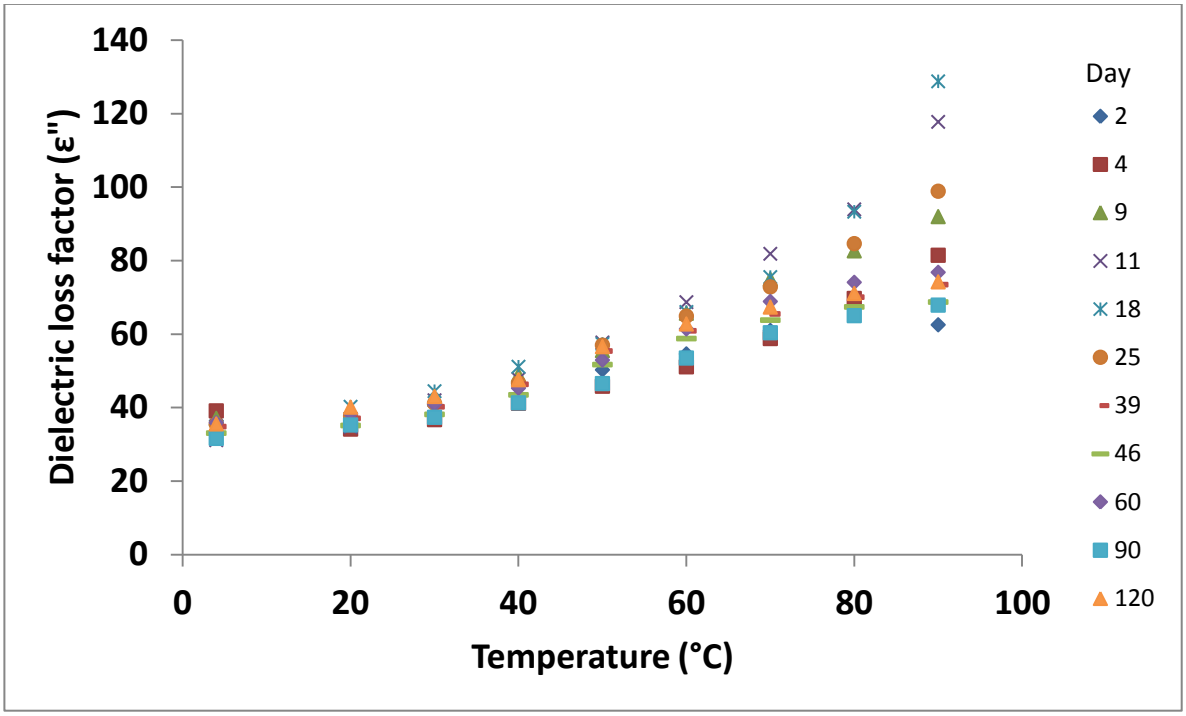


Figure 7.11: Change in the dielectric loss factor with temperature over the storage trial [244 MHz].

The dielectric loss factor exhibited a similar trend to the dielectric constant, with an initial curvature followed by a roughly linear period and then a deviation from linearity at high temperatures. As with the dielectric constant, a frequency sweep from 200 to 1300 MHz was conducted at each temperature that the experiment was conducted at. A frequency of 244 MHz was chosen to display the relationship between the dielectric loss factor and

temperature over the 120 day trial. This frequency was chosen due to the dominance of ionic conduction at lower frequencies (Fagan et al., 2005) and it is near the lower limit of the network analyser used for the experiment.

The dielectric loss factor has been associated with the salt content of foods (Green, 1997; Kudra et al., 1992) as well as being influenced by the moisture content (Ryynänen, 1995). The dielectric loss factor was found to exhibit curvature at high temperatures (above 70°C) in a similar manner to the dielectric constant data. Figure 7.12 below further examines the effect of temperature over the storage trial.

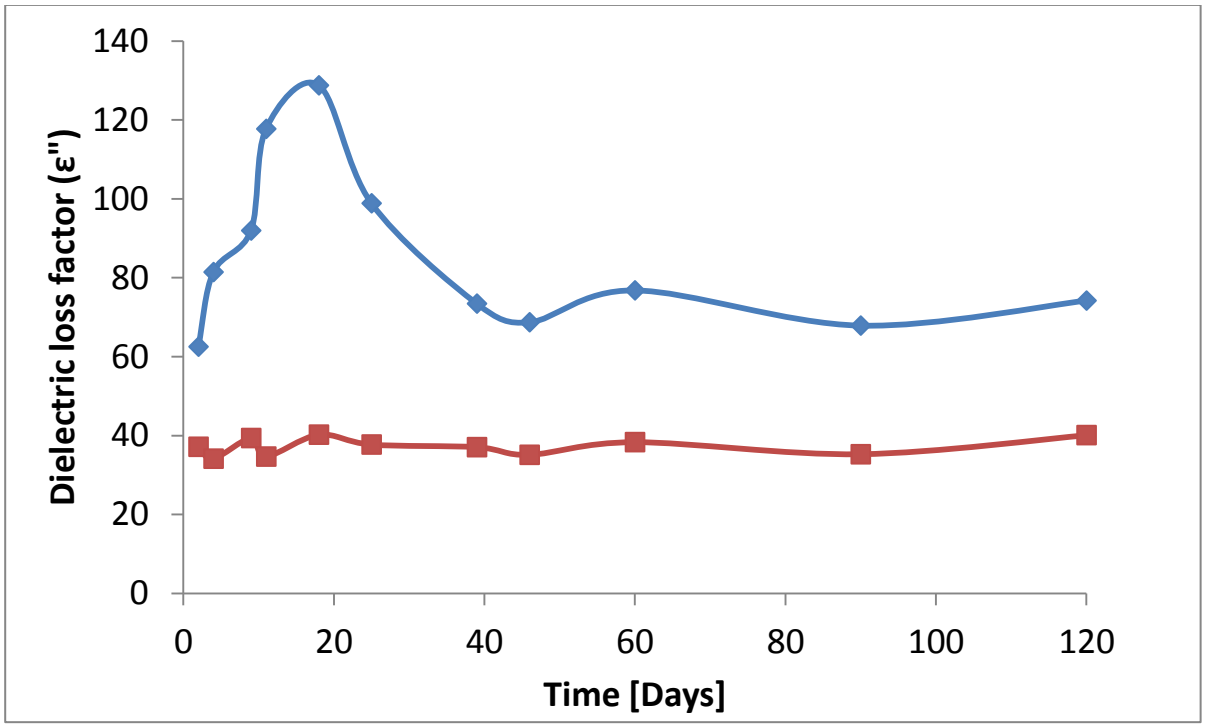


Figure 7.12: Change in the dielectric loss factor over the 120 day storage trial at ■ 20°C and ◆ 90°C [244 MHz].

At 20°C no obvious trend was evident in the dielectric loss factor data over the storage trial. However, a trend in the data was identified at higher temperatures. At 90°C the dielectric loss factor was found to increase in magnitude following manufacture up until

day 18. Following this peak the dielectric loss factor diminished by day 40 where it levelled off.

This is similar to the trend identified in the dielectric constant at 90°C, shown in Figure 7.10. One reason for this is that unlike the dielectric constant, which is dominated by the moisture content of a material, the dielectric loss factor is influenced by both the salt and moisture contents of a food (Fagan et al., 2005; Ryynänen, 1995). The molecular mobility of water within a material is also likely to affect the dielectric loss factor, as it has an impact on the dielectric constant (Tsoubeli et al., 1995). This is a result of the free ions being dispersed in the aqueous phase and as the proportion of the aqueous phase existing in a free state increases, there is likely to be a corresponding increase in the dielectric loss factor as the ions become can interact to a greater extent. Therefore any trends exhibited in the dielectric loss factor data are likely to be a combination of changes in the molecular mobility of the water in the system as well as variation in the ionic interactions.

7.3.3 General Discussion

The rheological and dielectric properties of Mozzarella were found to vary with temperature. The crossover of the storage and loss moduli from the rheological data was found to occur at a progressively lower temperature during maturation. This trend is consistent with observations made by Govindassamy-Lucey *et al* (2005b) where aging resulted in a decrease in the crossover temperature. This point, tanδ=1, has been linked to the point at which cheese begins to flow (Prow & Metzger, 2005; Sutheerawattananonda & Bastian, 2007) and the trend in the data was found to be inversely proportional to the spread observed in the modified Schreiber test used in Section 6.3.7. This indicates that melting occurred at a progressively lower temperature with maturation (Govindasamy-Lucey et al., 2005b) allowing a greater time where the cheese could flow. A lower melting temperature also would result in a lower viscosity, allowing cheese to flow to a greater extent.

The Arrhenius plot of the complex viscosity was found to change during the maturation, in particular the inflection points in the curve. A novel approach of conducting a differential analysis of the Arrhenius plot of the complex viscosity was taken, so that the rate of change occurring with temperature could be assessed. This indicated a number of peaks where the structure was changing over specific temperature ranges. Two peaks were identified between 20 and 40°C. Over this temperature range the fat is transitioning from a mixture of approximately 38% solid and 62% liquid (according to the data for Emmental cheese of Lopez et al. (2006)) to be entirely molten (Tunick, 2010). Over the course of the maturation period the secondary peak occurring at the higher temperature of approximately 35°C was found to increase in magnitude. One possible cause of this is a progressive change in the polymorphic state of fat within the Mozzarella during maturation. This phenomenon has been observed in Emmental cheese using a differential scanning calorimeter, where the quantity of higher melting point fat (30-36°C) increased during maturation (Lopez et al., 2006). The milk fat, which exists within cheese, is present in a mixture of different crystal forms with various melting temperatures (Lopez, Bourgaux, Lesieur, & Ollivon, 2007). In a system containing a mixture of fat crystals the crystals can dissolve and transform into a more stable polymorphic state (Small, 1986). This change in the polymorphic state of fat within the cheese to a more stable, high melting point form is likely to be responsible for the change in the complex viscosity over this temperature range. In order to further investigate changes in this temperature range the use of equipment such as a differential scanning calorimeter should be used in parallel to the rheological temperature sweep. This would allow additional information relating to the state of fat within the cheese samples to be gained in conjunction with the rheological temperature sweep. Unfortunately such a study was outside the scope of the current work due to time constraints.

The strengthening hydrophobic interactions during heating (Bryant & McClements, 1998) were possibly partly responsible for a decrease in the slope of the Arrhenius plot between

50 and 55°C. As the hydrophobic interactions within the protein matrix become stronger it could result in the contraction of the casein network forcing out free water entrapped within. Initially this could result in a partial dehydration of the protein matrix, causing a slight decrease in the slope of the complex viscosity. Further contraction may enable the protein fibres to move, lubricated by the fat, and begin to flow (Tunick, Malin, et al., 1993). This coincides with the increase in the slope as the cheese melts.

Unlike the trends identified in the rheological data, no obvious trends were identified in the dielectric temperature sweeps during the maturation period. The only discernible difference identified by inspection of the temperature sweeps both the dielectric constant and dielectric loss factor over the maturation period was a slight change in curvature at high temperatures. These changes at elevated temperatures are possibly related to changes in the water mobility within the cheese samples. To further elicit information about the state of water within the cheese at elevated temperatures T2 relaxation measurements could be conducted using NMR, as investigated in Chapters 8, 9 & **Error! Reference source not found.**. Another possible reason for this is the release of free oil at high temperatures increasing during maturation and interfering with the measurements. As fat acts by depressing the dielectric properties of a system (Ryynänen, 1995), free oil expressed at high temperatures may influence the dielectric properties of the cheese at high temperatures.

One issue with the temperature sweeps conducted with dielectric spectroscopy is that a number of different factors influence the dielectric properties of a material. These include the composition of the system (Zhang et al., 2004) and changes in ionic and water mobility (Brunton, Lyng, Zhang, & Jacquier, 2006; Roebuck, Goldblith, & Westphal, 2006). The data and discussion presented in Section 5.3.6 indicated that the proportion of free water in the cheese structure affected the dielectric parameters. Also the previous chapter showed that the heating of the Mozzarella curd was responsible for the free water that was present in the cheese. Therefore it is likely that during the heating of the cheese, during

the temperature sweeps, there will be a progressive increase in free water within the cheese structure. Therefore any information gained from the use of dielectric spectroscopy on cheese at elevated temperatures would not only be influenced by the moisture and salt content of the cheese, but it would also be affected by the disassociation of ions and the state that water exists within the system (Feng et al., 2002) due to heating. This means that there are a number of different factors confounding the interpretation of results ascertained from temperature sweeps of cheese using dielectric spectroscopy. In order to utilize the data obtained from dielectric spectroscopy, the data could be adjusted if the amount of free water present in a sample was known as well as the moisture content. A method that could be utilised to gain a greater understanding of the dynamics of water within cheese during heating, if this experiment was to be repeated, is T2 relaxation using NMR.

Another potential problem of using dielectric spectroscopy at elevated temperatures is the fat within the cheese samples could potentially interfere with the results. This is due to the dielectric properties of fat being very low and having a dilution effect on the dielectric properties of a system (Datta et al., 2005). Therefore fat can act by depressing the dielectric properties of foods (Ryynänen, 1995). This could happen in two ways. The first is that fat could be expressed from the structure in the form of free oil. The free oil may pool around the contact probe and interfere with the signal. The other possibility is that with an increase in temperature the fat within the cheese may move more freely, forming larger pockets of fat. As the cheese matures the casein matrix weakens, due to proteolysis (Tunick, Malin, et al., 1993) and solubilisation of calcium (O'Mahony et al., 2005), which means the fat can move freely through the matrix as it is heated, resulting in larger pockets of fat forming, as seen in Section 6.3.2. The presence of free fat or large pockets of fat within the cheese would result in the depression of the dielectric properties.

Based on the results from the dielectric properties of the cheese during maturation and heating it is difficult to come to any solid conclusions about changes in component mobility within the cheese. This is due to the dielectric properties being influenced by a number of different factors that could affect the results. However, collectively the data compiled over the storage trial indicated that the rheological properties of Mozzarella were influenced by a number of transitions affecting the cheese structure that may include: an increase in the quantity of a high melting polymorph of fat, the melting of fat, strengthening hydrophobic interaction resulting in free water, and the flow of casein fibres.

7.4 Conclusion

The results from the temperature sweeps conducted on the Mozzarella indicated that significant structural variation occurred during heating over the maturation trial. A number of key changes occurred in the rheological properties of the cheese as transitions in the state of components within the system were affected by heating. The study suggests that although trends do exist in the dielectric data, solid conclusions could not be made as the results were affected by a number of mechanisms occurring in the cheese during both heating and maturation.

Based on the findings of this work, a more detailed understanding of changes occurring during heating are required. In particular an investigation into the effect of heating on the free water within cheese is required. NMR has been identified as a powerful tool capable not only of shedding light on the level of water mobility but also has the capability of measuring how temperature affects the diffusion of water within cheese.

8 Assessment of the changes in the structure and component mobility of Mozzarella and Cheddar cheese during heating

8.1 Introduction

This thesis is about trying to understand the structure of Mozzarella cheese and finding tools for assessing changes occurring in the structure. As Mozzarella is usually consumed hot (Bertola et al., 1996a), it is important to try and characterise the changes occurring during heating. A number of changes occur within the structure of cheese during heating including: strengthening hydrophobic and electrostatic interactions (Bryant & McClements, 1998); weakening casein-to-casein interactions (Lucey, Johnson, & Horne, 2003; and the melting of fat (Tunick, 2010). The previous chapter used dielectric spectroscopy and rheology to explore changes occurring in the structure of Mozzarella during heating. Dielectric spectroscopy was identified as being a relatively insensitive technique for assessing changes during heating, as a number of factors confound measurement of the dielectric properties. Thus an alternative measurement technique for monitoring changes was sought. The opportunity arose to complete collaborative work at Montana State University using magnetic resonance tools to explore the structure of commercial samples of Mozzarella and Cheddar cheese.

Mozzarella and Cheddar, two of the most consumed cheeses in the world, differ in structure due to the processing of Mozzarella whereby the curd is stretched (Kindstedt et al., 2004). This results in Mozzarella having a structure where the protein is aligned into roughly parallel fibres interrupted by channels of fat and serum (Kindstedt, 2007), whereas Cheddar consists of a continuous gel network inter-dispersed with fat globules (Rogers et al., 2010).

Magnetic resonance is a powerful tool for assessing changes in structure and component mobility in food (Mariette, 2009). Different pulse sequences and parameters can be utilized to ascertain information relating to a number of diverse properties of a material.

Spin relaxation measurements can be used to study molecular interactions as well as gaining information regarding molecular tumbling (Godefroy, Creamer, et al., 2003). Previous studies of cheese that have used T2 relaxation measurements include: the assessment of changes in the water mobility of Mozzarella cheese following maturation (Kuo et al., 2001), monitoring changes in Feta during brining (Altan et al., 2011), measuring moisture in cheese analogues (Budiman, Stroshine, & Cornillon, 2002), and studying the effect of freezing on Mozzarella (Kuo et al., 2003).

The translational motion of molecules within a system can be assessed using Pulsed Gradient Spin Echo (PGSE) techniques to investigate their diffusion properties (Callaghan, 1991). The diffusion properties of solvent molecules in porous media are an area that is gaining increasing attention. At very short time scales the solvent molecules can freely diffuse, however, at long time scales the molecules are restricted by the gel network (Fridjonsson et al., 2011). There are two aspects associated with porous media having reduced diffusion movement of molecules (Song, 2009). The first, at short time scales, is due to solvent molecules near the surface of the pores having their movement restricted by the surface to volume ratio of the pores (Latour et al., 1995; Mitra et al., 1992). The second, at long time scales, is limited by the tortuosity of the network (Song, 2009). The tortuosity of a porous media is governed by the connectivity of the pores (Latour et al., 1995).

The objectives of this investigation were to:

- Develop a NMR method for studying cheese at elevated temperatures.
- Monitor changes in the relaxation and diffusion properties of water within cheese as it is heated.

8.2 Material & Methods

This study was carried out at Montana State University in Bozeman, Montana, USA when I visited in 2012.

8.2.1 Materials

Commercial samples of a low moisture part skim (LMPS) Mozzarella, a medium Cheddar (2 months aged) and a 9 month old Cheddar were obtained from a local supermarket (information and composition of the cheese samples in Table 8-1 and Table 8-2 below).

Table 8-1: Cheese varieties

Cheese Type	Details	Description
LMPS Mozzarella	LMPS Mozzarella Cheese: Associated Milk Producers Inc., Wisconsin	Low moisture part skim
Medium Cheddar	Medium Cheddar Cheese: Tillamook Country Creamery Association, Oregon	Aged over 60 days
Aged Cheddar	Sharp Cheddar Cheese: Tillamook Country Creamery Association, Oregon	Aged over 9 months

Table 8-2: Composition of commercial cheeses (from nutritional information panel)

	Protein (%)	Moisture (%)	Fat (%)	Calcium (%)	Sodium (%)
Mozzarella (LMPS)	25	50	21	0.71	0.54
Medium Cheddar	25	42	32	0.71	0.61
Aged Cheddar	25	42	32	0.71	0.61

8.2.2 Rheology

Small amplitude oscillatory rheology was conducted on a TA AR G2 rheometer (TA Instruments, New Castle, DE, USA). A 20 mm parallel plate geometry was used and the flat Peltier plate had sand paper attached to them with superglue. Disks of cheese, 2.8 mm in height and 20 mm in diameter, were cut using a cork borer and a cheese wire. The cheese samples were placed on the Peltier plate and had the 20 mm top plate lowered onto it until a normal force of 0.4 N was applied. In order to prevent the cheese samples drying out over the course of the temperature sweep a solvent trap was utilized.

A frequency sweep was carried out on a sample of each of the three cheeses to identify the linear viscoelastic regions. Based on this a strain rate for the temperature sweeps was chosen. The cheese samples were heated from 20 to 70°C at a rate of 1°C per minute. The samples were subjected to a frequency of 1 Hz at a strain rate of 0.03% (which was within the linear viscoelastic region for all three cheese samples). The complex viscosity (η*) was monitored, using the rheometer, to identify how each of the cheese samples responded to heating. The temperature sweeps were replicated at least three times.

8.2.3 Magnetic resonance

Cylindrical samples of cheese, measuring 12 mm in diameter and 70 mm in height, were cut with a cork borer. The samples were tightly inserted in a cut glass tube and sealed with Teflon tape. The samples were placed inside a Bruker Advance300 NMR (Bruker, Rheinstetten, Germany). The temperature was progressively increased in 15°C increments from 20°C to 65°C before being cooled back to 20°C. The heating was applied at a rate of 3°C/minute and an equilibration period of 10 minutes was allowed so that the cheese sample could reach the required temperature throughout. Relaxation and diffusion measurements were taken at each temperature following a gradual temperature increase (1.5°C/min) and a 10 minute equilibration period. Heating and cooling occurred over the

entire surface of the cylinder so that the maximum heat transfer distance was 6mm. Measurements for each variety of cheese were conducted in duplicate.

8.2.3.1 *Images*

MR images were obtained using a standard spin warp imaging pulse sequence (Callaghan 1991). The field of view was 30 x 15 mm with 256 x 128 pixels resulting in an isotropic resolution of 0.117 mm x 0.117 mm. 16 images were obtained with an echo time of 10.567 ms to obtain T2 weighted images. The images collected from the MRI used T2 relaxation maps to produce an image of the sample in the magnet. This uses the T2 intensity of a sample to construct an image.

8.2.3.2 *CPMG*

T2 relaxation measurements were performed using the Carr Purcell Meiboom Gill (CPMG) spin echo pulse sequence (Carr & Purcell, 1954; Meiboom & Gill, 1958). The standard sequence was resolved based on the two different frequency values for fat and water. Echo decay curves were then obtained for the fat and water components and analysed separately.

The relaxation constant of the water present in each cheese was fitted to a bi-exponential model based on Equation 8-1 below (Chaland et al., 2000; Kuo et al., 2001):

$$I = Ae^{(\frac{-t}{T2a})} + Be^{(\frac{-t}{T2b})}$$

Equation 8-1

The T2 component relates to the molecular mobility of the water with the T2a component relating to the fraction that has the shorter relaxation time due to being associated with the protein, while the T2b relates to the water in a free state which has a longer relaxation time. The NMR measurements were conducted in duplicate.

8.2.3.3 *Diffusion*

Diffusion measurements were obtained using a standard pulsed gradient stimulated echo sequence (PGSE). The echo time was kept constant for all observation times; and the pulse duration (δ) was 1 ms for all experiments. Observation times (Δ) of 10, 20, 50, 80, 100, 150, 300 and 500 ms were obtained for each type of cheese at each temperature. The Pade approximate, Equation 8-2 below, was used to calculate the tortuosity (α) and the surface to volume ratio (S/V_p) of the samples (Mair et al., 2001).

$$\frac{D(t)}{D_0} = 1 - (1 - \frac{1}{\alpha}) \frac{\left(4\sqrt{\frac{D_0 t}{9}}\sqrt{\pi}\right)\left(\frac{S}{V_p}\right) + \left(1 - \frac{1}{\alpha}\right)(\frac{D_0 t}{D_0 \theta})}{\left(1 - \frac{1}{\alpha}\right) + \left(4\sqrt{\frac{D_0 t}{9}}\sqrt{\pi}\right)\left(\frac{S}{V_p}\right) + \left(1 - \frac{1}{\alpha}\right)(\frac{D_0 t}{D_0 \theta})}$$

Equation 8-2: Pade approximate for diffusion measurements

D_0 was taken from literature values for the diffusion of water as a function of temperature. S/V_p is estimated from the short time slope of the $D(t)/D_0$ curve (Brown et al., 2012; Latour et al., 1995). The time constant (θ) describes the approach to the long-time restricted diffusion behaviour (Mair et al., 2001). Porous media are characterized by the relationship between the surface to volume ratio and the tortuosity (Latour et al., 1995).

The diffusion measurements were normalised using the diffusion of free water at each temperature assessed, $D(t)/D_0$. The normalised diffusion was plotted against the square root of the observation time multiplied by the diffusion of free water at each temperature, $(\Delta D_0)^{0.5}$. Two linear regression lines were fitted to each of the decay curves, the first used to find the surface to volume ratio by fitting the first two diffusion points and the diffusion at $D=D_0$ (Brown et al., 2012). The horizontal linear fit represents the asymptote equal to the inverse of the tortuosity (1/α) (Latour et al., 1995).

8.3 Results & Discussion

The cheeses used in this investigation differ from those used in previous studies. Factors that may be different include the maturity, composition and manufacturing process.

8.3.1 Rheology

The testing procedure used for the rheological analysis differed slightly from that used in Chapter 7. At Montana State University the equipment available for assessing rheology was different to that used in previous work. The key differences included: the rheometer; the use of sandpaper on the geometry and Peltier plate; and heating rate. Sandpaper was used on the geometry and Peltier plate as the rheometer being utilised did not have a serrated plate configuration available. Sandpaper has been used in a number of studies to prevent slippage in samples (Hemar, Hall, Munro, & Singh, 2002; Pereira et al., 2001). The heating rate used in this work was slower than in Chapter 7. One reason for this was due to the cheese samples not sitting directly on the Peltier plate as in the previous experiment in Chapter 7, as the sandpaper was between the sample and the plate. The sandpaper will decrease the heat transfer to the cheese sample as paper has a thermal conductivity of about 0.05 compared to steel which has a thermal conductivity of >16 $Wm^{-1}K^{-1}$ (Toolbox) so a slower heating rate (1°C/min) was used to ensure that the sample reached the desired temperatures.

Based on the results of the temperature sweeps, an Arrhenius plot of the complex viscosity against the temperature was constructed for each of cheese samples. This showed that all three cheeses decreased in viscosity with increasing temperature (Figure 8.1).

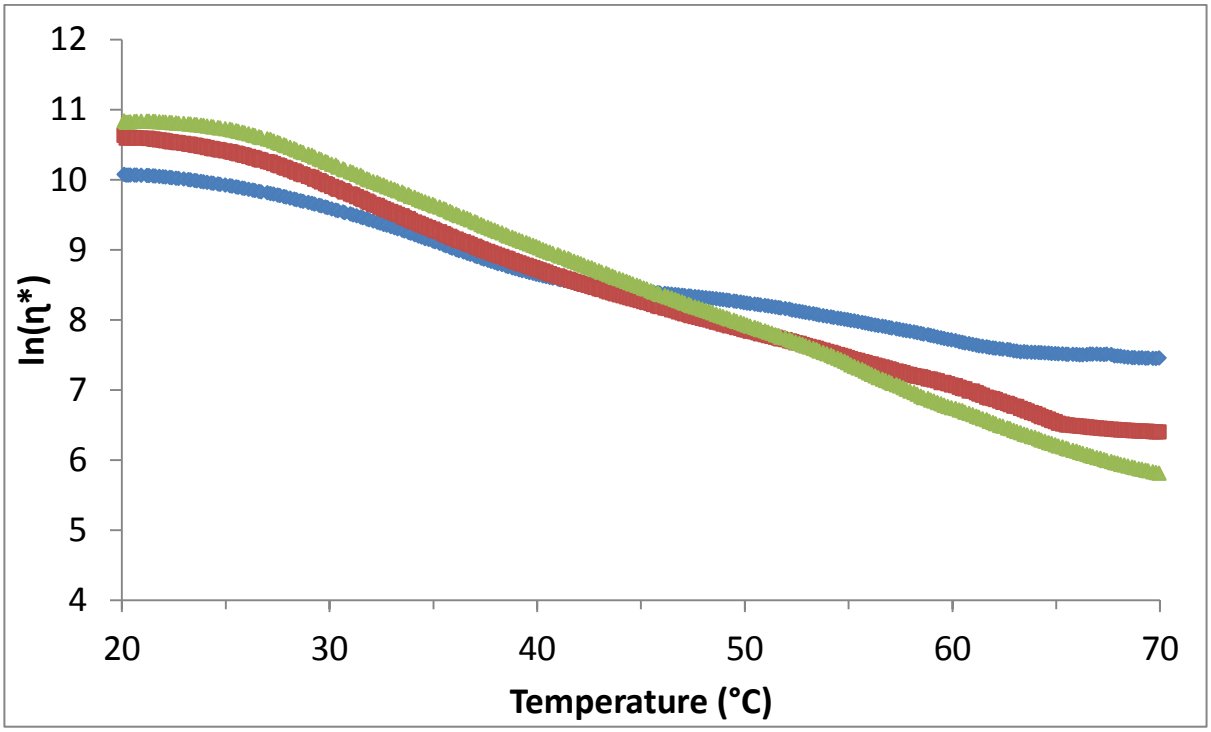


Figure 8.1: Arrhenius plots of complex viscosity and time over a temperature range of 20 to 70°C. [◆ Mozzarella, ■ Medium Cheddar and ▲ Aged Cheddar].

The complex viscosity for all three cheese samples was found to decrease as the temperature increased. The complex viscosities of the Cheddar samples were observed to decrease at a greater rate than the Mozzarella sample.

The two Cheddar samples were identified as having higher complex viscosities than Mozzarella over the temperature range of 20 to 40°C. This is likely due to the higher quantity of fat present in the Cheddar samples in comparison to Mozzarella, while having the same protein content (Table 8-2). As the temperature increases from 20 to 40°C, the ratio of solid to liquid fat decreases (Tunick, 2010). As liquid fat is less viscous than solid fat (Shukla et al., 1994), the contribution of the fat to the overall viscosity of the cheese samples diminishes as the fat melts. This results in a progressive decrease in the fat's contribution to the elastic properties of the cheese (Guinee, 2011). As Cheddar has a higher fat content than LMPS Mozzarella, the contribution that the solid fat imparts to the structure is higher. Thus the complex viscosity is greater in Cheddar than Mozzarella at

temperatures between 20 and 40°C. Following this, all three cheeses continued to have decreasing complex viscosities with the increasing temperature. The rate of decrease was found to be greatest for the aged Cheddar sample followed by the medium Cheddar and then the Mozzarella sample. At high temperatures Mozzarella was found to have a higher complex viscosity than either of the two Cheddars. This is similar to findings by Tunick (2010) who stated that this is likely to be due to the alignment of the casein fibres in Mozzarella giving it a greater degree of structural organisation. Additionally one would expect to have a higher complex viscosity by virtue of the fact that Mozzarella has a higher mass fraction of protein water matrix compared to Cheddar i.e. the complex viscosity of Mozzarella is not as adversely affected by the mass fraction of liquid fat as Cheddar.

Another factor that will affect the structure of the cheese samples is the extent of casein breakdown that has occurred during storage. During storage the cheese undergoes proteolysis whereby the casein is progressively broken down into fragments by residual enzymes (Coker et al., 2005). The aged Cheddar is likely to have undergone a greater amount of proteolytic breakdown than the medium Cheddar due to an increased storage time, however, the extent of proteolysis for the cheeses in this study were not measured. This results in the amount of intact casein present within the aged Cheddar to be reduced further, weakening the casein matrix (Tunick, Malin, et al., 1993) which may be responsible for the lower complex viscosity at elevated temperatures for the samples.

The proteolytic breakdown of Mozzarella differs from that of Cheddar. One of the key reasons for this is due to the heat treatment that Mozzarella is subjected to during stretching which has been shown to affect proteolysis (Lawrence, Creamer, & Gilles, 1987). Another factor that affects the rate of proteolysis is the composition of the cheese (Yun, Barbano, et al., 1993). The differing composition of the Mozzarella sample from the two Cheddar samples, including higher moisture and lower salt contents, will impact on the rate of proteolysis in the cheese (Lawrence et al., 1987).

The inflection points on these curves indicate where significant changes in the structure of the cheese are occurring. Therefore a differential analysis was conducted on the data from the Arrhenius plot (Figure 8.1), as done in Section 7.3.1.

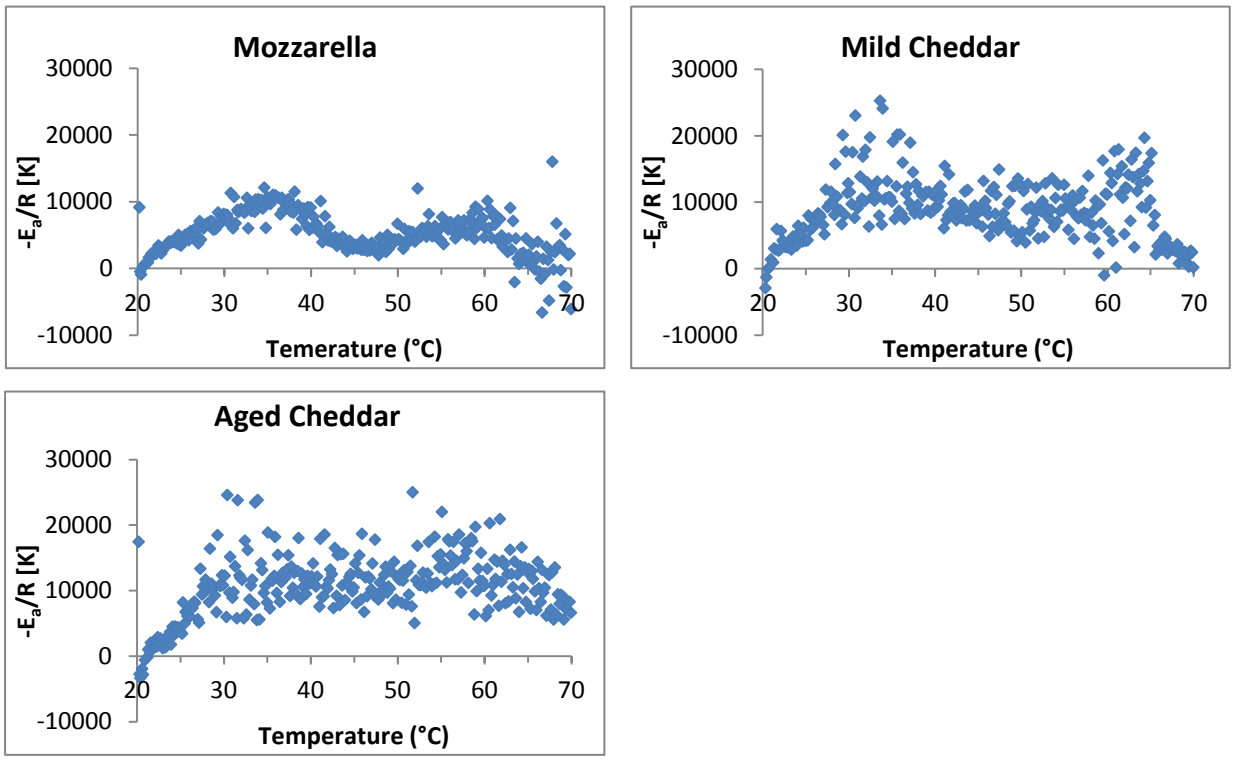


Figure 8.2: Differentiated Arrhenius plots for the three cheese samples over a temperature range from 20 to 70°C.

The graphs in Figure 8.2 show the rate of change in complex viscosity during the temperature sweep. Both of the Cheddar samples exhibited a greater degree of variation than the Mozzarella. The organised structure of Mozzarella may be responsible for the reduced variation in comparison to the Cheddar samples. Another possible reason for this variation includes the lower quantity of intact casein likely in the Cheddar samples and the difference in the structure of Cheddar in comparison to Mozzarella. However, the Arrhenius plots of the two Cheddar samples were observed to show near linearity between 30 and 60°C suggesting the variation may be a result of noise.

The shape of the Mozzarella curve was similar to that observed in the differential analysis in Section 7.3.1, apart from the mid-range peak. The magnitude of the peaks was also similar to those in the previous experiment. Mozzarella was found to have an initial increase in slope between 20 and 35°C. This is likely due to melting of fat within the cheese system corresponding with a decrease in the viscosity. The fat present within cheese melts between temperatures of -40 and 40°C (Tunick, 2010). As fat become progressively more liquid than solid, the rigidity that it imparts to the cheese structure lessens. One possible explanation for the decrease in the slope between 35 and 45°C is possibly due to a partial dehydration of the protein network. As the temperature increases, the hydrophobic interactions within the casein matrix become stronger (Bryant & McClements, 1998), forcing water out from within the protein. This would result in the protein phase becoming marginally stiffer causing a slight increase in the complex viscosity and a corresponding decrease in slope. Such temperatures are used in casein manufacturing and in cheese cooking to increase the mechanical strength of curd.

A second increase in slope, observed between 48 and 60°C, indicates a sharpening decrease in the viscosity in this temperature range. This decrease in viscosity occurs over the temperature range that the cheese flows. This temperature range is when the hydrophobic interactions are at their maximum strength (Nemethy & Scheraga, 1962). As the caseins contract due to their strengthening hydrophobic interior, more water will be squeezed out and contact between particles will lessen, beyond that in the previous temperature range. As the number of points of contact decreases the particles are able to move more freely and eventually flow. The flow that occurs will lead to a greater rate of decrease in viscosity and an increase in the slope.

A final decrease in the slope of the Arrhenius plot was found to occur above 60°C, indicating a decrease in the rate of change in viscosity. One possible cause of this is that as the cheese is flowing, free oil and moisture can leave the cheese structure. The expression of fat during heating is common in cheese and is considered a key functional property of

Mozzarella in its use as a pizza topping (Kindstedt & Fox, 1991). This loss of fat and moisture would result in the rheological properties of the remaining cheese become more dominated by the protein as the fat is progressively lost.

Both of the differential Arrhenius plots of the Cheddar samples were observed to exhibit a similar pattern to the Mozzarella sample. However, the greater amount of variation within the data limits the amount of interpretation that can be made from the graphs.

The differential analysis of the Arrhenius plot in Section 7.3.1 was useful as it showed how the slope changed over the course of the storage trial. However, without a reference point for each cheese sample, the differential analysis is less useful and open to a greater degree of interpretation as any deviation from normal behaviour may not be identified.

8.3.2 Magnetic resonance

8.3.2.1 *Method development*

The imaging capability of the MRI allowed the samples to be viewed during experimental work. Initially measurements were made on shredded cheese samples that were packed into a glass tube. Imaging during the heating experiment (from 20 to 65°C) revealed the presence of a number of air bubbles within the sample that would interfere with the NMR measurements. The bubbles were likely due to small pockets of air between the shreds of cheese packed together. Thus a solid plug of cheese was inserted into the tube and trialled. Imaging of this sample during heating revealed one large air pocket within the sample, rather than the multiple bubbles present in the shredded sample. It was theorised that this bubble may be the result of the curvature of the glass test tube where a small quantity of air could get trapped when the plug of cheese was inserted in the tube. In order to test this hypothesis the curved end of the tube was cut off, converting it into a cylinder. The plug of cheese was then inserted into the glass cylinder and a plastic cap fitted to the sample holder and fixed in place using Teflon tape. In this arrangement no

bubbles were identified in the cheese samples during heating when the samples were imaged.

Based on the results from this preliminary examination of the testing procedure a plug of cheese was used in a glass cylinder for all further measurements.

8.3.2.2 *Images*

Images were taken before heating to ensure that the sample was sitting in the sample holder correctly. Following the heating regime whereby the cheese samples were heated to 65°C an additional image was taken after cooling the sample to 20°C following an equilibration period of an hour. This was done to identify whether any significant changes could be observed in the structure of the cheese samples.

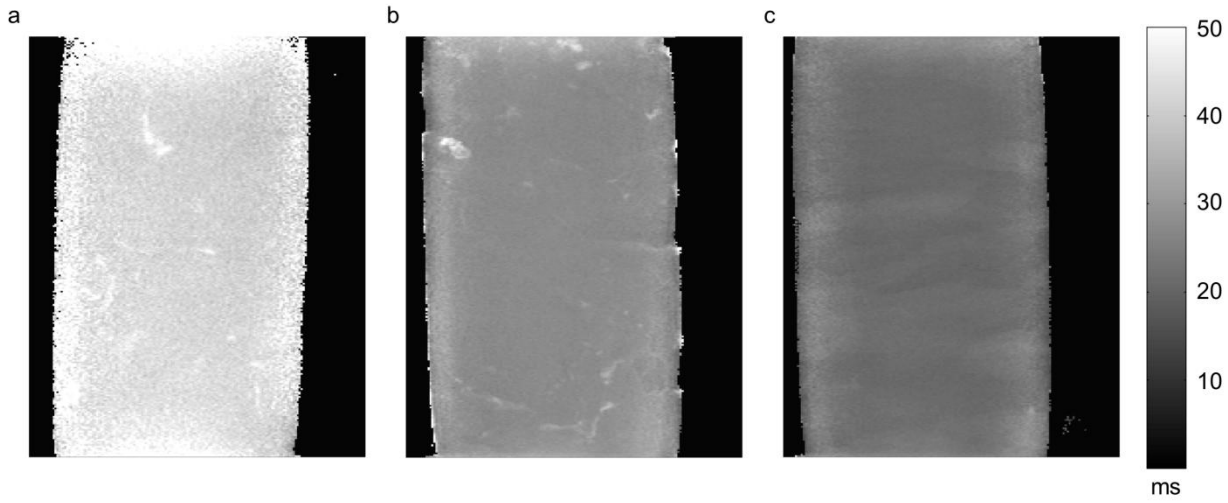


Figure 8.3: T2 maps of cheeses before heating. a) aged Cheddar, b) medium Cheddar, c) Mozzarella.

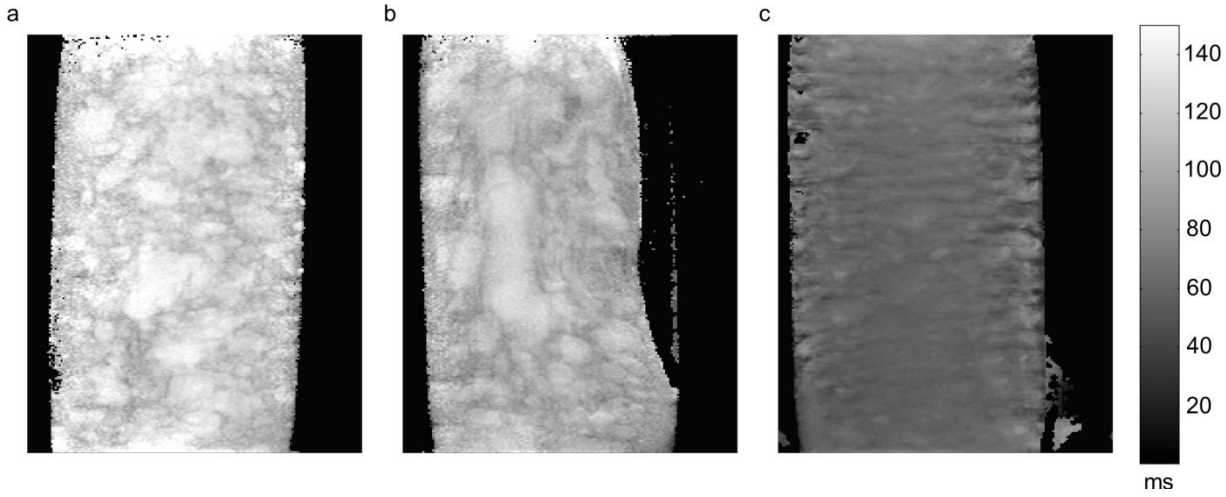


Figure 8.4: T2 maps of cheese after heating. a) aged Cheddar, b) medium Cheddar, c) Mozzarella.

The images collected from the MRI used T2 relaxation maps to produce an image of the sample in the magnet. They indicate a cross section of the middle of the samples. The lighter areas indicate longer relaxation times and darker areas indicate shorter relaxation times. Fat has a longer relaxation time than protein and will appear as light areas in the images.

The images of all three different cheeses before heating were similar each resembling a relatively homogeneous block. The difference in appearance prior to heating is likely to be due to the distribution and quantity of fat within the different cheeses. Section 6.3.2 indicated that as Cheddar matures the pockets of fat increase in size, larger pockets will show a more intense longer T2 signal (image will appear whiter). After heating, the two Cheddar samples were observed to have markedly different structures than prior to heating. The images showed large areas of fat aggregations within the Cheddar structure. The image of the Mozzarella sample following heating was identified as being less homogeneous than prior to heating, but no fat clusters were evident. The reason for the observed differences between the Cheddar and Mozzarella samples following heating is likely to be a combination of a more organised protein structure, lower fat and less

proteolysis in the Mozzarella sample. However, the resolution of the images obtained limits the insights gained from them.

8.3.2.3 *Relaxation*

All three cheese samples were identified as having a lengthening in the T2 relaxation times (from a range between 10 to 158 ms at 20°C to between 63 and 1260 at 65°C) of fat between 20 and 65°C. Over this temperature range the fat present within the cheese undergoes a transition from a solid/liquid mixture to being almost entirely liquid (Tunick, 2010). The increase in the relaxation time for the fat is consistent with this transition, as liquid fat is known to have a longer relaxation time than solid fat (Song, 2009). The signal for solid fat is below 100 ms while the relaxation time for liquid fat will be longer (Song, 2009).

Relaxation measurements were used to assess water mobility, monitoring changes in the quantity of free water and casein associated water during heating.

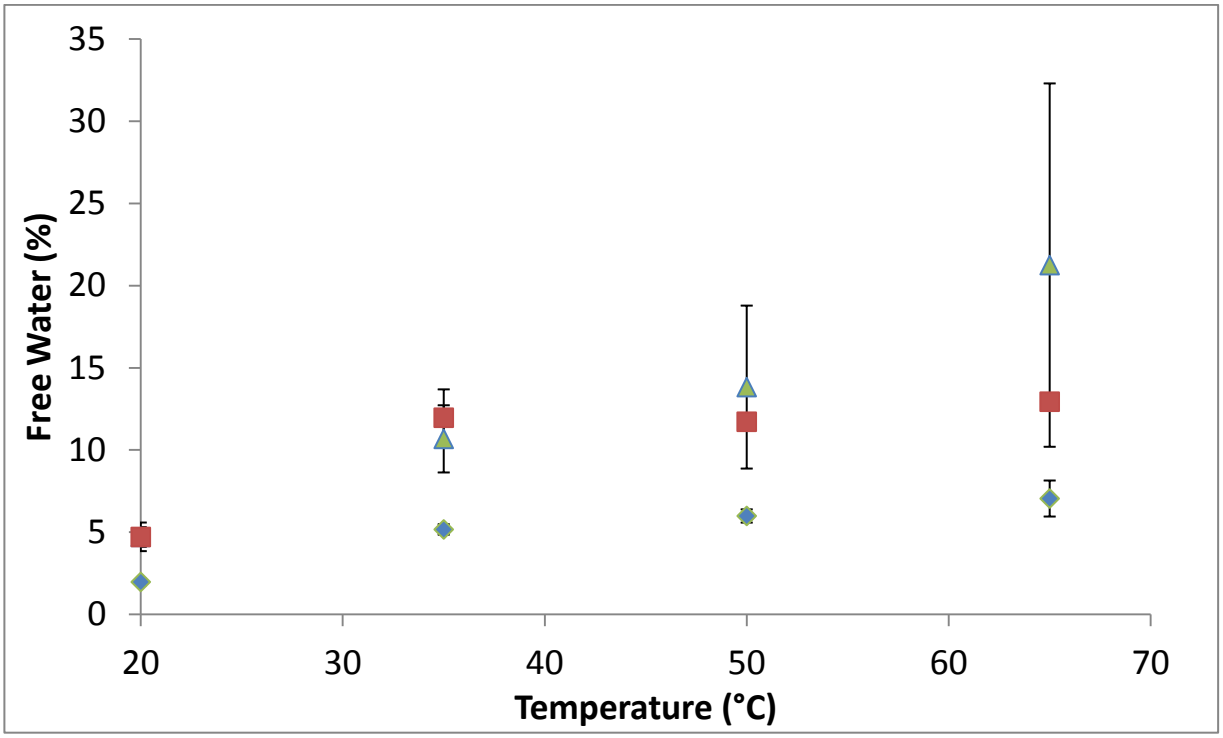


Figure 8.5: Effect of temperature on the quantity of free water (A in Equation 8-1) present in the cheese samples. [◆ Mozzarella, ■ Medium Cheddar and ▲ Aged Cheddar] (Error bars = ±1 SD)

The quantity of free water in all three cheese samples increased as they were heated from 20 to 65°C (Figure 8.5). The quantity of free water was found to increase from 2 to 7% in Mozzarella, 5 to 13% in the medium Cheddar and 5 to 20% in the aged Cheddar. As the temperature of the samples increase, the balance of forces within the cheese structure shifts as hydrophobic and electrostatic interactions increase in strength while protein-to-protein interactions decrease. The hydrophobic interactions increase in strength (Bryant & McClements, 1998) up until 50 to 60°C where they are at their strongest (Nemethy & Scheraga, 1962). This increase in strength is likely to cause the protein network to contract, changing the distribution of the water contained within the network. he reduction in protein-to-protein interactions that also occurs with increasing temperature will reduce the connectivity of the protein matrix, limiting its ability to resist flow.

Despite having a higher total moisture to protein ratio (Table 8-2) Mozzarella was identified as having less free water in its structure than the two Cheddars at each temperature studied. One possible reason for this is the difference in the quantity and distribution of the fat within the cheeses. When heated the protein matrix contracts due to increasing hydrophobic interactions. The water within the protein will be forced out into a space which is not being constricted by protein interactions i.e. the space surrounding the fat or voids. From Section 5.3.3 we saw that although the fat is maintained in a globule structure similar in size to native fat globules the fat globule membrane material did not form part of the protein network. With a greater quantity of fat in Cheddar, there is both a greater total volume of fat, and thus surface area for water to migrate to and occupy, and also a decrease in the average distance that a water molecule would have to travel in order to pool. The contraction of the protein fibres in Mozzarella may also differ from the contraction of the casein matrix in Cheddar. As the same temperature is applied to the samples, the driving force leading to contraction in all of the cheese samples should be the same. This will lead to the approximately spherical aggregated micelles within the Cheddar sample experiencing an equal contraction in all dimensions, while the fibrous structure within Mozzarella may experience a lessened contraction in the elongated longitudinal plane and may not expel as high a quantity of water as the Cheddar samples.

The extent of proteolysis is possibly the reason for the scatter in the aged Cheddar samples at elevated temperatures; however, the extent of breakdown was not evaluated in this experiment. The weaker gel network will allow large pools of fat to form in the cheese at elevated temperatures, as identified in Figure 8.4. Between samples the formation of these fat pools may vary, leading to the error in the aged Cheddar. However, further measurement would need to be conducted to gain a better understanding of what may be responsible for the variation in the sample. Due to the limited number of samples that could be run during this experiment, it would be advantageous to repeat the study

with a greater number of samples to gain a clearer picture of changes occurring in various cheese systems during heating.

The increase in free water could be responsible for the decrease in the energy of flow observed in Mozzarella between 35 and 45°C. The expulsion of water into a free state will result in the protein matrix becoming slightly tougher. As the majority of the fat has melted over this temperature and the cheese has not melted, the increase in free water with its corresponding slight dehydration of the protein phase may be the dominant mechanism impacting the complex viscosity.

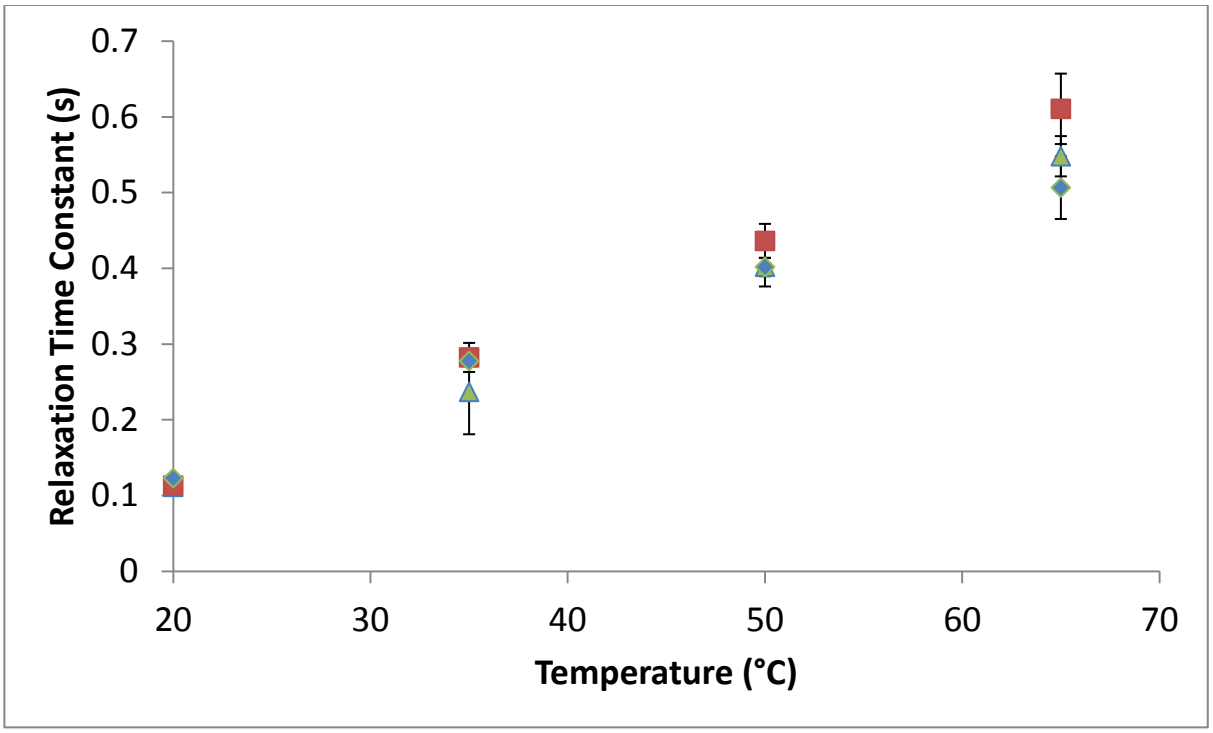


Figure 8.6: Relaxation time constants (T_{2B} in Equation 8-1) for free water between 20 and 65°C [◆ Mozzarella, ■ Medium Cheddar and ▲ Aged Cheddar].

The T2 relaxation time constant is a measure of the relative molecular mobility (Kuo et al., 2001) of water in this cheese system. The time constant of free water for each of cheese samples was found to progressively increase with temperature, as indicated in Figure 8.6. This increase in the relaxation time constant T2A for each of the cheeses with

temperature indicates that the free water within the structure of the cheese is becoming more mobile (Kuo et al., 2001). This is as expected as when heat is applied to free water it gains more energy and becomes more mobile. The increase with temperature was found to be significant with p values of 0.00079 for the aged Cheddar, 0.00023 for the mild Cheddar and 0.00024 for the Mozzarella. However, no significant difference was identified between the three cheeses, p = 0.19. This indicates that the application of heat affected the relaxation rate of free water in an identical manner in each of the three cheeses.

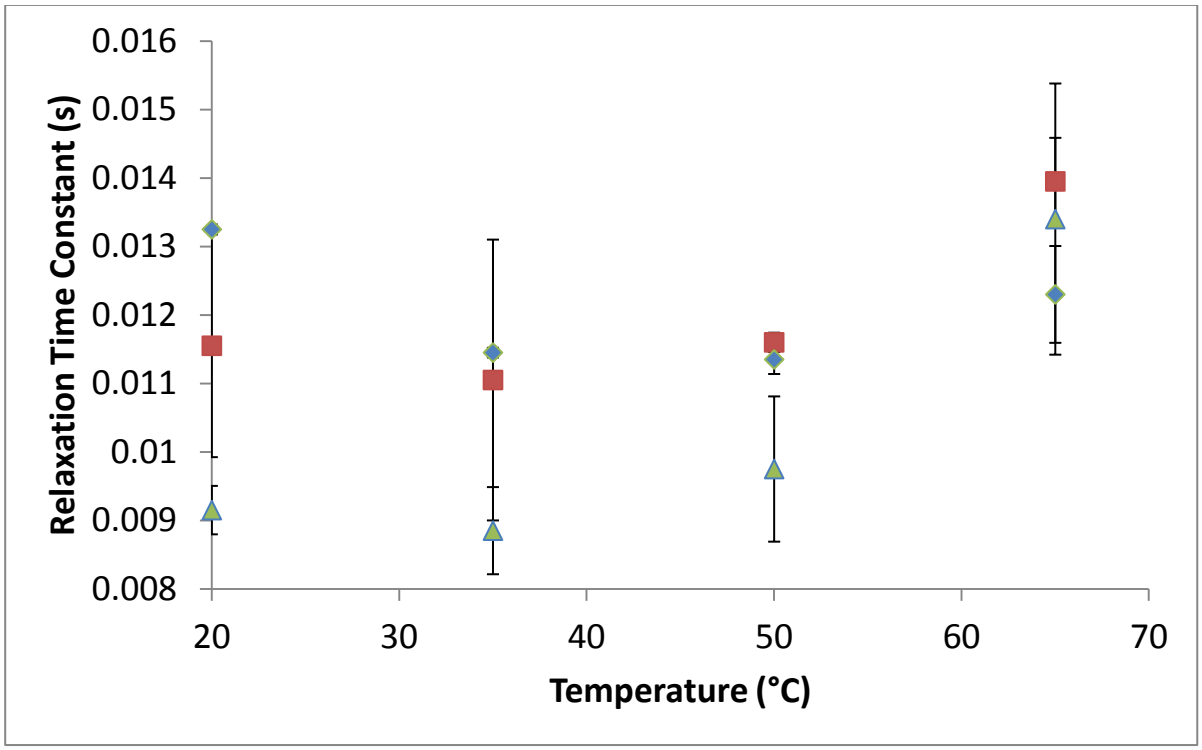


Figure 8.7: Relaxation time constant (T_{2A} in Equation 8-1) for casein associated water between 20 and 65°C [◆ Mozzarella, ■ Medium Cheddar and ▲ Aged Cheddar].

Unlike the relaxation time constant for free water which exhibited a linear increase with temperature, curvature was observed in the T_{2A} measurements for the water associated with the protein in the cheese, as shown in Figure 8.7. The curvature in the results differed between the three different cheese samples. Initially the relaxation time constant

of all three cheeses was found to decrease as the temperature was raised from 20 to 35°C, with the most pronounced decrease observed for the Mozzarella sample. As the temperature was raised from 35°C the relaxation time constant for the two Cheddar samples was observed to progressively increase. However, very little change was observed in Mozzarella between 35 and 50°C before the relaxation time constant increase between 50 and 65°C.

The curvature suggests that a number of competing mechanisms are occurring impacting on the molecular mobility of the casein associated water during the heating of the cheese samples. As with the free water, the increase in temperature will give the water molecules a greater amount of energy allowing them to become more mobile. However, heating will also have an effect on the environment that this water exists in as it is associated with the protein phase. With the increasing temperature the hydrophobic interactions will increase in strength (Bryant & McClements, 1998) within the protein matrix. As the hydrophobic regions of the caseins get closer in proximity the protein matrix will contract. This will likely cause a greater degree of restriction for the water molecules associated with the protein. This restriction in mobility due to changes in the protein matrix in conjunction with the increase in molecular mobility caused by the addition energy that the molecules receive at higher temperatures will impact on the net change in the mobility of casein-associated water.

The error displayed in the relaxation data represents one standard deviation. This error is likely to be due to sample to sample variation within the block of cheese sampled compounded by the limited number of samples that could be assessed in the experiment. When assessed statistically, no significant difference was identified between the three cheese samples, $p=0.216$. A significant difference was identified between the relaxation rate of the casein associated water at the different temperatures of testing in the Mozzarella cheese samples, $p=0.02$. However, no significant difference was identified in either the aged or mild Cheddar samples at a 95% confidence interval with p statistics of

0.053 and 0.278 respectively. To reduce this error in the results further replicates of each sample could be run to gain a greater degree of accuracy.

8.3.2.4 *Diffusion*

The change in the diffusion properties of water within each of the cheese samples was assessed from 20 to 65°C. This was done to identify how the diffusion of water within each of the cheeses changed with temperature.

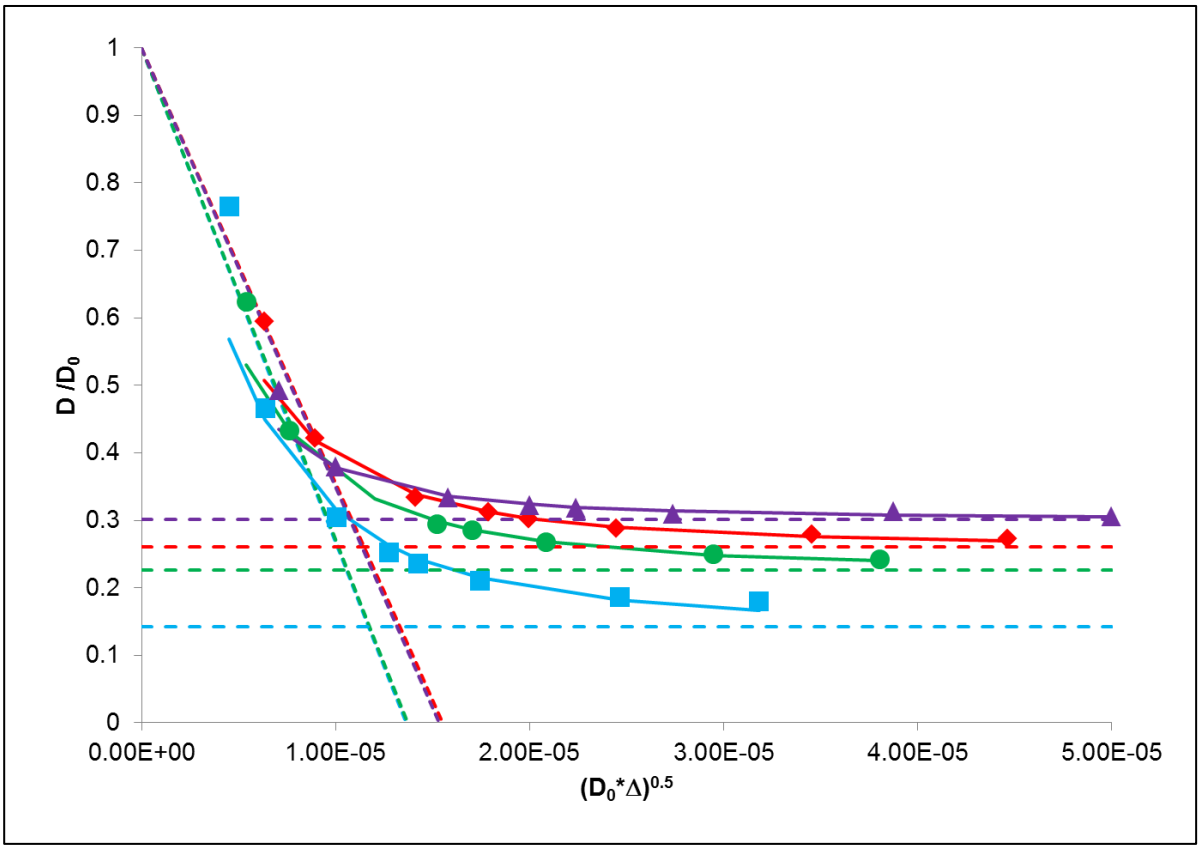


Figure 8.8: Diffusion decay of water in Mozzarella measured using a stimulated echo PGSE sequence in the transverse direction as a function of the observation time. [■ 20°C, ● 35°C, ◆ 50°C and ▲ 65°C] (Dashed lines represent the fitting from the Pade approximate with the horizontal asymptote indicating the tortuosity and the sloped line indicating the surface to volume ratio).

The diffusion properties of water within Mozzarella cheese were observed to change with temperature, as illustrated in Figure 8.8. As the temperature increased the diffusion at the longer time scales of the experiment was found to increase, from a normalised diffusion of approximately 0.16 to 0.3. At these longer times the diffusion is limited by the tortuosity of the system (Song, 2009) and approaches the tortuosity asymptote. As the tortuosity of porous media is related to the connectivity of the pores (Latour et al., 1995), the diffusion results for Mozzarella indicate that the connectivity within the casein matrix decreases with temperature. As a result, the complex path that a water molecule within the cheese takes over a given time period decreases in complexity as the temperature rises.

At a short time scale the diffusion is proportional to the surface to volume ratio of the pores (Latour et al., 1995; Mitra et al., 1992; Song, 2009). In Mozzarella there was a shift in the surface to volume ratio between 35 and 50°C as calculated using Pade approximate. This indicates a modification in the size of the pores that the water occupies within the cheese.

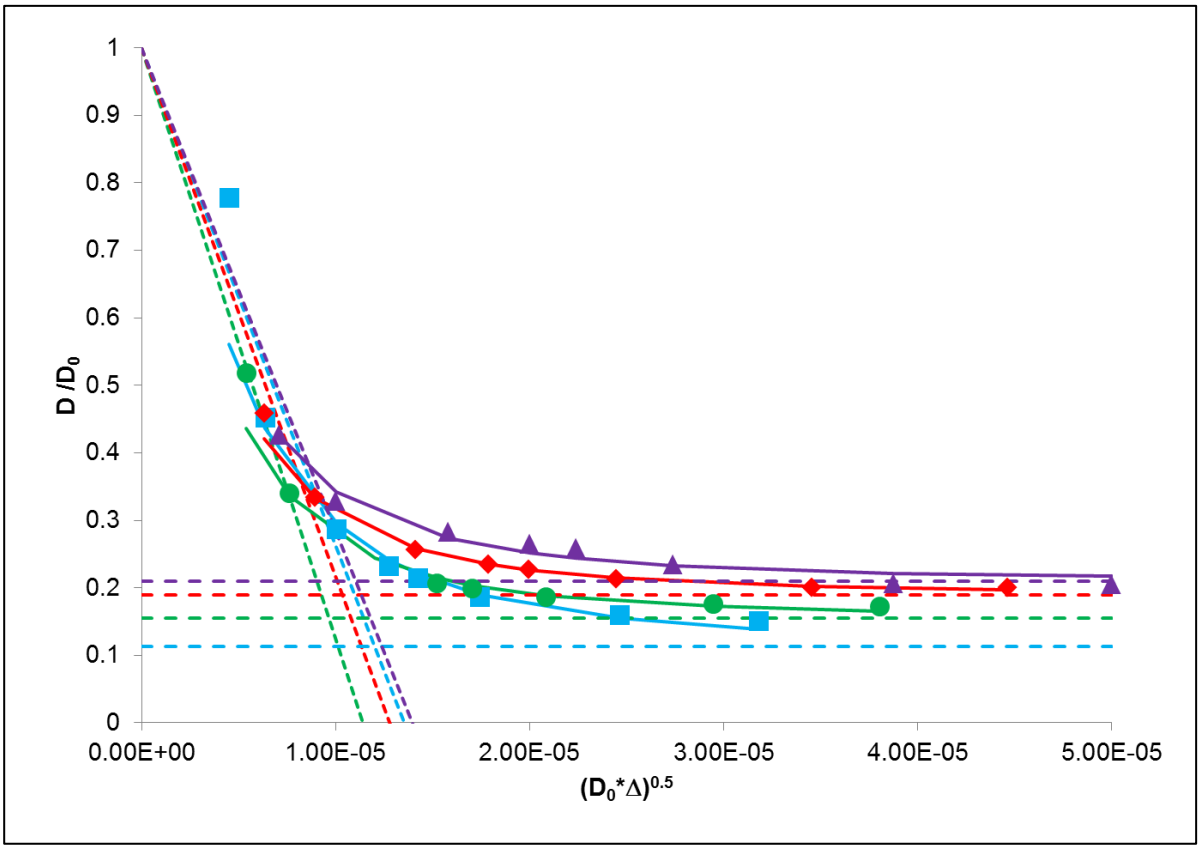


Figure 8.9: Diffusion decay of water in medium Cheddar measured using a stimulated echo PGSE sequence in the transverse direction as a function of the observation time. [■ 20°C, ● 35°C, ♦ 50°C and ▲ 65°C]

The diffusion of water within the samples of medium Cheddar was also found to be temperature dependant, as shown in Figure 8.9. As the temperature was raised the diffusion was observed to progressively increase, most notably at a longer time scale. This difference in the diffusion at a longer time scale indicates that the tortuosity is decreasing with temperature, as was the case for Mozzarella, however, the change is less pronounced.

The slope at short time scales, used to predict the surface to volume ratio, indicated a greater degree of variation between the different temperatures for the medium Cheddar than the Mozzarella samples assessed. This is indicative of greater changes in the surface to volume ratio of the pores within the medium Cheddar sample during heating in

comparison to the Mozzarella sample. This is likely a result of the differing structures of the two cheese types influencing the nature of the pores where the water resided.

It is conceivable that the surface to volume ratio is dominated by the pores that the free water is present in, the interstitial space surrounding the fat caused by the contraction of the protein, due to the size of these pores. Surface to volume ratio = pores surrounding fat + pores within protein; with the pores surrounding the fat being much larger than those within the protein matrix. The key difference between the two cheese varieties in this regard is the presence of channels in the Mozzarella in comparison to the more globular fat clusters within Cheddar. The difference in the structure of the two cheeses is likely to have an impact on how the pore size changes with temperature, as discussed in more detail later.

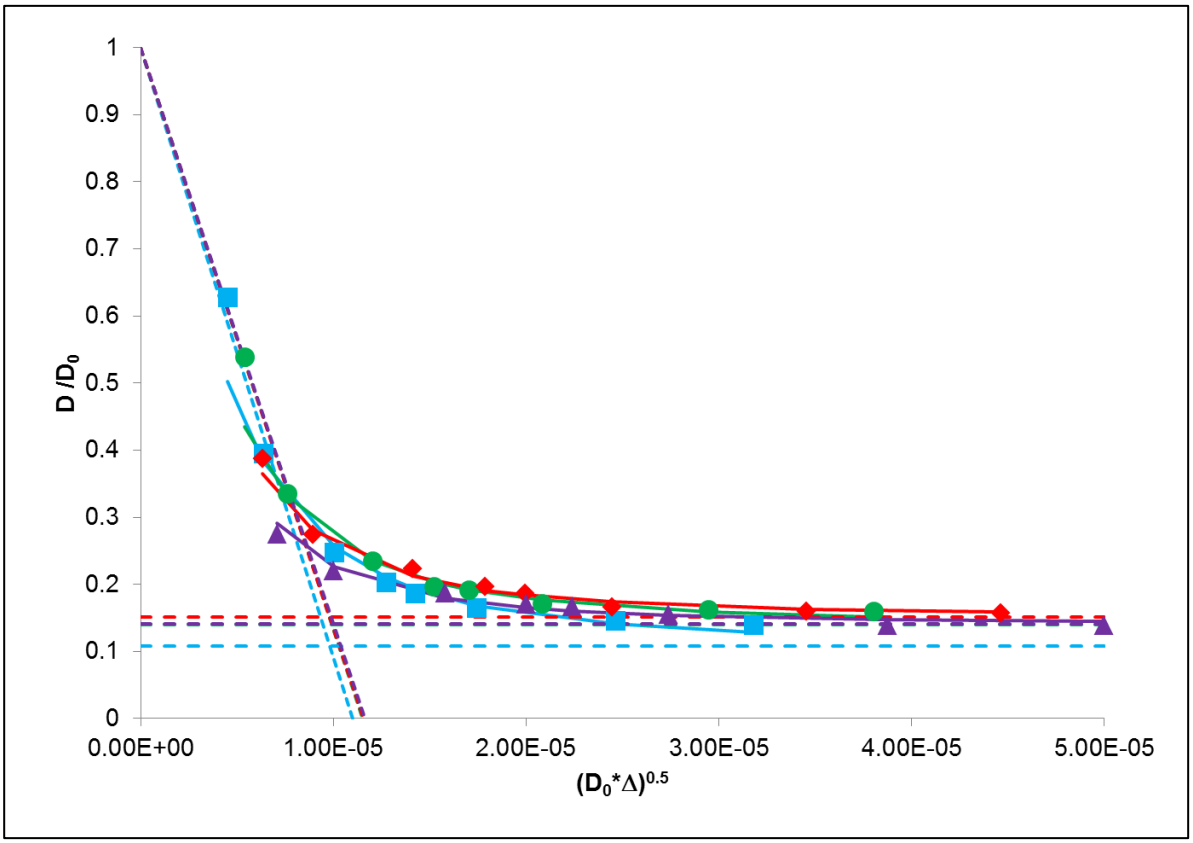


Figure 8.10: Diffusion decay of water in aged Cheddar measured using a stimulated echo PGSE sequence in the transverse direction as a function of the observation time. [■ 20°C, ● 35°C, ♦ 50°C and ▲ 65°C]

By inspection, the diffusion curves for the aged Cheddar sample (Figure 8.10) were identified as being very similar at each temperature, with much less separation apparent on inspection in comparison with the other two cheeses. Unlike Mozzarella and the medium Cheddar samples, the progressive change in the tortuosity was much smaller in the aged Cheddar sample. The fitted tortuosity asymptote ($1/\alpha$) at the different temperatures were found to be closer in proximity than the other two cheeses. This is possibly related to the weaker gel structure that is a result of proteolysis (Tunick, Malin, et al., 1993) making the cheese less susceptible to temperature induced changes. This would result from having fewer connections within the casein matrix limiting the degree of contraction that can occur at elevated temperatures. Based on the linear fitting from the

Pade approximate a change in tortuosity was evident between 20 and 35°C. However, above this temperature very little difference was noted.

The slope at short time scales for the aged Cheddar sample was found to be similar at the different temperatures. This indicates that the surface-to-volume ratios of the pores within the aged Cheddar did not vary greatly with temperature. Additionally, the surface to volume ratio was significantly different to the medium Cheddar sample which did exhibit variation between the different temperatures.

The diffusion properties of the three cheeses were compared at each of the temperatures studied (Figure 8.11 and Figure 8.12).

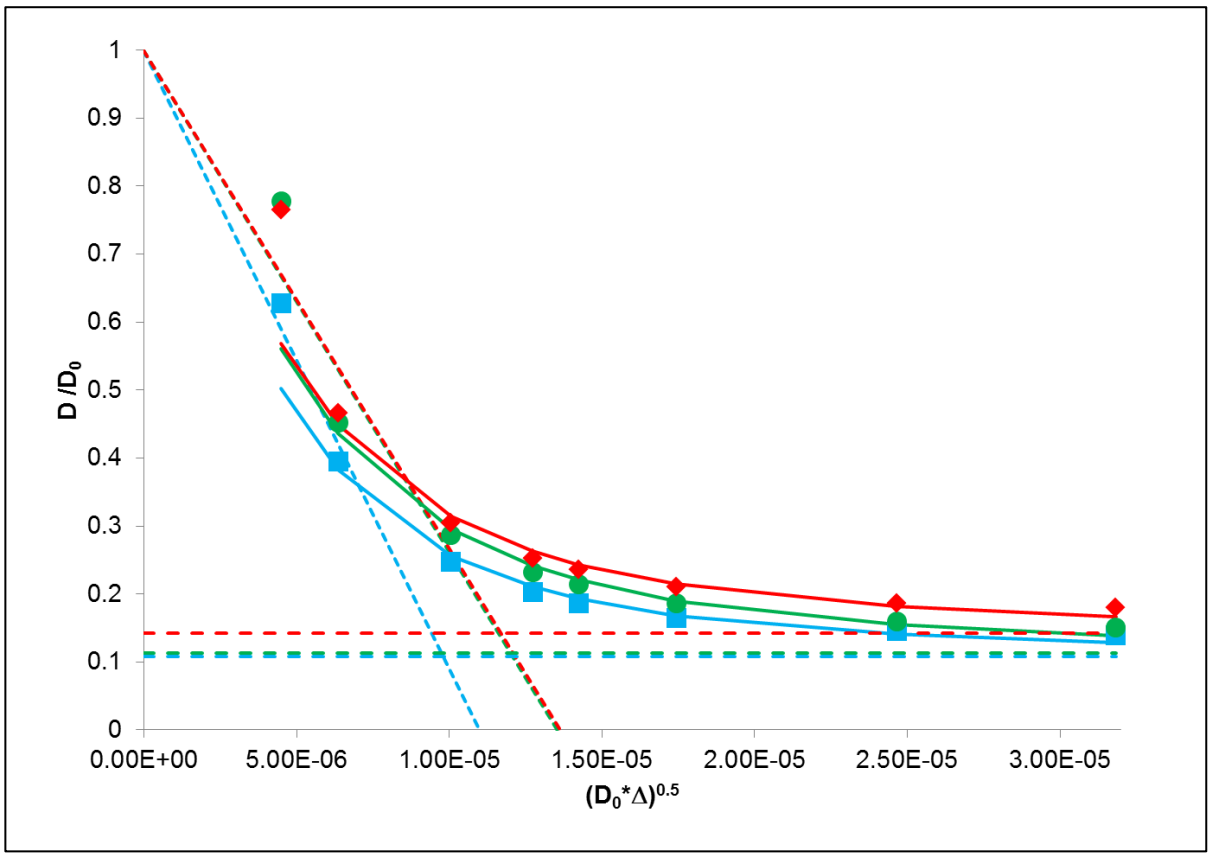


Figure 8.11: Diffusion decay of water in the three cheese samples at 20°C using D_0 = 2.03e^{-9} m^{2}s^{-1}. [■ Aged Cheddar, ● Medium Cheddar and ♦ Mozzarella]

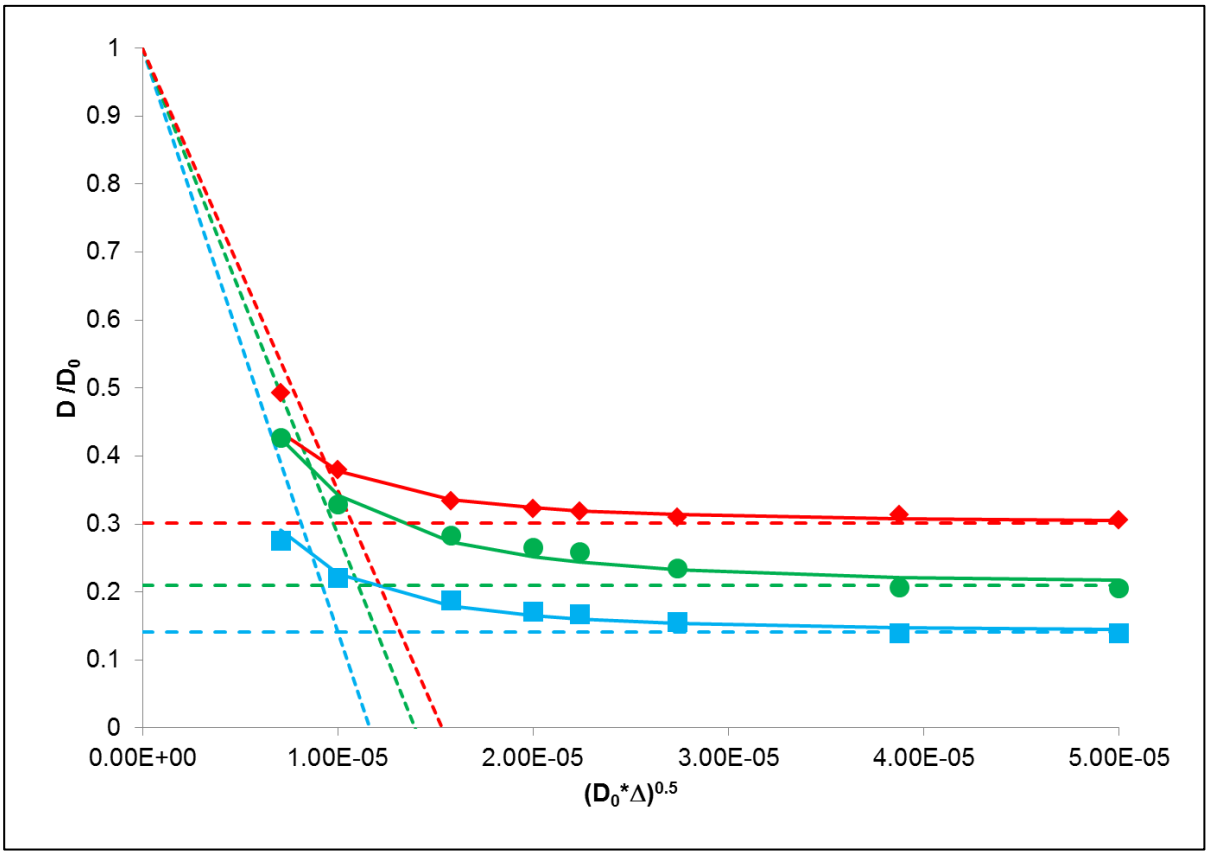


Figure 8.12: Diffusion decay of water in the three cheese samples at 65°C. Fittings using estimated $D_0 = 5.00e^{-9}$ m^2s^{-1}. [■ Aged Cheddar, ● Medium Cheddar and ♦ Mozzarella]

An analysis of the diffusion data collected at 20°C the for the three cheese samples shows that the Mozzarella data is slightly higher than that of the medium Cheddar, which in turn is slightly higher than the aged Cheddar. Even with the differences that exist in the structure of the cheeses, Mozzarella and Cheddar had similar diffusion curves at 20°C. Also no discernible difference was noted between the diffusion at a long time scale for medium and aged Cheddar samples. This suggests that the proteolytic breakdown, where by the casein is broken into fragments (Coker et al., 2005), has little effect on the diffusion of water at 20°C in cheese at a long time scale. However, it may be responsible for the differences in the surface to volume ratio between the medium and aged Cheddars.

The diffusion curve for Mozzarella was observed to be slightly higher than the two cheddar samples. A possible explanation for this behaviour is that a relationship exists between water self-diffusion and dry matter concentration (Métais, Cambert, Riaublanc, & Mariette, 2004). As the Cheddar cheese samples have a greater quantity of dry matter than the Mozzarella, the ability of water to diffuse is restricted.

As the temperature increased from 20 to 65°C a progressive separation of Mozzarella's diffusion curve was identified from the Cheddar samples, as the diffusion of water within Mozzarella increased with temperature. At both 50 and 65°C a separation was observed in the diffusion curve of the medium Cheddar from the aged Cheddar samples. The increase in diffusion indicates that the water within the cheese structure can diffuse more easily.

At 65°C the diffusion decay for the three cheese samples was found to differ markedly (Figure 8.12). At the longer time scale of the experiment the diffusion is higher in the Mozzarella than the other two cheese samples. This means that the water molecules within the Mozzarella sample have a less tortuous path when diffusing through the cheese structure than the Cheddar samples. This is possibly due to the organised fibrous structure that exists within Mozzarella with the presence of channels where water can pool. When the cheese is heated, the hydrophobic interactions increase in strength (Bryant & McClements, 1998) causing the casein matrix to contract and forcing out some water into a free state. This free water forms pools that surround the fat within the channels (Kindstedt, 2007). The long channels of free water are likely to increase the ability of water to diffuse within the cheese structure. The lower solids content in the Mozzarella may also influence the diffusion of water at this temperature due to a higher dry matter percentage increasing the tortuosity of a sample (Métais, Cambert, Riaublanc, & Mariette, 2004).

The water in the medium Cheddar was identified as having a higher rate of diffusion than the aged Cheddar sample. The primary difference between these two cheeses is age: aged

Cheddar is approximately 9 months old while the medium Cheddar is 2 months old. With the extended period of maturation the aged Cheddar will likely have undergone a greater amount of proteolysis; however, the extent of proteolysis was not measured. The greater period of proteolysis will result in a weakening of the casein matrix (Tunick, Malin, et al., 1993) allowing the fat within the cheese to pool together and form larger clusters. The larger fat clusters in the aged Cheddar may hinder the movement of water molecules through the cheese matrix causing the reduced level of diffusion. Additionally, extended proteolysis results in an increase in soluble peptides in the aqueous phase and therefore the water with pores in an aged Cheddar will be structured by the peptides and have a higher viscosity compared to medium Cheddar. An increase in viscosity of the aqueous phase would influence the Pade approximate as it would depress the value of D_0.

At the shorter time scale of the experiment a separation was also identified in the linear fitting related to the surface to volume ratio. This indicates that all three cheese samples have a different surface to volume ratio at 65°C. To further interpret the changes occurring in the tortuosity and porosity of the system, the results obtained from the Pade approximate, Equation 8-2, are displayed below.

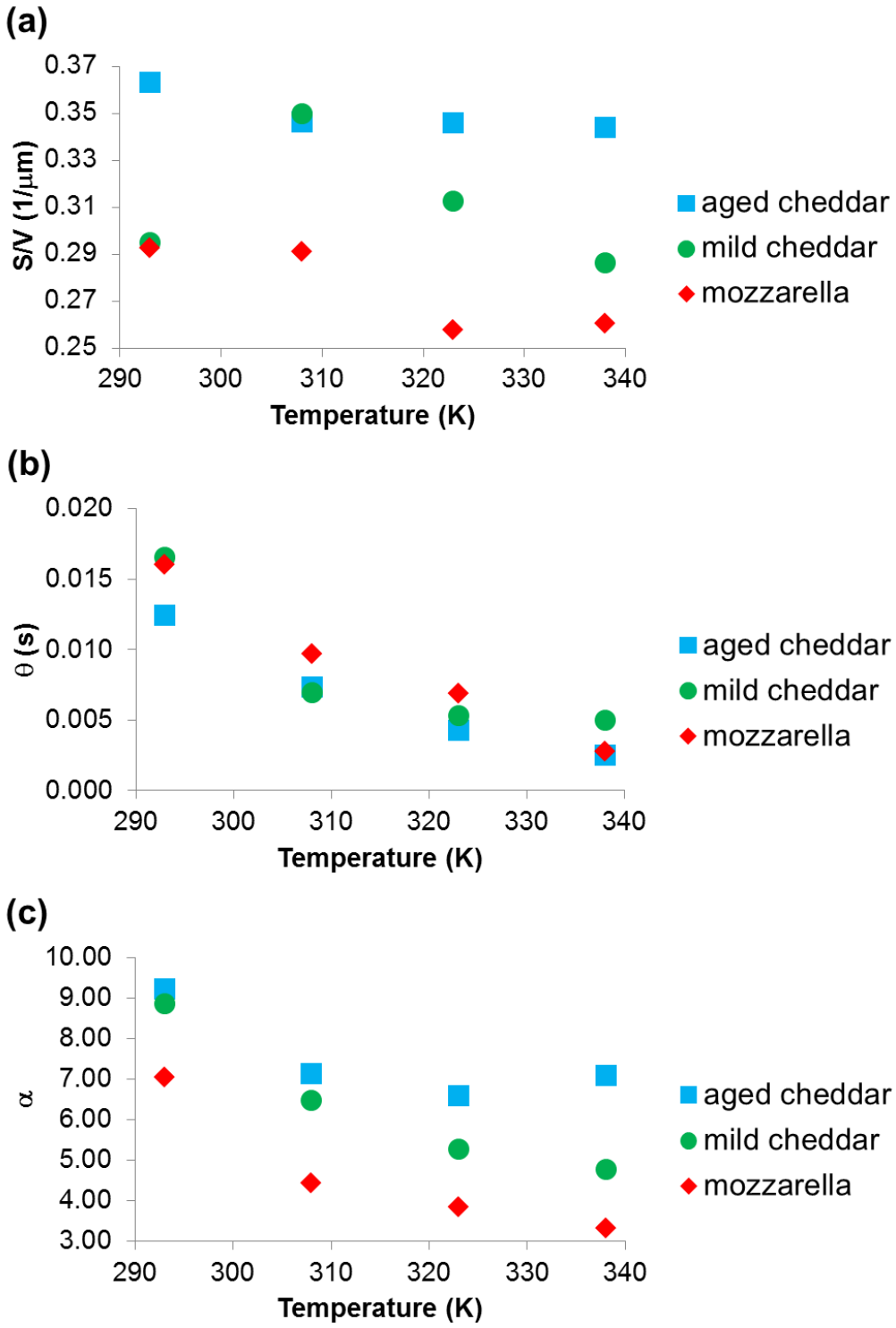


Figure 8.13: Change in the parameters for the Pade aproximate with temperature:a) Surface to volume ratio (S/V), b) time constant (θ) and c) tortuosity (α). [■ Aged Cheddar, ● medium Cheddar and ◆ Mozzarella]

Figure 8.13 shows the change in the surface to volume ratio (S/V_p), time constant (θ) and tortuosity (α) for the three different cheeses at each of the temperatures investigated.

The surface to volume ratio/temperature profile varied between each of the three cheeses. These variations are likely to be dominated by the pores occupied by both the free water and fat. In the aged Cheddar sample, a decrease in S/Vp was observed between 20 and 35°C after which the surface to volume ratio remained relatively constant. The surface to volume ratio for the medium Cheddar sample initially increased from 20 to 35°C followed by a decrease as the temperature was further increased. A decrease in the surface to volume ratio was observed in Mozzarella between 35 and 50°C, however, between 20 and 35°C as well as 50 and 65°C very little change was evident. The surface to volume ratio is related to the size of the pores within a system (Brown et al., 2012; Mitra et al., 1992), with the surface to volume ratio being inversely proportional to pore size. As Mozzarella was observed to have the lowest surface to volume ratio at temperatures above 20°C, it indicates that the size of the pores within the cheese is larger. This suggests that the surface to volume ratio is dominated by the size of the interstitial space that free water occupies, surrounding the fat, due to the contracting protein matrix. This pore space surrounding the fat within Mozzarella is much larger than that of Cheddar due to the channel structure that exists.

The increase in the surface to volume ratio in medium Cheddar between 20 and 35°C is possibly due to a relaxing of the protein gel network as the fat melts. A relaxation in the protein network could result in the pore size, which the water exists in, decreasing slightly. The reason that this behaviour may not be exhibited in the older Cheddar sample is due to proteolysis. As the casein matrix is broken down into fragments (Coker et al., 2005), the matrix weakens (Tunick, Malin, et al., 1993) so may not relax to the same extent as the medium Cheddar. The decrease in the surface to volume ratio with temperature could be a result of the hydrophobic interactions strengthening with temperature (Bryant & McClements, 1998). The strengthening hydrophobic interactions

could cause the protein matrix to contract leaving a greater pore space for the free water to occupy.

The fitting parameter (θ) value for each of the three cheeses was found to decrease with temperature. This indicates that the time required for a particle to diffuse the distance required to reach the tortuosity asymptote (Brown et al., 2012) decreases as the temperature increases.

The tortuosity decreased from 20 to 65°C in all of the cheese samples. The rate of this decrease in all three samples was greatest between 20 and 35°C. The calculated tortuosity of each of the Cheddar samples was higher than the Mozzarella sample at each temperature in the study. The tortuosity relates to the degree of hindrance that a particle experiences when diffusing (Hrabe, Hrabětová, & Segeth, 2004; Price, 2009). A greater degree of curvature and constrictions in a porous space will result in a higher tortuosity (Heil & Holz, 1998; Hizi & Bergman, 2000). This implies that the pathway that a water molecule diffuses through is more complex in Cheddar than Mozzarella at each temperature studied due to a greater degree of hindrance. This is expected when one considers the random aggregation nature of curd formation in Cheddar compared to the structure aligning that occurs in Mozzarella through stretching.

The tortuosity of all three cheeses decreased as the temperature was increased during heating. The decreasing tortuosity with temperature is possibly due to a greater quantity of water within the pores surrounding the fat, resulting in a decrease in the overall hindrance of water molecules as they diffuse. The tortuosity in both the medium Cheddar and Mozzarella samples progressively decreased as the temperature was increased over the entire temperature range studied. The tortuosity of the aged Cheddar however decreased from 20 to 50°C but then increased slightly with temperature as heating continued to 65°C.

Another factor to consider when interpreting these results is the level of insoluble calcium in the different cheese samples. As insoluble calcium within the micelle mediates protein-to-protein interactions within the casein matrix (Lawrence et al., 1987; Lucey & Fox, 1993), lower quantities will lead to a weaker matrix with fewer connections (Joshi, Muthukumarappan, et al., 2004a). Therefore differences in the quantity of insoluble calcium between the different cheeses will impact on the structure of the cheeses and possibly the diffusion of water within each sample. However, as the quantity of insoluble calcium was not measured in these samples any differences are not known.

Factors that could influence the Pade approximate calculations of the diffusion data include 'impurities' (compounds in the aqueous phase that are not water) within the aqueous phase of the cheese. This is because the D_0 value used in the equation is based on pure water diffusing freely. However, impurities in the water, such as lactose and salts in cheese, modify the viscosity of water (Heinrich-Schramm, Buttersack, & Lüdemann, 1996). The release of peptides as a result of proteolysis may also have an impact on the actual D_0 value for the water in the cheese. The diffusion of a solvent is influenced by the viscosity, with diffusion being inversely proportional to viscosity (Einstein, 1905) as defined by the Stokes-Einstein equation. Therefore the impurities in the water will have an effect on the D_0 value used in the experiments by depressing it.

8.3.3 General discussion

Both NMR and rheological analysis identified changes in Mozzarella and Cheddar during heating. The complex viscosity of all of the cheese samples was found to progressively decrease as the samples were heated. This is due to structural changes occurring in the cheese as they were heated resulting in the cheese samples becoming less viscous.

The differential analysis of the Arrhenius plot identified the rate of change in the complex viscosity with temperature. The Cheddar samples both had a greater degree of variation

than the Mozzarella samples, possibly due to the less ordered structure in comparison to the Mozzarella. However, between 30 and 60°C the Arrhenius plots for the two Cheddar samples behaved in a linear manner and without a reference point it was difficult to draw solid conclusions from the trends or variation. The Mozzarella sample had a similar overall shape to the differential analysis of the functional Mozzarella in Section 7.3.1. The curve did, however, differ from those in Section 7.3.1 with the peaks being slightly different. This is likely to be the result of the different: composition of cheeses, maturity of cheese samples and a slightly different method used to assess the samples including a slower heating rate.

All three cheeses had an increase in the percentage free water as they were heated from 20 to 65°C. This increase in free water is likely driven by the increase in the strength of hydrophobic interactions as the temperature was raised (Bryant & McClements, 1998; Joshi, Muthukumarappan, et al., 2004a). The increase in the hydrophobic interactions within the casein matrix will result in the hydrophobic regions becoming closer in proximity and thus decreasing the space that water can occupy within the protein. The two Cheddar samples had a greater quantity of free water than the Mozzarella, possibly as a result of the difference in the quantity and distribution of fat in the Cheddars allowing a greater number of sites for water to pool.

The increasing quantity of free water during heating may be linked to the slight decrease in the slope of the Arrhenius plot of the Mozzarella sample between 35°C and 45°C. As more free water is expressed from the protein matrix it will become more rigid which may result in the reduction in the rate of decrease of the complex viscosity. The relaxation time constant for the free water in each of the cheese samples was also identified as increasing with temperature. This increase in the relaxation time constant indicates that the free water within the cheese is becoming more mobile (Kuo et al., 2001).

The diffusion properties of water within the cheese was found to vary with temperature and between the different cheeses studied. The change in the diffusion curves indicated that the diffusion of water within Mozzarella was influenced by temperature to a greater extent than the Cheddar samples. It also indicated that the diffusion of water within the medium Cheddar had a greater amount of temperature dependency than the aged Cheddar sample, with the diffusion in medium Cheddar increasing with temperature.

At a longer time scale the diffusion is limited by the tortuosity of the system (Song, 2009), while at a shorter time scale the diffusion is proportional to the surface to volume ratio of the pores in a medium (Latour et al., 1995; Mitra et al., 1992). The Pade approximate, Equation 8-2, allowed the tortuosity, surface to volume ratio and time fitting parameter to be estimated (Brown et al., 2012; Mair et al., 2001). This allowed the temperature dependency of these parameters in each of the cheese samples to be assessed.

The surface to volume ratio of the pores within the cheese samples were found to vary with temperature. The ratio is likely to be dominated by the pore space that the free water occupies around the fat. This is due to the size of these pores being an order of magnitude larger than those existing in the protein matrix. The decrease in the surface to volume ratio for the cheeses could be a result of the strengthening hydrophobic interactions as the temperature was progressively elevated (Bryant & McClements, 1998). The strengthening hydrophobic interactions will result in a contraction of the casein matrix, resulting in an increase in the size of the pores that the free water can occupy. The decreasing trend in the surface to volume ratio indicates that the size of the pores is increasing. The lower surface to volume ratio of Mozzarella at temperature above 20°C indicates that the pore size is larger, likely due to the pores in which free water resides being long channels. An increase in the surface to volume ratio in the medium Cheddar sample between 20 and 35°C, which was not observed in the aged Cheddar, could be the result of a relaxation in the protein matrix as the fat melts. As the aged Cheddar has undergone a greater amount of proteolysis, resulting in a weaker gel structure (Tunick,

Malin, et al., 1993) due to fewer connections, the melting of fat does not have the same impact on the weaker protein matrix. This along with a possible reduction in ionic calcium in comparison with the medium Cheddar will weaken the casein matrix making it less susceptible to temperature, with less impact on the surface to volume ratio.

The time constant (θ) for each of the three cheese samples was found to progressively decrease with temperature. As this parameter is related to the time required to reach the tortuosity asymptote of a system (Brown et al., 2012), it indicates that the water molecules diffusing within the cheese require less time to reach this limit as the temperature is increased.

The difference in the tortuosity between the cheese samples at each temperature showed that Mozzarella was consistently less tortuous than the other cheeses. A number of factors could be responsible for this difference in tortuosity including: compositional factors such as fat and moisture as well as differences in the level of maturity. Fat has an effect on the tortuosity of a system causing a decrease in the diffusion of water (Métais et al., 2004). This is due to fat hindering the movement of water through the pores within the cheese. As the Cheddar samples have a higher fat content than Mozzarella (32% compared to 21%), the fat content is one factor resulting in their higher tortuosity. The percentage dry matter in the Cheddar samples will also result in a higher tortuosity, as a greater amount of dry matter restricts diffusion (Métais, Cambert, Riaublanc, & Mariette, 2004).

One of the key factors influencing the difference between the Mozzarella and the two Cheddar samples is the fibrous structure of Mozzarella. The thermo-mechanical processing step aligns the casein into roughly parallel fibres interrupted by fat and serum (Kindstedt, 2007). The stretching process is likely to disrupt the structure on the proteins that make up the gel network, renneted casein micelles. The force applied during stretching could result in the deformation of the roughly spherical micelles so that they

become elongated. These smeared micelles are jammed together during the stretching process so that long channels are formed, with fat globules and free water present within the channels. Over time most of the free water is absorbed into the protein matrix leaving the fat in contact with the protein fibres. This fibrous structure is responsible for the behaviour of components in Mozzarella during heating. At elevated temperatures strengthening hydrophobic interactions will force free water out of the casein matrix into the channels. This is as a result of a contraction of the casein matrix as the hydrophobic regions of the proteins get closer in proximity. The contraction of the protein reduces the points of contact and lubricated by the molten fat in the channels, the cheese can flow.

A number of factors differ in terms of the rheological temperature sweep and that conducted using NMR. One key difference is the flow and fat loss that occurs in rheology as the cheese is melted. This differed from the NMR measurements, were samples of cheese were in the form of a plug that completely filled the sample holder and thus is constrained within the sample holder preventing fat losses and flow. Another difference between the two measurement techniques was in regards to heating. In the rheological assessment a temperature ramp was used, whereas in NMR measurements were taken at specific temperatures after the sample had reached the desired temperature and allowed to equilibrate. However, information gained from the NMR data can be used to help interpret changes in the rheological profile of the cheese samples.

This preliminary investigation identified some important changes in the relaxation and diffusion properties in cheese. However, in order to gain a greater degree of insight from these experiments I would recommend that they be repeated with a larger number of samples and replicates. Additionally using samples where a greater amount of information regarding composition, manufacture and degree of proteolysis would be advantageous.

8.4 Conclusion

During heating the structure of cheese changes which impacts on the functional properties. The complex viscosity of Mozzarella and the two Cheddar samples changed with temperature, with a number of structural changes responsible for changes in the rate of change in viscosity. Magnetic resonance indicated that as cheese is heated there is a progressive increase in the quantity of free water present in the cheeses structure. The diffusion properties of water within the structure allowed information relating to the tortuosity and surface to volume ratio of pores, indicating that these properties are temperature dependant. These results indicate that significant information regarding the structural dynamics of various constituents of cheese can be monitored during heating using magnetic resonance.

As Mozzarella undergoes significant structural rearrangement following manufacture whereby the distribution of water changes, as shown in Chapter 5, a short shelf life study using NMR and heating would be advantageous.

9 Probing water migration and heat induced changes in Mozzarella cheese during early shelf life using magnetic resonance techniques

9.1 Introduction

The physical state of water within a food system influences both its structural and functional properties (Godefroy, Korb, et al., 2003). The water in cheese is either associated with the protein (casein) or located in pools near or within the fat globule aggregations (Godefroy, Korb, et al., 2003). The distribution of water within Mozzarella differs from other cheeses due to the stretching process during manufacture (Guo & Kindstedt, 1995). Following manufacture free water is present within the channels, where it surrounds the fat, and is gradually reabsorbed into the protein matrix over the first few weeks of storage (Kuo et al., 2001).

The previous experiment indicated that magnetic resonance techniques are useful for characterising the relaxation and diffusion properties of water within cheese during heating. As the distribution of water changes in Mozzarella over the first few weeks of storage, the evaluation of the changes in diffusion and relaxation properties over this time would be advantageous.

There have been numerous studies using NMR to assess changes in Mozzarella cheese systems, the majority of which have investigated changes in the relaxation properties of water. These have included studies investigating changes in water mobility during storage (Kuo et al., 2001), the effect of freezing (Kuo et al., 2003) as well as studies investigating age related changes in water mobility in traditional buffalo Mozzarella (Gianferri, D'Aiuto, et al., 2007). The use of NMR techniques to study the diffusion of components within cheese has received limited attention, with the noticeable exception of Callaghan, Jolley, and Humphrey (1983) who assessed the diffusion of water and fat in Cheddar and Swiss cheese. The majority of work related to diffusion in cheese has involved the use of

relaxation diffusion correlations to investigate structural dynamics of cheese. These studies include the comparison of various dairy products (Hurlimann et al., 2006), investigating the effect of salt contents in Mozzarella with aging (Hubbard et al., 2005) and examining the effect of age on the dynamics of Mozzarella and Gouda at 5 and 40°C (Godefroy, Korb, et al., 2003). These correlation techniques utilize 2D inverse Laplace inversion NMR to probe the intricate nature of heterogeneous systems, assessing the complex multi-exponential behaviour of their diffusion and relaxation rates (Hubbard et al., 2005).

The objectives of this study were to:

- Use magnetic resonance to monitor changes in the relaxation and diffusion properties of water in a brine salt Mozzarella during the first 18 days following manufacture.
- Assess heat induced changes in the diffusion and relaxation properties of water present within Mozzarella cheese using NMR over an 18 day period.

9.2 Material & Methods

9.2.1 Material

A 5 lb (2.27 kg) block of low moisture part skim (LMPS) Mozzarella was provided by Foremost Farms (Foremost Farms USA, Baraboo, WI), immediately following the brine salting step of manufacture.

Table 9-1: Composition of LMPS Mozzarella according to product labelling.

	Moisture (%)	Fat (%)	Protein (%)	Sodium (%)	Calcium (%)
LMPS Mozzarella	46.0	23.1	26.9	0.7	0.7

9.2.2 Magnetic Resonance

Sample preparation for the magnetic resonance experiment was based on the method described in Section 8.2.3 using the same Bruker Advance300 NMR (Bruker, Rheinstetten, Germany). As with Chapter 8, the temperature was progressively increased in 15°C increments from 20°C to 65°C before being cooled back to 20°C. Measurements were taken at each temperature following a gradual temperature increase and a 10 minute equilibration period. Experiments were conducted in duplicate over an 18 day storage trial.

9.2.2.1 *Relaxation*

The changes in the relaxation properties of water were monitored over the storage trial. T2 relaxation measurements were performed using the Carr Purcell Meiboom Gill (CPMG) spin echo pulse sequence (Carr & Purcell, 1954; Meiboom & Gill, 1958), as done in Section 8.2.3.2, using Equation 9-1 below.

$$\boldsymbol{I = Ae^{(\frac{-t}{T2a})} + Be^{(\frac{-t}{T2b})}}$$

Equation 9-1

A and B represent the proton intensity that is proportional to the amount of water in the sample, with A being the representing the proportion of water associated with the protein and B representing the proportion that is free. T_{2a} and T_{2b} represent the relaxation time constant for the two fractions of water present in the cheese (Kuo et al., 2001).

9.2.2.2 *Diffusion*

The diffusion properties of water within the Mozzarella sample were monitored over the 18 day storage trial. This was done using the technique described in Section 8.2.3.3.

However, a three point diffusion experiment was run at each temperature at observation times (Δ) of 50, 150 and 300 ms and a gradient pulse duration (δ) of 1 ms.

9.2.2.3 *Diffusion T2 relaxation correlations (DT2)*

The diffusion/relaxation correlations were produced by using a Pulsed Gradient Spin Echo (PGSE) followed by a Carr-Purcell-Meiboom-Gill (CPMG) echo train (Godefroy, Creamer, et al., 2003; Hubbard et al., 2005). The gradient was varied between 0 Tm^{-1} and 1.482 Tm^{-1} in 16 linearly spaced increments. The tau (τ) between the 180 degree pulses used for these experiments was 128 ms. The data collected has the form of Equation 9-2 below.

$$M(g,t_2)=\iint M_0(D,T_2)\exp\left[-\gamma^2 g^2 \delta^2 D\left(\Delta-\frac{\delta}{3}\right)\right]\exp\left(\frac{-t_2}{T_2}\right)dDdT_2+E(g,t_2)$$

Equation 9-2

A two-dimensional Laplace inversion was used to obtain the $M_0(D,T_2)$, the spectra of molecules as a function of both diffusion and T2 (Callaghan, Godefroy, & Ryland, 2003). The T2 measurements were determined using a "one-shot" CPMG sequence. In this experiment, spectral information is sacrificed in favour of experimental time. Rather than obtaining the full spectral information after varying numbers of refocusing pulses, the amplitude of the echo signal between each refocusing pulse is collected.

The D_0 values of free water used at each temperature to normalise the diffusion coefficient are displayed in Table 9-2 below.

Table 9-2: D_0 at each temperature of the study

Temperature (°C)	D_0 ($x10^{-9}$ m^2s^{-1})
20	2.03
35	2.90
50	3.98
65	5.00

9.3 Results & Discussion

As with the previous experiment in Chapter 8 this work was carried out at Montana State University in the Magnetic Resonance Group. Based on the results of the previous chapter a freshly manufactured Mozzarella was sought to monitor over an 18 day period. The cheese that was obtained differs from fresh Mozzarella studied in New Zealand due to being a brine salted cheese. Details of the differences are discussed in Section 2.2.1.6. These cheeses typically have salt and moisture gradients that exist within them immediately following manufacture which eventually reach equilibrium. The presence of these gradients within the cheese sample not only results in compositional inhomogeneities, but also influence factors associated with structural changes including proteolysis. In order to gain a representative sample of the cheese block, duplicate samples were taken to maximise the effect of variation within the block. This was done by taking a sample 2 cm from the edge of the cheese block and a sample 2 cm from the centre of the block on each day of testing.

Due to the number of measurements that were conducted at each temperature the number of samples that could be analysed on each day of the storage trial was limited to two. This was due to the full temperature sweep with all of the measurements taking close to 12 hours to run per sample. To reduce the duration of the experiments to the 12

hour period a number of concessions were made in the design of the methods used including reducing the number of diffusion times to three per temperature. This still allowed information about the diffusion of water within the cheese to be ascertained, however, the number of data points was reduced. Additionally, the relaxation pulses used for the DT2 mapping were done using a CPMG 'one shot' technique. This sacrifices spectral information for experimental time. The down side of this is that free water could not be distinguished; rather only a peak for the fat and a single peak for water in the cheese samples could be resolved.

9.3.1 Relaxation

9.3.1.1 *Relaxation at room temperature*

The quantity of free water present in the Mozzarella sample at 20°C was monitored using T2 relaxation measurements. This was calculated as the percentage of water that exists in the more slowly relaxing of the two populations identified using CPMG, as free water has a longer relaxation time than water associated with protein (Altan et al., 2011).

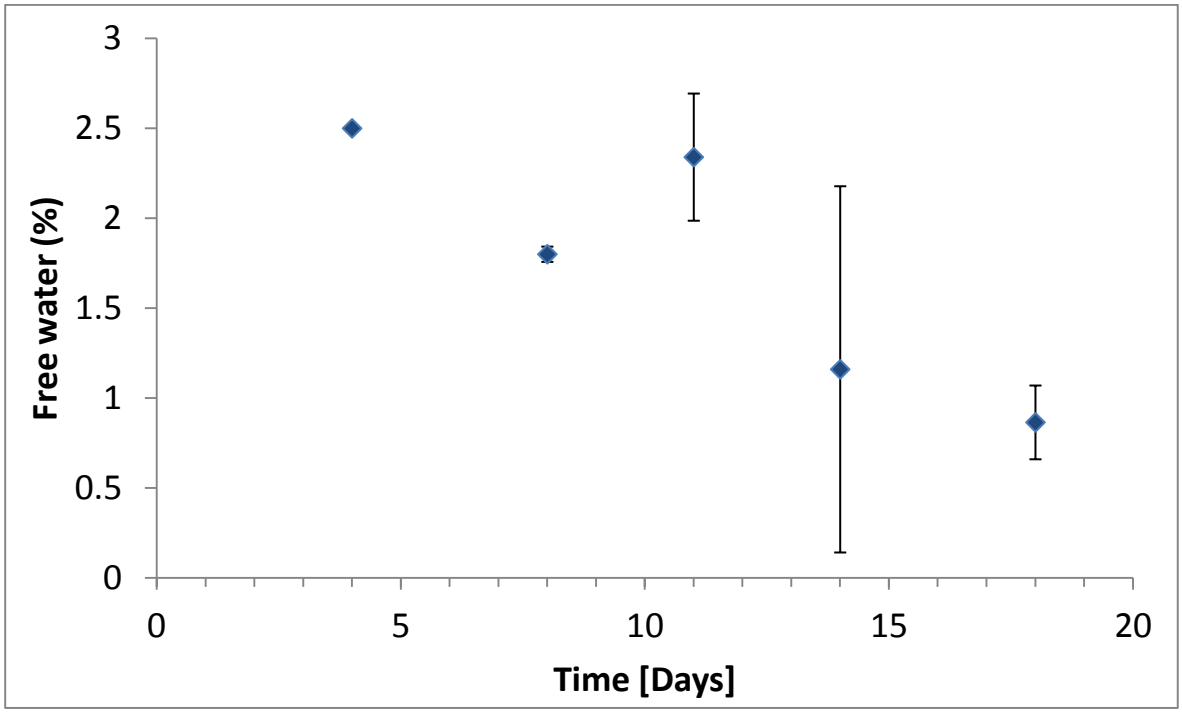


Figure 9.1: Change in free water in Mozzarella over 18 days. (Error bars = ± 1 standard deviation).

Figure 9.1 indicates a decreasing relationship between the quantity of free water present in Mozzarella and storage time, similar to the trend identified by (Kuo et al., 2001) and that identified in Section 5.3.4. This corresponds to a progressive increase in the quantity of water associated with the protein within the cheese. As discussed previously (Section 5.3.7), a number of mechanisms could be responsible for this change in the distribution of water within Mozzarella including the change in the strength of the hydrophobic interactions within the cheese following manufacture. The hydrophobic interactions increase in strength during stretching as a result of the concomitant high temperatures, forcing out some of the water entrapped within the casein. During refrigerated storage the hydrophobic interactions within the cheese weaken (Everett, 2007). As the protein relaxes, the water that exists in a free state can migrate back into the protein matrix.

Another factor that has been suggested to influence the re-absorption of water within Mozzarella is the change in the distribution of calcium (McMahon & Oberg, 2011). This

involves the solubilisation of calcium within the cheese resulting in a decrease in calcium-mediated protein-to-protein interactions. A decrease in the number of these interactions will lead to a looser gel network (Joshi, Muthukumarappan, et al., 2004a) that may influence the distribution of water within the cheese. This solubilisation of calcium during early storage has been suggested to be the result of the attainment of a pseudoequilibrium between the soluble and insoluble calcium present within cheese (Lee et al., 2010; O'Mahony et al., 2005). An ANOVA analysis was conducted to compare the level of free water on each day of the maturation trial. This gave a p-value of 0.067 which indicates that no significant difference exists between the quantity of free water on each day of the trial at a 95% confidence interval. However, a significant difference exists in the quantity of free water on the different days of the trial if α=0.1. This suggests that further replicates in a trial such as this would allow a more robust analysis of the significance of the trend. This was compromised in the case of this experiment for further pulse sequences to elicit further information regarding the dynamic changes in the state of water within the cheese. However, the earlier work (5.3.4) indicated a significant difference in the level of free water in Mozzarella during the maturation process.

Changes were also identified in the fraction of water associated with the protein in the cheese. Not only did the quantity of water associated with protein increase as the free water decreased, the range of relaxation times decreased as indicated by a shaper peak, suggesting a more homogeneous distribution of water within the cheese. The decrease in the distribution was in the form of shortening of relaxation times. This indicates that, not only is there movement of water from pooled areas to the protein, as has been discussed in Section 5.3.7, but from these experiments we have additionally shown that with storage the water within Mozzarella becomes more strongly associated with the protein phase. This indicates that there is probably a subtle rearrangement of the protein structure itself that allows the associated water to increase its degree of association. A factor that could be responsible for this greater level of association of water with the casein is proteolysis.

As the casein is progressively hydrolysed by residual enzymes within the cheese there is a cleavage of peptide bonds which results in the liberation of free carboxyl and amino groups that can structure water, reducing the water activity of cheese (Fox & McSweeney, 1996).

9.3.1.2 *Relaxation at elevated temperatures*

As Mozzarella is generally consumed in a molten state (Bertola et al., 1996b), the relaxation properties of water in the Mozzarella structure were investigated during heating.

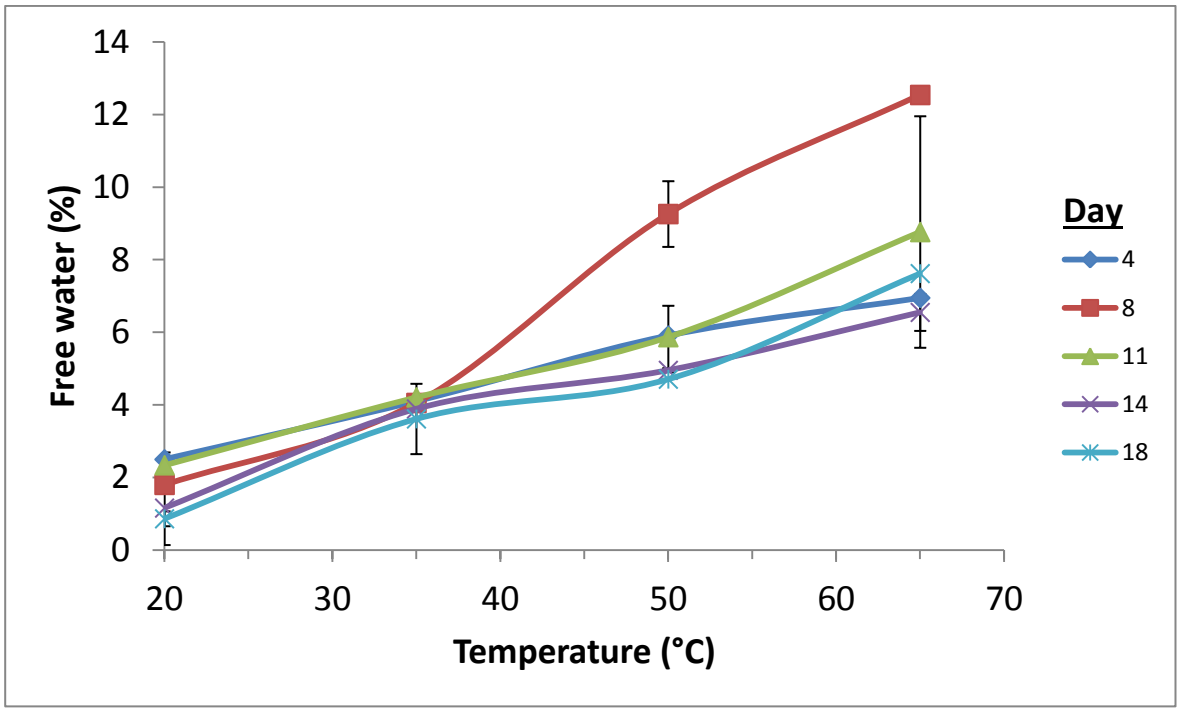


Figure 9.2: Effect of temperature on the quantity of free water in Mozzarella during storage. (Error bars = 1 standard deviation).

A progressive increase in free water was identified as the temperature was raised on each day studied, as shown in Figure 9.2. The data obtained on day 8 following manufacture indicated a significantly greater quantity of free water at 50 and 65°C in comparison to the other days studied. In addition to this a large error was identified in the data collected at 65°C on Day 11 of the trial. This was due to the first of the two measurements taken on

the day having a much higher quantity of free water than the second measurement. The first measurement identified the quantity of free water in the cheese to be similar in magnitude to that found on day 8 of the trial, while the second measurement indicated a lower quantity of free water in the system. This high level of free water at 65°C on day 8 of the trial is similar to trends identified in work in 10.3.10.1. As the temperature sweep and full cycle of measurements for each sample took approximately 12 hours to be carried out, this meant that half a day had elapsed between the two measurements. Over this time structural rearrangement within the cheese sample may have occurred resulting in the decreased level of free water expression.

The increase in free water as a result of temperature is likely to be due to strengthening hydrophobic interactions. Hydrophobic interactions increase in strength with temperature (Bryant & McClements, 1998). This increase in the strength of hydrophobic interactions will likely cause the casein matrix to contract as the hydrophobic areas within the protein get closer together. As the casein matrix in cheese is highly hydrated, the contraction will force some of the water contained within the casein out of the matrix. This water, forced out of the casein matrix, exists in a free state surrounding the fat within the system (Kindstedt, 2007).

Not only was there an increase in the quantity of water with a longer relaxation time (free water) but there was also a shift in the range of relaxation times for this population of water. This is characterised by the T2 relaxation time constant which relates to the relative molecular mobility of water molecules within a system compared with free water (Kuo et al., 2001).

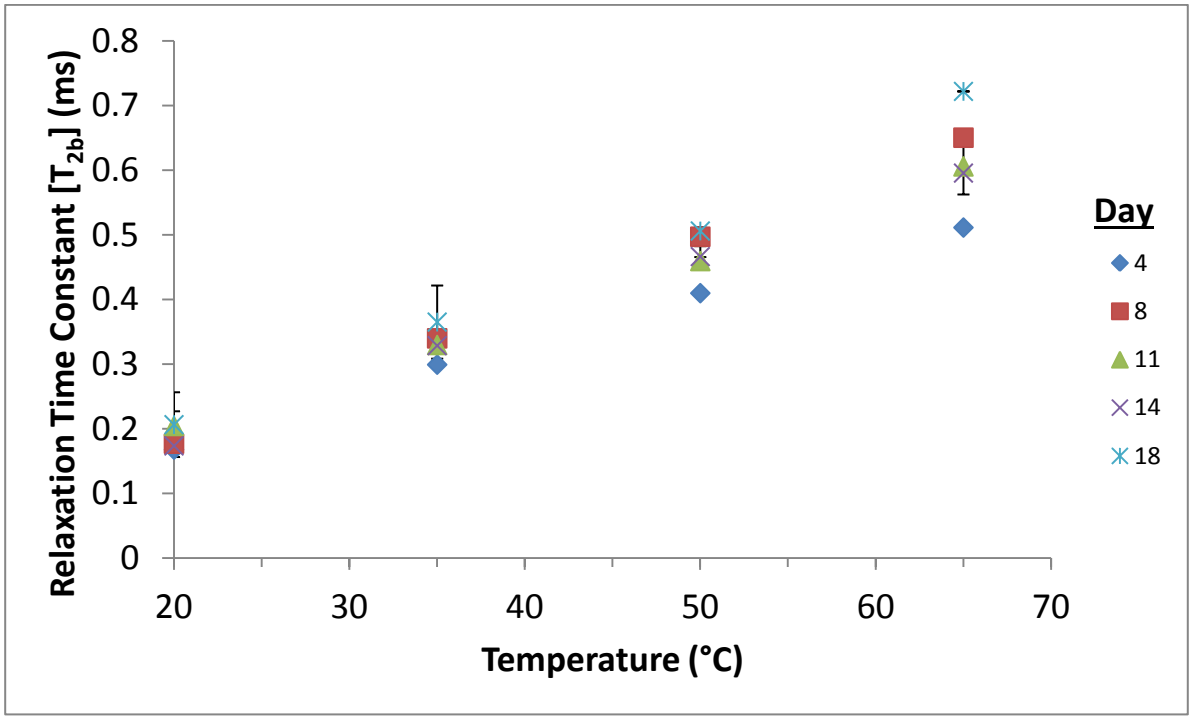


Figure 9.3: Change in the relaxation time constant for the free water in Mozzarella with temperature and storage. (Error bars = ±1 standard deviation).

The progressive increase in the relaxation time constant (T_{22}), illustrated in Figure 9.3, indicates that the free water within the Mozzarella is becoming increasingly more mobile with temperature. As the temperature is raised, the water molecules gain a greater amount of energy and thus a greater degree of mobility. Another factor that could influence the increase in the relaxation times is in regards to the structural changes occurring within the cheese. As the temperature is raised a greater amount of free water can occupy the channels. This expansion of the channels will result in a larger proportion of water existing in the free state, where it is not hindered by the protein or fat interfaces.

The increase in the quantity of free water within the cheese samples was also coupled with an increase in the range of relaxation times for the water associated with the casein. This was in the form of a lengthening in the relaxation times that the population occupied, indicating the water was becoming more mobile. To illustrate this, the T2 relaxation time constant for the water associated with protein was assessed over the temperature sweep.

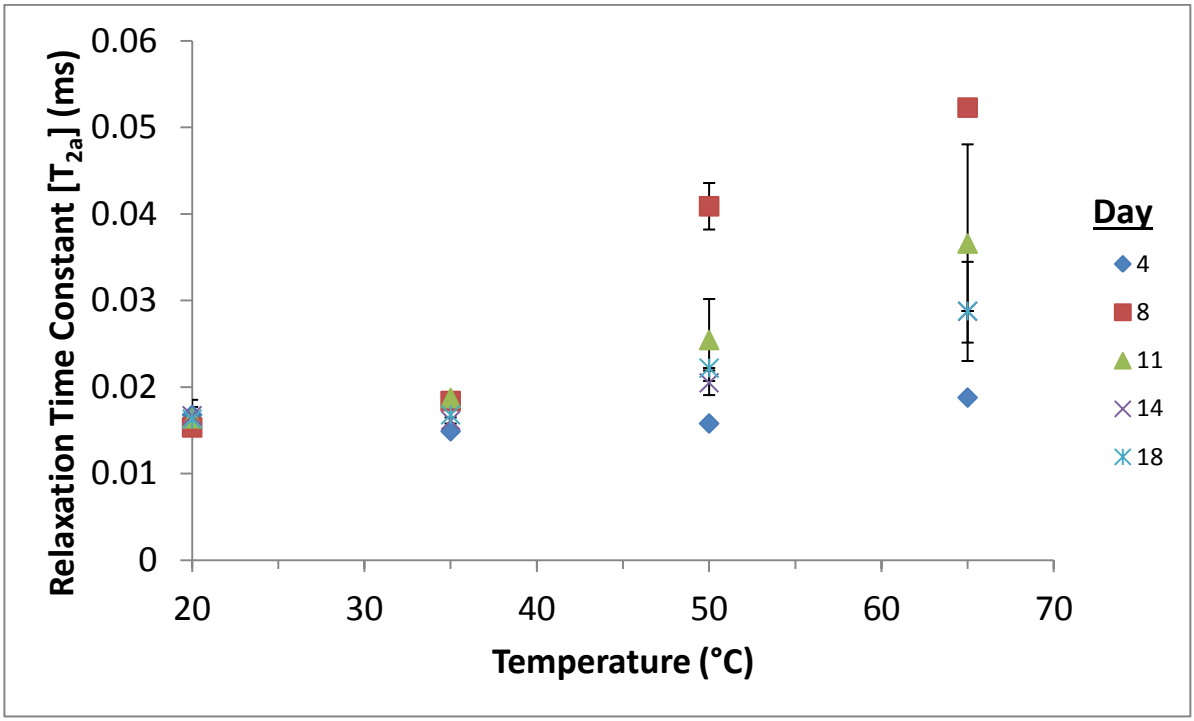


Figure 9.4: Change in the relaxation time constant for the water associated with protein within Mozzarella during heating and storage. (Error bars = ±1 standard deviation).

The T2 relaxation time constant was found to increase as the temperature increased on each day of the storage trial, as shown in Figure 9.4. This increase varied during the storage trial, with a more pronounced increase on day 8 and the first measurement on day 11 while the increase was lowest on day 2. The day 8 and 11 data exhibited a similar trend to the quantity of free water present on these days at 50 and 65°C. This suggests that not only is there a greater quantity of free water at these temperatures on day 8 and 11, but the water associated with the casein is more mobile.

This increase in mobility is likely to be a result of the water molecules having more energy as the temperature increased. This suggests that the heat not only results in free water within the cheese structure, but some of the water associated with protein has a greater degree of freedom as the temperature increased. This is in contrast to the result from the low temperature analysis in the previous section (9.3.1.1) which demonstrated an increase in homogeneity of association of the casein associated water. This current result

is likely showing a reversal of the maturation and gives a picture of the original state of water mobility immediately after stretching. The implication is that the kinetics to achieve equilibrium at molten temperatures are considerably faster than the relaxation kinetics that occur when the cheese is returned to a cold state.

9.3.2 Diffusion

The diffusion of water in the Mozzarella samples at different test temperatures was monitored over the storage trial. This allows information relating to the movement of water through the porous cheese structure to be studied.

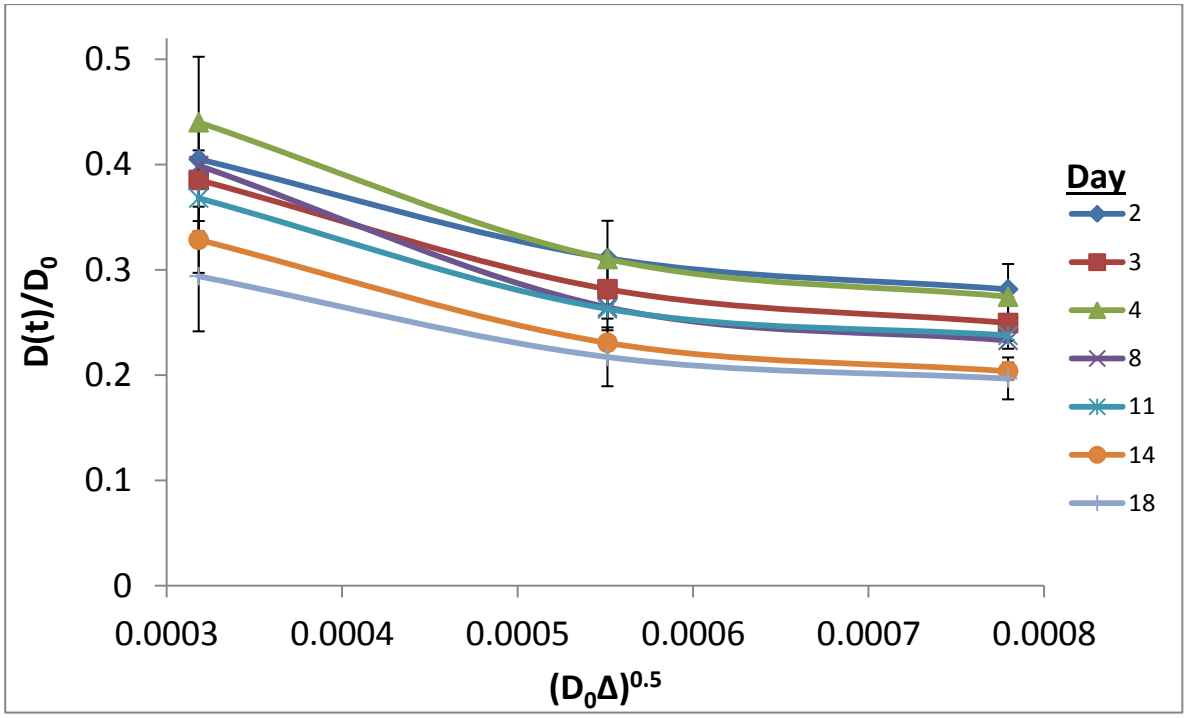


Figure 9.5: Diffusion coefficients of water at 20°C over the storage trial measured using a stimulated echo PGSE sequence in the transverse direction as a function of the observation time. The diffusion coefficients are normalised using the diffusion of free water at 20°C estimated at D_0 = 2.03e-9 m^2s^{-1}.

At 20°C a decreasing trend was observed in the diffusion of water within the Mozzarella samples over the course of the storage trial, as illustrated in Figure 9.5. At the longer

relaxation times the water is able to diffuse greater distances through the casein matrix. The diffusion at these time scales allows information about the tortuosity of the system to be assessed (Song, 2009). The decrease in diffusion at this longer time scale indicates that the tortuosity of the cheese is increasing during storage. This indicates that the path that water molecules diffuse over, during this time frame, is becoming more complex.

One possible explanation for this is that over the course of the storage trial we observed a decrease in the level of free water present within the cheese structure. Immediately following manufacture free water in Mozzarella is present in pools surrounding the fat channels (Kindstedt, 2007; Kuo et al., 2001). The water present within these pools can diffuse at a greater rate than water that is entrapped within the casein matrix as it is less hindered by the protein. As these pools of free water in the cheese system decrease in size, their contribution to the diffusion of water in the system is reduced.

Less difference was identified between the diffusion properties at day 14 and 18. This is possibly due to the limited change in the quantity of free water present in the cheese at 20°C between the samples assessed on these days of the trial.

In order to examine the possible interaction between free water and the diffusion at 20°C, the changing quantity of free water was assessed against the normalised diffusion coefficient.

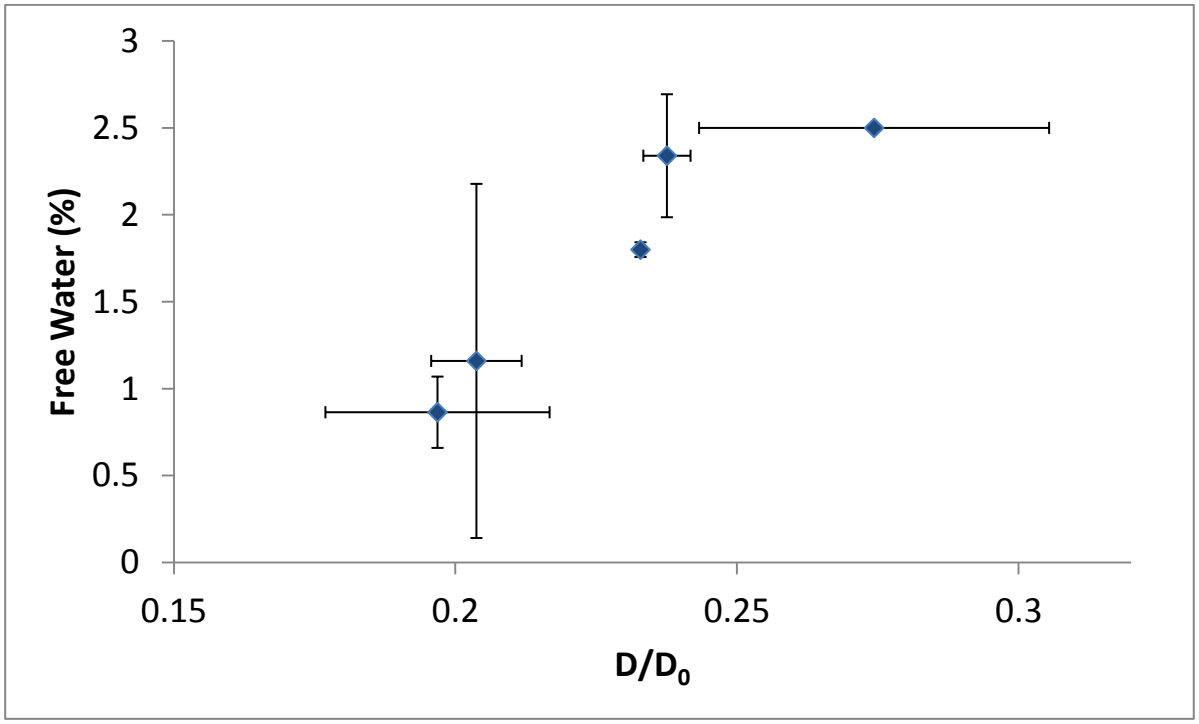


Figure 9.6: Relationship at 20°C between the quantity of free water in the sample and the normalised diffusion coefficient at 300 ms.

Figure 9.6 indicates a trend between the quantity of free water present in the Mozzarella sample during storage and the diffusion coefficient at 20°C. This suggests that the free water present in the channels within Mozzarella has a higher diffusion coefficient than that of water entrapped within the protein matrix. As the free water is slowly reabsorbed into the protein matrix during storage (Kuo et al., 2001; McMahon et al., 1999), the quantity of water in the channels decreases which in turn reduces the diffusion coefficient. This suggests that the change in the environment that the water exists in within the cheese influences its diffusion properties.

The diffusion of water within the Mozzarella samples was also measured at elevated temperatures to identify changes over the storage trial.

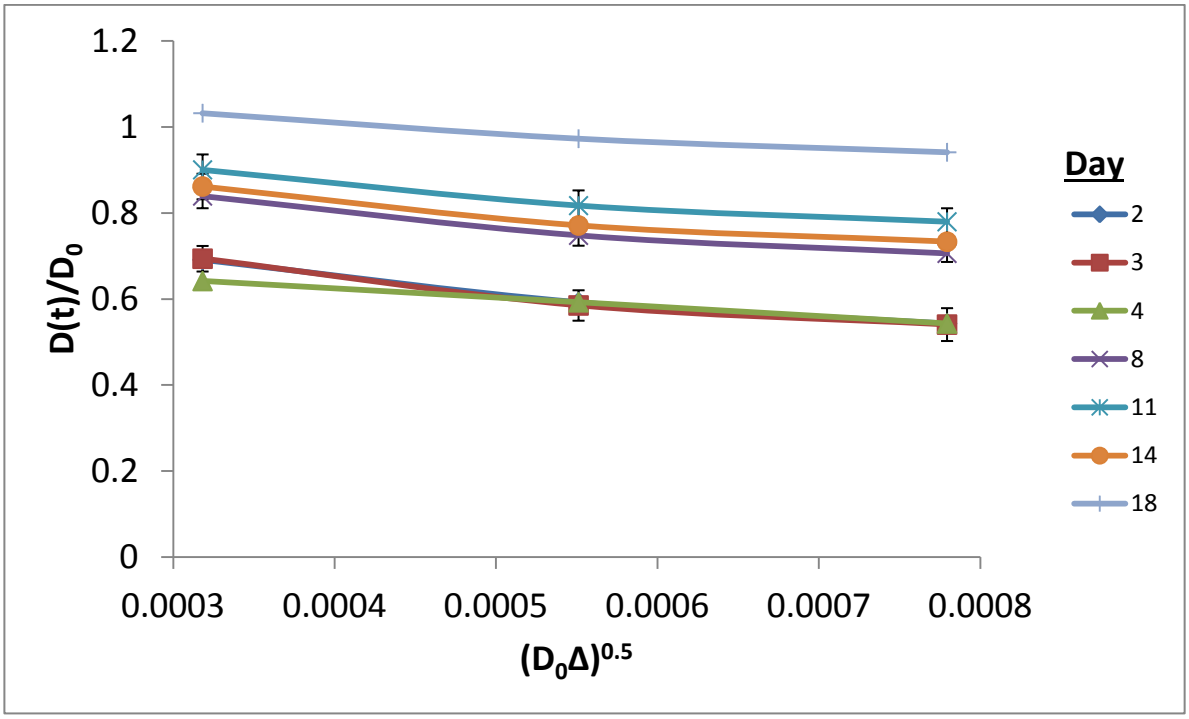


Figure 9.7: Diffusion coefficients of water at 65°C over the storage trial measured using a stimulated echo PGSE sequence in the transverse direction as a function of the observation time. The diffusion coefficients are normalised using the diffusion of free water at 65°C estimated at D_0 = 5.00e-9 m^2s^{-1}.

The diffusion of water at 65°C was found to increase over the course of the storage trial, as illustrated in Figure 9.7. This indicates that the water is able to move more freely throughout the cheese structure when heated as the cheese matures. Based on these results, the tortuosity of the cheese was identified as decreasing during storage. This is indicated by the diffusion at long time scales increasing with age. The implication of this is that the connectivity of the pores within the casein matrix is decreasing with time, as the tortuosity in porous media is governed by how well the pores are connected (Latour et al., 1995). During maturation, the casein within Mozzarella is subjected to proteolytic breakdown (Costabel et al., 2007; Farkye et al., 1991). This is due to residual enzymes within the cheese progressively hydrolysing the casein (Coker et al., 2005), breaking them into peptides. This progressive breakdown of the casein matrix has a significant effect on the functional properties of the cheese (Yun, Barbano, Kindstedt, & Larose, 1995) due to a

weakening in the matrix as a result of fewer connections. This reduction in the connectivity of the casein matrix will influence the degree of hindrance that water entrapped within the protein experiences. It is likely that the proteolytic breakdown of casein influences the diffusion of water within Mozzarella at elevated temperatures.

To gain a better understanding of the diffusion properties at the longer time scale, diffusion at 300 ms was plotted as a function of time at each temperature assessed.

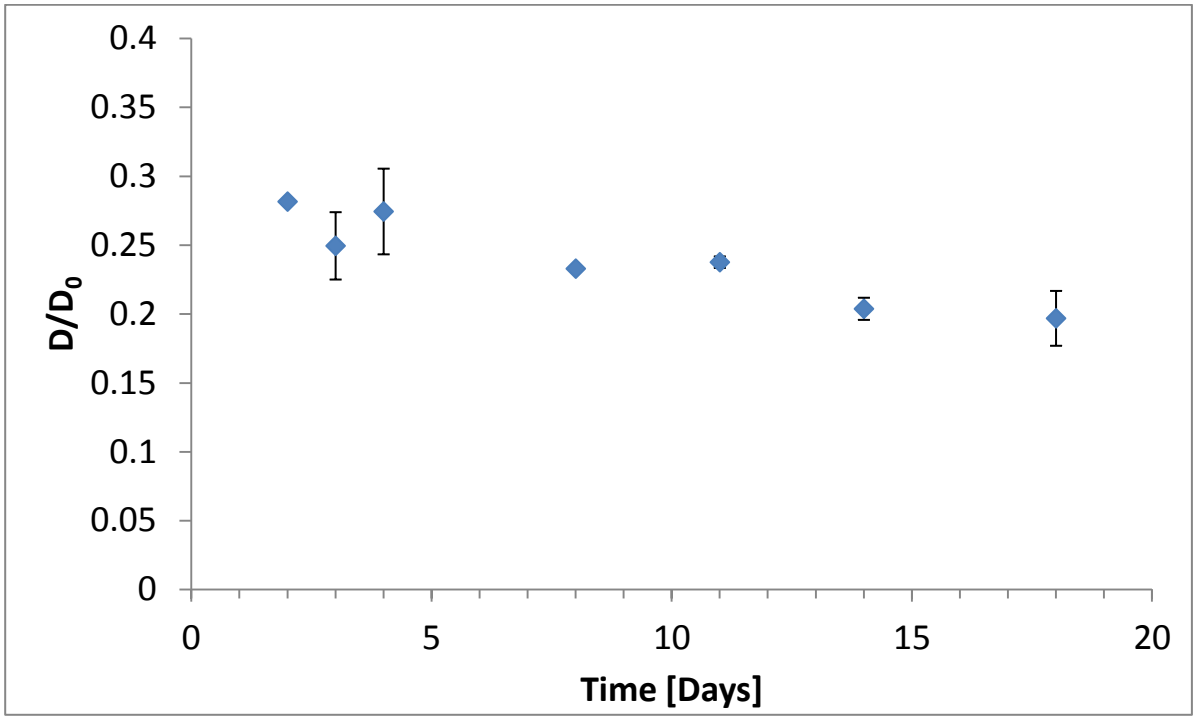


Figure 9.8: Normalised diffusion coefficient at 300 ms at a temperature of 20°C.

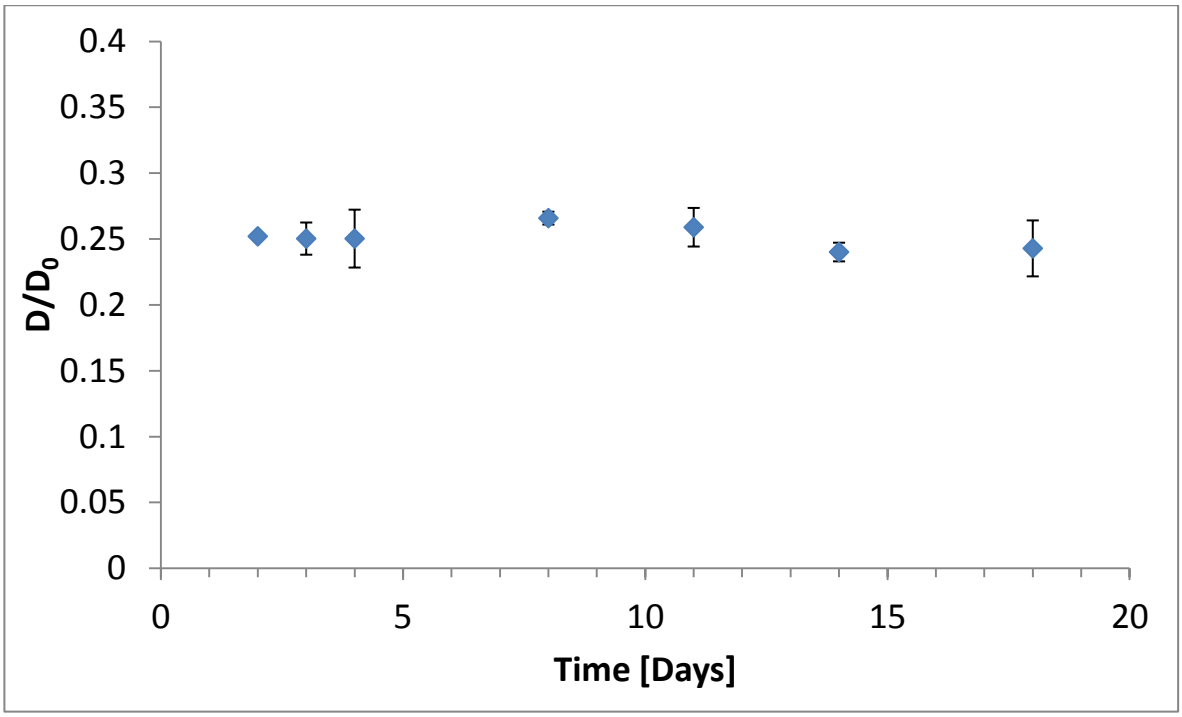


Figure 9.9: Normalised diffusion coefficient at 300 ms at a temperature of 35°C.

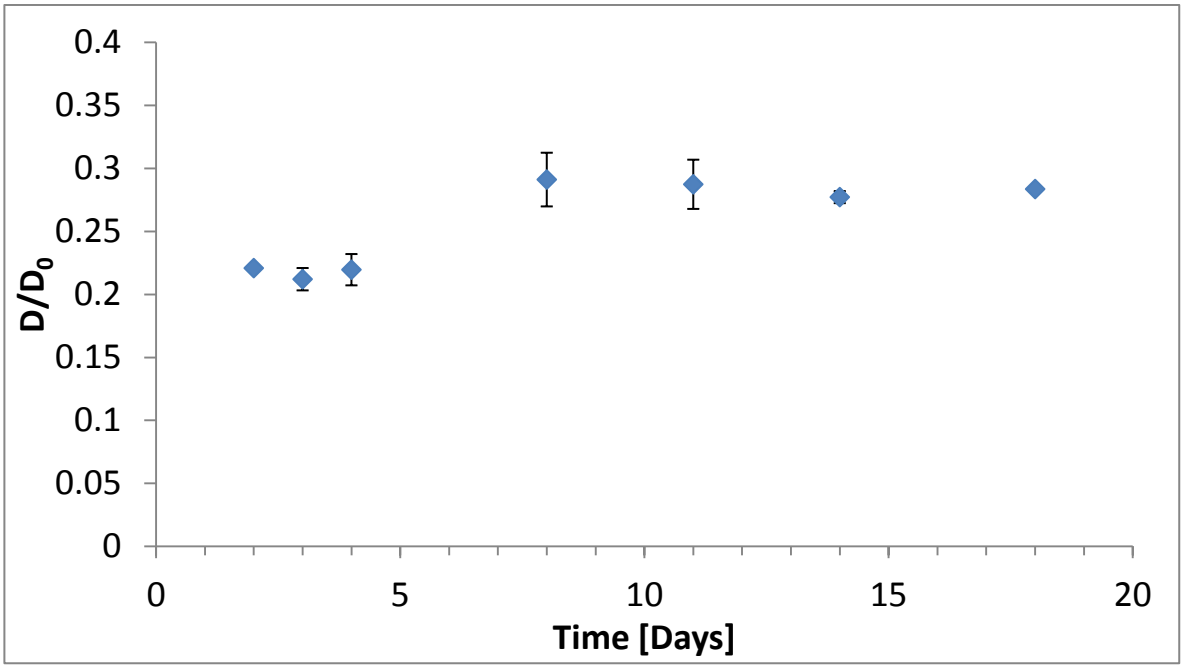


Figure 9.10: Normalised diffusion coefficient at 300 ms at a temperature of 50°C.

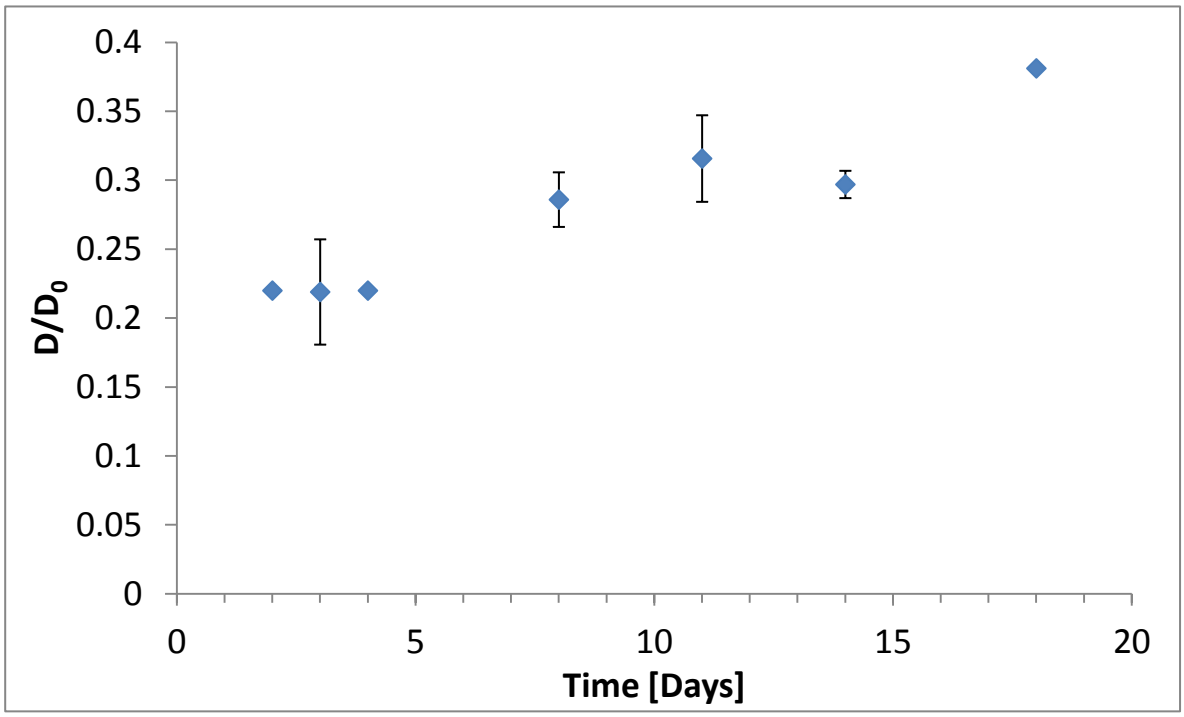


Figure 9.11: Normalised diffusion coefficient at 300 ms at a temperature of 65°C.

At the longer time scale of the experiment the diffusion approaches the tortuosity asymptote (Brown et al., 2012; Latour et al., 1995). Based on this, diffusion data at this time scale allows information relating to the tortuosity of the cheese system to be inferred.

At 20°C the diffusion at 300 ms decreases over the course of the storage trial, as shown in Figure 9.8. In Figure 9.6 this change in the diffusion at this time scale was related to the change in the free water at 20°C. This decrease in the diffusion indicates that the environment that the water exists in within the cheese is becoming more tortuous. As discussed earlier, the migration of the free water into the protein matrix will result in an increase in the tortuosity as the water molecules are hindered to a greater extent within the protein matrix than in the channels.

At 35°C the normalised diffusion at 300 ms (Figure 9.9) was identified as remaining relatively constant over the course of the storage trial. The diffusion coefficient at 50°C was observed to change over the storage trial, as shown in Figure 9.10. Initially very little variation was observed in the diffusion coefficients between day 2 and 4. However, an increase between day 4 and 8 was observed after which the diffusion coefficient remained relatively stable. At 65°C the diffusion at 300 ms (Figure 9.11) was found to increase over the storage trial. As with the diffusion coefficient at 50°C, very little variation was identified in the data collected on day 2, 3 and 4 followed by an increase by day 8. A further increase in the diffusion coefficient at 65°C was noted between day 14 and 18 of the storage trial.

The increase of the diffusion coefficient at this time scale indicates that there is a decrease in tortuosity. This decrease in tortuosity means that the degree of hindrance that a water molecule experiences when diffusing is decreasing. As discussed earlier, the decrease in tortuosity with storage at 65°C may be the result of the gradual weakening in the protein gel due to proteolysis (Tunick, Malin, et al., 1993).

The results from the diffusion experiments indicate that at high temperatures the diffusion of the water through the cheese matrix is likely to be influenced by factors relating to the integrity of the casein matrix. While diffusion at 20°C, over the period studied, was dominated by the free water present in the channels of the Mozzarella cheese, as it is in a less tortuous environment.

The error displayed in the diffusion experiments, as with the relaxation measurements, is possibly the result of a number of variables. One of the key factors that influence the error in the data is the time between the duplicate measurements. As the total time taken to run all of the experiments on an individual sample was 12 hours, half a day elapsed between the duplicates. Another factor that could introduce error in the results is inhomogeneity within the block of Mozzarella. As the cheese sample was a brine salt

Mozzarella, salt and moisture gradients are likely to be present through the cheese. Not only does this mean that there will be compositional inhomogeneity prior to the gradients reaching equilibrium, the gradients will also impact result in proteolysis occurring at differing rates throughout the cheese. Therefore it is more likely that any trend between two consecutive measurements would be obscured by the dominance of the effect from natural inhomogeneity. This was noted in the results, with the diffusion data showing no obvious effect of order between duplicate samples.

9.3.3 DT2

Diffusion T_2 correlation maps allow molecular motion to be differentiated from interactions experienced by the system of spins (Godefroy & Callaghan, 2003; Godefroy, Creamer, et al., 2003). This means that different components within the structure of a system can be separated out and assessed in relation to diffusion and relaxation.

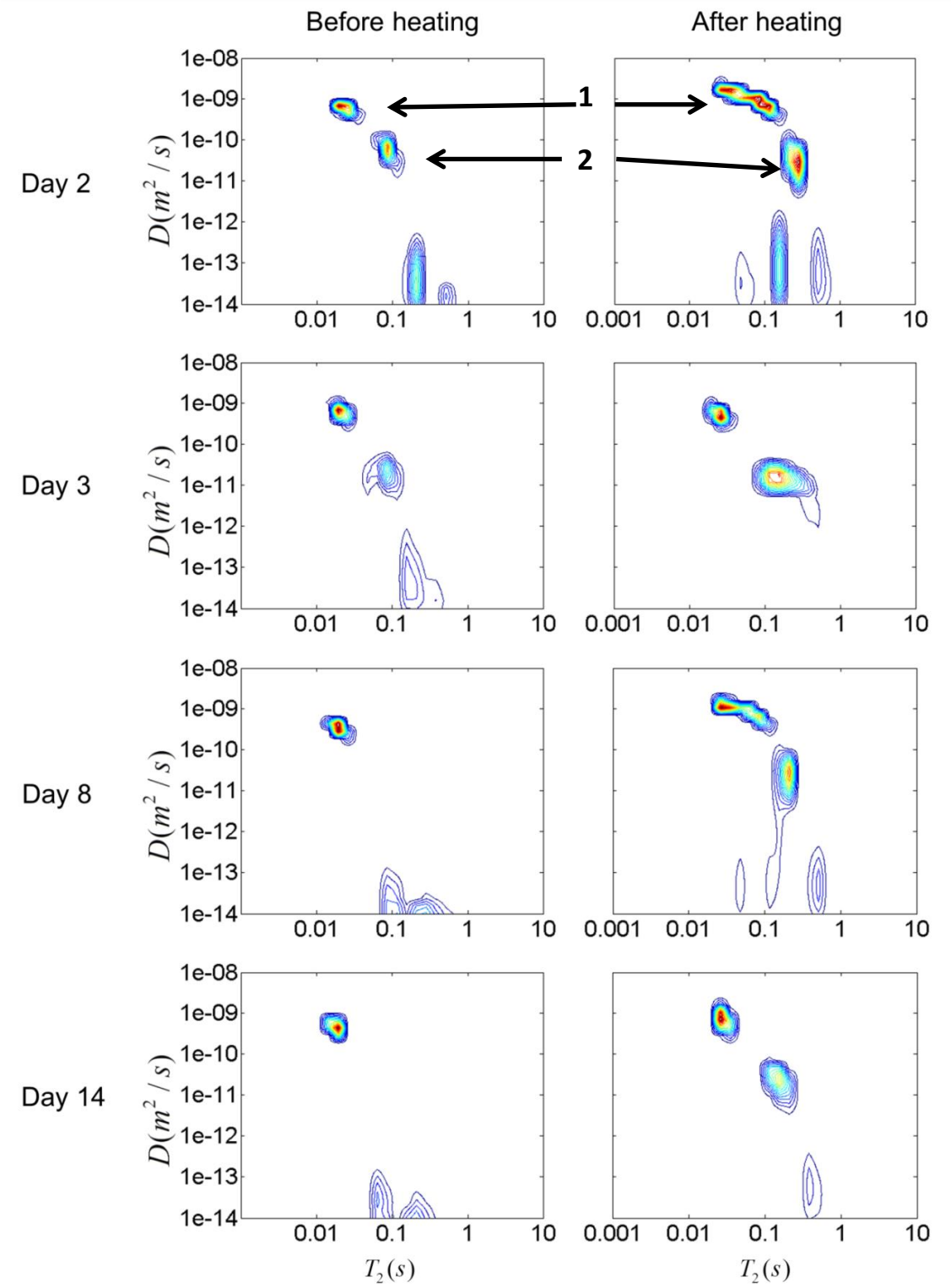


Figure 9.12: Diffusion relaxation correlation (DT2) maps for Mozzarella before and after heating at 20°C during storage [1 – Peak 1, 2 – Peak 2].

The vertical axis in the DT2 maps shows the diffusion of components within the cheese system, while the horizontal axis depicts T2 relaxation data obtained from the 'one shot' CPMG sequence used. This allows the various components within the Mozzarella to be distinguished based on their relative diffusion and relaxation rates. The DT2 maps are displayed in Figure 9.12.

As with previous DT2 correlation investigations of cheese, two key components were observable (Song, 2009) in the majority of maps (Peaks 1 and 2). The upper component, with a high diffusion and short relaxation time (Peak 1), is relatively sharp and has a diffusion coefficient similar to that of water so can be considered to be representative of the aqueous phase (Hurlimann et al., 2006). Over the storage trial the signal for the aqueous phase followed the same trend as that observed in the relaxation properties of water, with a slight reduction in the range of relaxation times that the water exists in. After heating, the water signal was observed to become slightly broader, both in the relaxation and diffusion parameters. The broadening in the relaxation direction was in the form of longer relaxation times, indicating that the water has a greater amount of mobility following heating.

Another feature of the DT2 results is the secondary peak (Peak 2) evident on all of the days assessed following heating and on the first few days before heating. This peak has a lower diffusion and longer relaxation to the peak related to the water. This is likely to be a signal from the fat present within the system. This is consistent with observations made by Song (2009) and Hurlimann et al. (2006) where the lower of the two peaks in their studies, with a diffusion coefficient in the vicinity of 10^{-10} m^2s^{-1}, was associated with the liquid fat within cheese. The more intense signal with a longer relaxation time following heating is probably due to a greater quantity of fat existing as a liquid. As fat melts the relaxation time lengthens as the fat becomes more mobile (Mariette, 2009). This lengthening of relaxation times was observed in the fat signal of the cheese after heating.

One possible explanation for the loss of this signal before heating over time is potentially due to the fat within Mozzarella progressively becoming more solid. As Mozzarella curd reaches temperatures of approximately 55-65°C during processing, as part of the stretching step (McMahon & Oberg, 2011), on the subsequent cooling operation the fat globules within the centre of the fat channels take time to cool and crystallise. As the fat becomes progressively more solid like, the signal lessens. This is similar to observations made by Godefroy, Creamer, et al. (2003) where during the early stages of storage of Mozzarella and Gouda (when monitoring changes at 5 and 40°C) no fat signal was observed at 5°C where the majority of the fat within the cheese was solid. However, they identified a peak at 40°C, at which point the fat would have been entirely liquid (Tunick, 2010).

The signal observed at the bottom of the correlation maps could possibly be a signal from the protons of the protein. This signal is at the edge of the measurement boundaries for the experiment and occupies a space with a very low diffusion coefficient, orders of magnitude lower than the signal from the aqueous phase. This indicates that the molecules associated with this peak have a very limited degree of translational motion that they exhibit, such as the protein matrix.

9.3.4 General discussion

Both the diffusion and relaxation properties of water within the Mozzarella sample were found to change with both temperature and early storage. The relaxation measurements demonstrated a similar trend to that identified by Kuo (2001) and Section 5.3.4, where the quantity of free water at room temperature was found to decrease with storage. The amount of free water was identified as being temperature dependent, with a progressive increase in free water occurring as the temperature was increased. A potential driver for this increase in free water is the strengthening of hydrophobic interactions that occurs

during heating (Bryant & McClements, 1998). This could result in the protein matrix contracting, forcing out water into the channels where it can exist in a free state.

Both the water in a free state and that associated with the casein within the cheese were found to increase in their mobility with temperature. This was indicated by the progressive increase in the relaxation time constant for both of the populations of water with temperature. A higher relaxation time constant indicates that the molecules are more mobile (Kuo et al., 2001), which is likely to be the result of them having more energy as the temperature is increased.

The diffusion of water within the sample at 20°C was found to decrease over the trial. This follows a similar trend to the quantity of free water present at this temperature. As relaxation properties may bias diffusion measurements (Gottwald et al., 2005), the free water content was plotted against the diffusion at 20°C. This indicated a trend between free water and the diffusion coefficient at 300 ms within the Mozzarella sample. This suggests that the contribution of free water to the diffusion coefficient is higher than that of water entrapped in the casein matrix i.e. the overall environment experienced by the water molecules within the cheese influences how the water molecules diffuse at 20°C. As the water is absorbed into the protein matrix, the environment that water molecules experience is more tortuous due to a greater degree of hindrance within the protein network than in the long channels.

A progressive increase in the ability of water molecules to diffuse through Mozzarella was observed at 65°C. At longer time scales the diffusion is limited by the tortuosity of the gel network (Song, 2009). As tortuosity is inversely proportional to the normalised diffusion coefficient at a long timescale (Brown et al., 2012), the increase in diffusion with time at 65°C indicates that the tortuosity of the cheese is diminishing during storage. This suggests that the complexity of the path that a water molecule can travel over a certain time interval is reducing. The tortuosity of a porous medium, such as cheese, is linked to

the connectivity of the pores in the system (Latour et al., 1995). In cheese the tortuosity would therefore be dictated by the level of structural integrity of the casein matrix. One of the key mechanisms affecting the structure of the protein network during storage is proteolysis (Fox & McSweeney, 1996). The residual enzymes present in the cheese gradually hydrolyse the caseins into fragments (Coker et al., 2005). This results in fewer points of connection within the gel matrix as the proteins are progressively broken down (Yun, Barbano, Kindstedt, et al., 1995). This reduction in the connectivity within the casein gel network will reduce the complexity of the path that water molecules can diffuse over.

Another change impacting on the gel structure of the cheese during storage is the solubilisation of calcium (McMahon & Oberg, 2011). This results in fewer calcium-mediated protein-to-protein interactions will lead to a looser gel network (Joshi, Muthukumarappan, et al., 2004a). This loosening of the casein matrix during storage will allow water within the cheese to diffuse with a reduced level of hindrance.

The two dimensional diffusion relaxation correlation maps of Mozzarella illustrated changes occurring within Mozzarella with storage. The correlation maps also identified changes in the properties of fat and the aqueous phase of Mozzarella before and after heating. The most notable change in the DT2 maps was in peak 2, which was attributed to the fat within the cheese. At the beginning of the storage trial this peak was evident before heating at 20°C. However, by day 8 the peak was no longer visible prior to heating. The peak became evident and more pronounced following the heating, indicating that the peak was likely to be due to the liquid fat in the cheese. This peak is consistent with the secondary peak observed by Hurlimann et al. (2006), who reported that the lower component of their DT2 maps was associated with liquid fat and had a diffusion in the vicinity of 10^{-11} m^2s^{-1}. The change observed in the water peak followed a similar trend to that of the protein associated water studied using the separate relaxation and diffusion experiments.

As mentioned in Section 8.3.2.4, an error that is inherent to this experiment is related to the estimation of D_0 for cheese. In this work we have used the diffusion coefficient at each temperature studied, however, in cheese the aqueous phase does not solely contain water. The presence of salts and lactose within the aqueous phase of the cheese will influence the viscosity. The diffusion of molecules is inversely proportional to the viscosity (Einstein, 1905), as described by the Stoke-Einstein equation. However assuming that the net change in soluble components is relatively low any concomitant change in viscosity will likely be minimal so therefore while the base viscosity may change the absolute numbers for the results calculated in this section, it is not expected that the general trends described will be affected.

9.4 Conclusion

The relaxation and diffusion properties of LMPS Mozzarella were found to change following manufacture. During storage the quantity of free water present in Mozzarella was found to progressively decrease at 20°C as it is absorbed into the protein matrix. Heating resulted in a progressive increase in the quantity of free water and molecular mobility of water within the cheese. At 20°C the diffusion properties of water in Mozzarella decreased with storage while at 65°C the trend was an increase in diffusion. Based on the results it was suggested that the diffusion properties of water within Mozzarella at 20°C is related to the change in the distribution of water during the initial stages of storage. At 65°C the diffusion properties of water were linked to the structural integrity of the casein matrix. The DT2 correlation maps identified changes in the diffusion and relaxation properties of both water and fat within the Mozzarella over the storage trial. The peak associated with the aqueous phase mirrored changes observed in the relaxation and diffusion results of the water associated with the protein. The behaviour in the peak related to the fat within the system suggested that the fat becomes more solid with storage and a greater quantity exists as liquid after heating.

The magnetic resonance experiments in this chapter and Chapter 8 showed promising results in the ability to assess changes occurring in cheese samples. However, these studies have been limited in regards to time constraints and availability of well characterised cheese samples. Therefore the next piece of work will utilise magnetic resonance techniques in conjunction with numerous other characterisation tools to monitor changes in the structure and component mobility of three different Mozzarella samples during storage.

A question that arises from this work is whether the structure of Mozzarella can be manipulated using compositional levers to modify the diffusion properties of water within the cheese.

10 Investigation into the role of calcium in the structure and component mobility of Mozzarella cheese

10.1 Introduction

The previous chapters have used a number of tools to characterise the structure and component mobility of Mozzarella cheese. One particular phenomena explored in these previous experiments has been the change in the distribution of water during the initial stages of storage and heating. Measurement techniques to evaluate changes in the distribution of water have included NMR and dielectric spectroscopy. These methods will be revisited in this work to evaluate the distribution and behaviour of water in three cheeses manufactured with differing calcium levels. In addition to water mobility, another important change that affects the structure of cheese is the solubilisation of colloidal calcium during the first few weeks of storage. The transformation of the state of calcium during storage has been examined in a number of cheese varieties including Cheddar (O'Mahony et al., 2005) and Colby (Lee et al., 2010). This phenomenon has been described as the attainment of a pseudoequilibrium between the insoluble and soluble calcium within the cheese (Hassan et al., 2004). The solubilisation of colloidal calcium is a key factor affecting the functionality of Mozzarella cheese (Mizuno, Matsuda, Lucey, & Ichihashi, 2009). However, the exact mechanism responsible for the pseudoequilibrium has not been elucidated. This shift in the distribution of calcium within cheese during storage has been correlated with changes in physical properties including hardness (O'Mahony et al., 2005).

The quantity of soluble calcium present in cheese has been measured using extraction methods (Metzger, Barbano, & Kindstedt, 2001). One common method involves the homogenisation of cheese in water, filtering and measuring the calcium content of the aqueous phase (Ayyash & Shah, 2011).

Other less common methods that may be of value in monitoring soluble calcium include dielectric spectroscopy and NMR. The dielectric loss factor is related to the ionic interactions (salts) within a material (Green, 1997; Kudra et al., 1992). This suggests that dielectric spectroscopy may give some insights into the change in the ionic state of calcium during storage.

NMR may also be useful to indirectly monitor the solubilisation of calcium. NMR can be used to monitor components, beyond protons as done for water distribution studies, within cheese. A particular component that is useful to measure in cheese systems is the ability of NMR to measure the mobility of phosphorus (Gobet et al., 2010). This can be achieved by utilising ^{31}P magic angle spinning solid state NMR (Rondeau-Mouro et al., 2009a). This method used with a combination of a ^{31}P single pulse, cross polarisation and dipolar dephasing enables the resonances of phosphates with different levels of mobility and proximity to protons to be distinguished (Bak et al., 2001). This allows the signals to be discriminated in regards to mobility, with immobile phosphorus in the insoluble phase and mobile phosphorus in the soluble phase (Gobet et al., 2010). As dissociation of calcium occurs from colloidal calcium phosphate, the phosphate groups making up the CCP will also solubilise and thus become more mobile.

As equilibration processes are important to the development of structure in Mozzarella during storage and the physical properties of the cheese, it is important to gain a detailed understanding of the associated mechanisms.

Therefore the objectives of this work were to:

- Manufacture Mozzarella with a number of different calcium levels whilst maintaining compositional uniformity amongst the other components.
- Monitor changes in the structure and physical properties of the Mozzarella over a 40 day storage trial.

- Monitor changes in the mobility of water and colloidal calcium phosphate within the three Mozzarella samples over a 40 day storage trial in order to elicit how these equilibration processes affect structure.
- Identify the effect of calcium on water mobility and equilibrium processes within Mozzarella during a 40 day storage trial.

10.2 Materials and Methods

10.2.1 Materials

Whole milk was delivered on the 25th of October 2012 to the Fonterra Research and Development Centre. Cheese manufacture was carried out using the following ingredients: starter cultures (Fonterra Cooperative Group, New Zealand), Fromase (DSM, Netherlands), acetic acid and salt (Dominion salt, NZ). Calcium Chloride, Sodium Chloride and Lactose were added to the water in the cooker/stretcher to prevent leaching from the curd during stretching.

10.2.2 Cheese manufacture

The method for manufacturing the three different Mozzarella cheeses with three different calcium levels whilst maintaining the same composition and pH was developed in conjunction with the cheese makers at the Fonterra Research and Development Centre in Palmerston North. The biggest challenge with cheese-making is trying to manipulate variables in isolation, as limited studies of this nature have been conducted in literature with none manipulating calcium and maintaining a constant pH. The lever used in these trials, was the pH at key stages of cheese manufacture.

The cheese samples manufactured for this piece of work were made in accordance with the parameters outlined in Table 10-2. The manufacture of the Mozzarella samples was

carried out at the Fonterra Research and Development Centre with assistance from the Pilot Plant Cheese Team and Cheese Foods Technologists.

Based on the experience of the Fonterra cheesemakers with low calcium cheese milk systems two low calcium vats were included to increase the chance of hitting all the composition targets. The calcium concentration targets are displayed Table 10-1 below.

Table 10-1: Target calcium levels

	Target calcium (mmol/kg)
High	170
Control	150
Low 1	130
Low 2	110

A direct acidified, dry salt method was used for the manufacture of the low moisture part skim (LMPS) Mozzarella, apart from the high calcium sample that relied solely on the acidification from the starter culture. The manufacture of the cheeses was conducted in the Fonterra Research and Development Centre pilot plant.

One potential issue with having a different set pH for the different vats was that the time required for the coagulum to set would vary. This would result in different rate of gel formation between the different vats and thus a potentially confounding variable. In order to keep the set time relatively constant between the various vats, the quantity of rennet was varied so that the higher the set pH the greater the quantity of rennet added to the cheese vat.

The run pH for the different vats was set to achieve the desired level of calcium retention in the curd. To achieve a lower concentration of calcium in the final cheese a lower run pH was used compared to the control sample, while the sample with the higher target

calcium concentration had a higher run pH. This was done as calcium progressively solubilises as the pH is reduced and when the whey is drained from the cheese curd the bulk of the soluble calcium is lost with it.

The mill pH and salting rate of all of the vats was kept constant to try and achieve a consistent final pH for each of the cheeses produced.

Table 10-2: Cheese manufacture

	Low 1	control	High	Low 2
Protein : Fat	1.3	1.3	1.3	1.3
Starter (%)	4	5	5.5	4
Direct acidification to pH	6.1	6.3	-	6.1
Set pH	6.05	6.2	6.55	6.00
Target Set Time (min)	18	18	18	18
Rennet – Fromase (mL)	3.5	5	7	3
Knife size (mm)	12	12	12	12
Cook (°C)	36	36	36	36
Run pH	5.75	5.9	6.2	5.6
Dry Stir	1	1	1	1
Mill pH	5.40	5.40	5.40	5.40
Salt (g/kg)	23.5	23.5	23.5	23.5
Mellow time (min)	20	20	20	20
Cooker/Stretcher temperature (°C)	59	59	59	59
Exit Temperature (°C)	77	77	77	77
Stretching time (min)	7	7	7	7

10.2.3 Composition

The composition of each of the different cheeses was assessed immediately following manufacture. The composition of the cheese samples was determined by the methods described in Section 5.2.7 by the Fonterra Research and Development Analytical Services team. Based on the composition of the samples, a decision could be reached as to which of the low calcium samples were closer in composition to the other samples. This low calcium sample along with the control and high calcium sample were characterised over the 40 day trial.

The moisture content of each of the cheeses was also measured on each day of testing using the method described in 5.2.1.

10.2.4 Uniaxial compression

Uniaxial compression was used to monitor the changes in the hardness of the three samples over the 40 days of storage. The method used to assess the samples is described in Section 4.2.4.

10.2.5 Meltability

The meltability of the three samples was assessed over the 40 day storage trial using a modified Schreiber test (Muthukumarappan et al., 1999) as described in Section 6.2.6.

10.2.6 Pizza baking evaluation

To assess how the three different cheeses performed during pizza baking, each of the samples were baked on days 1, 20 and 40 of the storage trial. Tomato paste (25 g) was mixed with water (25 g) and spread evenly onto a pizza base (0.15g.cm^{-1} coverage). Grated Mozzarella (100 g) was evenly distributed over the tomato paste mixture. The sample was then placed in a forced convection oven set at 250°C for a period of 5

minutes. The pizza was allowed to cool for 10 minutes and was then photographed to assess whether or not shreds were still evident.

10.2.7 Confocal microscopy

Confocal microscopy was used to assess changes in the distribution of fat and protein within the structure of the three different cheeses over the course of the storage trial. The confocal microscopy method used to examine the samples is stated in Section 4.2.2. The samples were all sectioned parallel to the protein fibres. Triplicate samples were assessed and images were collected at each magnification on days 1, 20 and 40. The images displayed in Figure 10.5 were selected as they were representative of those acquired on each day of analysis.

10.2.8 Scanning electron microscopy

Scanning electron microscopy (SEM) was used to monitor changes in the protein matrix over the course of the storage trial. The SEM method used to assess the samples is stated in Section 5.2.3. The samples were all sectioned parallel to the protein fibres. Triplicate samples were assessed and images were collected at 2000x, 1000x, 500x and 130x magnification on days 1, 20 and 40.

10.2.9 Urea PAGE

In order to monitor the proteolysis within each of the cheeses, gel electrophoresis was used, as described in Section 6.2.3. Samples were assessed on days 1, 20 and 40 following manufacture.

10.2.10 Magnetic resonance

Magnetic resonance measurements were conducted and analysed with help from Dr Jason Hindmarsh of the Institute of Food, Nutrition and Human Health at Massey University.

10.2.10.1 *Relaxation*

The quantity of free water within each of the cheese samples was assessed using T2 relaxation measurements conducted at both 25 and 65°C, as described in Section 5.2.4. The measurements were conducted on a 400 MHz Bruker NMR spectrometer (Bruker, Rheinstetten, Germany). The method used for assessing the relaxation properties of water within Mozzarella were changed slightly from the previous experiments. The new modified method used the data collected from Chapter 6 to try and optimise the separation of the two distribution signals associated with water within the cheese.

10.2.10.2 *Diffusion*

An 8 point diffusion measurement was carried out at 25 and 65°C over the storage trial as described in Section 8.2.3.3. However, unlike the work carried out at Montana State University in Chapters 8 and 9, the NMR unit used for this experiment did not have a diffusion probe. As a consequence, diffusion measurements required a greater period of time to complete. The measurements were conducted on the same 400 MHz Bruker NMR spectrometer as the relaxation measurements.

10.2.10.3 *^{31}P NMR*

Solid state phosphorus magic angle spinning (MAS) NMR experiments were conducted in accordance with the method disclosed by Gobet et al. (2010). These experiments were conducted on a Bruker Biospec 200 MHz NMR (Bruker, Rheinstetten, Germany)

using a 7 mm Bruker MAS probe (80.99 MHz ^{31}P). Further detail relating to ^{31}P NMR is noted in 2.4.3.2.3.

Samples of cheese (4 mm depth, 4 mm wide and 12 mm in height) were cut with a razor blade. The samples were inserted into a 5 mm ceramic NMR tube and then place in the NMR.

A single pulse excitation (SPE) sequence was performed using 5 µs $\frac{\pi}{2}$ ^{31}P pulse for an acquisition time of 30 ms during which a 45 kHz dipolar decoupling was applied. A recycling of 30 s was used for quantification conditions. The spectral information was deconvoluted in accordance with Gobet et al. (2010)

10.2.11 Soluble calcium

Soluble calcium was measured according to the methods described by Metzger, Barbano, and Kindstedt (2001) and Ayyash and Shah (2011), with some minor modifications. Finely grated cheese (10 g) was homogenised at 11,000 rpm with MilliQ water (90 g) at 60°C using an Ultraturrax homogeniser. The homogenisation was carried out in a metal flask sitting in a waterbath set at 60°C to ensure a constant temperature in the sample. The slurry was then centrifuged (4000 x g) at 20°C for 20 minutes and then filtered. The soluble calcium content of the filtrate was analysed using inductive coupled plasma ICP atomic emission spectrometry by the Analytical Services team at the Fonterra Research and Development Centre.

10.2.12 Dielectric spectroscopy

The dielectric properties of the cheese samples were assessed over the storage trial using the method given in Section 4.2.5. Quadruplicates of each sample were analysed on days 1, 7, 12, 20 and 40.

10.2.13 Statistical analysis

A statistical analysis was carried out on the data collected from this investigation using Minitab 16 statistical software (Minitab Inc., State College, PA, USA). Principle component analysis (PCA) was used to investigate the data obtained in both this chapter and the previous chapter.

10.3 Results and Discussion

10.3.1 Observations during processing

A visible difference was observable during the stretching of the different cheese curds. The highest calcium curd was observed to be the stiffest of the cheeses manufactured, building up behind the orifice at the end of the stretcher to a greater extent than the lower calcium cheeses. In comparison the lower calcium samples were the smoothest of the cheeses during stretching.

10.3.2 Composition

The composition of each of the manufactured cheese samples (Table 10-3) was assessed to determine whether the goal of the trial had been achieved (i.e. three cheeses with different calcium levels with similar compositions).

Table 10-3: Composition of Mozzarella samples

	Moisture (%)	Protein (%)	Fat (%)	Ash (%)	Lactose (%)	Calcium (mmol/kg)
Low1 (129)	49.4	25.2	20.6	2.93	1.52	129
Control (148)	49.0	25.4	20.2	3.17	1.52	148
High (171)	48.2	26.6	20.4	3.36	1.42	171
Low2 (107)	51.0	23.9	20.4	2.87	1.68	107

Based on the results of the compositional analysis, the second low calcium sample [Low (107)] was identified as not meeting the compositional requirements of this study so was dropped from the testing regime.

10.3.3 pH

The change in the pH of the three Mozzarella samples was monitored over the 40 day storage trial, as shown in Figure 10.1 below.

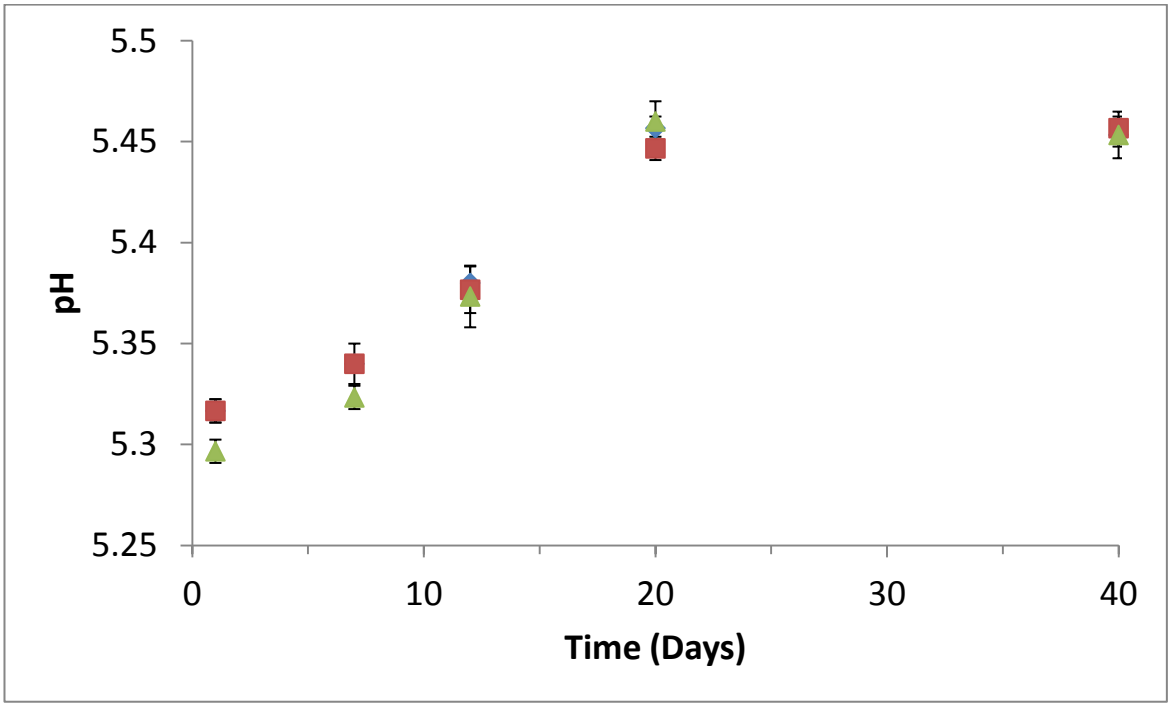


Figure 10.1: Change in the pH of the Mozzarella samples over the 40 day trial [◆ Low (129), ■ Control (148) and ▲ High (171)].

The pH of all three cheese samples was found to be relatively similar over the storage trial. As pH was a variable that we were trying to control, these results indicate that this was reasonably successful. Over the storage trial, the pH of the three different Mozzarella samples was found to initially increase in pH (by ~0.15) following manufacture followed by a levelling off after day 20. This is similar to the trend in the data obtained in Section 6.3.1, thus gives confidence that the data is consistent with commercial Mozzarella.

As discussed in Section 6.3.1, the change in the pH during storage is likely to be a result of an increase in the buffering capacity of the cheese. The increase in buffering capacity is a consequence of the disassociation of CCP which leads to an increase in the quantity of phosphate groups (Hassan et al., 2004; Johnson, 2002). This change in the proportion of soluble to insoluble calcium has been observed to predominantly occur over the first four weeks of storage in Cheddar (Hassan et al., 2004). If a similar trend occurs within Mozzarella, as investigated in 10.3.11 & 10.3.12, the solubilisation of colloidal calcium occurs in line with the observed increase in pH. The change in the distribution of water also takes place predominantly over the first 20 days following manufacture, which may also be linked to the changes in pH over the storage trial (as discussed in 10.4). Other factors affecting pH, as discussed in Section 6.3.1, including the hydrolysis of protein, which would cause a decrease in the pH of the system.

10.3.4 Compression

The hardness of the Mozzarella samples during storage was measured using uniaxial compression (Figure 10.2).

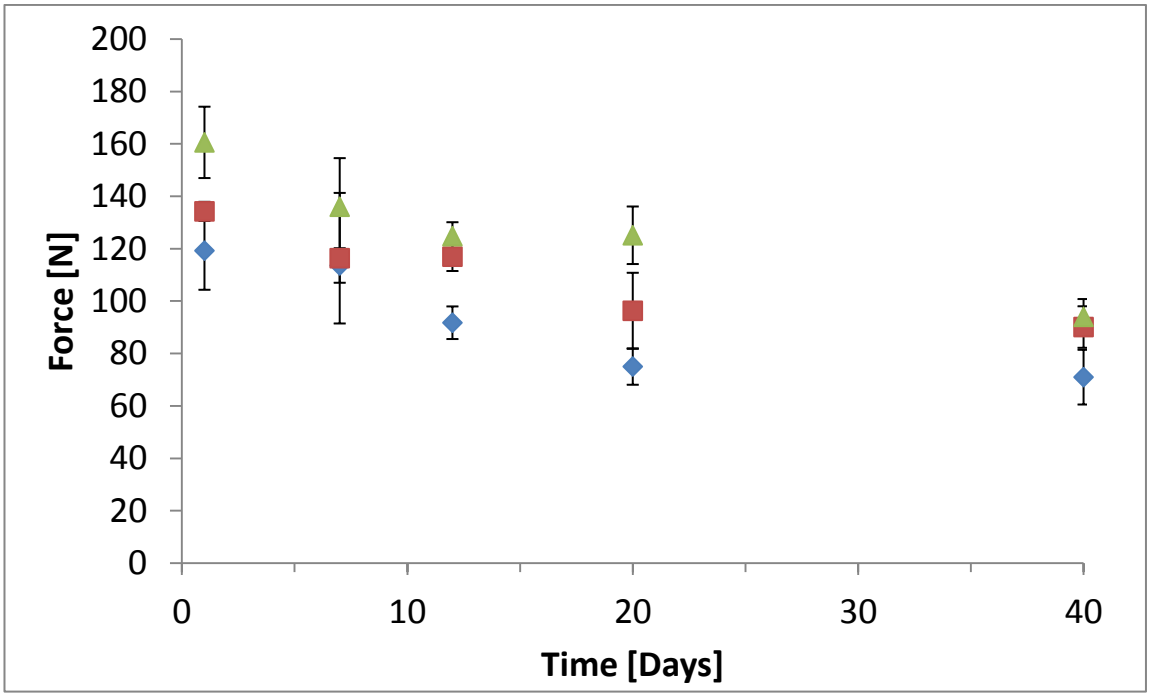


Figure 10.2: Change in the hardness of the Mozzarella samples during the storage trial [◆Low (129), ■ Control (148) and ▲High (171)].

All of the Mozzarella samples were found to significantly decrease in their hardness over the storage trial, p=0.001 for Low (129), p=0.0134 for the Control (148), and p=4.12x10^{-6} for High (171). This trend is consistent with what was observed in Section 6.3.6 where the hardness of the Mozzarella sample also decreased during storage. The level of calcium within the cheese samples was expected to affect the hardness, with more calcium resulting in a harder cheese throughout the duration of the trial. This is because calcium plays an essential role in the strength of the protein matrix in cheese, mediating protein-to-protein interactions (Joshi, Muthukumarappan, et al., 2004a). However, no significant difference was identified between the mean hardness's different samples at a 95% confidence interval, p=0.074 but significant at an α of 0.1.

The decrease in the hardness of each of the Mozzarella samples was identified as being more pronounced over the first 20 days of storage than the period between day 20 and 40. This is consistent with the observations made in Section 6.3.6 where the commercial Mozzarella sample dropped from a hardness of approximately 130 N to 100 N over the first 20 days following manufacture and had decreased to 84 N by day

40. The hardness values for the commercial sample are similar in magnitude to the control Mozzarella sample in this experiment and also had a similar composition. The more pronounced decrease in hardness over the first 20 days following manufacture may be the result of the change in the distribution of calcium that occurs following manufacture. O'Mahony et al. (2005) found that the softening of Cheddar over the first 21 days of ripening was more strongly correlated with the concentration of insoluble calcium than the level of intact α_{s1}-casein. This phenomenon in Mozzarella is explored in this work. An exploration into the possible influence of the ratio of soluble to insoluble calcium to the results is explored in 10.3.11 & 10.3.12.

It is possible that the slight difference in the moisture content (Table 10-3) may also influence the trend observed in the hardness data. In order to identify whether the variation in the moisture content would affect the different samples the moisture content of the samples compressed on each day of the storage trial were collected. An ANOVA was conducted to identify whether any difference existed in the moisture content of the samples over the storage trial. This returned a P value of 0.342, indicates that the null hypothesis, that mean moisture content of the three samples is the same, should be accepted at an α of 0.05. This indicates that no significant different exists between the moisture content of the three samples on each day of testing.

10.3.5 Melt

The melt properties of the three different Mozzarella samples were found to change over the 40 day storage trial, as shown in Figure 10.3.

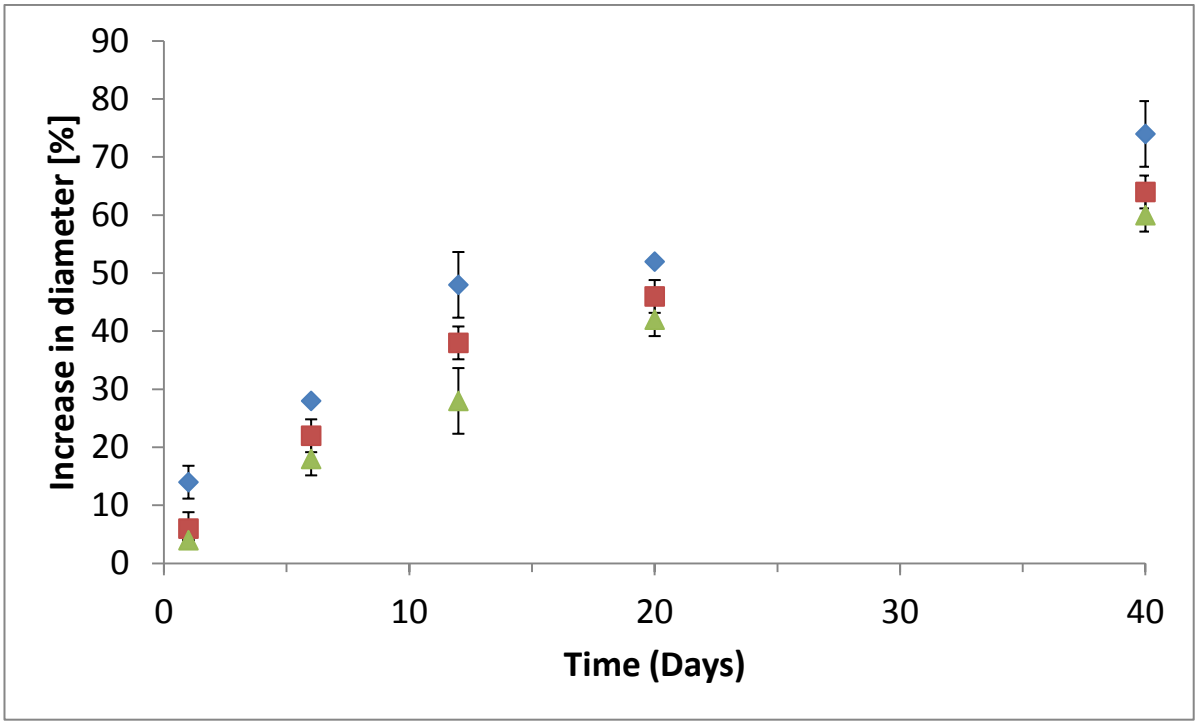


Figure 10.3: Change in meltability of the Mozzarella samples during the 40 day trial [◆Low (129), ■ Control (148) and ▲High (171)].

The Mozzarella samples were all found to increase in meltability over the storage trial. The low calcium Mozzarella sample was found to consistently melt to a greater extent than the other samples, while the high calcium sample melted the least on each day evaluated. It is well established that Mozzarella with lower calcium concentrations flow to a greater extent (Joshi, Muthukumarappan, & Dave, 2003b; McMahon & Oberg, 1999), these results are consistent with this observation.

Over time the melt of all three samples increased, likely the result of the proteolytic breakdown of casein that produces a weaker gel structure (Karami et al., 2009; Tunick, Malin, et al., 1993) resulting in a cheese that can flow more readily when heated. The solubilisation of colloidal calcium, as discussed in regards to the change in the hardness of the cheese samples, may also impact on the degree of melt (explored in 10.3.11 & 10.3.12).

10.3.6 Pizza baking performance

A key consumer attribute for Mozzarella is whether there is a presence or absence of shreds following pizza baking. This is evaluated qualitatively below by assessing photographs. The photographs illustrating the changes in the appearance of the pizzas following melting on the days 1, 20 and 40 are displayed in Figure 10.4 below.

Day	Low (129)	Control (148)	High (171)
Day 1			
Day 20			
Day 40			

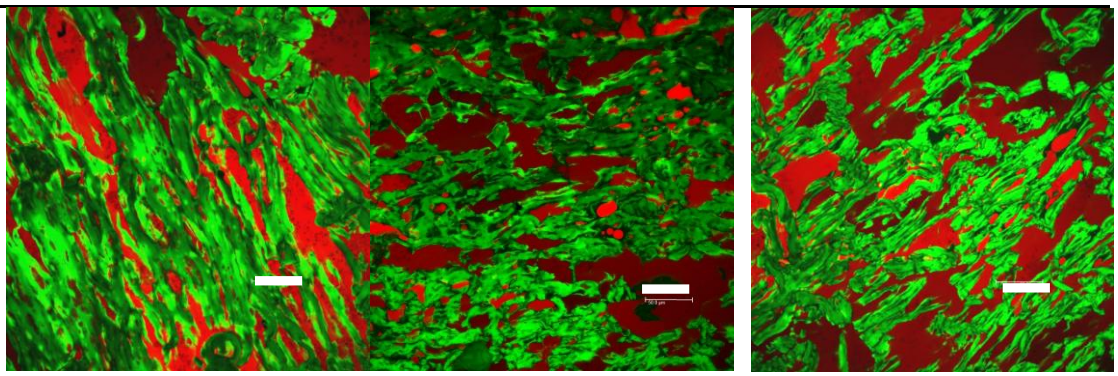
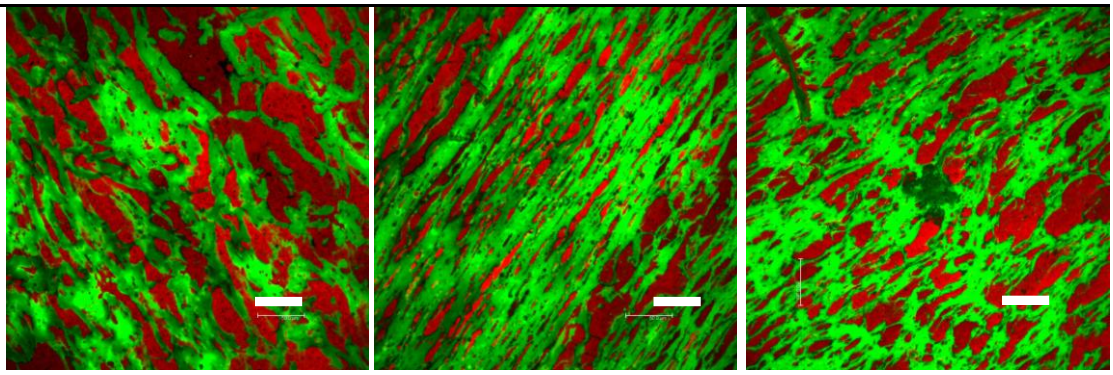
Figure 10.4: Evaluation of pizza bake performance during storage

Immediately following manufacture both the Mozzarella samples Control (148) and High (171) still had individual cheese shreds visible after baking. These samples were also found to burn slightly, probably due to the lack of free oil expressed. However, the low calcium cheese, Low (129), was found to melt better on the first day following manufacture.

On day 20 of the storage trial all three samples were observed to melt with no evidence of individual shreds. None of the samples exhibited burning as they all were observed to have a sheen of free oil on the surface of the cheese. By day 40 all three of the Mozzarella samples were found to melt in an analogous fashion, with no discernible difference being noted between the samples. These samples were found to differ slightly from the samples assessed on day 20 of the storage trial. The difference that exists in the appearance on these two days is that the more mature cheese was identified as having a lesser extent of surface oil pooling, a greater degree of browning and larger blisters.

10.3.7 Confocal microscopy

Confocal microscopy was used to monitor the change in the distribution of fat and protein within each of the Mozzarella samples over the storage trial.

	Low (129)	Control (148)	High (171)
Day 1			
Day 20			

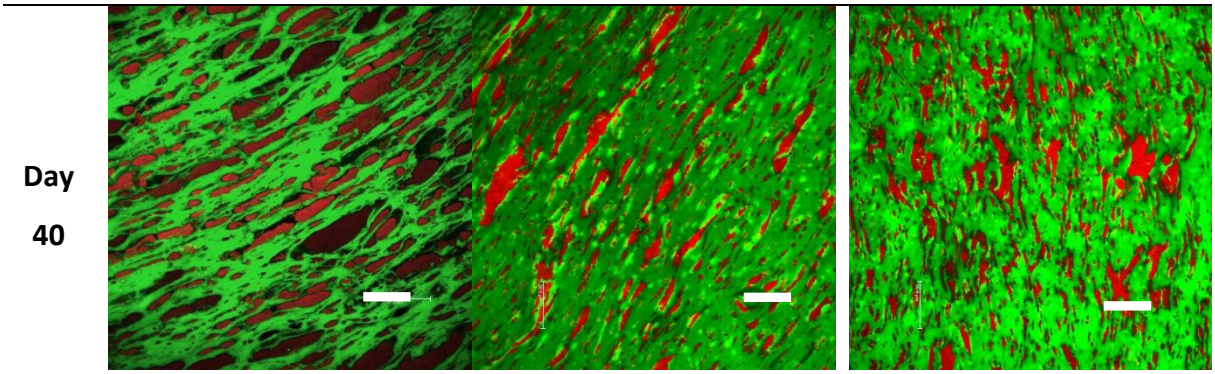


Figure 10.5: Confocal micrographs of the Mozzarella samples on day 1, 20 and 40 of the storage trial (scale bar = 50 µm).

The distribution of fat and protein within the three different Mozzarella samples varied on each day of the days that confocal micrographs were acquired, as displayed in Figure 10.5. On day 1 of the storage trial all three of the samples were observed to have some form of stretched structure, with elongated fat within the cheeses. However, the trends in regards to the formation of fat channels are less obvious than in later days. This is consistent with observations made by McMahon and Oberg (1999) who stated that the fat/serum channels become organised (more pronounced) during the storage of Mozzarella. On day 20, distinct differences were observed between the micrographs of the three different samples. The image of the Low (129) sample indicated that large channels of fat were present in the structure of the cheese. The Control (148) sample was observed to have long thin channels of fat within the structure of the cheese. The High (171) was identified as having much shorter 'channels' within the structure of the cheese. On day 40 of the storage trial, the confocal images of the three different samples were observed to be different. A similar trend to the day 20 micrographs were observed, with the fat channels within the Low (129) sample being large and the High (171) having shorter more 'pocket-like' channels.

Table 10-4: Analysis of confocal micrographs

Sample	Average axial ratio	Standard deviation – axial (µm)	Standard deviation – longitudinal (µm)
Day 1 – Low (129)	6.3	9.9	50
Day 40 – Low (129)	3.9	5.5	27.8
Day 1 – Control	4.1	7.6	23.9
Day 40 – Control	6.3	2.3	23
Day 1 – High	5.2	14	30
Day 40 - High	5.8	4.1	15.9

The analysis of the distribution of fat within the confocal micrographs is displayed in Table 10-4. This indicates the change in the average axial ratio of the fat from day 1 of the trial to day 40 of the trial, along with the standard deviation in both the axial and longitudinal dimensions. The average axial ratio of the Low (129) sample was found to decrease from day 1 to day 40, indicating that the fat within the cheese was becoming more spherical in nature. A change was also identified in the average axial ratio of the control sample, however, unlike the Low (129) sample an increase was observed increasing that the fat was becoming less spherical in nature. A decrease in the axial ratio of the High (171) sample was also identified, although the change was smaller than that of the control sample. The trends identified in the standard deviation in both the axial and longitudinal dimensions indicate that the fat within all three of the Mozzarella samples is becoming more uniformly distributed over the 40 day trial. This is indicated by a decrease in the standard deviation in both dimensions for all three cheese samples over the storage trial. However, to fully quantify the distribution on the fat within the Mozzarella samples multiple images would need to be analysed, beyond the triplicates for this assessment, to gain a more representative assessment of the cheese especially due to the anisotropic nature of Mozzarella.

Over the course of the storage trial each of the three different cheese samples were observed to undergo a change in the distribution of protein and fat. The evolution of

the structure over the 40 days of storage is likely due to the swelling of the casein matrix as free water is absorbed following manufacture. This is similar to the trend observed over the first 30 days following manufacture in the Mozzarella sample studied in Section 6.3.2. This is also consistent with observations made by Guinee et al. (2002) who noted that the fat pockets within Mozzarella diminished in size during a 70 day storage trial and that the microstructural differences that existed of Mozzarella with different calcium levels diminished when samples were aged past 20 days. Unlike the earlier confocal images in Section 5.2.2, free water was not observable in the confocal micrographs on day 1 of the storage trial, despite it being present (as discussed in 10.3.10).

Calcium was perceived to have an effect of reducing the size of the fat channels present in the Mozzarella samples based on the confocal micrographs obtained over the storage trial. This is in accordance with Joshi, Muthukumarappan, et al. (2004a), who observed higher calcium levels lead to a reduction in the size of fat pockets within their SEM micrographs. The Low (129) sample was observed to have the largest channels, while the High (171) sample had short, less channel-like pockets of fat within the cheese structure. This was particularly evident in the confocal images obtained on day 20 and 40 of the storage trial. As calcium acts as a crosslinking agent mediating protein-to-protein interactions (Joshi et al., 2003b), the higher calcium sample will have a stronger protein-protein interactions and/or more protein-protein connections within the casein micelles that make up the protein matrix. This will impact on the development of the fibrous structure that occurs as a result of stretching as the micelles may deform to a lesser extent when sheared. All of the samples were subjected to the same stretching regime. The higher calcium level may result in a greater connectivity of calcium mediated protein-to-protein interactions within the casein micelles giving it a greater level of rigidity. The more rigid micelle may resist the deformation that occurs during the stretching process, becoming less elongated than a reduced calcium micelle.

Prior to stretching the fat within Mozzarella curd is relatively isometric, as it resides in roughly spherical pockets, as identified previously in Figure 6.12. Stretching applies shear to the curd causing a deformation of the fat and elongates it in the direction of flow. This results in the fat pockets within the curd becoming more asymmetrical. The High (171) sample of Mozzarella, due to its higher calcium content, will have a higher viscosity within the cooker/stretcher due to the greater number of calcium mediated protein-to-protein interactions making the micelles that make up the curd tougher. The higher viscosity will reduce the degree of deformation that the fat experiences, thus the fat pockets remain more isometric than the more asymmetrical lower calcium Mozzarella samples.

One of the key issues that arises from microscopy, is gaining a representative image of the structure of a system. With Mozzarella this is compounded due to the anisotropic nature of the structure. By taking a number of images at a range of magnification allows the representative nature of the images to be assessed by comparison.

10.3.8 Scanning electron microscopy

Scanning electron microscopy (SEM) was used to examine the structure of the Mozzarella samples over the 40 day storage trial. However, it was difficult to distinguish any differences between the samples and during the storage trial, so the images are not included in the thesis.

When the SEM images were assessed at a higher magnification the dimpling of the protein channel walls was observed on day 20 of the storage trial, as observed in Section 5.3.3. An example of the dimpling is shown in Figure 10.6 below.

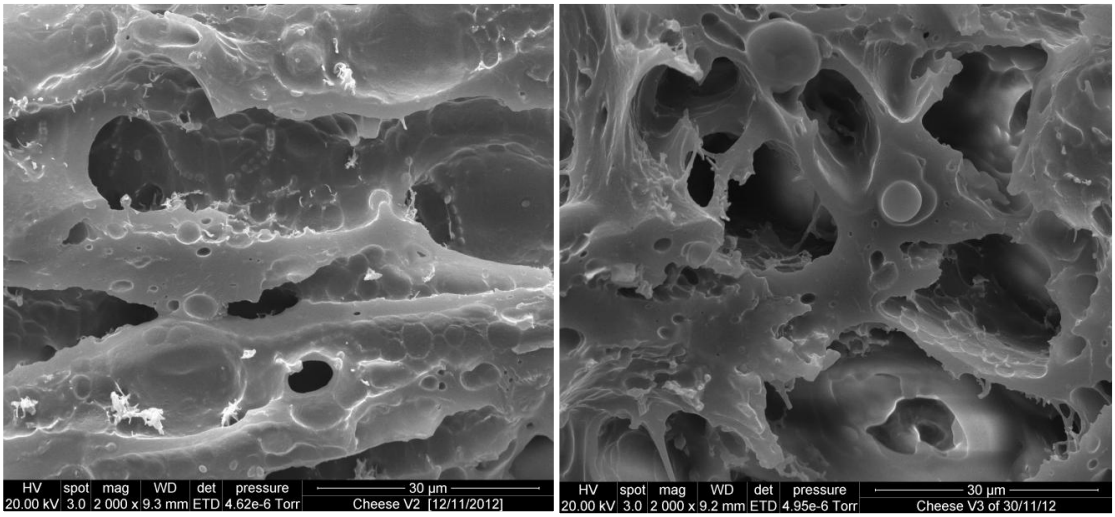


Figure 10.6: Example of the dimples present in Mozzarella cheese at day 20 (left) and day 40 (right) at 2000x magnification.

All of the images obtained on day 20 and 40 of the storage trial indicate that the walls of the protein fibres have indentations, in a similar size range to native fat globules (between 0.2 to 15 µm (Walstra, 1995)). This backs up previous observations (Section 5.3.3) made that as the free water present in the channels is absorbed into the protein matrix, the fibres swell applying pressure to the fat globules that are either in their native state or partially agglomerated.

10.3.9 Urea PAGE

Proteolysis of the three Mozzarella cheese samples was monitored over the storage trial using alkaline urea PAGE (Figure 10.7 & Figure 10.8).

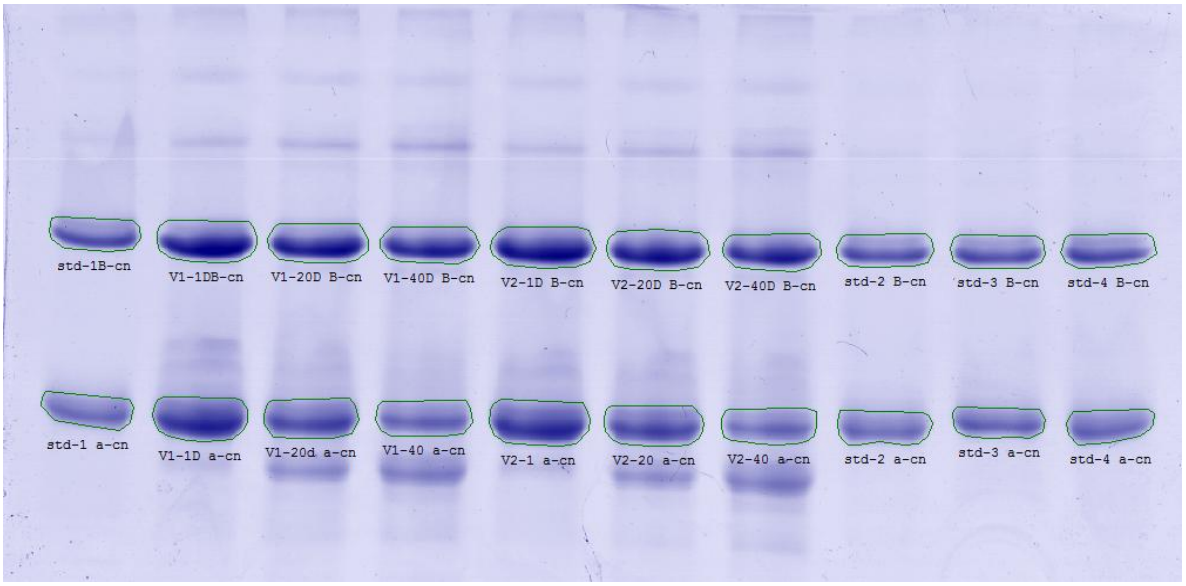


Figure 10.7: An example of the electrophoretogram used to monitor the breakdown of α_{s1}-casein (a-cn) and β-casein (b-cn) for the Mozzarella samples over the 40 day trial. Standards (std) were used for comparison, while V1 represents Low (129) and V2 represents the Control (148) sample. The breakdown profile shown is typical of all breakdown in this trial.

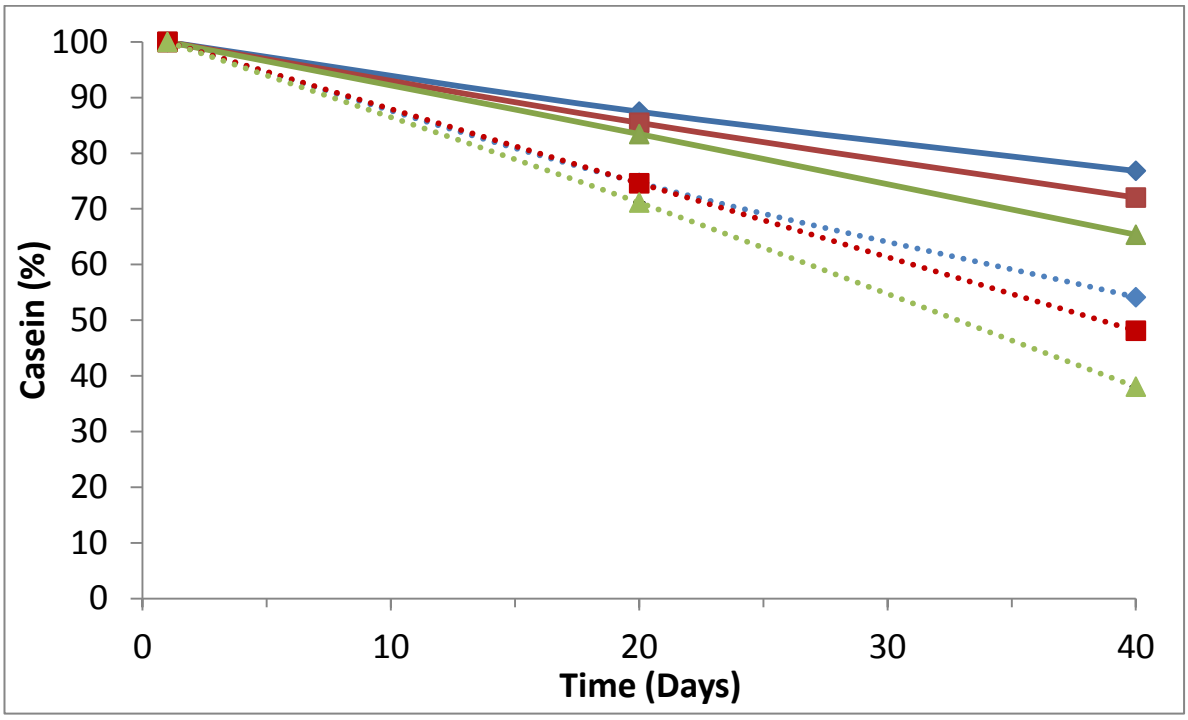


Figure 10.8: Casein breakdown in the Mozzarella samples with the solid line representing β-casein and the dashed line representing α_{s1}-casein [◆Low (129), ■ Control (148) and ▲High (171)].

All three of the Mozzarella samples were observed to decrease in the quantity of intact α_{s1} and β-casein over the course of the storage trial. The breakdown of α_{s1}-casein was found to be greater than β-casein in all three cheeses, similar to the trend exhibited in Section 6.3.4. This trend is unexpected, as discussed in Section 6.3.4, as the heat treatment that the Mozzarella curd is subjected to during stretching has been noted as inactivating chymosin (Lawrence et al., 1987) and inactivating the plasmin inhibitor. This would lead to the breakdown of casein within Mozzarella being dominated by the plasmin, and hence a greater degree of hydrolysis of β-casein than α_{s1}-casein.

A difference in the rate of protein breakdown was also observable in the data collected from the gels. The breakdown of casein was found to be greatest in the sample with the highest calcium content, High (171). One possible reason for this is the greater quantity of rennet used in the manufacture of the sample to obtain the desired set time. However, as mentioned previously, it would be expected that the rennet would be significantly inactivated during the stretching step during manufacture.

As both the breakdown profile and the differences between the three samples suggested that the Fromase was still active within the cheese samples during storage, a brief experiment was conducted to test the premise that Fromase was significantly inactivated during stretching. This involved the addition of Fromase to skim milk. The skim milk was then heated to 59°C with a 5 mL sample taken and chilled in an ice bath every minute for a 10 minute period. Each of the samples taken was added to 45 mL of skim milk with 0.02% (w/v) sodium azide. The skim milk samples were covered with cling wrap and placed in a water bath set to 30°C. The samples were left for an hour before being evaluated to identify whether a gel had formed. The presence of a gel would indicate that the Fromase was still active.

All of the samples, including the samples heat treated for 10 minutes, were identified as forming a gel. These results indicate that there was residual Fromase active in each of the milk samples resulting in the formation of a gel structure. This suggests that there is likely to be active Fromase in the Mozzarella cheese samples studied in this chapter. If this is the case then the activity of Fromase during maturation would

explain the results observed, where the breakdown of α_{s1}-casein was greater than β-casein in each of the samples. It also explains the difference observed in the breakdown of casein by day 40, where the cheese with the most Fromase added was also the cheese with the greatest degree of breakdown.

10.3.10 Magnetic resonance

10.3.10.1 *Relaxation*

The relaxation properties of water within the three Mozzarella samples were assessed using NMR. From this data the quantity of free water present in each of the cheese samples could be monitored during the storage trial (Figure 10.9).

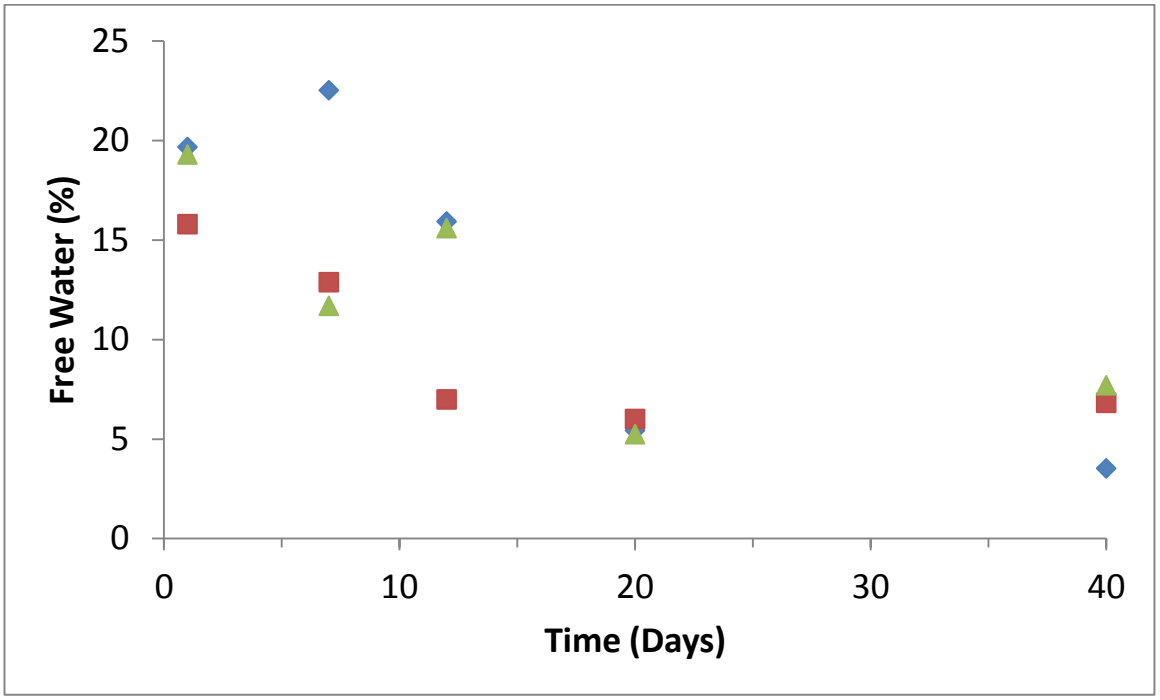


Figure 10.9: Free water at 25°C measured by NMR in the Mozzarella samples over 40 days [◆Low (129), ■ Control (148) and ▲High (171)].

Following manufacture the quantity of free water present in the cheese samples as determined by NMR was identified as decreasing from day 1 to day 20 of the storage trial. The trend identified in the data indicated a similar trend to that exhibited in Chapter 5, Chapter 9 and Kuo et al. (2001). However, no significant difference was

identified between the three different cheeses, p=0.664. This suggests that the application of heat on three samples with similar compositions has a similar effect on the quantity of free water expressed in the cheeses.

The quantity of free water identified within the cheese structure, using NMR, was found to be greater than either of the previous studies in Chapters 5 and 9. These previous studies recorded a maximum percentage free water in the vicinity of 7% for Mozzarella. One possible cause for this increased level of free water identified in the cheese using NMR is a result of the slight change in method used to analyse the sample. The method used in this experiment was optimised based on the data collected from Chapter 6 so that a greater time scale could be used to identify the entire range of relaxation times that water experienced. Additionally, when the samples were cut prior to insertion into the NMR tube they were observed to be 'wetter' during the first few days of testing in comparison to cheeses tested previously. This suggests that the water within the cheese was not physically constrained to a great extent.

Unfortunately due to the time constraints of the storage trial only one sample from each of the three cheeses could be assessed on each day of the storage trial due to both the relaxation and diffusion experiments being conducted on the same NMR instrument. However, based on previous experiments conducted at a similar temperature with duplicates, the error in the T2 measurements can be estimated to be up to 20%.

As in Chapter 8 and 9, the quantity of free water in the cheese samples was also assessed at 65°C.

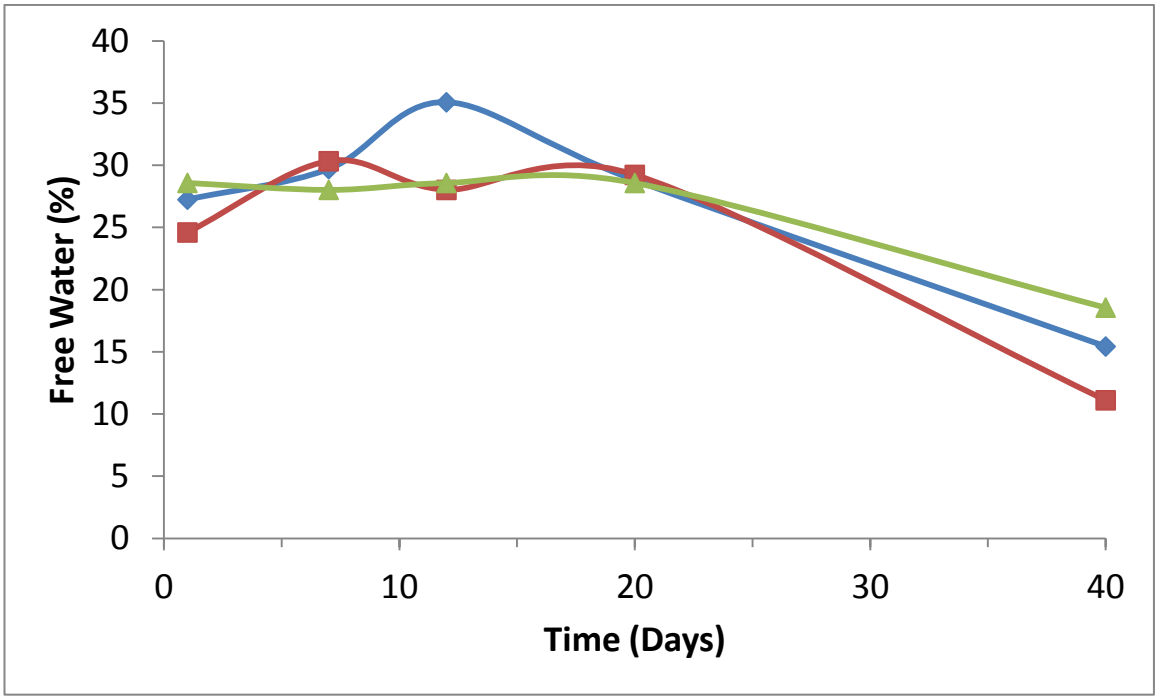


Figure 10.10: Free water at 65°C measured within each of the Mozzarella samples [◆ Low (129), ■ Control (148) and ▲High (171)].

The percentage of the water within each Mozzarella sample that existed in a free state at 65°C was identified as changing over the course of the maturation trial, as shown in Figure 10.10. The Low (129) calcium sample was found to have an initial increase in the quantity of free water at 65°C where it peaked on day 12, followed by a decrease. The Control (148) Mozzarella sample with the intermediate calcium level was also observed to initially increase in free water at 65°C following manufacture. However, he trends exhibited were found to not be significant with no significant difference identified between the three different cheese samples, p=0.82.

This investigation extended the period in which the cheese was monitored in comparison to the study in Montana. This indicated a decrease in the quantity of free water between day 20 and 40. A possible reason for this decrease is proteolysis. As the casein is progressively broken down into fragments (Coker et al., 2005) it results in the liberation of carboxyl and amino groups due to the cleaving of casein molecules which are able to structure water, reducing the water activity of cheese (Fox & McSweeney, 1996).

Another possibility is that proteolysis weakens the effect of hydrophobic interactions on the casein matrix which lessen the degree of contraction that occurs as a response to heating. The hydrophobic interactions are likely to increase in strength during maturation, as a response to the increasing ionic strength (Zangi & Berne, 2006) of the aqueous phase as the CCP solubilises. However, the connectivity of the proteins involved in the hydrophobic interactions decreases with the progressive proteolysis within the cheese. Therefore, even though the hydrophobic interactions are likely to increase in strength, their contribution to the overall casein matrix diminishes as the connectivity decreases. This would reduce the degree of contraction within the matrix during heating, diminishing the quantity of water that is forced out of the protein during heating.

As with the Low (129) and Control (148) measurements, a peak in the quantity of free water at 65°C was also observed at day 8 for the American LMPS Mozzarella studied in Montana in Section 9.3.1.2. One possible explanation for this peak is that there is a greater quantity of calcium in the aqueous phase, due to the solubilisation of the CCP, increasing the ionic strength of the aqueous phase and as a consequence increasing the strength of the hydrophobic interactions in the system. The stronger hydrophobic interactions may result in a greater degree of contraction occurring within the casein matrix during heating, resulting in a greater quantity of free water. As proteolysis is limited at this stage in the storage trial, the contribution the hydrophobic interactions have on the casein matrix is higher. However, as proteolysis continues over the storage trial, the contribution that the hydrophobic interactions impart to the casein matrix may diminish as the connectivity decreases, as mentioned earlier. The High (171) sample was not observed to have a peak in the quantity of free water present in the cheese structure at 65°C. However, as no duplicates could be measured in the time frame of experimentation, no error could be calculated for each sample.

Relaxation measurements at 65°C in Section 9.3.1.2 indicated that there was error between the duplicate measurements, possibly the result of the gradients existing within the brine salted cheese. However, as the experiment in Chapter 9 is the only

one of its kind known to investigate free water in Mozzarella at elevated temperatures, the error can be used as a estimate for the current experiment. If the average error in those results at 65°C (~12%) is applied to the current study, the difference between the samples over the first 20 days of the storage trial becomes insignificant. This suggests that the peaks observed in the data may not exist, with further experiments needed to confirm the results. The decrease in free water between day 20 and 40 is still significant even with the error from Section 9.3.1.2 applied to the data.

10.3.10.2 *Diffusion*

The diffusion properties of water were monitored within each of the three cheese samples during the storage trial to identify whether the different levels of calcium would impact on water movement.

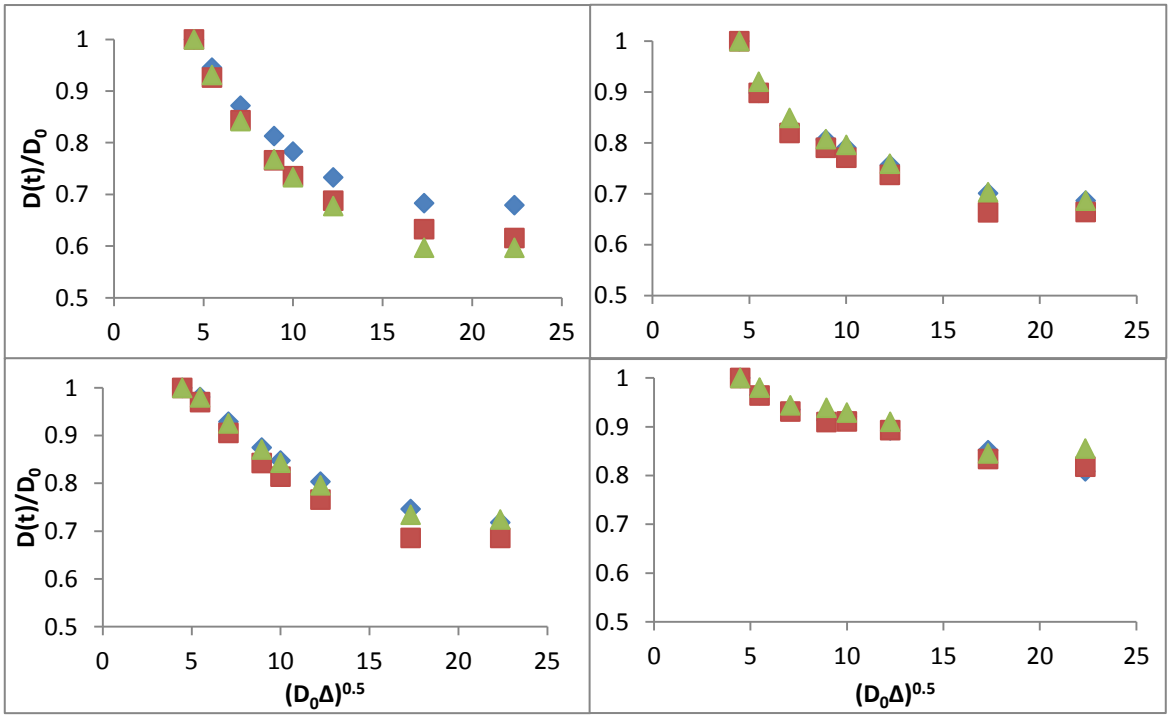


Figure 10.11: Diffusion of water within the Mozzarella samples on day 1 (top) and day 40 (bottom) of the storage trial at 25°C (left) and 65°C (right) [◇Low (129), ■ Control (148) and △High (171)].

The diffusion of water within the three different cheese systems were identified as increasing with temperature, as identified in Chapters 8 and 9. The effect of temperature was observed to be more pronounced at the end of the storage trial (day 40) than initially following manufacture, as shown in Figure 10.11. The diffusion properties of the three Mozzarella samples on the first day of the storage trial were found to be similar at 65°C, with no significant difference existing between the samples at a 95% confidence interval.

On day 40 very little difference was evident between the three different cheese samples at both 25 and 65°C. This indicates that even after 40 days of storage the diffusion properties of water within the three samples were difficult to differentiate between the cheeses. Although similar, there were slight differences in the diffusion data over the 40 day trial between the three samples. To identify how the individual samples changed over the storage trial the data collected at 25 and 65°C was displayed.

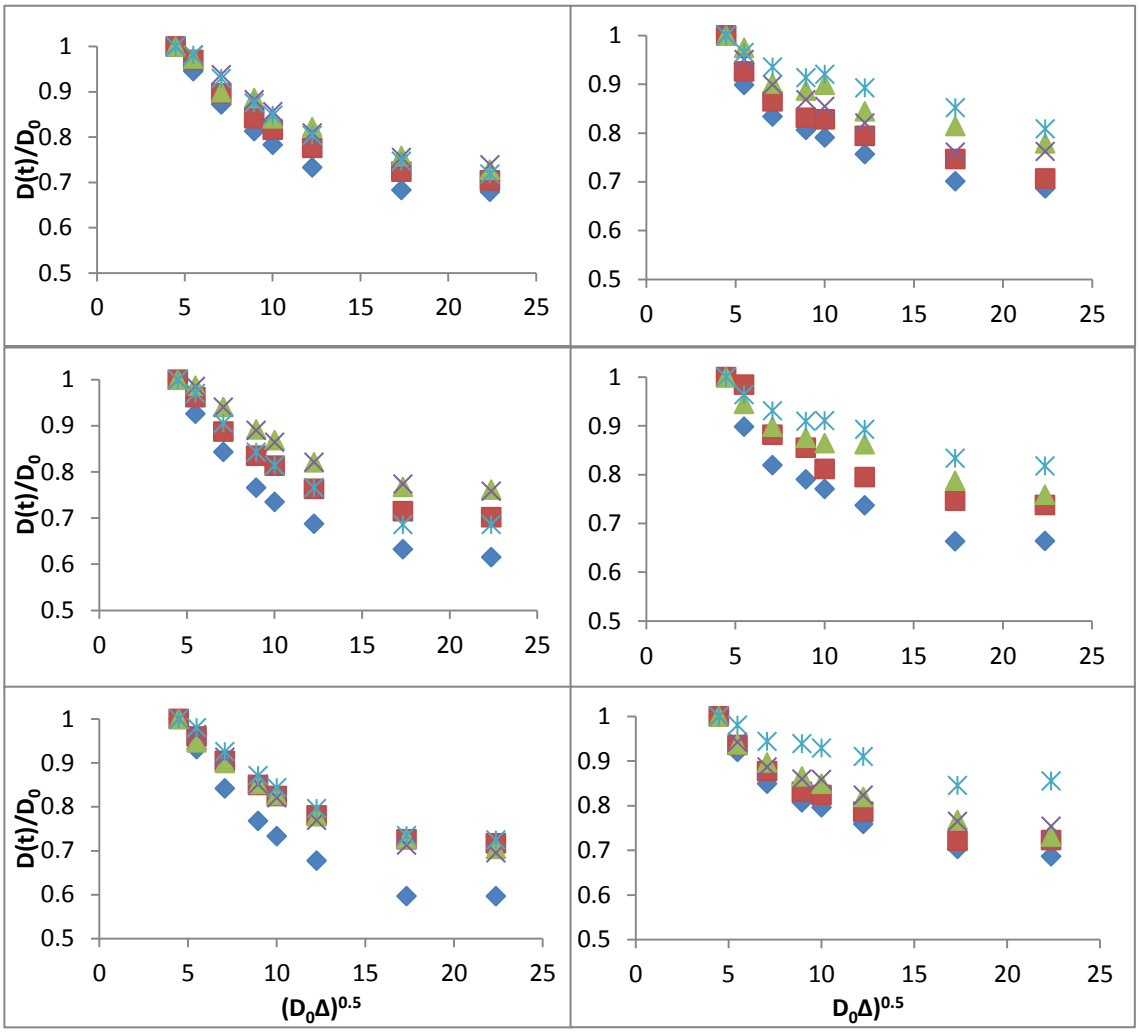


Figure 10.12: Diffusion of water at 25°C (left) and 65°C (right) for the Mozzarella samples: Low (top), Control (middle) and High (bottom) [◇ Day 1, ■ Day 7, △ Day 12, X Day 20 and * Day 40].

Over the 40 day storage trial the three samples exhibited a similar trend in the diffusion properties of water, as illustrated in Figure 10.12. At 25°C no significant difference was identified in any of the three cheese samples over the 40 day trial with p values of 0.7, 0.16 and 0.34 for the Low, Control and High samples respectively. At 65°C a significant difference was identified over the storage trial with p values of 0.03 for the Low (129) sample and 0.02 for both the Control and High (171) samples. A greater degree of separation in the diffusion data for the high calcium sample of Mozzarella over the storage trial was observed. However, this difference may be due to the rate of proteolysis, rather than being related to changes in the distribution of

calcium within the cheese. In Section 9.3.2 it was suggested that the change in the diffusion of water within the cheese measured at 65°C during the storage trial was due to the proteolytic breakdown of the casein matrix. As the previous chapter noted, there was a slight difference in the rate of proteolysis between the three different Mozzarella samples. This did show that the high calcium sample, High (171), had a greater degree of casein breakdown than the other two samples, due to the additional rennet added during processing to achieve the desired set time, still having a degree of activity. The greater quantity of casein breakdown at day 40 in this sample may be responsible for the divergence of the diffusion curve.

To further explore the changes occurring in the diffusion of water as it approaches the tortuosity asymptote, the diffusion at 500 ms was inspected (Figure 10.13).

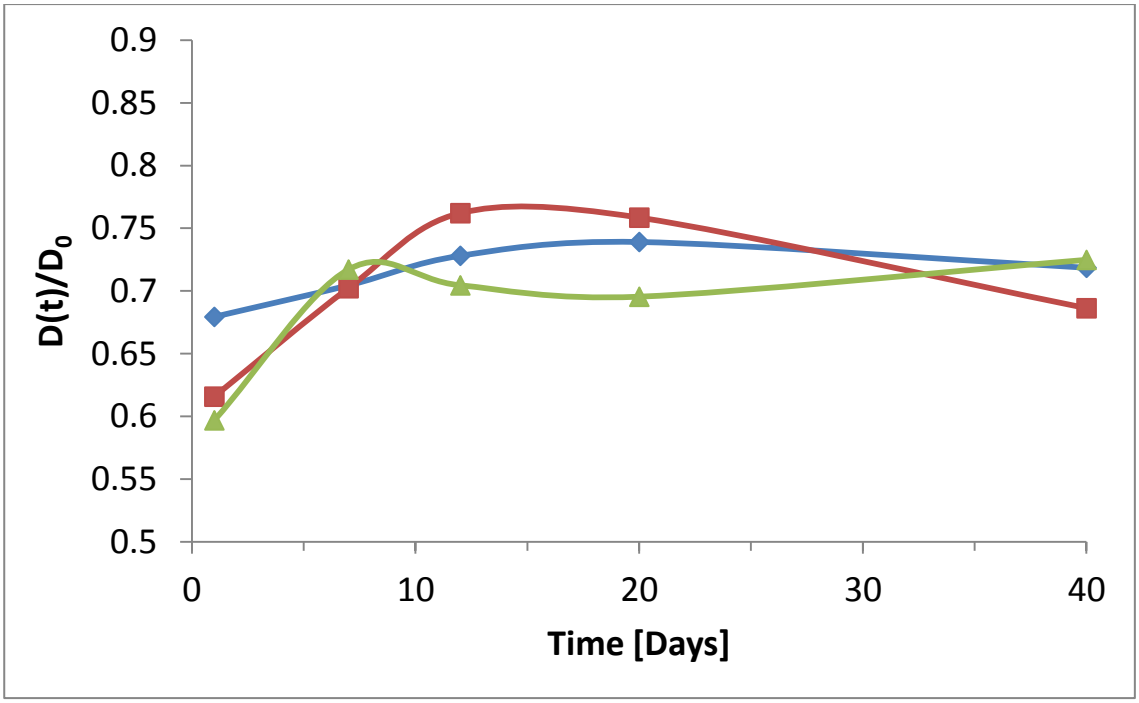


Figure 10.13: Normalised diffusion of water at 500 ms within the Mozzarella samples over the storage trial at 25°C [◆Low (129), ■ Control (148) and ▲High (171)].

Unlike the previous study monitoring the diffusion of water within Mozzarella in Section 9.3.2, a decreasing trend was not identified in the diffusion at a long scale over the storage trial at 25°C. Instead the diffusion of water in each of the Mozzarella samples was found to initially increase following manufacture. However, no significant

difference was identified between the samples, p =0.8. The variation in the data over the storage trial was found to be significant at a 95% confidence interval with a significant difference being identified between the days of the trial, p=0.009.

In the previous study, Chapter 9, the change in the diffusion at 20°C was linked to changes in the distribution of water during storage. This trend was not exhibited in the results from this trial, as no significant difference was identified in the current trial. This discrepancy between the two experiments could be due to a number of factors. These include the differences in the cheese samples including factors associated with the manufacture of the cheeses such as these samples being dry salt cheeses whereas the previous cheese examined was a brine salt cheese. The difference in brine salt and dry salt cheeses is explained in Section 2.2.1.6.

The diffusion of water in the three samples was also assessed at 65°C as it approached the tortuosity asymptote (Figure 10.14).

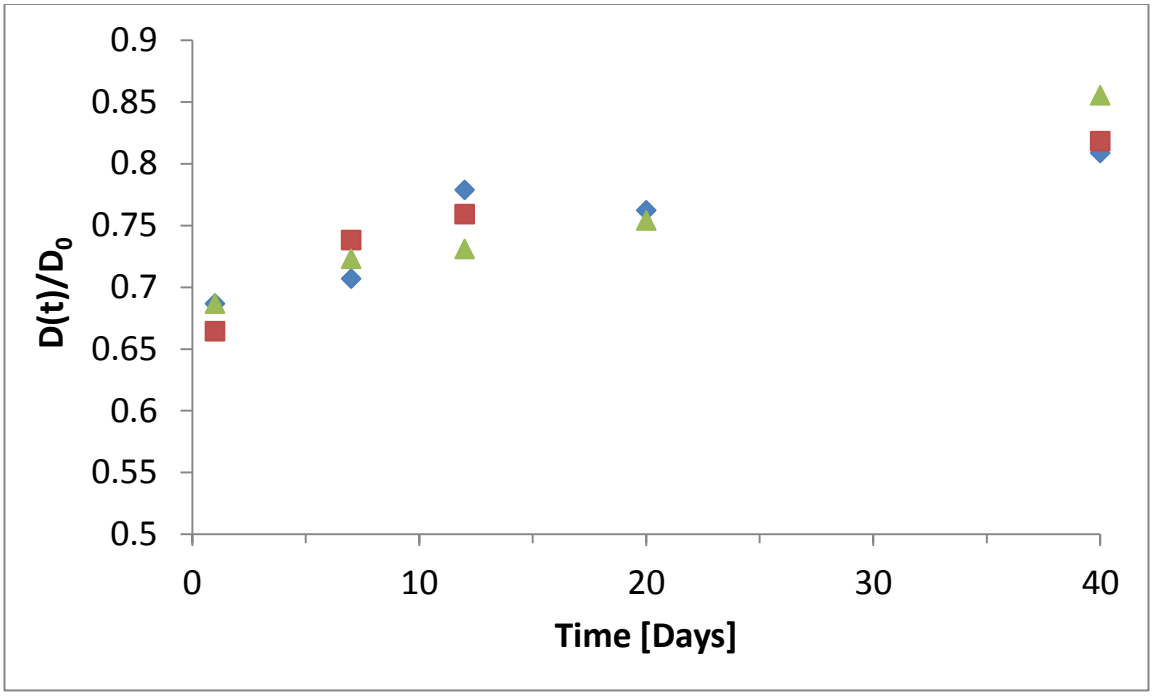


Figure 10.14: Normalised diffusion of water at 500 ms during the storage trial at 65°C [◆Low (129), ■ Control (148) and ▲High (171)].

The diffusion of water within the Mozzarella samples at 65°C at a long time scale for the diffusion experiment (500 ms) was found to progressively increase over the

storage trial. All three of the Mozzarella samples were identified as following a similar increasing trend. The increase in the diffusion coefficient over the storage trial was found to be significant, p=0.001, however no significant difference was identified between the three different samples.

This is similar to the trend identified in Section 9.3.1.2 where the diffusion of water at long time scales at 65°C was found to progressively increase over the 18 day storage trial. The change in the diffusion at long time scales indicates that there is a change in tortuosity. This is due the normalised diffusion being inversely proportional to the tortuosity at a long time scale (Brown et al., 2012; Latour et al., 1995). The increase in the diffusion with storage indicates that there is a corresponding decrease in the tortuosity of the system. The implication of this is that the connectivity of the pores within the casein matrix are decreasing with time, as the tortuosity in porous media is governed by how well the pores are connected (Latour et al., 1995). In cheese, the porous medium is the casein matrix, thus changes in the connectivity of the pores is related to the structural integrity of the matrix. During maturation, the casein within Mozzarella is subjected to proteolytic breakdown (Costabel et al., 2007; Farkye et al., 1991). This progressive breakdown of casein into fragments (Coker et al., 2005) reduces the connectivity of the matrix and thus reduce the tortuous path that water molecules diffuse over.

10.3.11 Soluble calcium

During storage the quantity of soluble calcium extracted was found to change, as shown in Figure 10.15 below.

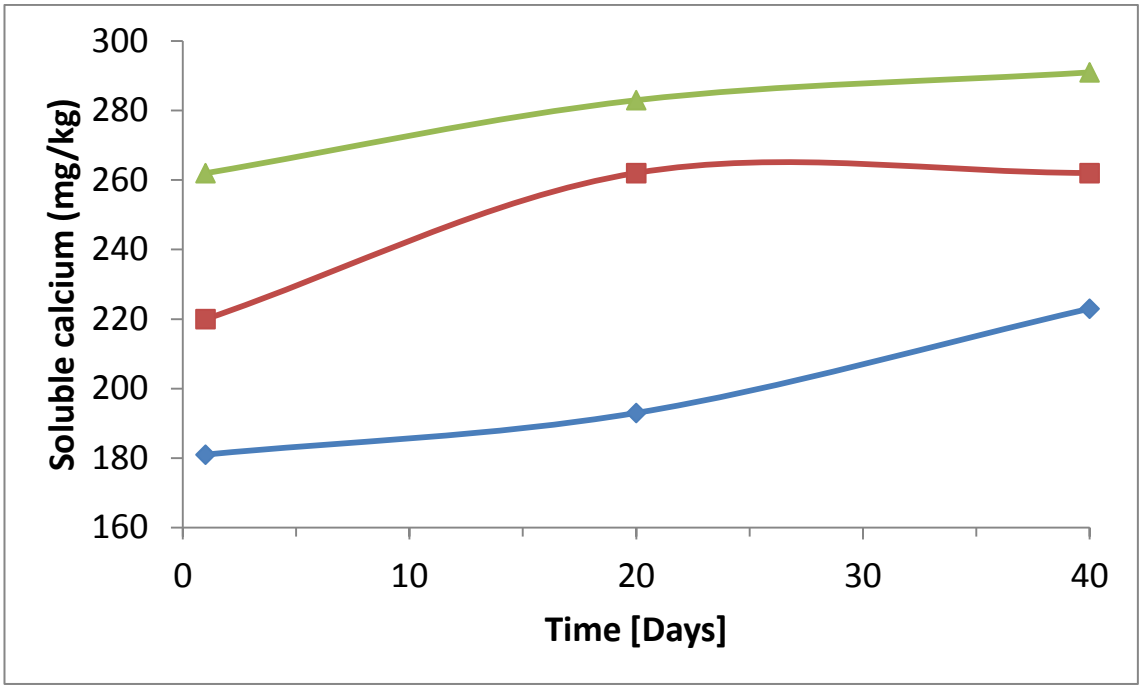


Figure 10.15: Change in the quantity of soluble calcium for the Mozzarella samples over the storage trial. [◇Low (129), ■ Control (148) and △High (171)].

The quantity of soluble calcium extracted in each of the Mozzarella samples was found to increase from day 1 to day 40. This was found to increase with the increasing calcium content of the Mozzarella samples, with High (171) having a higher calcium content and quantity of soluble calcium. This indicated that the quantity of soluble calcium present within each cheese sample was proportional to the total calcium concentration.

This solubilisation of calcium is not unique to Mozzarella with it being studied in Cheddar (Hassan et al., 2004; O'Mahony et al., 2005) and Colby cheese (Lee et al., 2010). The current theory for the solubilisation is a pseudoequilibrium between the soluble and insoluble calcium in cheese that is attained during storage (Hassan et al., 2004).

The change in the distribution of water that occurs during the storage of cheese may also influence the solubilisation of calcium. As the water becomes progressively more associated with the casein matrix, it modifies the local aqueous environment that the

CCP experiences. As the CCP finds itself exposed to a greater quantity of water, it may disassociate until a new equilibrium is reached.

The quantity of soluble calcium extracted is only a small fraction of the total calcium within the cheese. The change in the extracted soluble calcium as a fraction of the total calcium within the cheese was also evaluated (Figure 10.16).

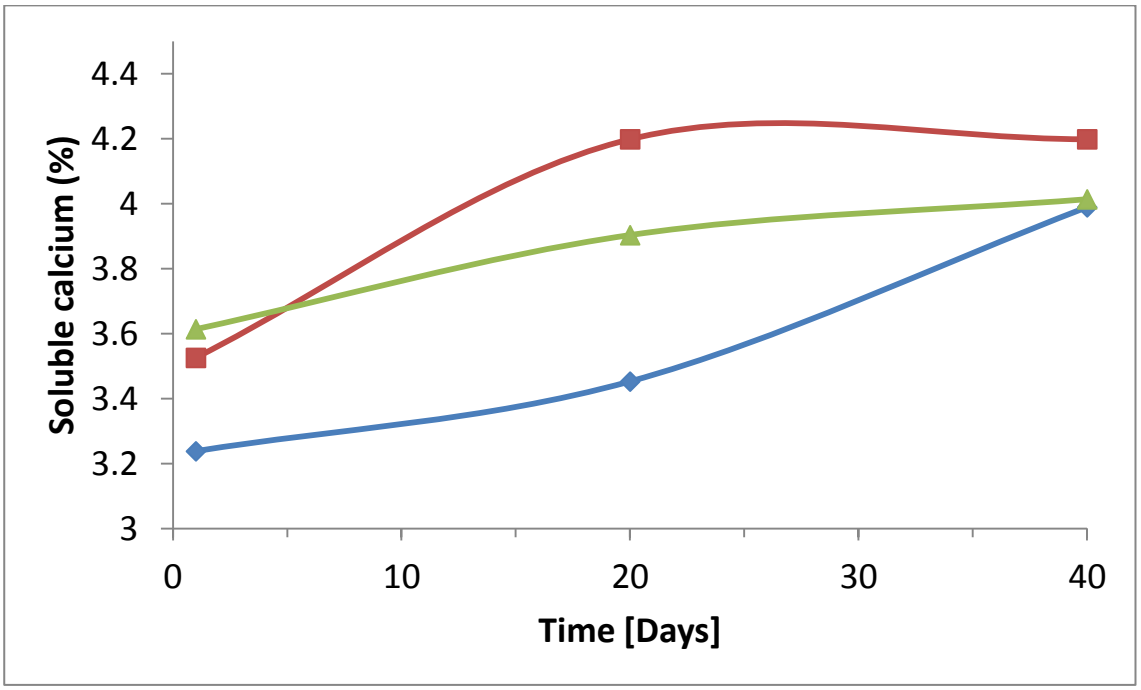


Figure 10.16: Change in the percentage of calcium within the Mozzarella samples that is soluble [◆Low (129), ■ Control (148) and ▲High (171)].

The percentage of the calcium that was soluble in each of the cheese samples was found to increase from day 1 to day 40. The trends exhibited in Figure 10.16 differed from those for the overall quantity of soluble calcium extracted from each cheese in Figure 10.15. The percentage of calcium that was soluble in the cheeses was initially identified as being lowest in the Low (129) while the higher calcium samples, Control (148) and High (171), had a similar percentage calcium that was soluble. On day 20 and 40 the sample with the intermediate level of calcium, Control (148), was found to have a higher percentage of calcium that was in a soluble state than the other two samples.

A limited number of samples could be analysed for soluble calcium so no duplicates could be repeated. As no error has been reported using this method in literature no

error can be associated with the technique. However, the extraction method used to measure the soluble calcium within the Mozzarella samples could influence the results. The use of MilliQ water for the extraction may influence the calcium equilibrium by changing the ionic strength and pH of the cheese (Hassan et al., 2004). It may also impact on the quantity of water in proximity to CCP which could modify the solubility of these nanoclusters. The temperature that the extraction was performed at was 60°C (Metzger, Barbano, & Kindstedt, 2001), which is also likely to influence the calcium equilibrium of the cheese.

Due to the limitations of the calcium extraction experiment, a further technique was sought to monitor the changes in the colloidal calcium phosphate (CCP).

10.3.12 Phosphorus NMR

Solid state ^{31}P NMR with magic angle spinning can be used to isolate different forms of phosphorus in cheese based on their nature and dynamics (Rondeau-Mouro et al., 2009a). Due to its relationship with calcium ions in a cheese system, changes in the phosphorus in the CCP can be related to the state of calcium in the cheese.

An example of the spectral information obtained in these experiments is indicated below.

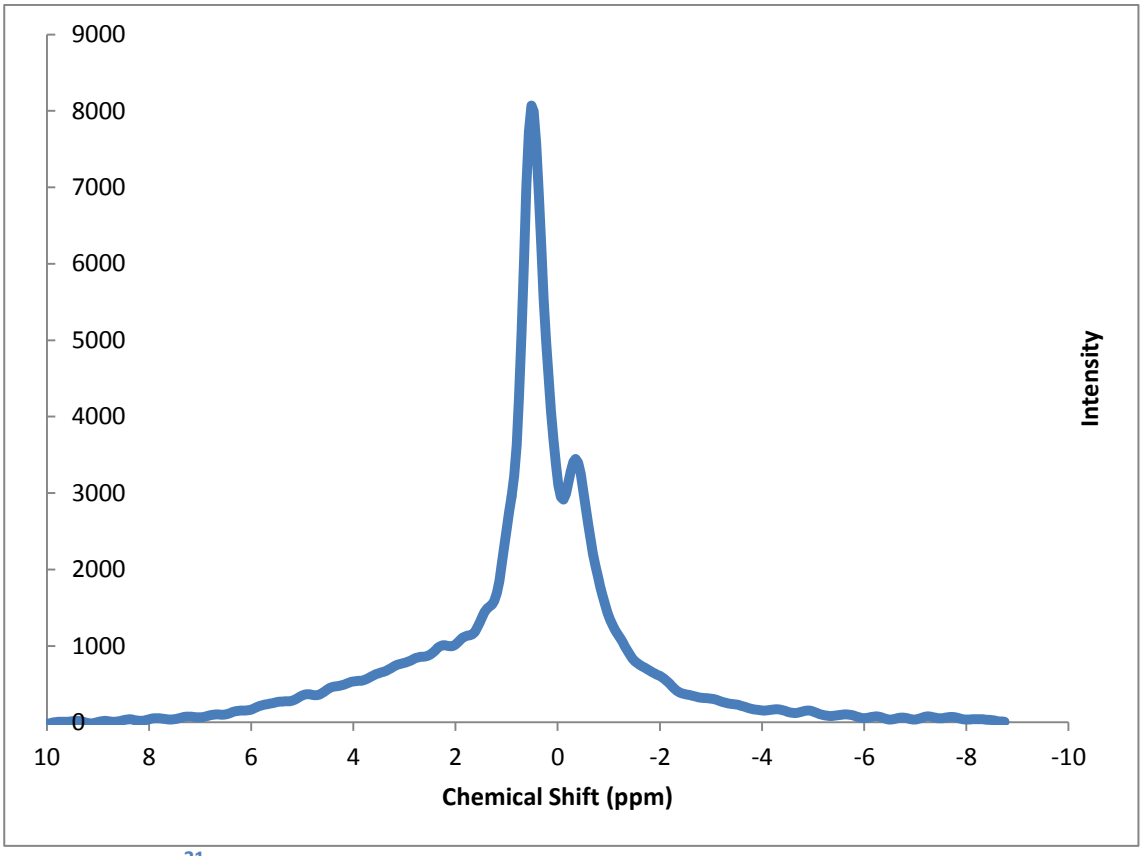


Figure 10.17: ^{31}P MAS spectrum.

The line width is inversely proportional to the mobility of the phosphorus, with narrow peaks indicating mobile phosphorus and broad peaks representing immobile phosphorus. Figure 10.17 shows the spectrum of signal for the phosphorus in cheese. The spectrum is made up of three peaks: a low broad peak and two narrow intense peaks. A broad weak signal has been attributed to the immobile phosphorus within the cheese, namely the colloidal calcium phosphate along with phosphoserine residues (Gobet et al., 2010). This broad peak contributes to the initial curvature of the spectral map but is not shown as a separate peak. The largest of the narrow intense signals represents the contribution from mobile phosphorus within the cheese. In cheese the mobile phosphate consists of inorganic phosphates as well as mobile phosphoserine residues (Rondeau-Mouro et al., 2009a).

Literature is less clear about what the secondary (smaller) narrow peak represents. Gobet et al. (2010) suggest that the two narrow peaks represent different states of mobile phosphorus, with the larger of the two peaks associated with mobile

phosphoserine residues and the secondary peak representing the inorganic phosphorus (Rondeau-Mouro, Gobet, Mietton, Buchin, & Moreau, 2009b). However, this secondary peak has also been described as an exchange peak (Gobet et al., 2010).

However, the change in the immobile phosphorus was of particular interest in this investigation. Hence the proportion of phosphorus described by the broad peak and the most intense narrow peak are compared over the storage trial in Figure 10.18 below.

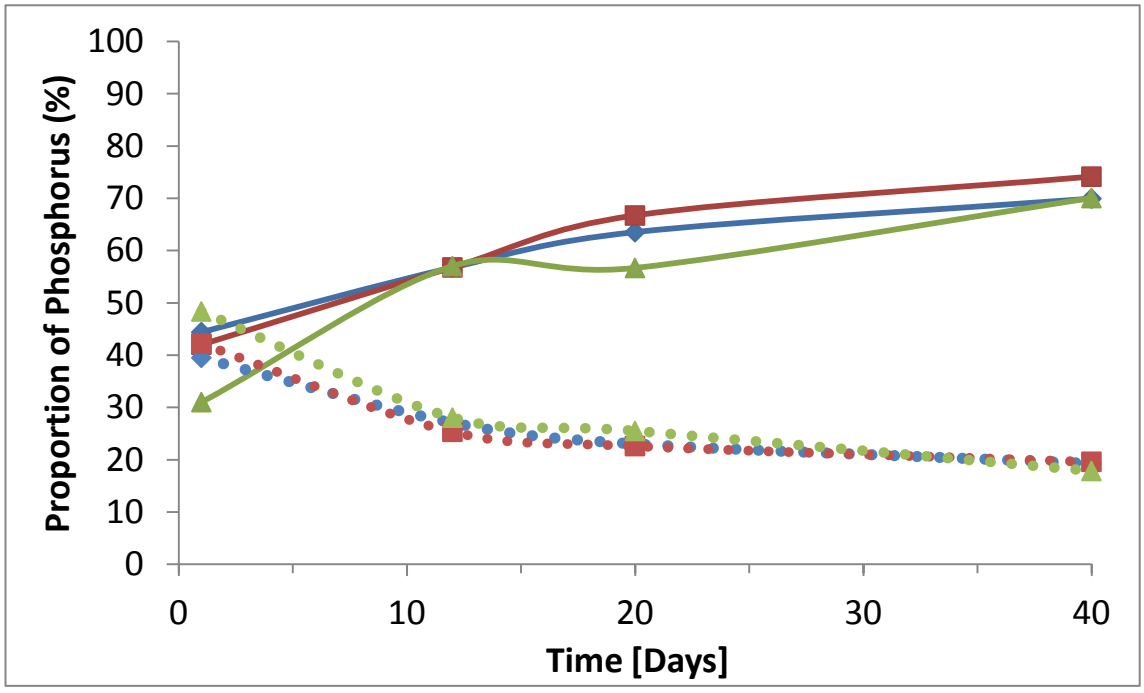


Figure 10.18: Change in the proportion of mobile phosphorus (solid line) and immobile phosphorus (dashed line) containing groups over the 40 day storage trial [◆Low (129), ■ Control (148) and ▲High (171)].

A decrease in the proportion of immobile phosphorus contained within the cheese was observed over the 40 day storage trial. This was coupled with a corresponding increase in the mobile phosphorus within the samples. The change in both mobile and immobile phosphorus was found to be greatest over the first 12 days of the storage trial, although this judgement is limited by the number of data points collected. The change over the storage trial in the mobile and immobile phosphorus was found to be significant, $p=0.0001$. However, no significant difference was identified between the samples ($p=0.8$).

This decrease in the proportion of immobile phosphorus present within the cheese samples suggests that there is a decrease in the immobile colloidal phosphorus groups. This corresponds with the solubilisation of calcium that occurs during the initial stages of storage. It is also possible that the change in the distribution of water within the cheese influences the mobility of phosphorus groups. This could occur as the protein matrix becomes more hydrated the phosposerine residues may become more mobile.

The change in the mobility of phosphorus within the cheese suggests that there is a corresponding change in calcium within the cheese. This is due to immobile phosphorus in cheese being located primarily in CCP nanoclusters distributed throughout the casein micelles (Holt, Wahlgren, & Drakenberg, 1996). The reduction in the proportion of immobile phosphorus suggests that the amount of CCP is decreasing, which would be coupled with an increase in the soluble calcium in the aqueous phase.

It also suggests that the phosphate groups are becoming more mobile which may then act to buffer the system to a greater extent, resulting in the increase in pH observed in the previous chapter.

This initial investigation into the use of ^{31}P NMR as a technique to monitor changes in phosphorus during maturation in cheese has shown encouraging results. However, to fully understand the transformation of phosphorus and its relation to the calcium within cheese it is recommended that further work be conducted using this technique. In particular the differentiation of organic and inorganic phosphate groups within the cheese may aid in gaining a better understanding of the change in CCP.

10.3.13 Dielectric spectroscopy

The dielectric constant of the three cheese samples was evaluated over the 40 day storage trial (Figure 10.19).

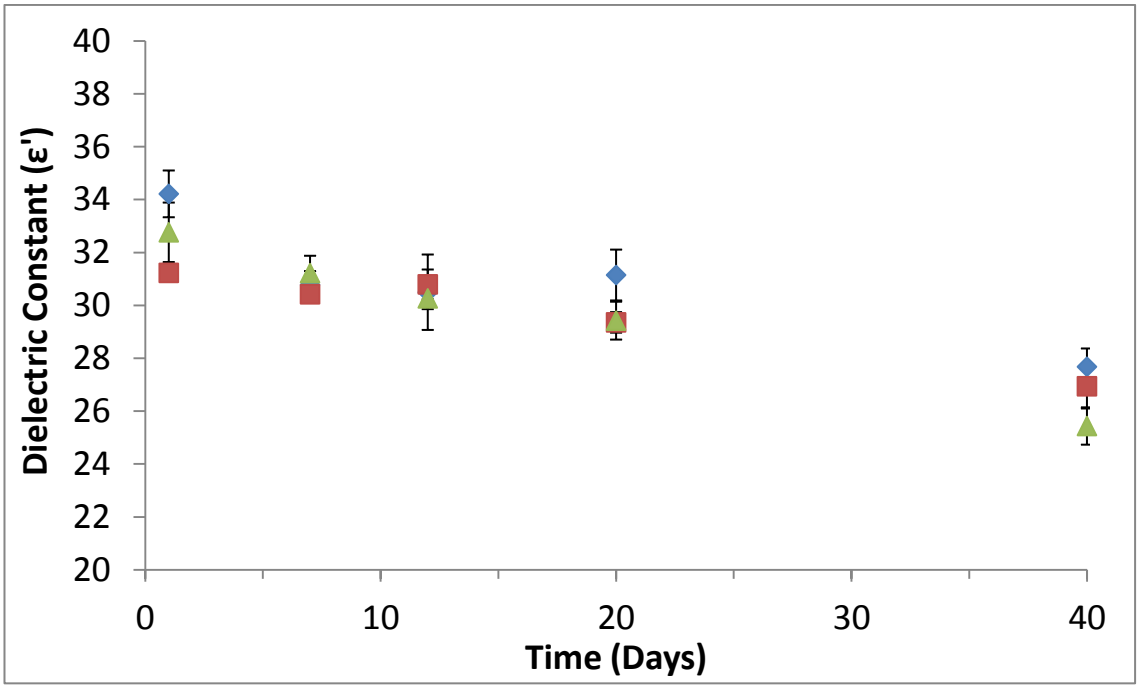


Figure 10.19: Dielectric constant of the Mozzarella samples over the storage trial at 1.26 GHz [◇Low (129), ■ Control (148) and △High (171)].

The dielectric constant of each of the Mozzarella samples was observed to decrease in magnitude over the course of the storage trial. As the dielectric constant has been related to water mobility in the previous research, including Feng et al. (2002) and Chapter 6, a decreasing trend suggests that the water is becoming less mobile in the cheese at 20°C over the storage trial. The drop in the dielectric constant over the first 20 days following manufacture may possibly be the result of the redistribution of free water identified using NMR. Following this the structuring of water that occurs as a result of proteolysis (Fox & McSweeney, 1996) may be responsible for the continued decrease in the dielectric constant, as the water becomes more associated with the casein and less mobile. However, this trend was not exhibited in the previous experiments where the dielectric constant was monitored during storage.

The dielectric constant was similar for all of the three Mozzarella samples throughout the storage trial. This indicates that little difference could be identified between the three different samples in terms of the dielectric constant. There were slight differences in the dielectric constant of the samples on various days of the study, but these differences were generally within the bounds of the error in the measurement.

However, as discussed in previous chapters, variation in the moisture content of the cheese samples along with the change in the distribution of water within the Mozzarella will impact on the dielectric constant.

The dielectric loss factor was found to vary over the course of the storage trial for the three different samples of Mozzarella (Figure 10.20).

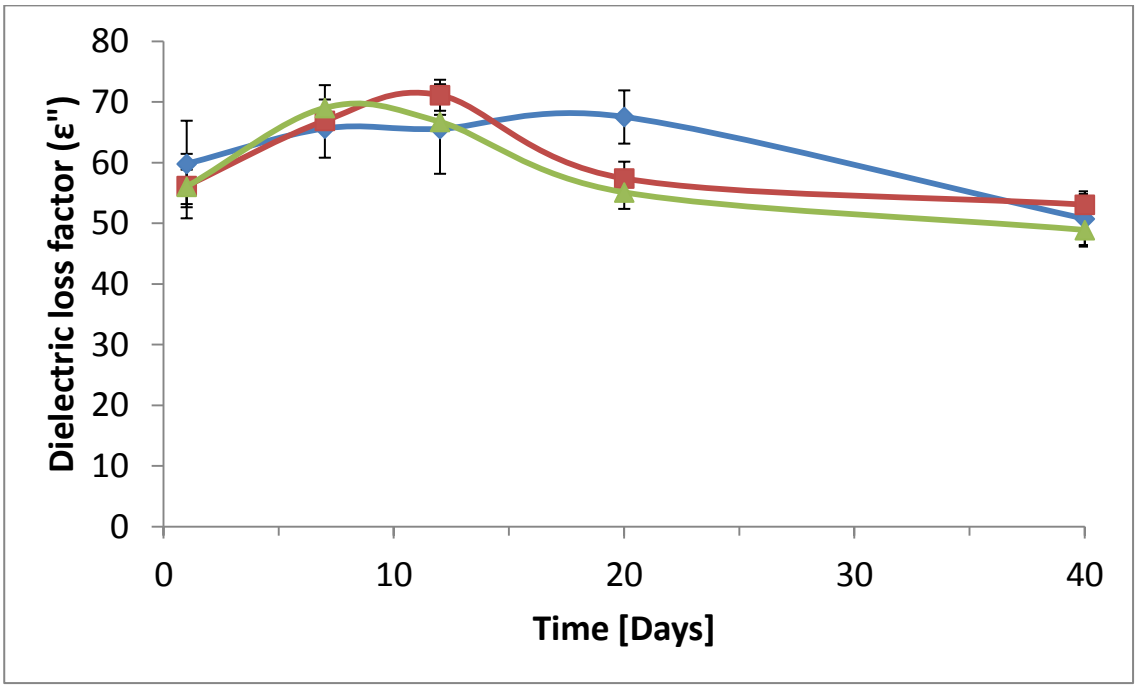


Figure 10.20: Change in the dielectric loss factor over the storage trial at 244 MHz [◆ Low (129), ■ Control (148) and ▲High (171)].

The loss factor was observed to initially increase following manufacture in all three of the Mozzarella samples during the 40 day storage trial. An order was evident within the different cheese samples as to when the peak dielectric loss was reached, with it occurring earlier at higher calcium concentrations. Although the low calcium sample, Low (129), was observed to have a peak on day 20 of the storage trial the change in the dielectric loss factor was within the range of error for the sample. However, as with the other two samples, the dielectric loss factor was found to decrease in magnitude following the initial increase.

As mentioned in previous chapters, the dielectric loss factor is influenced by a number of variables. Two key changes occurring in the Mozzarella samples that will influence

the dielectric loss factor over the storage trial are the solubilisation of calcium and the change in the distribution of water. The solubilisation of calcium will result in a greater quantity of ionic calcium in the aqueous phase. The change in the mobility of water within the Mozzarella samples will also impact on the dielectric loss factor; with the movement of the aqueous phase becoming more restricted the loss factor may also decrease. The peak observed in the dielectric loss data for each cheese may be the result of a transition of the dominance of mechanisms, with the solubilisation initially being the predominant mechanism affecting the dielectric loss factor until the movement of water dominates.

In Chapter 6 the dielectric constant was used to predict the quantity of free water present in Mozzarella. By applying the predictive model from the previous study to the results from this trial the percentage free water could be calculated on each day assessed, as shown in Figure 10.21.

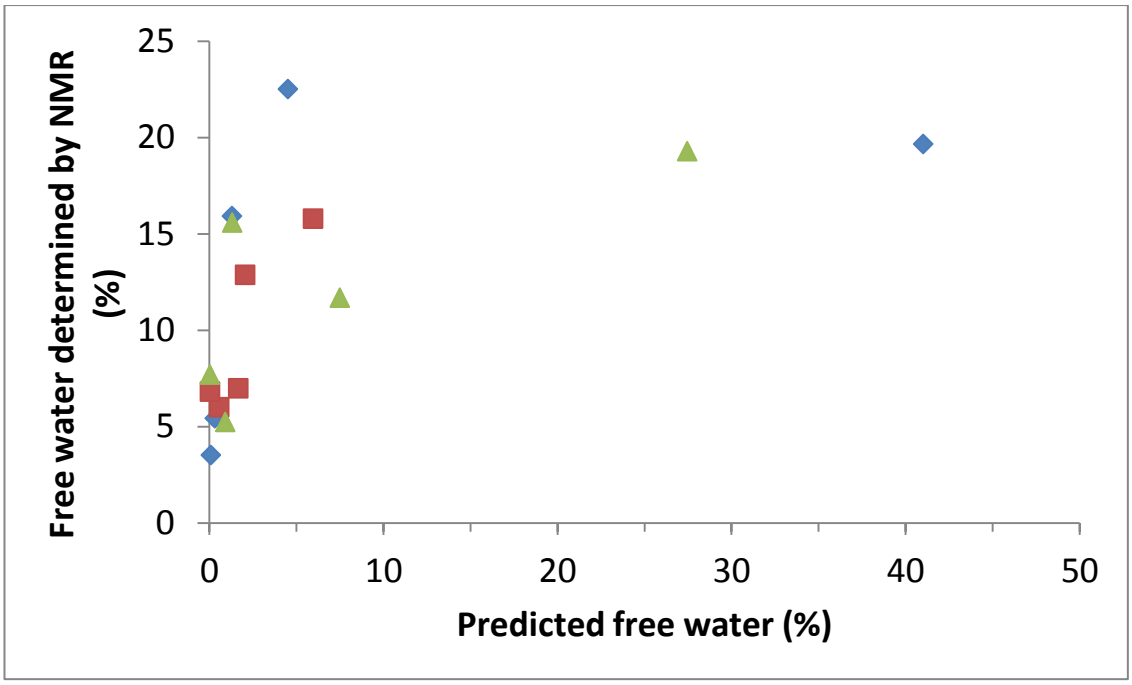


Figure 10.21: Comparison of the actual percentage free water with that predicted [◆ Low (129), ■ Control (148) and ▲High (171)].

Figure 10.21 shows the relationship (or lack thereof) between the quantity of free water determined using NMR and the quantity predicted based on the dielectric

constant. The figure indicated that the predictive model was incapable of matching the actual measured quantity of free water as determined by NMR. If the model was capable of predicting the free water content in the cheese samples a straight line would have been observed in the figure.

As the quantity of free water determined by NMR was higher in this study in comparison to previous studies, the plot used to create the predictive model in Chapter 6 was reconstructed including the data from the current study (Figure 10.22).

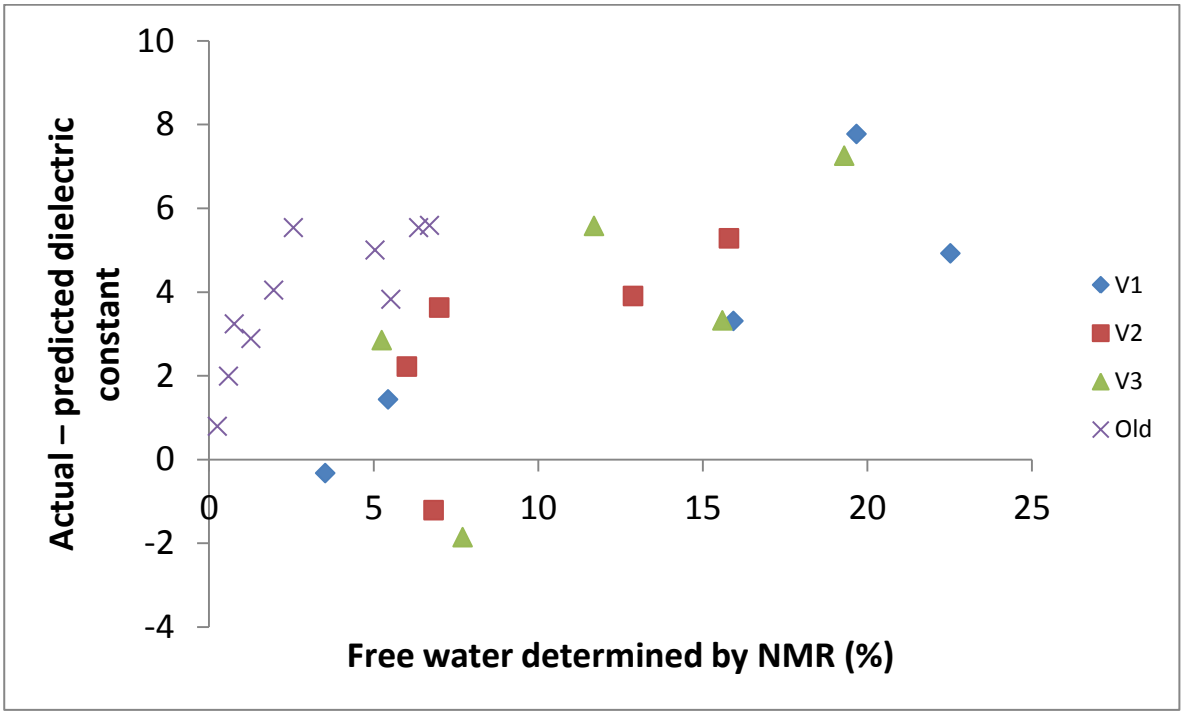


Figure 10.22: Difference in the actual and predicted dielectric constant versus free water determined by NMR [◆Low (129), ■ Control (148) and ▲High (171) and X data from Chapter 6].

When the plot comparing the difference in the measured and calculated dielectric constant with the quantity of free water for the three samples was compared to the results from Chapter 6, little crossover was noted. This indicates that the data from the two studies cannot be directly compared, based primarily on the disparity in the quantity of free water determined using NMR.

When comparing the three different cheese samples from the current study, no obvious grouping was evident distinguishing the different calcium levels. In order to

assess the degree of variation within the data collected from the current study, the data was replotted and the trends in the data evaluated (Figure 10.23).

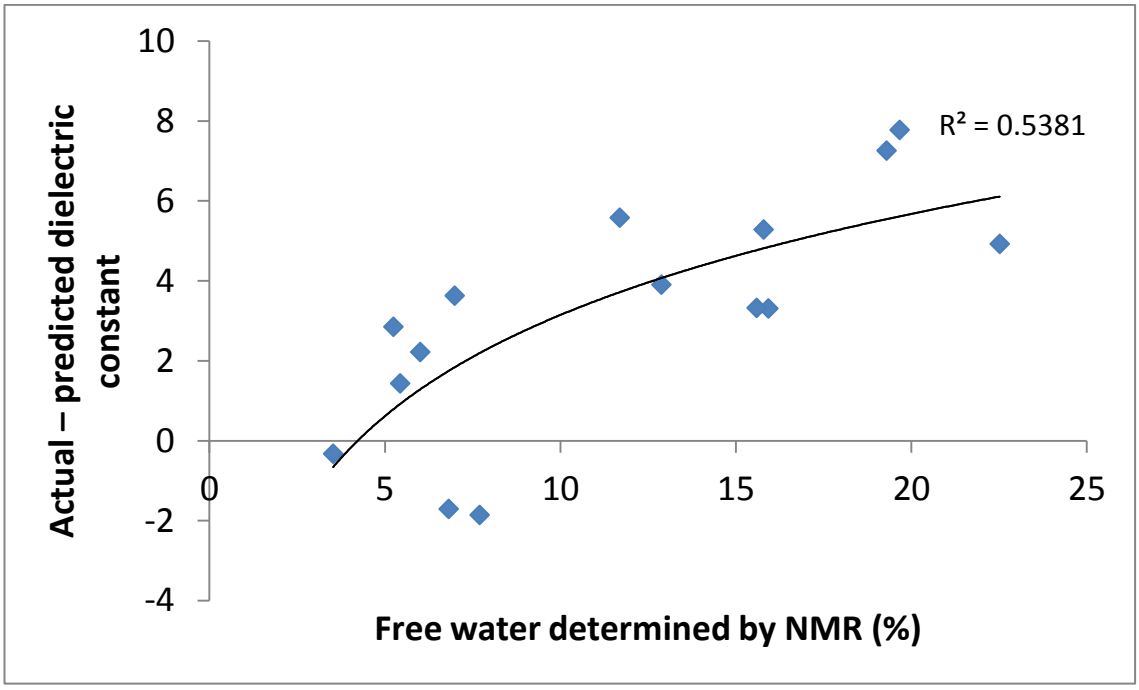


Figure 10.23: Difference in the actual and predicted dielectric constant versus free water determined by NMR.

A relationship was identified between the quantity of free water and the difference in the actual and predicted dielectric constant. However, the relationship was much weaker than that identified in Section 5.3.6. This is due to a greater degree of variation in the data than the previous study. This does suggest that there is a relationship between the dielectric constant and the quantity of free water within the cheese; however, the relationship is not as simple as previously hypothesised.

However, the decrease in the actual dielectric constant measurements collected over the storage trial did indicate a decreasing trend, suggesting a decrease in the degree of water mobility occurring within the different samples during storage.

10.3.14 Statistical analysis

In order to assess the data collected from the storage trial, in both this chapter and the previous chapter, Principle component analysis (PCA) was used.

Table 10-5: Eigenvector values for variables associated with the first two principle components.

	PC1 (69.8%)	PC2 (18.5)
Calcium	0.011	**-0.457**
Day	**-0.265**	-0.043
Moisture	-0.183	**0.347**
Melt	**-0.262**	0.079
pH	-0.246	0.072
Uniaxial comp	-0.221	-0.282
Free water 25	0.235	-0.081
Free water 65	0.200	0.111
Diffusion 25	-0.208	0.251
Diffusion 65	**-0.261**	-0.052
e' 1260 MHz	0.244	0.185
e" 244 MHz	0.116	**0.402**
Soluble Calcium	-0.101	**-0.448**
B casein	**0.262**	0.050
A casein	**0.264**	0.057
% of calcium soluble	-0.216	-0.274
P31 peak 1	**-0.261**	0.088
P31 peak 2	0.195	-0.005
P31 Peak 3	**0.260**	-0.080

The principle component analysis identified that the first two components accounted for 88.3% of the total variation in the results. The first principle component (PC1) was found to account for 69.8% of the total variation while the second (PC2) accounted for 18.5%.

The eigenvectors for PC1 were found to be relatively similar in magnitude for most of the variables assessed over the storage trial. However, if we consider all of the eigenvector values of 0.260 and above we can assess which variables are significant to PC1. Positive eigenvector values for PC1 included the breakdown of both caseins and the change in immobile phosphate groups (P31 peak 3). Negative eigenvectors were associated with the mobile phosphate groups (P31 peak 1), the diffusion of water at

65°C, melt and the variable of time (Day). The interrelationship between these variables is likely signified by their high eigenvectors for PC1. As the days increase, the colloidal calcium phosphate solubilises leading to a decrease in immobile phosphorus groups and the casein within the cheese is hydrolysed causing a decrease in α_{s1} and β casein. The result of the decrease in immobile calcium and intact α_{s1} and β casein is that the cheese melts to a greater extent and the water can diffuse through the molten cheese structure to a greater extent.

The second principle component (PC2) had fewer significant eigenvector values for the variables studied over the storage trial. Above values of ± 0.4, three variables existed that strongly influenced PC2. These variables were the calcium content, amount of soluble calcium and the dielectric loss factor (ε" 244 MHz). As the dielectric loss factor is associated with ionic interactions, it would be expected that it would vary with the calcium content and quantity of soluble calcium. However, both of the calcium variables contrasted the dielectric loss factor indicating that an inverse relationship exists between the variables. By delving further into the eigenvector values for PC2 it was determined that the next most significant variable was the moisture content of the cheese samples (0.347). The sample with the highest calcium content also was found to have the lowest moisture content, with a consistent inverse relationship existing between the two parameters. As the dielectric loss factor is also influenced by the quantity of water in the system, the lower moisture content will lead to a higher concentration of ions within the aqueous phase resulting in a higher dielectric loss factor.

10.4 General discussion

The Mozzarella manufacture produced three cheese samples with different levels of calcium. These samples were found to be relatively similar in composition and pH. This was achieved by manipulating the pH at various stages during the manufacture of the samples.

The differing calcium levels were found to behave as expected in regards to both hardness and melt tests. The hardness of the three cheese samples decreased following manufacture, with the High (171) found to be consistently harder than the lower calcium samples. This decrease is possibly the result of the change in the quantity of insoluble calcium within each cheese. The subsequent decrease in hardness over the storage trial is initially dominated by the solubilisation of colloidal calcium. The meltability of the three different cheeses also behaved as expected, with the low calcium sample [Low (129)] melting to a greater extent than the other samples. The progressive increase in melt is likely to be a result of the progressive breakdown of the casein matrix and may also be influenced by the solubilisation of colloidal calcium.

Confocal microscopy indicated that the pockets/channels of fat interrupting the casein matrix were more isometric in the High (171) sample than the samples with a lower calcium concentration. This suggests that the higher viscosity of the High (171) sample in the cooker/stretcher, due to the greater number of calcium mediated protein-to-protein interactions within the individual micellar casein building blocks, results in more rigid subunits making up the protein network. More rigid micelles would lead to a decreased level of deformation in the direction of stretch. The variation in the channel structure within the cheese samples may contribute to the melt behaviour. The Low (129) sample was observed to have the largest channels of fat within the structure of the cheese, while the High (171) sample had small pockets of fat. During heating, the protein fibres in Mozzarella are lubricated by the molten fat and can flow past each other (Tunick, Malin, et al., 1993). As the fat channels within the Low (129) sample are larger, they are likely to impart a greater degree of lubrication to aid flow than the pockets in the High (171) sample.

The breakdown of casein, as a result of proteolysis, in each of the samples suggested that there was residual coagulant still active within the cheese sample, even after the heating that occurs during the stretching process. This was based on the breakdown profile in all of the samples, where α_{s1}-casein was hydrolysed to a greater extent than β-casein, as well as the disparity between the samples manufactured being in

accordance with the quantity of coagulant added. A quick experiment confirmed this observation, where Fromase was not fully inactivated at the temperature the curd was stretched at after a 10 minute period. The implication of this is that there was a greater degree of casein breakdown in the Mozzarella than expected which would cause the cheeses to mature at a faster rate. A faster rate of breakdown would lead to a more rapid decrease in connectivity of the protein fibres which would likely lead to the tortuosity of the network decreasing (examined in more detail in Chapter **Error! Reference source not found.**) Also the difference in the breakdown profile may influence the functional properties of the Mozzarella, although functionality is not assessed to a great extent in this thesis.

As with past work using NMR, the quantity of free water within the three different cheese samples was found to decrease over the initial stages of the storage trial at 25°C. All three samples were found to then decrease in the quantity of free water present at 65°C between day 20 and day 40. This suggests that proteolysis is influencing the expression of water at elevated temperatures. This could occur for a couple of reasons including the greater degree of structuring of water that can occur and also the diminishing of the influence that hydrophobic interactions can impart onto the casein matrix at elevated temperatures due to a reduction in connectivity.

At 25°C the diffusion of water in all three of the cheese samples behaved in a different manner to the trend identified in Section 9.3.2, where the diffusion at long time scales decreased in magnitude during storage. This suggests that the mechanism responsible for the change in diffusion in the cheeses examined in the two different studies differed. A number of factors were different in the cheeses manufactured in this trial to the cheese used for the study in the United States, including the cheeses in this study being dry salted while the cheese in the previous study was a brine salt cheese. Brine salt cheeses are known to have greater compositional gradients existing in the cheese (Guinee & Fox, 2004b), this could perhaps alter the mechanism associated with water diffusion in the previous sample.

The residual active coagulant identified in the previous chapter was likely to influence the diffusion of water within the cheese samples at 65°C. As the samples with greater levels of coagulant added during manufacture had the greatest change in the diffusion at a long time scale during the storage. However, all three samples were observed to become less tortuous at 65°C as the storage trial progressed, in a similar manner to that observed in Section 9.3.2.

The diffusion of water within the Mozzarella is likely to be dominated by the free water in channels. Therefore the additional calcium partaking in mediating protein-to-protein interactions, may only have a very limited effect on the overall diffusion of water within the cheese. Additionally, higher calcium would be expected to produce a system with a greater level of connectivity and thus a more tortuous environment. However, the size of the water molecules in comparison to the highly porous casein micelle may mean that additional connectivity may not hinder the motion of the water molecules to a great extent.

Phosphorus NMR suggested that the mechanism responsible for the transfer of immobile phosphorus to mobile phosphorus in the cheese was occurring at approximately the same rate for the three different cheese samples. The reduction in the quantity of immobile phosphorus as determined by ^{31}P NMR in conjunction with the soluble calcium extraction indicates that the CCP diminishes during storage. It also indicated that the rate of transfer between the states of calcium was greatest over the initial 20 days of storage, in line with the shift in the distribution of water, the possible mechanisms for these changes have been discussed previously.

When the results from the ^{31}P NMR was assessed in conjunction with the other measurement techniques used in this investigation, a number of potential relationships were identified between changes in component mobility and changes in the physical properties of the cheese, for example the change in the hardness of the samples. These results suggested that the initial changes in the hardness of the Mozzarella samples are dominated by the decrease in the proportion of insoluble CCP. Following this initial change in the distribution of calcium, the change in hardness is

likely to be dominated by the progressive breakdown of the protein matrix as a result of proteolysis. This suggests that softening is a function of proteolysis and the water/calcium equilibration processes, over the first few weeks following manufacture following which the effect of the equilibration processes diminish and proteolysis becomes the driving force.

As a method ^{31}P NMR offers a powerful technique for assessing changes in the distribution of phosphorus within cheese. In particular, the technique promises to be a powerful tool for assessing immobile phosphate groups within cheese. Although it does not directly measure calcium, with calcium NMR being an unrealistic method due to its cost, it may assist in gaining a greater understanding of the dynamics of the phosphate groups within cheese, in particular the CCP. It has the advantage over other methods used to monitor the solubilisation of CCP due to being a non-destructive technique that does not involve any physical or chemical means of extraction that could impact on the results.

A trend was identified in the data collected from the dielectric constant, indicating a reduction over the storage trial in accordance with the decrease in water mobility. However, the model developed as part of Chapter 5 was incapable of predicting the quantity of free water within the different cheese samples. This suggested that the model was not a good fit for analysis the data beyond the original experiment that it was developed from. Dielectric spectroscopy has been used as a tool to assess cheese systems throughout this thesis. The results from these analyses have in some cases yielded results that suggest that relationships between parameters within the cheese samples and the dielectric parameters exist. However, due to the inconsistent nature of the results it suggests that the dynamic nature of the Mozzarella, where a number of different equilibration process occurring, the interpretation of results is complicated.

Principle component analysis suggested that 88.3% of the variation in the data from this and the previous chapter could be explained by the first two principle components. The first principle component (PC1) was dominated by time related

variables while the second principle component (PC2) was driven primarily by compositional parameters.

The results from this investigation suggest that the level of calcium present in the cheese is fundamental for the development of the structure of Mozzarella during the stretching process. However, following its role in the creation of the fibrous Mozzarella structure, the different calcium levels had little impact on further changes in the structure or component mobility within the cheese samples.

10.5 Conclusion

Three Mozzarella samples with differing calcium concentrations were manufactured with similar compositions and pHs. The effect of the calcium within the Mozzarella was as expected in regards to the hardness and melt properties of the three samples, with the sample with the lowest calcium content melting to the greatest extent and being the softest. The calcium content was observed to influence the distribution of fat within the Mozzarella structure, based on the confocal micrographs collected.

The investigation into the component mobility within the three different Mozzarella samples identified similar trends between the three samples. This indicated that the difference in the concentration of calcium in the cheese samples did not have a marked effect on the mechanisms involved with water and ion migration. This suggests that the calcium plays a critical role in the development of the structure in Mozzarella during manufacture; however, it does not have a large influence on the mechanisms involved in changes during storage.

Phosphorus NMR showed promising results as a potential method for evaluating the change in CCP during the maturation process. Further work using ^{31}P NMR to further characterise the changes in the state of phosphate and its possible use to infer information about the state of calcium should be conducted.

Dielectric spectroscopy was found to exhibit trends in the data, however, these were inconsistent with those seen in past experiments. Although it has been proven to be a

useful technique for assessing a number of different systems, including processed cheese, the interpretation of results in the dynamic Mozzarella system, where a number of different equilibration processes are occurring simultaneously, is not straight forward.

11 Concluding Discussion and Future work

The discussion that follows in this chapter places the insights gained in this study in regards to changes in structure and mobility within the context of a conceptual model of the entire Mozzarella product lifecycle.

11.1 Evaluation of techniques used to characterise component mobility

This study investigated several novel techniques to probe component mobility within Mozzarella cheese. These new techniques in combination with well-established methods gave further insights into structural changes occurring within the dynamic Mozzarella structure.

Dielectric spectroscopy, although showing promise based on work carried out on process cheese in literature and in the initial work in this thesis, was confounded by water movement during maturation and heating. This indicated that although dielectric spectroscopy was a powerful, non-destructive tool to characterise other systems, it was not a straightforward means of characterising component mobility within Mozzarella.

The use of NMR T2 relaxation measurements at elevated temperatures extended the use of a well-established method to exploring the effect of temperature on the cheese structure. It confirmed observations made using microscopy to analyse the Mozzarella structure during the stretching process, that free water is present at high temperatures. This added credence to the theory that the heating that occurs as part of the stretching step causes a strengthening in the hydrophobic interactions within the casein matrix resulting in the expression of free water. The extension of this work further into determining how the mobility of water changed at elevated temperatures during maturation provided new information related to dynamic changes within Mozzarella.

NMR diffusion measurements to study water movement provided new insights into the dynamics of water mobility in a cheese matrix, in particular in relation to both

maturation and heating. The DT2 correlation maps provided insights into the state of component before and after heating.

^{31}P NMR was identified as a potential technique to investigate changes in CCP within Mozzarella cheese during maturation. This is beneficial as previous work carried out in literature to monitor these changes have employed destructive methods that may impact on the distribution of components within the cheese system. The use of ^{31}P NMR in combination with other techniques used in the maturation trial provided insights into the potential mechanisms responsible for the change in CCP that occurs during maturation. This preliminary investigation into ^{31}P NMR and its use to study dynamic changes in Mozzarella cheese showed that the technique has the potential to be a powerful tool for studying component mobility.

The limitations identified in using NMR to study the dynamic Mozzarella system was in regards to the time required to carry out the measurements, limiting the number of tests that could be conducted in a day (to get a snapshot of the structure during dynamic change). However, these experiments have highlighted the different test methods as being potential powerful tools for probing component mobility in greater detail than current methods allow.

Based on the results of the experiments conducted as part of this thesis, a discussion regarding the structure and component mobility within Mozzarella follows:

11.2 Constituents and their role in structure

11.2.1 Protein

The protein matrix that makes up the continuous phase in Mozzarella is composed of renneted casein micelles. The coagulation of the renneted micelles is facilitated by calcium, allowing the micelles to come together by reducing electrostatic repulsions. During the Mozzarella making process, the acidification of the milk results in the dissociation of colloidal calcium from the casein micelles. This is done to achieve the desired level of insoluble colloidal calcium in the micelles which impacts on the curd during stretching as well as on other physical properties related to the cheese. During

processing the Mozzarella curd is kneaded, which results in the formation of protein fibres aligned in the direction of stretching.

11.2.2 Fat

Entrapped within the protein matrix resides fat. As the protein matrix is kneaded, the distribution of fat goes from roughly round pockets to long channels. The distribution of fat continues to change during the storage of the cheese. Scanning electron microscopy indicated that the fat globules within Mozzarella are either in their native state or partially agglomerated following manufacture. This is based on the identification of dimples in the channel walls within Mozzarella following the absorption of free water into the casein matrix.

11.2.3 Aqueous phase

The protein matrix is predominantly composed of an aqueous phase that exists within the protein subunits (micelles) making up the casein fibres. The aqueous phase within Mozzarella is comprised primarily of water and solubilised ions. The distribution of the aqueous phase is altered as a result of the stretching step, whereby the curd is heated and then stretched. This results in some of the aqueous phase existing in a free state outside of the protein matrix immediately following manufacture. During the first few weeks following manufacture it is reabsorbed into the protein.

11.3 The development of structure

The stretching of the Mozzarella curd is the unit operation that imparts the unique fibrous structure to the cheese. The stretching is commercially achieved by running the curd through what is commonly referred to as a cooker/stretcher. The process can be broken down into two key parts: the heating of the curd within the cooker/stretcher and the kneading that the curd is subjected to by the augers.

11.3.1 Cooker

During this process the Mozzarella curd is placed in hot brine prior to stretching. During this time the hydrophobic interactions within the protein matrix strengthen. This increase in hydrophobic interactions results in the protein matrix contracting

forcing some of the water entrapped within the matrix out into the interstitial space around the fat.

The temperature that the curd is stretched at will have a profound effect on the final cheese produced. The temperature will affect a number of key variables associated with the structure and evolution of structure during storage. Firstly, the temperature will impact the enzyme activity in the cheese which will dictate the proteolytic breakdown that occurs during storage. As chymosin and both the plasminogen activator inhibitor and plasmin inhibitor are all heat labile (Ismail & Nielsen, 2010), high temperatures in the cooker/stretcher will result in plasmin becoming increasingly more dominant than chymosin. As a consequence, the breakdown of β-casein will become more dominant than the hydrolysis of α-casein.

Secondly, the temperature that the curd reaches in the cooker/stretcher will influence the behaviour of the curd during stretching and therefore the degree of stretching. This is due to temperature influencing the viscosity of the curd, which will impact the structure of the final cheese. At higher temperatures the viscosity of the curd decreases which will cause a greater degree of deformation during stretching. This will result in an increase in the amount of elongation that the protein and fat undergoes during stretching.

Caseins soften and flow at temperatures above 55°C which results in the plasticising of the curd. This allows the curd to be kneaded without resulting in tearing.

11.3.2 Stretching

The second stage of this process involves the plasticised curd being kneaded by augers. The stretching elongates the protein and fat in the direction of flow. Due to the contraction of the protein, the fat can pool within the curd structure to form larger pockets. The amount of insoluble calcium within the system will influence the degree of asymmetry in the fat that is created. This is due to higher insoluble calcium levels leading to a more viscous curd, reducing the degree of deformation that the stretching imparts to the curd.

The shear exerted on the protein matrix may possibly result in the deformation of the roughly spherical micelles, elongating them in the direction of flow. These smeared micelles are jammed together during the stretching process so that long channels are formed, with fat globules and free water present within the channels.

11.4 Structural evolution during storage

11.4.1 Equilibrium processes

Over the first few weeks following manufacture the development of structure during storage is dominated by equilibration processes affecting the distribution of water and ions in Mozzarella.

11.4.1.1 *Water migration*

The free water present within the structure of the Mozzarella progressively migrates back to within the protein matrix during storage. This process occurs over the first few weeks following manufacture. This is a result of the lower temperature of storage causing the hydrophobic interactions to weaken; the casein matrix slowly relaxes allowing water to migrate back within the protein.

The initial quantity of free water is likely to be dependent on the temperature that the curd is stretched at and the residence time of stretching. It is possible that the fat content of the cheese may also impact on the quantity of free water within cheese, as it creates a greater number of spaces for water to pool during heating as the casein contracts.

This water migration was characterised by the use of both NMR and expressible serum in this research in Sections 5.3.4, 5.3.5, 6.3.5, 9.3.1.1 and 10.3.10.1.

11.4.1.2 *Ionic equilibrium*

During the initial few weeks following manufacture some of the colloidal calcium phosphate within the casein matrix disassociates and becomes soluble. This has been related to a pseudoequilibrium existing between the soluble and insoluble fractions of calcium present within cheese (Lee et al., 2010).

It is possible that the solubilisation of calcium that occurs within Mozzarella cheese occurs in some part due to the water migration that occurs during the initial stages of maturation. As the water migrates into the casein matrix the CCP nanoclusters find themselves in an 'excess' of water for the localised calcium environment. This results in a solubilisation of nanoclusters as a new equilibrium is reached. As the majority of calcium precipitates in the form of brushite ($CaHPO_4.2H_2O$), the phosphate groups dissolve and become ionic. Due to phosphate ions being unstable they react with H+ ions to form dihydrogen phosphate ions ($H_2PO_4^-$) and in the process decrease H+ concentration and thus increase pH.

The dissociation of colloidal calcium has repercussions in regards to the structural integrity of the cheese. As the quantity of colloidal calcium phosphate diminishes the rigidity of the casein matrix reduces. This is a result of the reduction in the connectivity of the matrix due to the lower level of calcium mediated protein to protein interactions. This depletion of colloidal calcium phosphate also changes the ionic environment of the aqueous phase as a greater quantity of ions exist in a soluble form.

The disassociation of calcium was characterised using an extraction technique as well as using ^{31}P NMR as discussed in Section 10.3.12.

11.4.2 Proteolysis

During storage residual enzymes present in Mozzarella progressively break down the protein into fragments (Coker et al., 2005). As mentioned earlier, the temperature that the Mozzarella curd is stretched at impacts on the type of breakdown that occurs in the cheese during storage. This will affect the amount of breakdown that occurs as well as the preferential hydrolysis of α or β casein. The progressive breakdown of the caseins causes a weakening in the protein matrix during storage impacting on a number of different structural and functional properties. This weakening is the result of a reduction in the connectivity of the casein matrix as the protein is progressively hydrolysed. Depending on what enzymes are active within the cheese during storage, the degree of structural breakdown will vary.

The hydrolysis of proteins releases free amino and carboxyl groups that can structure water (Creamer & Olson, 1982; Lawrence et al., 1987; Lucey et al., 2003). This structuring of water will result in the distribution of water to continue to change during storage, but to a much lesser extent than the initial migration. As proteolysis continues the water will become more strongly associated with the casein. Proteolysis was monitored and discussed in Sections 6.3.4 and 10.3.9.

11.4.3 Effect of structural change

11.4.3.1 *pH*

The pH of all of the Mozzarella samples studied as part of this work showed an initial increase in pH following manufacture followed by a levelling off by day 20. There are a number of factors that will influence the pH during the storage of Mozzarella. The most dominant factor influencing the pH is likely to be the solubilisation of the colloidal calcium phosphate. This solubilisation results in an increase in ionic phosphate, likely to exist as dihydrogen phosphate ions, within the aqueous phase will cause the system to be more buffered and thus the pH increases (Hassan et al., 2004; Johnson, 2002).

Other factors that are likely to influence the pH of Mozzarella during storage include proteolysis and lipolysis. The hydrolysis of casein due to proteolysis will result in a greater number of free amino and carboxyl groups (Creamer & Olson, 1982; Lawrence et al., 1987). The cleavage of peptide bonds, producing these free amino and carboxyl groups, causes a liberation of protons into the surrounding media resulting in a reduction in pH (Spellman et al., 2003). Lipolysis, although limited in Mozzarella may also cause a decrease in the pH, as free fatty acids are liberated from triacylglycerides.

However, no long term decreasing trend in pH was observed in any of the storage trials conducted. This suggests that there is a balance between the mechanisms. The fact that the serum pH increased indicates that the solubilisation of colloidal calcium phosphate dominates the overall variation in pH, particularly during the moisture migration/equilibration phase of maturation.

11.4.3.2 *Protein interactions*

The hydrophobic interactions within the protein matrix will weaken following manufacture as the temperature of the cheese falls. As a result this work has shown that the protein matrix swells as the hydrophobic regions become further apart due to the reduction in attractive forces.

The solubilisation of colloidal calcium phosphate will reduce the number of calcium mediated protein-to-protein interactions within the casein micelles that make up the protein matrix. As a result the micelles will become less rigid and more flexible, which will affect the physical properties of the cheese.

The solubilisation of calcium will also affect how the proteins interact due to the increase in the ionic strength within the cheese. As a result of this the hydrophobic interactions within the system will increase in strength. However, due to the decrease in the calcium mediated protein-to-protein interactions within the micelles, the casein network is looser due to the reduction in connectivity. As proteolysis progresses during storage, the contribution that hydrophobic interactions impart to the casein matrix will lessen due to the decrease in protein connectivity.

11.4.3.3 *Fat distribution*

The fat entrapped within the casein matrix undergoes a transformation in its distribution within the cheese. Initially following manufacture the fat exists in loose channels, as a result of the stretching process, where it is surrounded by free water. As the free water is absorbed in to the casein matrix, the protein fibres swell. The swelling of the protein fibres applies pressure to the fat particles forcing them into forming long thin channels. Following the initial modification in the distribution of fat, the pockets of fat were observed to become more spherical in nature.

Proteolysis will result in a weakening of the protein matrix which may cause a deformation in the channelled fat structure allowing them to become more spherical with time.

Alternatively, if the micelles are smeared during stretching the elongated state that they form is unlikely to be thermodynamically stable. During storage it is likely that the micelles will revert to a more energetically favourable state, of a more spherical conformation. This relaxation in the conformation of the micelles will apply a force to the fat residing in the channels, causing a deformation in the shape of the pockets that the fat globules occupy.

An additional mechanism that could facilitate the change in the distribution may occur if the micellar subunits that make up the protein phase interact. If interactions exist between adjacent micelles within a protein fibre that diverges around a fat channel, the protein interactions may progressively increase. This may result in a 'zipping' effect whereby the fat channel is forced into a shorter more spherical pocket as the protein fibres come together.

The transition in the distribution of fat within the structure of Mozzarella was monitored and discussed in Sections 5.3.2, 6.3.2 and 10.3.7.

11.5 Structural transformation during heating

11.5.1 Cold structure

The micelles that make up the protein fibres within Mozzarella are either interacting or jammed, whereby they are sterically held in place by the other micelles making up the casein matrix as well as by the fat.

At storage temperatures, the majority of the water within the Mozzarella resides within the protein phase.

In the work laid out in this thesis temperature sweeps were carried out generally with an initial temperature of 20°C, so the discussion regarding the structural changes during heating will begin at 20°C.

11.5.2 20-35°C

At 20°C the fat within the Mozzarella exists as a mixture of solid and liquid, with approximately 40% existing in a solid state (Lopez et al., 2006). As the temperature is

increased, the solid fat gradually melts until only 3% is solid at 35°C. This progressive transition in the state of the fat within the cheese will result in a lessening in the rigidity that it imparts to the cheese structure.

The increase in the temperature will also increase the strength of the hydrophobic interactions within the protein matrix. The strengthening hydrophobic interactions will result in the protein matrix contracting as the hydrophobic regions within the interior of the protein get closer in proximity.

11.5.3 35-55°C

The hydrophobic interactions will continue to increase in strength as the temperature is increased causing a progressive contraction of the protein matrix. The contraction of the protein matrix will result in water being forced out into regions where it is constrained to a lesser extent by the protein, in the void space caused by the contraction of the protein surrounding the fat. If the micelles are smeared, with a greater number of hydrophobic regions in close proximity between micelles that make up the protein fibres, the increasing temperature will cause the hydrophobic interactions to become stronger.

The contraction of the protein may also allow the fat within the cheese to pool into larger pockets prior to the cheese melting.

11.5.4 55°C and above

The contraction of the protein matrix continues as the hydrophobic interactions continue to strengthen. The progressive contraction causes fewer points of contact between adjacent protein fibres so that the structure is no longer jammed. A transition can occur from touching protein fibres to a state where they can slide past each other, lubricated by the molten fat and free water present within the structure of the cheese.

As the cheese matures, the solubilisation of calcium (over the first few weeks) and proteolytic breakdown of the casein will reduce connectivity, decreasing the resistance of the cheese to flow.

Based on the investigation carried out as part of this thesis, the structure and mobility of components within Mozzarella cheese was studied. This used a combination of techniques to examine the cheese at multiple length scales to assist in the identification of mechanisms related to structural development. The conceptual model for the structure of Mozzarella and the cascading effects related to the changes occurring during storage as described in this chapter is summarised in Figure 11.1 below.

	Heating	Stretch	Cold Storage	
Primary Driving Force	• Contraction of protein	• Alignment of structure	• Water migration	• Proteolysis
Secondary Driving Force			• Proteolysis	• Protein relaxation
Effects	• Water migration	• Channel formation	• Protein swelling -> fat deformation • CCP solubilisation - $Ca^{2+} + PO_4^{2-}$ → Ionic strength ↑ - $H_2PO_4^-$ → pH ↑	• fat deformation

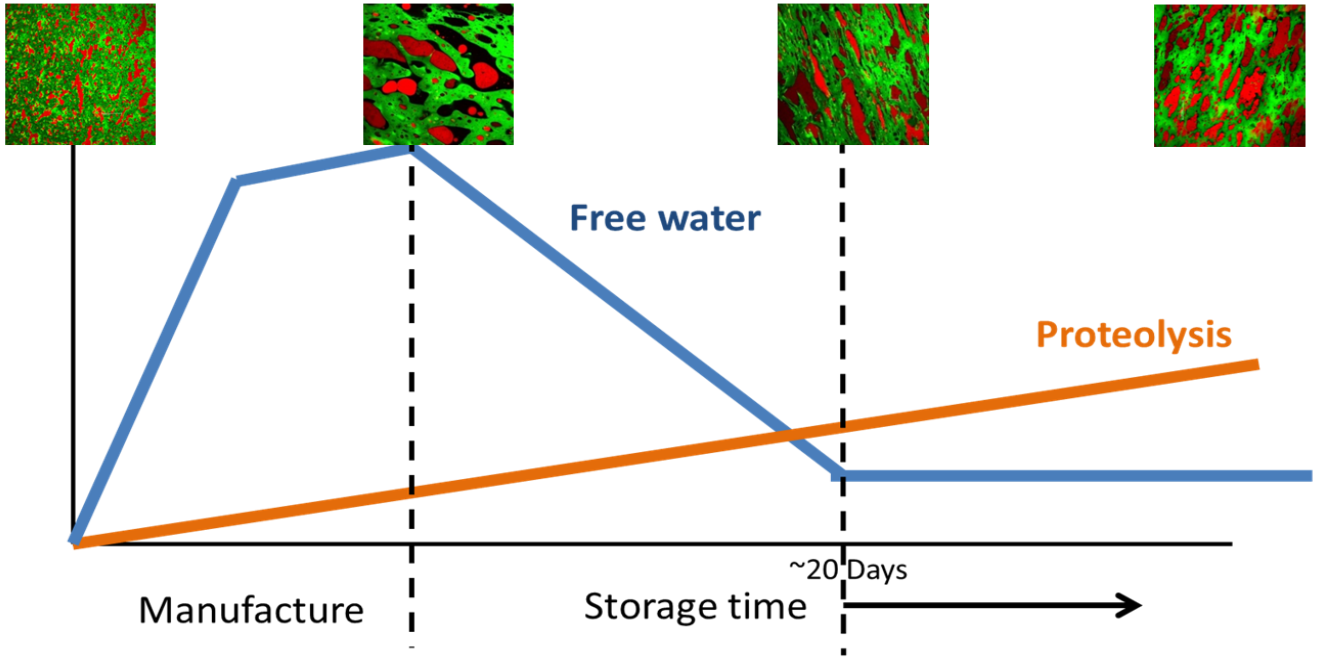


Figure 11.1: Summary of the drivers associated with structural change in Mozzarella.

11.6 Future work

The movement of components within Mozzarella was found to have a significant effect on the structure and physical properties of the cheese. It raises the question of whether model systems undergo equilibration processes following manufacture that may impact measurements. Therefore a brief study to investigate the stability of model systems would be advantageous to identify whether an equilibration period is necessary prior to measurements being made.

The use of NMR was explored in this thesis as a means to monitor component mobility in the dynamic Mozzarella system during both storage and heating. Although these preliminary studies indicated that there was potential for these methods to be applied to monitor the mobility of components within a Mozzarella cheese, a more detailed investigation of these methods to explore the bounds of the associated error is required.

Conduct measurements of the diffusion of water within cheese using the Pade approximate and use expressible serum at different temperatures to assess the salt content of the serum to give a better prediction of the D_0 in the cheese samples and thus enable a more accurate prediction of tortuosity and the surface to volume ratio.

The use of ^{31}P NMR at elevated temperatures is recommended to aid in the understanding of the thermal dependence of the system on colloidal calcium phosphate.

Repeat the calcium trial ensuring that the coagulant is inactivated so that the Mozzarella matures with a similar breakdown profile to commercial samples. This would allow the development of the structure of the cheese samples manufactured in the pilot plant to be related to the structural evolution observed in commercial samples.

Conduct the structural measurements in conjunction with functional testing to investigate correlations that exist between changes in the various testing methods during storage and heating.

Explore the use of both ^{1}H and ^{31}P NMR to characterise the movements of components during gel formation as part of the coagulation process. Coupling this with Rheo-NMR would not only allow the monitoring of the components within the system, it would also allow information related to gel formation and strength to be ascertained.

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