The Renneting Properties of Skim Milk Solutions Supplemented with Milk Protein Concentrate: The Effect of Hydration and Storage of the Milk Protein Concentrate.

Massey University

A thesis presented in partial fulfillment of the requirements for the degree of Master of Science in Physics at Massey University, Palmerston North, New Zealand.

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2004

Abstract

The purpose of this study was to examine the effect of storage and hydration of milk protein concentrate with 85% protein (MPC85) on the renneting properties of skim milk solutions supplemented with MPC85. The following techniques were used in this investigation: solubility testing, rheology, polyacrylamide gel electrophoresis (PAGE), and mass spectrometry.

The solubility of MPC85 samples which had been stored for different periods at temperatures ranging from 30°C to 50°C was found to decrease as storage time increased. In addition, as the storage temperature increased, so did the rate at which solubility decreased. This decrease in solubility dropped to approximately 22% of its original amount. Similar experiments were also performed on samples stored at 20°C (ie approximate room temperature), but showed no change over the time frame of the experiment.

Rheology experiments were performed on 10% (w/w) skim milk supplemented with 2.5% MPC85 (w/w). The experimental conditions, such as temperature (30°C), reconstitution time frame, and rennet concentration (100 μL per 50 g sample), remained constant throughout testing.

The rheological properties of the samples showed a large decrease in the formed gel strength of the renneted samples, and an increase in gelation time, as storage time and/or temperature were increased. Additionally, the viscoelastic moduli (G' and G'') and fracture stress also decreased as storage time and/or temperature were increased. This again excludes samples prepared from MPC85 stored at 20°C which showed no change.

The rheological properties of skim milk solutions supplemented with MPC85, with respect to hydration time was studied using three MPC85 powders of low, medium, and high solubility. Hydration time was varied between 1 and 24 hours, and results showed that samples made from high and medium solubility MPC85 increased gel strength with hydration, with high solubility MPC85 producing the stronger gels. Samples prepared with low solubility MPC85, produced

very weak gels which only changed minimally with hydration time. Aggregation and gelation times for each set of samples were different, but did not change with hydration time.

PAGE was used to analyse the composition of MPC85 to attempt to gain an understanding of what caused the change in properties with storage time and temperature. Experiments revealed that a large decrease in the solubility of caseins, and whey proteins decreased in solubility only minimally.

Mass spectrometry was used to analyse samples stored at 50°C, and it was found that the casein proteins suffered glycation. Whey proteins were also analysed, but data proved too noisy for any conclusions.

In general this thesis aims to provide useful information on the effects of storage and hydration of MPC85, especially with regard to the production of skim milk solutions supplemented with MPC85.

Acknowledgements

My most sincere thanks to the three best supervisors in town: Associate Professor Neil Pinder, Dr Skelte Anema, and Dr Yacine Hemar. Your guidance and encouragement were invaluable on what seemed a very long journey.

Many thanks to Buck Rogers who organized funding for this project, and also to Alan Baldwin for supplying a large volume of MPC, without which the study could not have taken place.

Thank you to Don Otter for assistance with sample preparation and running of the mass spectrometer, and also to Alistair Carr and Tasa Havea for useful advice on experimental procedure.

Thank you to Micelle Tamehana and Chris Hall for their encouragement and friendship during the early stages of the project.

"Oh Lord" where would I be without my good friend Thérèse Considine! Thank you very much for all the encouragement, and sharing of your office space during the write-up. Also a big thank you to Steve, Debbie, Aveenash, Leyton, Hayden, Bert, and everyone else who helped keep me sane throughout the duration of the project.

Thank you to the Fonterra Cooperative group for the resources and funding for this project.

Finally, thank you to my parents who were always there to support and encourage me when I needed it.

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1 Introduction

1.1 Background Information

The food industry produces many products to supply an ever changing market. In most countries dairy goods are an important component of the human diet, but milk is a perishable substance due to its high water content and near neutral pH. So if it is not intended for immediate consumption, it requires processing into other products such as milk powder or cheese to prolong shelf life.

Traditionally conversion of milk into cheese was a way of preserving milk and the nutrients it contained. Cheese can be made by many methods, one of which is through the addition of rennet to milk, which in turn causes aggregation to the point where it becomes a gel. The gel (often referred to as the curd) is separated from the whey, and the curd is the basis of the cheese. Cheese is a very important product to the New Zealand dairy industry, it is the second highest export product after milk powder. The cheese making process that is the topic of research for this thesis involves the fortification of milk with milk protein concentrates (MPC's).

MPC's are ultrafiltered/diafiltered concentrates of skim milk with high protein content (dry basis ranging from ~37% (skim milk powder) to 85% (MPC85)). These MPC powders, particularly MPC85, are often used as milk protein sources in cheese applications (eg as a cheese extender to improve the yield of cheese during each production run). As such, MPC, when added to milk must provide adequate properties during rennet treatment (gelation time and strength).

There has been extensive research carried out on cheese making, the goals of which were a better understanding of the physical interactions and chemistry of milk gelation. There has been a lot of work carried out in the recent past to collect information on the effects of milk renneting conditions such as pH, temperature, ionic strength, calcium concentration, casein concentration,

and the temperature history of milk on the final property of the cheese product. In contrast, there have been few literature reports on the renneting properties of reconstituted MPC either alone, or on its addition to skim milk, although this remains an area of commercial interest.

The aim of this study is to systematically investigate the effect of hydration time and storage conditions of MPC on the renneting properties of skim milk supplemented with MPC. MPC85 is used in preference to other MPC products as it is a key product for cheese milk extension due to its high protein content which reduces the level of whey by-products.

1.2 Thesis Outline

The goal of this thesis is to provide an understanding of the effects of variation of storage conditions, and variation in hydration times, on the properties of rennet induced skim milk gels fortified with MPC85.

Chapter 2 is a review of the literature relevant to this work. This includes skim milk, MPC, and the effects of rennet on skim milk.

Chapter 3 is a description of the experimental procedures used to collect information, and gives brief background information on the methods employed throughout the research.

Chapter 4 is an investigation of the solubility properties of MPC85 that have been stored under a variety of storage temperatures for varying amounts of time.

Chapter 5 is a description of the rheological study of the renneting properties of skim milk gels supplemented with MPC85. The MPC has also been stored at different storage times and temperatures.

Chapter 6 examines the effects of hydration time on samples using three different MPC85 powders which have high, medium, or low solubility.

Chapter 7 is a study of the composition of MPC85 powders that have been stored for different storage times at different temperatures, to gain insight into the changing properties of samples made with the MPC85.

Chapter 8 explains the use of mass spectrometry to study MPC85 stored at 50°C and covers the full decrease of MPC85 solubility found during this study. Mass spectrometry can identify the changes to individual proteins with storage time.

Chapter 9 is an overall discussion, and examines the relationship between different testing methods.

Chapter 10 is a presentation of the conclusions and lists recommendations for further work.

The appendix gives an overview of the preliminary work performed before the commencement of the full study. The preliminary experiments were initially presented separately as a progress report, and remain in this form.

2 Literature Review

2.1 Milk

Milk is the secretion from the mammary gland of female mammals. It is a readily digested nutritious food for feeding offspring being high in protein and minerals. With the domestication of animals, milk has also become part of the diet for adult humans, with many different foods incorporating it as an ingredient.

Milk is a versatile raw material and all dairy products originate from it. It is a perishable substance having a limited shelf life even when refrigerated. Milk however is a unique biological fluid with regard to its stability. This stability allows it to be processed to greatly prolong shelf life, as well as to extract valuable components, and reduce water. This study and associated literature is concerned only with bovine milk, but other dairy industries are based on other types (ie goats, sheep, buffalo, etc).

The composition of milk is variable and dependent on factors such as breed, season, climate, feed, stage of lactation, and the health of the cow. A typical bulk composition of milk for water, fat, protein, lactose, and ash (minerals) is approximately 86.6%, 4.1%, 3.6%, 5%, and 0.7% respectively. A more detailed composition can be viewed in table 2.1 (Swaisgood, 1996).

Table 2.1 Composition of Bovine Milk from Western Cattle (Swaisgood, 1996). Note that bold numbers represent totals.

Component	Sub-component	Average Percentage	Concentration (g/L)
Water		**86.6**	
Total Solids		**15.4**	
	Solids not Fat	11.3	
Fat		**4.1**	
Lactose		**5**	
Protein		**3.6**	
	Total Casein	**2.88**	24-28
	α_{s1}-Casein		12-15
	α_{s2}-Casein		3-4
	β-Casein		9-11
	κ-Casein		3-4
	Total Whey Protein	**0.72**	1-2
	β-Lactoglobulin		2-4
	α-Lactabumin		1-1.5
	Proteose-peptones		0.6-1.8
	BSA		0.1-0.4
	Immunoglobulins		0.6-1.0
Minerals (ash)	Total Minerals	**0.7**	
	Total Calcium		1.21
	Calcium ion		0.08
	Magnesium		0.125
	Citrate		1.81
	Inorganic-Phosphorus		0.65
	Sodium		0.60
	Potassium		1.44
	Chloride		1.08

2.1.1 Proteins

Proteins are important to sustain life. They are made from different combinations of 20 amino acid residues (note: this is a total, and all 20 acids are not necessarily found in all proteins). Proteins control the chemical and biochemical processes in an organism, as well as functioning as structural components of cells and complex organisms.

Milk proteins can be divided into two major groups, caseins and the whey or serum proteins (Walstra & Jenness, 1984; Dalgleish, 1992). Caseins are more abundant, making up about 80% of the total protein. Casein is also the most important for the manufacture of cheese, as these are the proteins that form the gel matrix on which cheese manufacture is based. In normal cheese manufacture the whey proteins are of little relevance, but may become important if the cheese milk is ultrafiltered or heated at temperatures greater than 60°C. In these cases whey proteins become incorporated into the curd.

2.1.1.1 Caseins

Caseins are a group of milk specific proteins that can be precipitated upon acidification to pH 4.6 (acid casein), or by the addition of rennet to milk (rennet casein). Representing approximately 80% of total milk proteins in milk, they are the principal protein component when milk is transformed into a curd such as cheese (Schmidt, 1982; Walstra & Jenness, 1984). There are four main casein proteins, which can be distinguished as: α_{s1}-casein (mol wt~23,600 daltons), α_{s2}-casein (mol wt~25,200 daltons), β-casein (mol wt~24,000 daltons), and κ-casein (mol wt~19,000 daltons) (Swaisgood, 1996). Caseins have an open structure due to the number and distribution of proline residues, which disrupt secondary protein structure. As such caseins are stable to heat and do not denature. The caseins do not exist as individual molecules in milk, but instead form large spherical micellar aggregates. Caseins are phosphoproteins, meaning they have phosphate attached to serine (and sometimes threonine) residues. These phosphate residues are important as they are able to bind large amounts of calcium, which enables the formation of

casein micelles. Note that casein micelles also contain many other minerals (Schmidt, 1982; Walstra & Jenness, 1984). κ-casein is the only casein that contains carbohydrate, and is therefore glycosylated. All caseins have both hydrophobic and hydrophilic regions as is illustrated in figure 2.1. Figure 2.1 shows that caseins have open structures, as opposed to whey proteins which have globular structures.

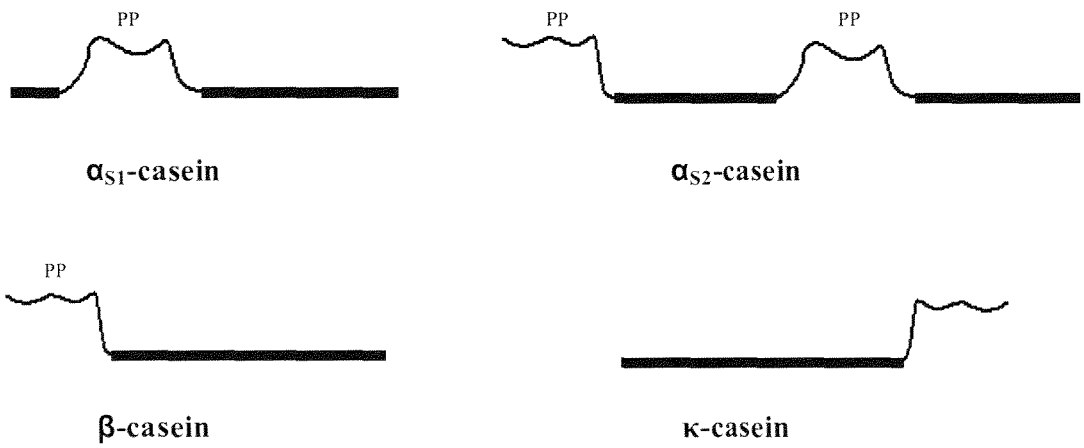


Figure 2.1 Schematic diagrams of the 4 main caseins. The loose structure denotes negatively charged regions, and PP denotes phosphate centres. The bars denote hydrophobic regions and do not imply rigidity (Horne, 1998).

When rennet is added to milk (section 2.2.2), κ-casein is cleaved into 2 components: the hydrophobic para-κ-casein, that will stay attached to the casein micelle, and the very hydrophilic glycomacropeptide (GMP) which will diffuse away in the whey.

2.1.1.2 Whey Proteins

Whey proteins are defined as the milk proteins that remain in the whey once the precipitation and removal of the casein proteins has occurred by either acidification to pH 4.6 (acid whey), or by rennet addition (rennet/sweet whey). Differences between acid and rennet whey are that rennet whey has GMP as a protein component, whereas acid whey does not. Whey proteins make up 20% of the protein found in milk, and, unlike caseins, are susceptible to protein denaturation (Walstra and Jenness, 1984a). There are different whey proteins including β-

lactoglobulin (β-Lg), α-lactalbumin (α-La), bovine serum albumin (BSA), immunoglobulins (Ig), and low molecular weight peptides, some of which are derived from proteolysis of caseins (Walstra and Jenness, 1984a).

β-Lactoglobulin (β-Lg)

β-Lactoglobulin is the most abundant of the whey proteins, making up approximately 50% of the total whey proteins. Several genetic variants have been identified (Swaisgood, 1982). β-Lg has a monomer molecular weight ~ 18,000 daltons, but exists as a stable dimer between pH 5.5 and 7.5. β-Lg has a single free thiol group which is of great importance for changes occurring in milk during heating since it is involved in reactions with other proteins, notably κ-casein and α-La (Walstra and Jenness, 1984a). Heat is required for this reaction to take place as β-Lg has a globular structure that must first be denatured, exposing the free thiol group.

α-Lactalbumin (α-La)

α-Lactalbumin is the second most common of the whey proteins making up approximately 20% of the total amount. Like the other whey proteins, α-La is a globular protein and has a molecular weight of 14,000 daltons.

Other Whey Proteins

There exist several other whey proteins that may be found in the serum in small quantities, including two iron binding proteins, lactoferin and transferin, and a group of glycoproteins. Note that once renneting has occurred in milk, GMP diffuses away from the casein micelle and becomes part of the rennet whey.

2.1.2 Milk Salts

Many different salts exist in milk (Table 2.1). The practical importance of the salts arises from their influence on the condition and stability of the milk proteins, particularly the caseins.

Some of the major salts (eg calcium and phosphate) found in normal bovine milk at natural pH exist partly in dissolved and partly insoluble (colloidal) form in close association with the caseins. The reason that calcium and phosphate exist in two phases is due to milk being supersaturated with these two salts. The amount of calcium phosphate found in either of these two phases is very important since it greatly influences the properties of milk depending on the phase in which it is situated.

Colloidal calcium phosphate (CCP) can be viewed as hydrated clusters of calcium and phosphate ions surrounded by casein phosphate centres (Holt, 1997). Calcium phosphate clusters, via the casein phosphate centres can crosslink caseins in the micelle. Also, α_S-casein has multiple phosphate centres, and so may crosslink the clusters. Holt (1997) suggested that the biological functions of casein are to transport calcium.

2.1.3 The Casein Micelle

Casein micelles are large colloidal particles varying in size from 20 to 600 nm with a large voluminosity (ie the ratio between hydrodynamic radius and mass). On a dry basis the micelle consists of approximately 92% protein (of which the individual caseins α_{s1}-casein, α_{s2}-casein, β-casein and κ-casein make up ~38, ~10, ~36 and ~13% respectively), and 8% inorganic salt, mainly calcium, phosphate, and citrate (Schmidt, 1982; Swaisgood, 1982). The structure and behaviour of the casein micelle is a topic of continuing research, and insight into its behaviour is very important to the advancement of dairy technology. Models that represent the structure of

the casein micelle fall into two categories: Sub-micelle models, and more recently non sub-micelle models.

The sub-micelle model is based on the idea that the casein micelle is built of smaller units, the sub-micelle. The theory was based on electron micrographic evidence which show casein micelles composed of what appeared to be sub units about 8 to 20 nm in diameter (Shimmin and Hill, 1964). In the Schmidt model (1982) two types of sub-micelles are found, firstly sub-micelles comprised of hydrophobic α_{S1}-casein, α_{S2}-casein, and β-casein (almost no κ-casein being present), and secondly those coated with high levels of κ-casein and a hydrophobic core similar to that of the first type of sub-micelle. Sub-micelles with high κ-casein levels are found at the surface of the micelle, while those with low κ-casein levels are in the centre. This structure was suggested by labelling studies, which involved the caseins being labelled with small gold granules and detected using electron micrographs. These labelling studies gave κ-casein a surface location, whereas the other caseins were uniformly distributed throughout the micelle. Micelles were formed by aggregating sub-micelles connected by finely divided CCP binding to the phosphoserine residues of α_S-casein and β-casein, but not phosphoserine poor κ-casein. The micelle growth ends when the surface is covered by κ-casein. A representation of the Schmidt model can be seen in figure 2.2.

Walstra (1990) expanded on the model proposed by Schmidt and introduced the concept of the hairy casein micelle. The model proposes that while α_S-casein and β-casein are bonded within the casein micelle by CCP, κ-casein acts as flexible hair that extends out into the solution and adds stability to the system by steric repulsion. Evidence for κ-casein acting as a flexible hair came from NMR studies which showed considerable freedom of movement for this casein. Evidence was also found from the size and viscosity reduction upon the addition of rennet, which is known to cleave κ-casein (Walstra, 1990), as well as size reduction caused by the addition of ethanol. Micelles having hairs have become accepted for most casein models. A representation of the model proposed by Walstra can be seen in figure 2.3.

Holt (1996) argued that the sub-micelle model was not consistent with all experimental evidence. He suggested that since casein undergoes indefinite self association to form

voluminous structures, the interior of the casein micelle may be no different to the exterior. The inside of the casein micelle would be "cemented" together with CCP, leaving κ-casein as a flexible hair at the exterior. This was known as the tangled web model, and is shown in figure 2.4.

Horne (1998) put forward the idea of a dual binding model of the casein micelle. The two types of binding possible in this model are the linkage of hydrophobic regions of the caseins, and the bonding permitted by charge neutralisation and cross linking by CCP via serine phosphate centres. As κ-casein has only one phosphoserine residue in the hydrophobic region (and is therefore not a phosphate centre), it is bound to the micelle by hydrophobic bonding. κ-casein will also limit further growth of the micelle since the flexible tail extending into solution does not have a hydrophobic region or phosphoserine residue. This model can be seen in figure 2.5.

Walstra (1999) discussed the pros and cons surrounding this concept in an effort to incorporate new experimental evidence into a modified casein sub-micelle model. He suggested that the process by which micelles are produced by cows comes about through sub-micelles of about 12-20 nm forming in the golgi vessels of the mammary gland. These particles are in various stages of aggregation and it was shown that each golgi vessel could hold up to 1 or 2 micelles. As such he argued that casein micelles were formed via this method of aggregating sub-micelles.

McMahon and McManus (1998) studied the methods used by other researchers to obtain electron micrographs of the casein micelle and found that the appearance of sub-micelles could be an artefact of sample preparation. A new sample preparation method was developed to study the casein micelle in its native form with as little damage to the micelle during preparation as possible. Samples prepared using this new protocol showed no sub-micelle structure, or at least none larger than 2 nm, and these may be just clusters of protein. Using this evidence an argument was presented against the existence of sub-micelle structure.

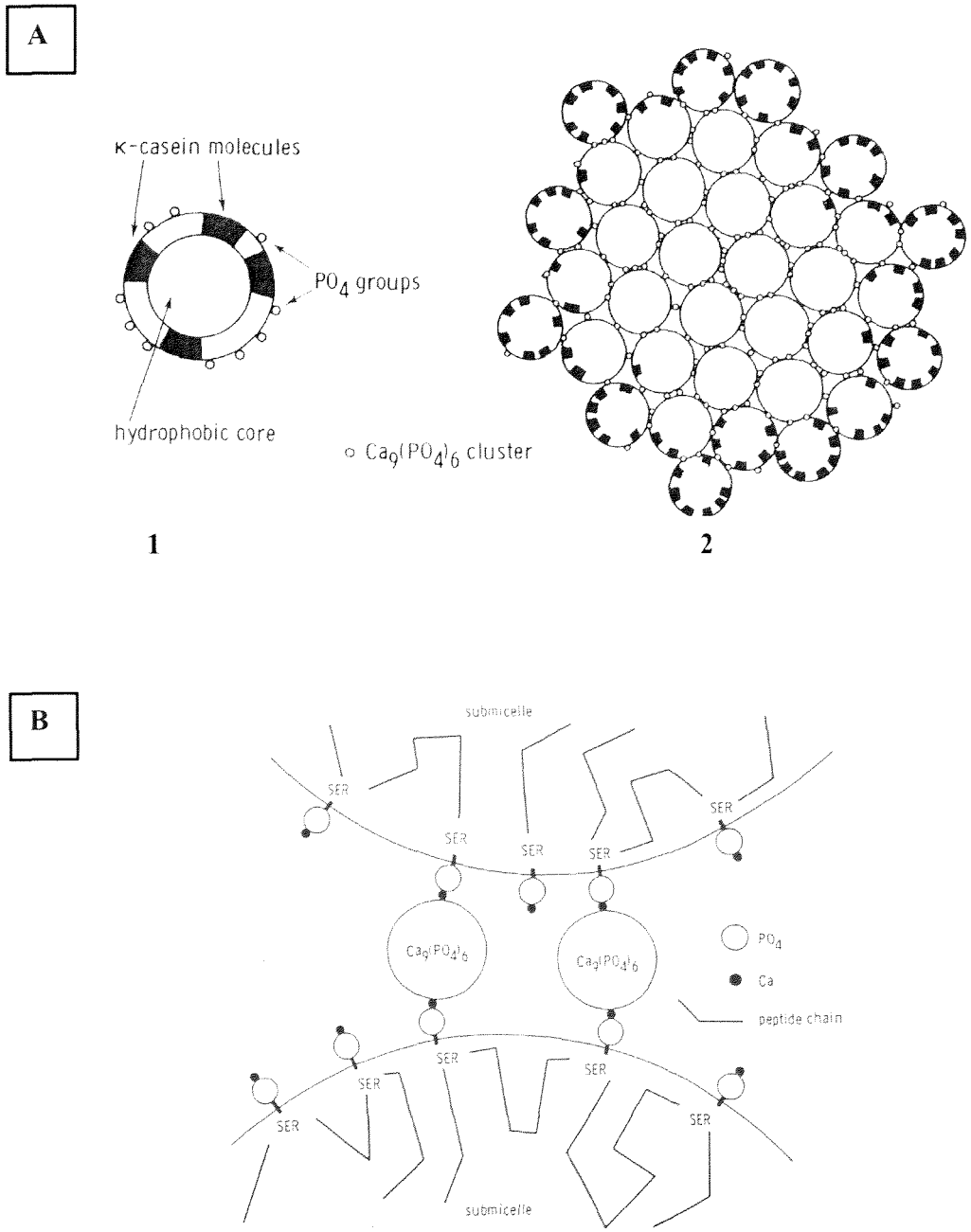


Figure 2.2 A) Schematic representation of the sub-micelle (1) and a casein micelle composed of sub-micelles (2). B) Schematic diagram of the binding of two sub-micelles via $Ca_9(Po_4)_6$ (CCP) clusters (Schmidt, 1982).

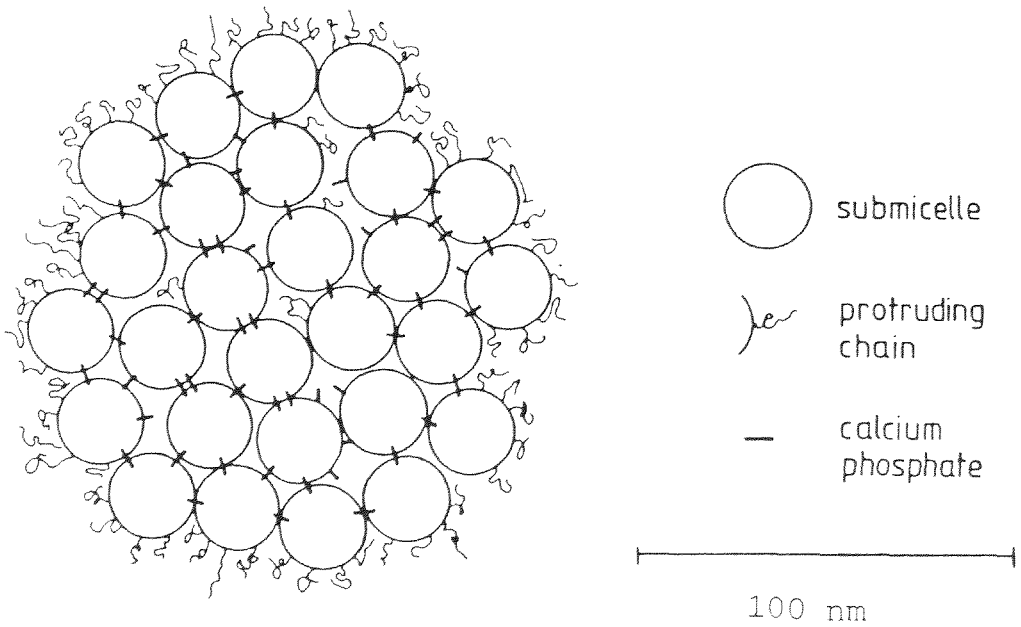


Figure 2.3 Representation of the casein sub-micelle model (Walstra, 1990).

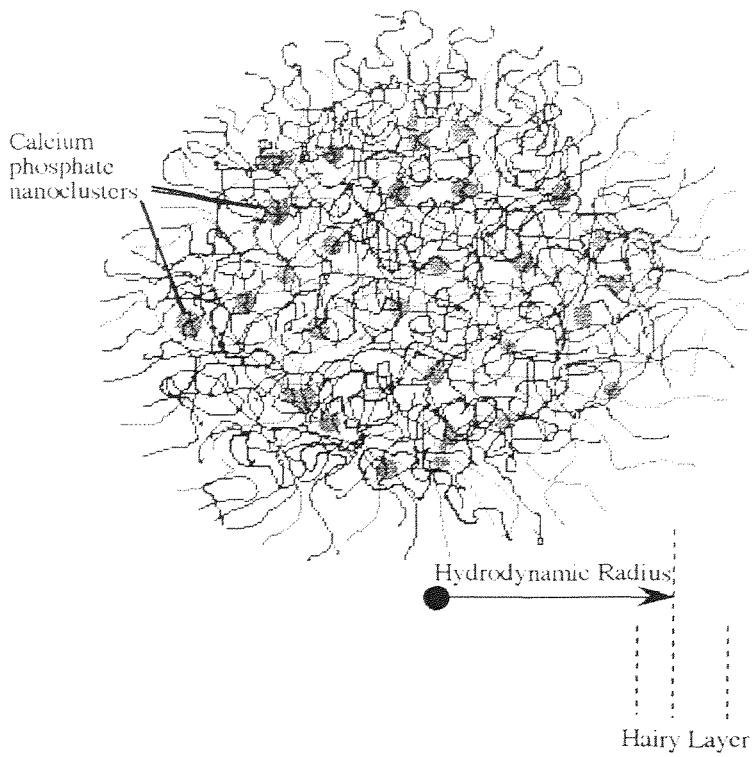


Figure 2.4 Tangled web model of the casein micelle (Holt, 1996).

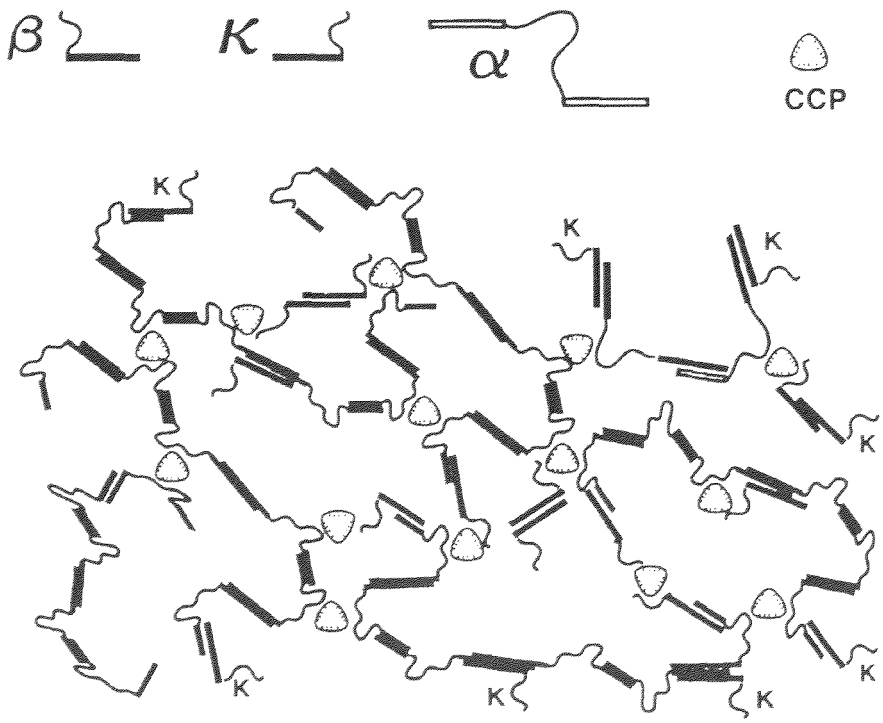


Figure 2.5 Dual bonding model of casein micelle structure (Horne, 1998). Key for figure: β = β-casein, κ = κ-casein, and α = α-casein.

2.1.3.1 Casein Micelle Stability

Classically the stability of the casein micelle was modelled on the Deryagin Landau Verwey Overbeek (DLVO) theory, which balanced the electrostatic repulsion of charged molecules against the attractive van der Waals forces of the caseins (Walstra, 1990). However it has been agreed by many researchers (Walstra, 1990; Holt and Horne, 1996; Horne, 1998, de Kruif, 1999) that the DLVO theory does not explain the stability of casein micelles, with regard to such processes as renneting, and they proposed that κ-casein (located on the surface of the micelle) is responsible for the high stability of the casein due to steric repulsion. As previously stated this stability is often exploited in milk processing. This stability however can be disturbed by processes such as: renneting (as in this study); acidification which lowers the pH of milk to the isoelectric point causing micelle aggregation; heating which can cause dissociation of casein from the micelle; lowering the solvent quality of milk through the addition of ethanol; or ageing. Each of these processes will, by different mechanisms, affect the κ-casein, disturbing the stability resulting in aggregation.

2.1.3.2 Changes to the Micelle on Heating

This process is not important to the current work, and as such will not be discussed in detail. The heating of milk causes the denaturation of whey proteins such as β-lactoglobulin and α-lactalbumin. Upon heating the thiol group, which was previously concealed in the globular structure of β-Lg, is exposed via protein denaturation and this causes covalent bonding with κ-casein (Walstra and Jenness, 1984b; Vasbinder and de Kruif, 2003). Note that this thermal denaturation of the whey proteins is irreversible. Heating increases the level of CCP and reduces pH. However these changes are reversible on subsequent cooling to a certain extent depending on the severity of the heat treatment. Lowering the temperature has the effect of solubilizing some of the binding CCP from the casein micelles. Bonding whey proteins to κ-casein is often used to increase the yield during cheese making, but also has other implication that will be discussed later.

2.2 Renneting of Milk

The clotting of milk by the specific action of selected proteolytic enzymes forms the basis for the manufacture of most cheese varieties. It is one of the oldest operations in food technology, having a history of some thousands of years.

Many proteolytic enzymes, taken from a wide range of plants and animals, are able to cause aggregation in milk. The most commonly used coagulant is calf rennet, the active component of which is the enzyme chymosin. Rennets consist of the enzymes chymosin and pepsin, and are traditionally prepared from extracts from the stomach of animals (although recently the genes for chymosin have also been cloned and expressed in micro-organisms). Chymosin contributes approximately 87% to the specific proteolytic activity under normal conditions, with pepsin providing the rest (van Hooydonk and Walstra, 1987). Both enzymes are from the group known as acid proteinases.

2.2.1 Chymosin

The Chymosin molecule (sometimes called rennin) has a rod like shape of the dimensions 2.5 nm and 4.5 nm. It consists of two domains separated by a deep cleft running parallel to the smallest diameter. The cleft is the active site of the molecule where two aspartic acid residues (which take part in the catalytic mechanism) are located. The enzyme may be deactivated by esterifying either of these two residues (Foltmann, 1981).

The unit of activity for renneting enzymes is the rennet unit (RU). Ruettimann and Ladisch (1987) defined the RU as the activity able to clot 10 ml of substrate (12 g skim milk dissolved in 0.01 M $CaCl_2$) in 100 seconds at 30°C.

2.2.2 Mechanism of Coagulation by Rennet

The rennet coagulation process may be divided into three separate but overlapping stages: (1) primary enzymatic proteolysis phase where κ-casein, which stabilises the casein micelle, is attacked by the proteolytic enzyme chymosin which is found in rennet, (2) aggregation phase which is the subsequent coagulation of the rennet-altered micelles, (3) tertiary stages such as syneresis (Dalgleish, 1983; Dalgleish, 1992). Phases 1 and 2 can be schematically seen in figure 2.6.

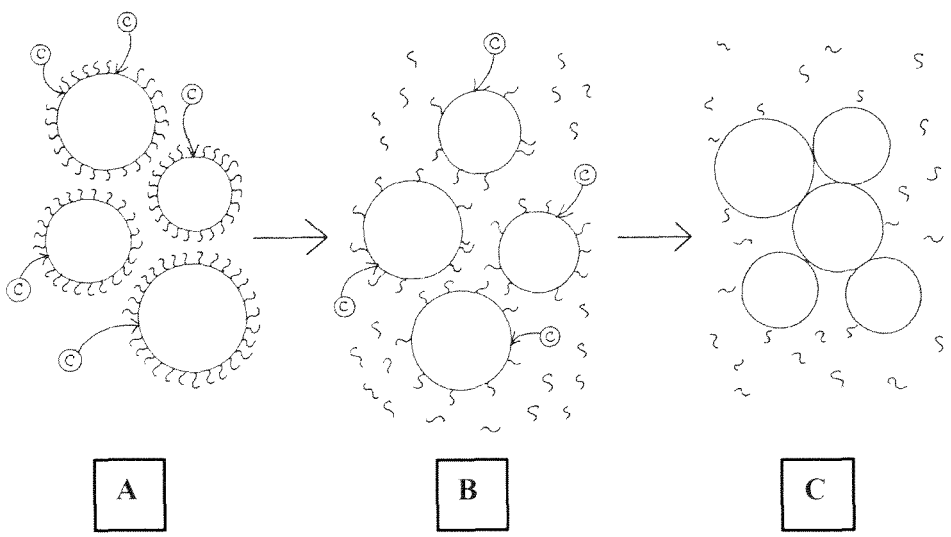


Figure 2.6 Schematic diagram of the action of chymosin (shown as C) on casein micelles. Three different points in the reaction are illustrated: (a) the κ-casein coat of the micelles is intact, and the chymosin has just been added; (b) some time later, much of the κ-casein has been hydrolysed but sufficient remain to prevent aggregation; (c) at a later time still, nearly all of the κ-casein has been hydrolysed and the micelles have started to aggregate (Dalgleish, 1992).

2.2.2.1 Primary Phase of Rennet Coagulation

The primary phase of rennet coagulation of milk involves an "attack" by the rennet on κ-casein, or more specifically the hydrolysis of κ-casein at the connection between para-κ-casein and glycomacropeptide (GMP) moieties. This occurs at the $Phenylalanine_{105}$ – $Methionine_{106}$ bond (Dalgleish, 1992), as chymosin is very specific for this site. GMP is negatively charged, hydrophilic and soluble, so once hydrolysed from the κ-casein is able to diffuse away from the casein micelle into the serum. In contrast the para-κ-casein is strongly hydrophobic and remains attached to the micelles.

The conversion of κ-casein, is essentially a single step enzymatic reaction, which was assumed to follow the standard Michaelis-Menten reaction kinetics for this type of reaction (Dalgleish, 1992). Therefore, the instantaneous rate of reaction, v (the rate at which substrate $[S]$ is converted into product), can be expressed as:

$$v = -\frac{d[S]}{dt} = V_{\max} \cdot \frac{[S]}{(K_m + [S])} \qquad \textbf{(2.1)}$$

In the above equation, V_{max} is the maximum rate of reaction at infinite concentration of substrate, and K_m is the dissociation constant for the enzyme-substrate complex.

The rennet clotting time (the time taken for visual coagulation of the milk under defined conditions of temperature) of milk has been found to be inversely proportional to the concentration of rennet. This would be expected since the rate of enzymatic reaction increases linearly with the concentration of the enzyme. Zoon *et al.* (1988a) and Lomholt and Qvist (1999) showed that an increase in rennet concentration resulted in shorter gelation times and higher gel strengths. The amount of rennet normally added to milk in commercial cheese making in New Zealand is reported to be 0.16 ml/L (Waungana, 1995).

2.2.2.2 Secondary Phase of Rennet Coagulation

The secondary phase of the renneting process involves aggregation of the casein micelles once their stability has been reduced via hydrolysis of the κ-casein. It was reported by Dalgleish (1983) that when approximately 85% of the total κ-casein has been converted to para-κ casein and GMP, the micelles begin to aggregate, but an individual micelle cannot participate in gelation until approximately 97% of it's κ-casein has been hydrolysed. In other words, some micelles may be completely hydrolysed, while others are only partially hydrolysed. The action of the rennet can therefore be seen to create 'reactive zones' where large enough amounts of the GMP moiety have been 'shaved off' the casein micelles and aggregation may take place. As the proteolysis of all the casein micelles in solution becomes complete, the aggregation, and the rate of aggregate formation accelerates (Dalgleish, 1992).

Aggregation requires the presence of calcium ions. It is thought that the aggregation of casein micelles is able to take place possibly due to the formation of calcium 'bridges' between the aggregating micelles and reducing the net negative charge of casein. Based on electron microscopic observations, explanations have been offered by Fox (1987), as to the process of gel assembly during the second phase of rennet coagulation. It was concluded that the initial stages of gelation involved the formation of small aggregates with micelles linked in chain like structures rather than clumps. The chains eventually form a network that slowly becomes more extensive. Therefore, the gel is assumed to assemble by the linkage of smaller aggregates rather than the addition of single particles to preformed chains. This process probably progressively strengthens the junctions between micelles, explaining the rise in gel firmness after coagulation.

2.2.2.3 Tertiary Phase of Rennet Coagulation

The process of renneting is often described as having a tertiary stage, which involves processes such as syneresis, non-specific proteolysis of the caseins in the rennet curd, and structural rearrangements of the renneted gel network once it has been formed (Dalgleish, 1983). No further detail will be given on this phase of coagulation as it is not important to this study as it takes place after gelation.

The coagulation process has a large effect on the physical properties of the final gel, particularly its porosity, permeability and thickness of the strands, as well as on further stages of cheese development.

2.2.3 Factors Affecting Coagulation

The enzymatic coagulation of milk is a complicated reaction, and as such there are many factors that may have an effect on the process and resulting product. Many authors (Cheryan *et al.*, 1974; Dalgleish, 1983; Zoon *et al.*, 1988a, b, and c, and 1989a, and b; Dalgleish, 1992; de Kruif *et al.*, 1992; Lomholt and Qvist, 1999) have studied the mechanisms as well as some of the factors that influence the formation and structure of rennet-induced milk gels.

2.2.3.1 Effect of Temperature

It is common practice to store milk at cooler temperatures to reduce spoiling. However Zoon *et al.* (1988a) showed that the lowering of milk storage temperatures resulted in retardation of coagulation. This was attributed to an irreversible increase in pH, a dissociation of casein, and the solubilization of the colloidal calcium phosphate. The effects of cold storage may be partially reversed by holding the milk at 60 - 65°C, which reduces the clotting time and improves curd quality.

Preheating milk to high temperatures causes the denaturation of the whey proteins that exist in the milk serum. When this occurs reactive thiol groups within β-lactoglobulin are exposed which then react covalently with κ-casein at the casein surface (Vasbinder *et al.*, 2003a and b). This in turn increases the rennet clotting time (RCT), and reduces the eventual gel strength (Waungana, 1995). Vasbinder (2003a) showed that milk heated for 10 minutes at 90°C denatures approximately 90% of whey proteins, resulting in the changes noted above. Milk heated in the absence of whey proteins showed minimal changes in RCT or gel strength. Note that the addition of $CaCl_2$ to milk can partially reverse the effects of heating.

Coagulation is highly temperature dependent, with almost no clotting occurring at 15°C, and the RCT decreasing to a minimum at approximately 35°C. Above 35°C a longer RCT is noticed due to heat inactivation of chymosin (Zoon *et al.*, 1988b). Higher temperatures also result in a higher rate of firming although a lower modulus.

2.2.3.2 Influence of Calcium Concentration

The secondary phase of rennet coagulation of milk samples has been found to be dependent on a critical Ca^{2+} concentration, above which the RCT decreases with an increased amount of added calcium (Walstra & Jenness, 1984). The RCT will decrease to a minimum as temperature increases from 15 to 40°C, with increasing calcium addition, but above 40°C coagulation becomes independent of calcium concentration (Dalgleish, 1983). CCP and Ca^{2+} activity increase with the addition of $CaCl_2$ to milk (Zoon *et al.*, 1988c). The effects that the addition of Ca^{2+} has on the mechanism of rennet induced coagulation are not fully understood, but several suggestions have been made. Green (1982) claimed that the rate of aggregation is increased by absorbed cations shielding the negatively charged groups of the casein. Dalgleish (1983) explained the increase in the rate of aggregation from calcium addition to be a specific interaction of unknown nature and not due to the simple charge neutralisation of renneted micelles.

2.2.3.3 Influence of Ionic Strength

The ionic strength must fall between a minimum and maximum level for aggregation to occur. The addition of NaCl causes a decrease in both pH and Ca^{2+} activity, and will necessitate an increase in rennet addition to keep the RCT constant. Na^{+} has also been found to replace Ca^{2+} in the casein micelle upon increased ionic strength (Zoon *et al.*, 1989a). Dalgleish (1983) found that the rate of coagulation decreased at all temperatures with NaCl addition.

2.2.3.4 Influence of pH

If the pH of a milk solution is decreased from the natural pH of 6.7 to 5.6, the rate of coagulation increases by a factor of 30 (Cheryan *et al.*, 1974). The effect of lowering pH is an increase in enzyme activity as well as a reduction in the charge repulsion between micelles and an increase in soluble calcium. Hence the rates of reaction of both primary and secondary stages of coagulation are increased at a lower pH. It should be noted that although both phases of coagulation were increased, it was the rate of the secondary stage (aggregation) that was most noticeable (Cheryan *et al.*, 1974). These results were confirmed by Zoon *et al.*, (1989b), who reported that a decrease in the pH of milk from pH 6.7 to 5.7 resulted in a maximum value of the elastic modulus G' near pH 6.15. Zoon (1989b) also found that CCP and micellar calcium were dissolved into the milk serum as the pH was lowered. This resulted in aggregation taking place at lower net κ-casein conversion.

2.2.4 Acid Coagulation

Casein micelles can also be destabilised by acidification to pH 4.6 which is the isoelectric point of casein where the proteins have no net charge. Destabilisation by acid is not directly relevant to this thesis and will only be briefly discussed. Lowering the pH of milk causes the calcium and CCP bound to the micelle to dissolve into the serum. Lowering the pH reduces the electric charge on the micelles, so that at their isoelectric point, they lose their stability, and precipitate.

2.3 Milk Protein Concentrates

Milk protein concentrate (MPC) is a high protein spray-dried powder manufactured from skim milk by means of membrane separation. There is a wide range of terms used to describe MPCs, which include: 'retentate powders', 'native milk protein concentrate', 'ultrafiltered milk protein concentrate', 'milk powder from ultrafiltered skim milk', skim milk retentate powder', and 'high-protein lactose-free milk powder'. Development over recent years has resulted in MPCs of different compositions being produced and incorporated into a wide range of products. One such use is an alternative to skim milk powder in countries with insufficient milk production, especially where long distance transport is involved. MPC powders are also often used to standardise the protein content in normal milk, and is also utilised in the recombined cheese industry.

2.3.1 Manufacture of MPC

The manufacture of MPC is based on protein concentration by ultrafiltration (UF), and if necessary diafiltration, preservation by heat treatment, and water removal by evaporation and drying. By using UF technology (and diafiltration for high protein products) at relatively low temperatures, MPCs of 50-90% protein content (dry basis) can be produced at the natural pH of milk (~6.7), which means caseins and whey proteins are present in their original ratios. A general process flow diagram for the manufacture of MPC is shown in figure 2.7.

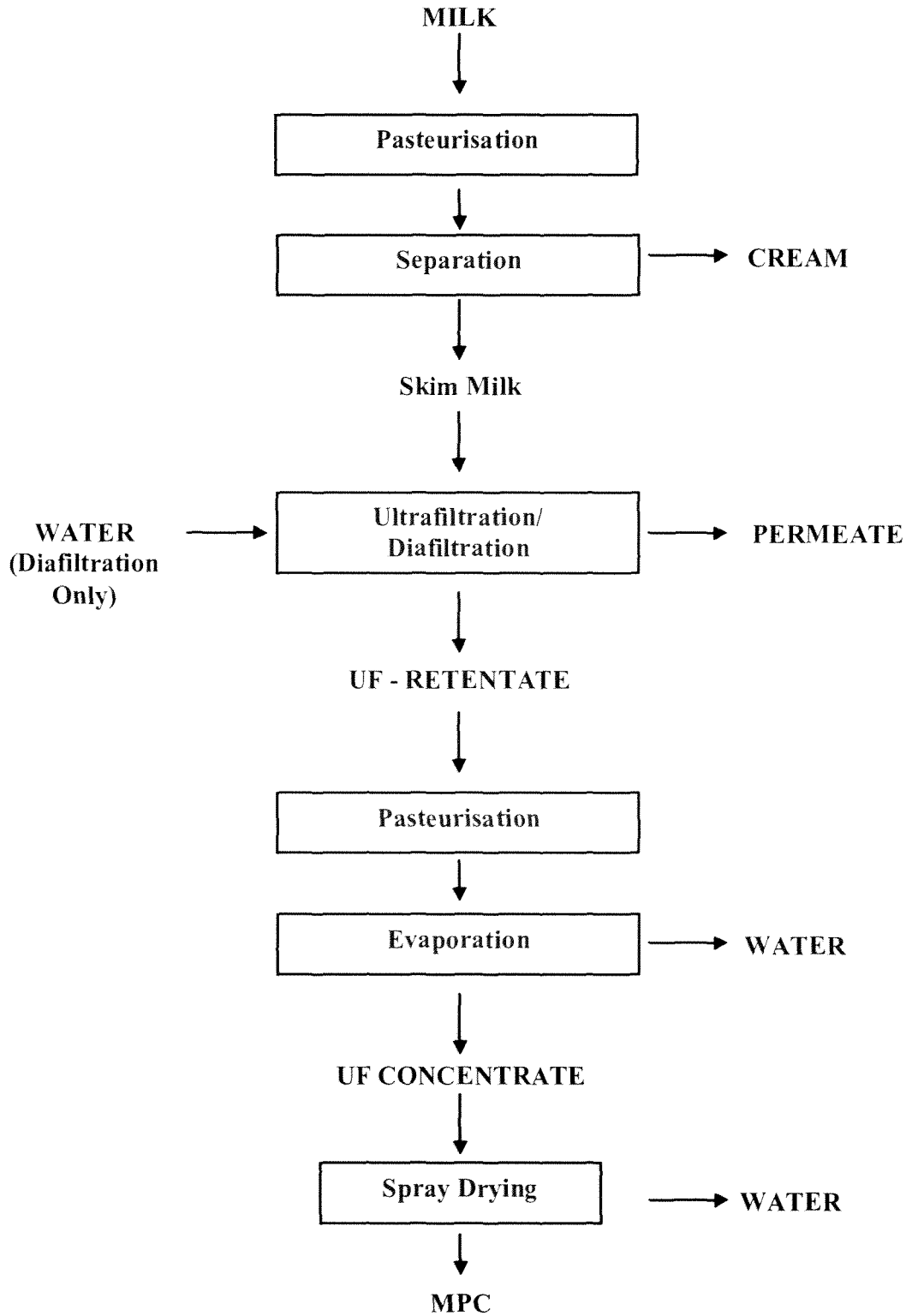


Figure 2.7 Schematic diagram of the MPC manufacturing process (Novak, 1991).

2.3.1.1 Membrane processing

The basic process of ultrafiltration involves the pumping of a feed solution over a chosen membrane surface under pressure. The idea behind this set-up is that the smaller species are forced through the membranes pores, while the larger molecules are retained. The part of the solution that is retained (also known as the 'retentate' or 'concentrate'), will become concentrated with the retained molecules, while the permeate flow will flush out the bulk of the unwanted smaller particles. The size of rejected species can be varied by the choice of membrane and corresponding pore size.

The quality of separation may be expressed by the volume concentration ratio (VCR):

$$VCR = \frac{\text{Initial feed volume } (V_0)}{\text{Retentate volume } (V_R)} \quad \textbf{(2.3)}$$

VCR may also be referred to as the 'concentration factor' (CF).

There are three main advantages to membrane concentration as opposed to concentration by evaporation. These are:

1. There is no need for a heating step to increase concentration, so there is minimal loss of nutritional or eating quality and particularly less loss of volatiles. This advantage is especially important to dairy products since heat induced protein denaturation is avoided.

2. Membrane concentration is more energy efficient than evaporation (boiling) as the food in question does not require a phase change.

3. A controlled separation of particles from the process fluid may be achieved through control of the pore size, and pressure drop over the membrane.

When higher protein products are produced, such as MPC85, the process of ultrafiltration needs to be performed in conjunction with diafiltration. This technique is required to increase the

protein concentration to high levels (such as MPC85 used in this study) by removing excess lactose. Diafiltration involves the addition of water to the retentate obtained from ultrafiltration, followed by the further separation of permeate species along with the water during ultrafiltration.

2.3.2 Composition and Structure of Milk Protein Concentrates

A table of typical compositions of commercially produced milk protein concentrates is shown in table 2.2.

A study was made by Mistry and Hassan (1991) into the microstructure of different dairy powders, including skim milk and MPC. Despite being formed from the same raw product there are many differences between these two powders. Electron micrographs of both are shown in figure 2.8 and figure 2.9.

The surface of MPC85 powder particles (as shown in figure 2.8) is smooth with large dents. The interior of the particles are hollow with walls approximately 2μm thick. In contrast to these observations, the surfaces of skim milk powder particles produced under the exact same method as those of MPC (figure 2.9) had a wrinkly surface. In addition to this there was less variation in particle size, although the dents remained. There are several explanations for the difference in surface morphology. Mistry and Hassan (1991) claimed the difference in protein composition (85% vs 37%) may cause this difference. However, Warburton and Pixton (1978) stated that moisture content of the particles and the presence of lactose could account for the differences in morphology. Their explanation was that as the moisture content of dried particles increased to 7%, the surface of the particles become wrinkled, with the number of wrinkled particles increasing with increasing moisture content. The cause of surface wrinkles has been related to the presence of anhydrous lactose in a glassy form (Saito, 1985). Note that MPC85 (used in this study) has low lactose levels. Above 7% moisture content, the lactose becomes sufficiently dilute for crystallisation to occur (Warburton and Pixton, 1978). The occurrence of wrinkles

becomes greater at high inlet temperatures during the spray drying process, and also when large temperature differences occur between the hot air and the milk particles (Caric and Kalab, 1987).

Table 2.2 Composition of commercially produced milk protein concentrates. In this table data on MPC85 and MPC56 are typical compositions from New Zealand (New Zealand Dairy Board product sheet), and the data on the MPC80 and MPC75 powders are Hungarian (Novak, 1991).

	MPC85	MPC80	MPC75	MPC56
Protein % dry Basis	85.4	79.4	74.8	56.0
True Protein	84.8	78.4	73.5	54.3
Non-Protein Nitrogen		1.6	1.5	
Moisture [%]	4.1	5.0	5.0	3.8
Fat [%]	1.7	1.7	1.5	1.2
Lactose [%]	4.6	5.5	10.9	31.0
Ash [%]	7.3	7.8	7.6	8.0
Calcium [mg/100g]	2100	2450-2550	2300-2400	
Potassium [mg/100g]		250-300	450-550	
Phosphorous [mg/100g]		1500-1700	1450-1650	
Sodium [mg/100g]	120	120-150	130-180	
pH [5% at 20°]	7.0			6.8

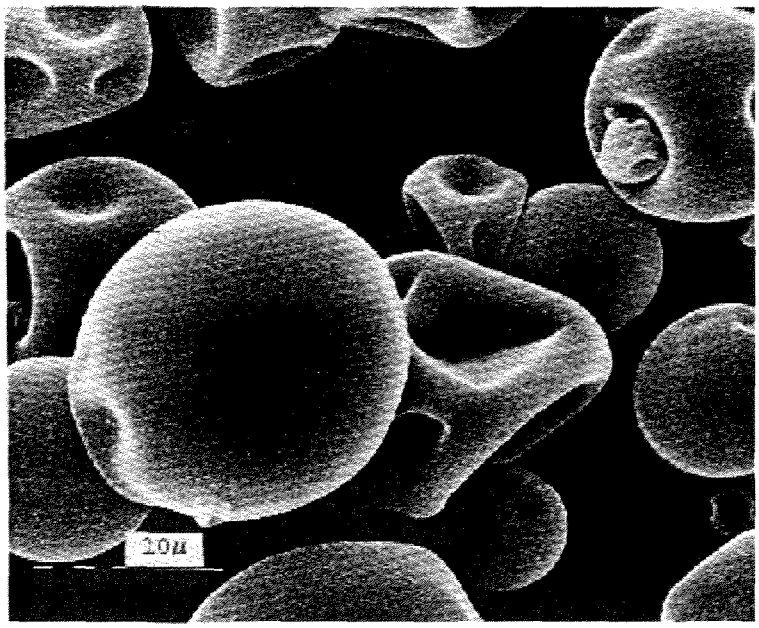


Figure 2.8 Scanning electron micrograph of MPC85 powder showing smooth surface and dents on the particles. A wide range of particle sizes are evident (Mistry and Hassan, 1991).

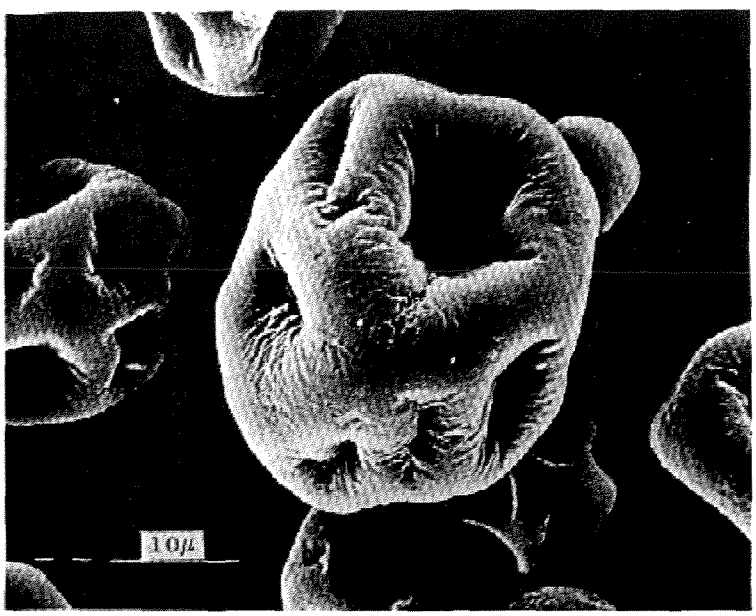


Figure 2.9 Scanning electron micrograph of spray-dried skim milk powder. Powder particles are characterised by a wrinkled surface with dents. A narrow range of particle sizes are evident (Mistry and Hassan, 1991).

2.3.3 Functional Properties of MPC

There are many conflicting reports available in the literature that address the problem of MPC solubility. Zwijgers (1992) found that the solubility of MPC could be increased by raising the temperature at which the powders were dissolved. The problem with solubility is the slowing of water transfer towards the interior of the grain of powder. This was confirmed by Davenal *et al.* (1997) who used NMR techniques to follow the reconstitution of native micellar phosphocaseins (NMC). Note that NMC is basically whey protein free MPC. They showed that two phenomena were occurring during the NMC rehydration: the strong and almost instantaneous absorption of water by powder particles, followed by a further slow absorption leading to acceleration of the solubilisation of particles.

MPC is widely employed in Hungary for use in the meat and canning industry. MPC has an emulsifying capacity (EC) which is very useful in these industries. Babella (1989) found the EC decreased with the degree of heat treatment during manufacture. This study also revealed that increasing the calcium ratio (calcium:total mineral) would decrease the solubility and EC of MPC. Increasing the calcium: total mineral ratio does not affect the water binding capacity, but decreases the solubility and EC of MPC. However an increase in the potassium or sodium ratio, increases the water binding capacity considerably, decreases the EC, with no change in the heat stability or solubility of MPC.

2.3.4 Recent Research into MPC

Milk protein concentrates are still a relatively new food product available in the international market, and as such many of their properties are still unknown. During the course of the current research, reports have been produced by various groups discussing newly discovered functionalities of MPC.

Research performed by Kuo and Harper (2003a), investigated the rennet gel properties of MPC. It was found that MPC with a higher protein level required the addition of calcium to form rennet induced gels. An increase in rennet concentration to MPC70 resulted in a stronger gel, whilst this treatment did not affect the gel formed by MPC85. Instead additional processes such as ultrasonication, heat, holding, and homogenization were required to produce such an effect. An increase in the amount of MPC (from 3.5% to 10%) also resulted in a stronger gel being formed. In general, a negative correlation between particle size of the hydrated MPC and rennet gel hardness was noted.

The effect of hydration time of milk protein concentrates on cast feta cheese texture (Kuo and Harper, 2003**b**) was recorded using several commercial MPC samples. Methods described a hydration time of 1, 3 and 5 hours before being made into cheese. General results recorded a decrease in pH, with an increase in hardness of the cheeses over a 1 to 7 day period. Overall, satisfactory cheeses could be produced using most MPC although some did require the addition of lactose to develop a more desirable texture.

De Castro-Morel and Harper (2002) collected samples of 37 different MPCs from 10 different countries to examine the basic functionality of these milk protein concentrates. Findings showed that there was no correlation between protein content and solubility, viscosity, or foaming. It was found that when only a small sub-group of the MPCs with high protein levels (82 – 86%) were considered, then a correlation could be found between solubility and protein content, pH and viscosity. Unfortunately there was no experimental basis determined to account for the wide variable functionality. These researchers concluded that the variability of the commercial products of similar protein content could be expected to impact negatively on MPCs achieving full market potential.

Kameswaren and Smith (1999) investigated the rennet clotting times of skim milk based rennet gels supplemented with an ultrafiltered milk protein concentrate. In this study the MPC being examined was subjected to 2 different pasteurization conditions (73 and 85°C for 17s). The solutions being tested were made by adding 4.65% to 6.25% protein by weight to skim milk solutions. The rennet clotting times were not significantly affected by the heat treatment

suggesting the extent of whey denaturation was insufficient to alter these times. However if the retentate was pH-adjusted during manufacture then a significant reduction of clotting times was noticed.

Pomprasirt *et al.* (1998) found that the effect of heat treatment of MPCs added to milk resulted in a slower rate of increase in gel strength (measured from the storage modulus G'). This treatment also caused a reduction in the gelation time, as well as a decrease in the force required to fracture the gels. The extent of whey protein denaturation (resulting from the heat treatment), was related to the decrease in G' values as well as the yield force.

2.4 Summary

This literature review was compiled to gain an understanding of the fundamental properties of the raw materials used in this study, as found from past research. The experimental methods used in this study were performed based on information gained from the reviewed material. The amount of literature available for MPCs was minimal compared with that found for milk and rennet, so this study is important to further the understanding of dairy science, and especially milk protein concentrates.

3 Experimental Methods

3.1 Introduction

The aim of this study was to systematically investigate the effects of hydration time and storage conditions of milk protein concentrate with 85% protein (MPC85), on the renneting properties of skim milk fortified with MPC85, which from this point will be stated as skim milk/MPC85. Preliminary experiments were carried out (see appendix) to characterise the skim milk powder, MPC85, and rennet used in this study, and to develop a suitable experimental method.

3.2 Materials

3.2.1 Low Heat Skim Milk Powder and Milk Protein Concentrate 85

The skim milk powder used was a commercial grade, low heat skim milk powder (SMP). The milk protein concentrate (MPC85) was manufactured by the Fonterra Co-operative Group Ltd, Palmerston North, New Zealand. The method of MPC85 manufacture cannot be disclosed as it was prepared by a propriety method to achieve high solubility. Composition details are shown in Table 3.1. Note the high moisture content of the MPC85 powder. While this value is higher than levels found in traditional MPC powders, it is not believed to affect the powders characteristic trends.

Table 3.1 Composition of skim milk and MPC85 used in study

	SMP	MPC85
Non Casein Nitrogen % w/w	0.968	1.98
Non Protein Nitrogen % w/w	0.319	0.093
Total Nitrogen % w/w	5.49	12.79
Chloride mmol/kg	342	12.6
Lactose Monohydrate % w/w	56.9	1.9
Moisture % w/w	3.91	8.96
Inorganic Phosphorous mmol/kg	229	264
Calcium mg/kg	12200	21400
Sodium mg/kg	3810	472

3.2.2 Rennet

Australian double strength (ADS) rennet (Rennet Company, Eltham, New Zealand) was used in all rheology experiments. Rennet concentration was 80 RU.

3.2.3 Chemical Reagents

Sodium Azide (NaN_3, BDH laboratory, Poole, England) was of a technical reagent grade. Water purified by reverse osmosis was used in some tests; while in others further filtration through MilliQ apparatus was used (MilliQ is a registered trademark of Millipore Corporation).

3.3 Sample Preparation

3.3.1 Treatment of MPC85

The MPC85 used in this study was frozen to -18°C immediately after manufacture. MPC85 samples were stored at one of five different temperatures (50°C, 40°C, 35°C, 30°C, and 20°C) for periods up to 60 days. Samples were tested on a 1 or 2 day basis. Individual samples were sealed in airtight bags and placed in heated rooms/incubators that were regulated at the above temperatures. The variation of properties of the powders kept under these conditions were monitored for an extended period of time using the methods described in the following sections to study the effects of storage temperature on MPC85. Samples were frozen to -18°C after storage to allow for re-analysis. Preliminary studies showed the properties of MPC85 did not change once frozen.

3.3.2 Reconstitution of Skim Milk Powder

Base skim milk solutions were prepared by reconstituting skim milk powder to 10% (w/w) total solids in milliQ water (with a concentration of 0.02% NaN_3). During reconstitution, the mixture was covered with parafilm to minimise change in moisture content and stirred at room temperature (~20°C) for a period of two hours to ensure complete dissolution.

3.3.3 Rennet Preparation

The rennet was diluted to reduce percentage errors resulting from the transference of small volumes. The rennet used in all tests was diluted to 10% (w/w) in milliQ water. Rennet was stored at 4°C for a maximum of two days and then discarded if not used.

3.4 Experimental methods

3.4.1 MPC85 Solubility Testing

3.4.1.1 Sample Preparation for Solubility Testing

The solubility tests require a 5% reconstituted MPC85 solution. These were prepared using milliQ water. During reconstitution the solution was stirred with a propeller-blade attached to an Heidolph stirrer motor (Heidolph RZR 2050 Overhead Stirrer, Heidolph Elektro GmbH and Co. KG, Kelheim, Germany) suspended over a water bath at 30°C. This solution was stirred for 30 minutes to allow for complete dispersion before tests were commenced.

3.4.1.2 Experimental Method for Solubility Testing

A bulk solution was prepared as described above (section 3.4.1.1). After half an hour dissolution time, a sub-sample was taken and centrifuged using an MSE Mistral 1000 Centrifuge (Sanyo-Gallenkamp, Global Science and Technology Ltd, Auckland, New Zealand), at 700xg for 10 minutes. A sample of the supernatant was placed in a pre weighed moisture dish, and weighed. The moisture dish was dried overnight in an oven at 105°C, pre-cooled in a desiccator (to avoid condensation affecting the results) and then reweighed. The fraction of soluble material in the MPC85 was calculated as:

$$\text{Fraction of Soluble Material} = \frac{[\textit{weight of dry material in the dish}]}{[\textit{weight of the solution added to the dish}]} \times 100 \qquad \textbf{(3.1)}$$

Note: all results were normalised to an initial 100% soluble material. To do this the maximum solubility of the MPC85 under study was defined as 100%. This was done to aid in the reviewing of results, as well as to protect commercially sensitive information.

3.4.2 Rheology of Skim Milk/MPC85 Solutions

3.4.2.1 Sample Preparation for Rheology Experiments

A 50 g sample was prepared by adding 2.5% (w/w) MPC85 to skim milk (which was prepared as described in section 3.3.2). Skim milk/MPC85 samples that were used to test the effects of storage time and temperature had MPC85 added at room temperature (20°C). The solutions were stirred for half an hour. Samples were then left for 24 hours at 4°C to allow equilibration. Samples that were used to test hydration time had MPC85 added without removal from refrigeration (4°C), and were stirred for half an hour during this addition. They were then left for the appropriate hydration time (between 1 and 24 hours) before commencement of experiments.

The samples were removed from the refrigerator and allowed to equilibrate at room temperature for one hour before testing began.

3.4.2.2 Experimental Methods for Rheology Experiments

The rheological properties of renneted skim milk/MPC85 samples were determined using a controlled strain AR2000 Rheometer (TA instruments Ltd., New Castle, Delaware, United States of America). A cone and plate geometry, consisting of a peltier system (cooled by water bath), and a 4°, 4 cm diameter cone with solvent trap (part number 511407.901, TA instruments), was used for all rheology experiments.

Three sequential tests were performed in each rheology experiment of a skim milk/MPC85 solution.

Firstly a dynamic time sweep was employed, whereby a strain of 0.5% at an oscillation frequency of 0.1 Hz was used. Measurements of the samples stress, produced in response to the strain applied by the rheometer, were taken every 60 seconds for three hours.

Secondly a frequency sweep was performed where a constant strain of 0.5% was maintained as the applied frequency was varied from 0.01 to10Hz.

Thirdly a strain sweep was made, in which the applied strain was increased from 0.5 to 50%, at a frequency of 0.1 Hz. The goal of this last test was to find the strain necessary to break the formed structure and so obtain the strength of the gel. This will be referred to later in this thesis as the fracture strain.

All experiments were performed at 30°C.

3.4.4 Polyacrylamide Gel Electrophoresis

3.4.4.1 Sample Preparation for PAGE

The basic procedure described by Laemelli (1970) was followed. The resolving gel was prepared by combining 5.3 ml of a 30% stock solution of Acrylamide/Bis (mixed in a 37.5:1 proportion), 2.5 ml TRIS/HCl (1.5M, pH 8.8), and 2 ml MilliQ water. The solution was degassed by evacuation for 15 minutes, then 100 µl of 10% SDS stock solution, 5 µl of tetramethylenediamine (TEMED), and 50 µl of 10% ($^{W}/_{V}$) Ammonium Persulphate (APS) were added. Then 3.3 ml of gel solution was poured between two glass casting plates (Bio-Rad Mini Protean, Bio-Rad Richmond, Ca, USA), and left to polymerise with a small amount of milliQ water placed on top. This water was removed before adding the stacking gel. The stacking gel was prepared by combining 0.65 ml of the 30% stock solution of Acrylamide/Bis, 1.25 TRIS/HCl (0.5M, pH 6.8), and 3.05 ml milliQ water. The solution was then degassed by evacuation for 15 minutes. Just prior to being poured 50 µl 10% SDS stock solution, 5µl TEMED, and 25µl 10% ($^{W}/_{V}$) APS were added to the solution. This solution was then pipetted on top of the resolving gel to the point of overflow. Lanes were created in the gel using a 15-slot comb.

Each MPC85 sample to be analysed was first diluted as 0.436 g powder in 40g milliQ water (Note: this dilution was derived from protein levels found in 10% skim milk solutions (3.7% protein), which are then further diluted 1:40 times in sample buffer (Anema and Klostermeyer, 1997)). Once combined, this sample was stirred for half an hour at room temperature to complete dissolution. Then samples to be centrifuged had 1ml sub-sampled into an Ependorf tube and centrifuged for 4 minutes at 14000 rpm, at which point 100 µl were combined with 900 µl of sample buffer (containing 13% 0.5 M TRIS/HCl buffer (pH 6.8), 10.5% Glycerol, 21% of 10% ($^{W}/_{V}$) SDS, 2.6% of 0.4% ($^{W}/_{V}$) Bromophenol Blue (tracking dye), and 52% MilliQ water). Samples that were not centrifuged were combined at a ratio of 1:10 with sample buffer without further preparation.

Note that prior to experiments commencing, samples that were to be reduced had 20 µl of 2-mercaptoethanol added to them at 2%, and were heated to 100°C for 4 minutes.

3.4.4.2 Experimental Methods for PAGE

Gels were run in pairs, fitted into a Mini-Protean II slab Electrophoresis system (Bio-Rad, Richmond, Ca, USA). SDS Electrode buffer (A 5X concentration electrode stock buffer (containing TRIS, Glycerine, SDS, and milliQ water in the ratio 12:72:5:1000 respectively) diluted 1:4 in milliQ water) completely filled the inner buffer chamber and partially filled the outer buffer chamber. The MPC85 sample was added to an unoccupied lane in the gel by a Hamilton syringe delivering 10 µl. Once ready, gel experiments were run with power supplies set to 6.5 watts, with a maximum of 210Volts, and 70 milliamps for a time period of 1 hour.

Gels were stained using 50 ml Amido black solution (0.1% Amido Black 10B Dye, 25% isopropanol, 10% glacial acetic acid, 65% milliQ water) whilst being agitated for one hour. The Amido Black solution was then removed, and replaced with 100 ml of de-staining solution (10%

($^{V}/_{V}$) glacial acetic acid in milliQ water). The de-staining solution was replaced every hour until a clear background was achieved.

Gels were scanned using a Personal Densitometer SI (Molecular Dynamics Inc., Molecular Dynamics World Headquarters, Sunnyvale, CA, USA). Imagequant software was used to evaluate the amount of protein left in the MPC85 powders after their respective heated storage times, and a report was produced assigning numerical values to stained areas as the intensity of a protein band increased linearly with the amount of protein present (Hill and Lowe, 1997).

3.4.5 Mass Spectrometry

3.4.5.1 Sample Preparation for Mass Spectrometry

MPC85 samples to be analysed were prepared by reconstituting MPC85 to 10% (w/w) total solids in milliQ water. Once dissolved 250 μL was sub-sampled and added to 250 μL of 0.1% trifloroacetic acid (TFA), and 10 μL 2-mercaptoethanol, and left to equilibrate for one hour at room temperature. Then the sample was diluted by 1 mL of a 0.09% TFA, 90% acetonitrile buffer, and centrifuged at 21,000 x g for 5 minutes, and then filtered through a 0.45 μm syringe into an HPLC vial.

3.4.5.2 Experimental Methods for Mass Spectrometry

-High Performance Liquid Chromatography (HPLC)

The milk proteins were separated using an Alliance HPLC (Model 2690, Waters, Milford, MA, USA), with a UV detector, with a refrigerated auto-sampler (set to 5°C). The column used was a C18 Hi-Pore RP-318 silica based column (Bio-Rad, Hercules, CA, USA), protected with a guard

column containing the same column packing (Bio-Rad, Hercules, CA, USA). The column details and running conditions can be seen in tables 3.2 and 3.3.

Table 3.2 Configuration details of HPLC column

	Column Details
Separating Column	250 mm x 4.6 mm id, particle size: 5μm, pore size: 300 Å, Catalogue No. 125-0551
Guard Column	30mm x 4.6mm id, No. 125-0134

Table 3.3 HPLC column running details

	Running Conditions
Column Temperature	40°C
Run Time	55 minutes
Flow Rate	1.0 ml/min
Detection Wavelength	280 nm
Injection Volume	standards 25, 50, 75 and 100 μL
Sample Size	25 to 50 μL

-HPLC-Mass Spectrometry

The mass spectrometry of the proteins was analysed using a Perkin Elmer Sciex Triple Quadrupole API300 LC/MS/MS. The HPLC output (1mL/min) was sub-sampled at approximately 10 μL/min for the mass spectrometer using an electrospray API Perkin Elmer Sciex Ionspray inlet. The running conditions are shown in table 3.4. The mass spectrometer had

been calibrated using standards outlined in the manufacturers manual (Perkin Elmer Sciex API 300 manual).

Table 3.4 Mass spectrometer running details

	Running Conditions
Positive Ion Spray Voltage	6000V
Detection	52 to 3000 amu
Orifice Voltage	35V
Ring Voltage	-15 to -90V
IQ2 Voltage	140 to 280V

4 Solubility Testing of Stored MPC85

4.1 Introduction

Much research has been reported on the conditions (heat treatment, protein concentration, protein type, temperature, Ca^{2+} addition, rennet concentration) that govern such properties as formation time, rate of firming, structure and rheological characteristics of renneted milk gels (Zoon *et al.*, 1988a, b, c and 1989a and b). The current investigation has the aim of understanding the influence of MPC85, which has undergone a variety of storage conditions that could be met in industry, on rennet gels under defined experimental conditions. One of the main functional properties studied in this work is the solubility of MPC85. This property is important because it is believed to relate to the functional properties of the MPC85, particularly in applications such as liquid beverages and in cheese milk extension (such is the case in this study) where solubility of the MPC is an important attribute.

4.2 Results

Methodology for determining the total solids of MPC solutions has been described in chapter 3. The solubility tests were performed on MPC85 powder that had been stored at one of five different temperatures: 50°C, 40°C, 35°C, 30°C, or 20°C. A solubility test was also performed on MPC85 that had not been exposed to high temperatures, but instead acidified to pH 4.6 (conditions which caused casein proteins to precipitate). All experiments were performed in duplicate. Individual results can be viewed in figure 4.1.

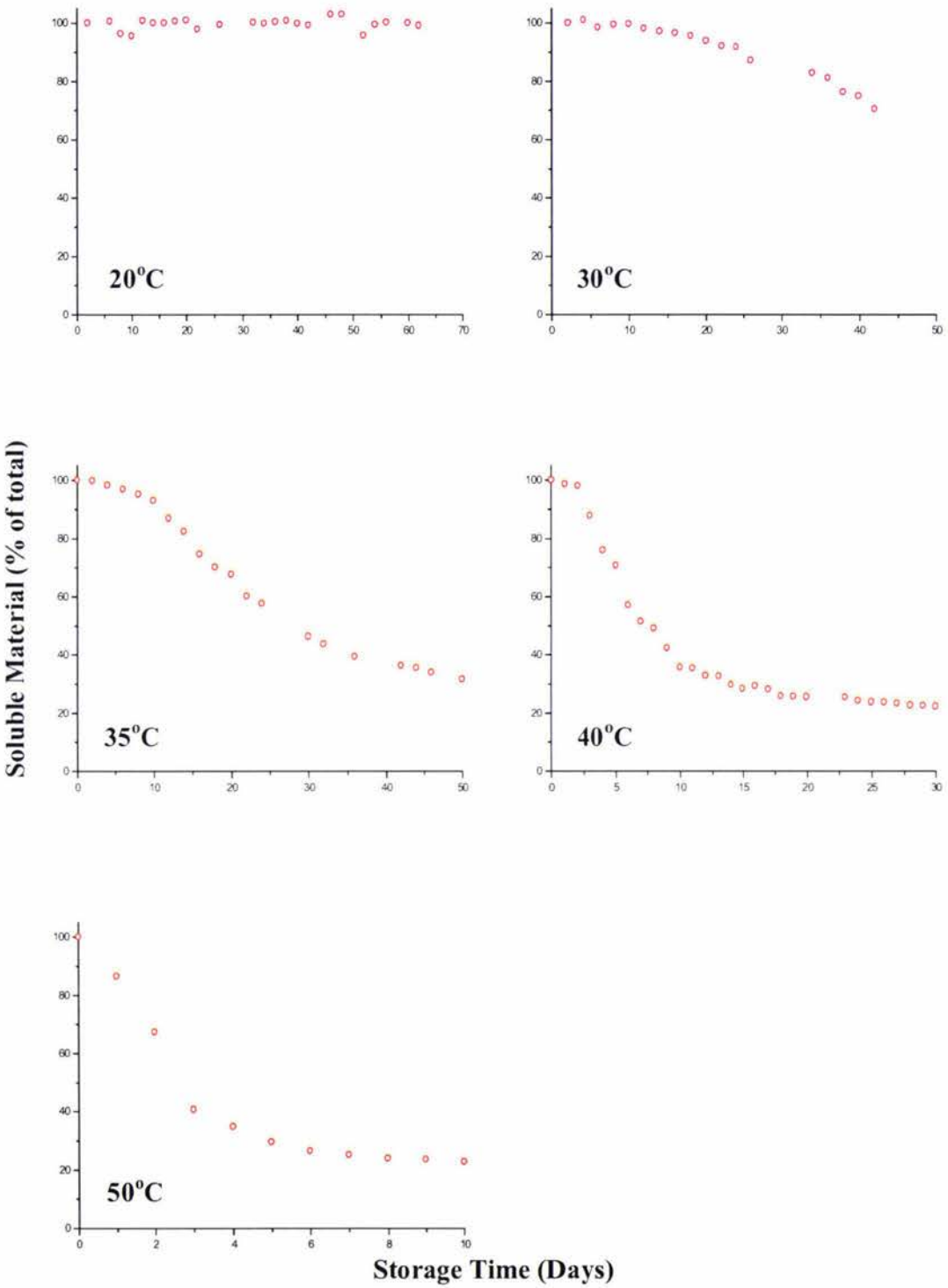


Figure 4.1 Concentration of soluble MPC85 as a function of storage time, for MPC85 stored at different temperatures. Note that error bars are not shown due to the uncertainties being smaller than the data points used in the graphs.

The results produced in this section all seem to follow a similar general trend and each curve can be sectioned into three regions as shown in figure 4.2. Region 1 can be considered as a shoulder in the curve, where a small decrease in values may be noticeable, but the major decrease in solubility has not occurred. In region 2, the solubility of the MPC85 decreases rapidly with time. In region 3, the solubility is close to its minimum value and little or no further change is observed on storage

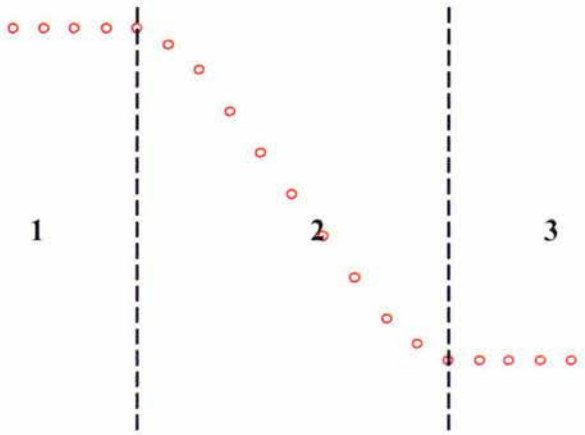


Figure 4.2 General trend of solubility curves. Region 1 represents a shoulder in data where solubility remains constant or close to it. Region 2 represents a major decrease in sample solubility. In region 3 the solubility has decreased to its minimum amount and remains constant.

The following is a discussion of the results shown in figure 4.1. MPC85 stored at 20°C showed little change in solubility and therefore remained in region "1" throughout the two month trial. MPC85 stored at 30°C remained in region "1" for 10 days before moving into region "2". MPC85 powder stored at 35°C initially has good solubility and therefore the solubility curve remained in region "1" for up to 10 days. After this, the solubility decreased rapidly with storage time (region "2"). After 30 days, smaller changes were observed as the solubility approached the minimum value (region "3"). MPC85 stored at 40°C has its solubility curve remain in region "1" for 2 days before moving into region "2". The solubility decreased rapidly for 30 days when

data had reached a plateau of 22% concentration of soluble material (region "3"). The soluble material curve for MPC85 stored at 50°C showed no region "1", although one may have occurred in less than one day. After 9 days the curve reaches a plateau of 22% concentration of soluble material (region "3").

Figure 4.3 displays a least squares fit of the time when the solubility of MPC85 has decreased by 39%, which will be defined as the solubility half life. Figure 4.3 could serve as an approximate guide for future testing of solubility. It may be useful to researchers as an indication of the solubility of a MPC85 powder that has been stored at high temperatures (i.e. an MPC85 that falls above the line will have decreased in solubility by more than 39%).

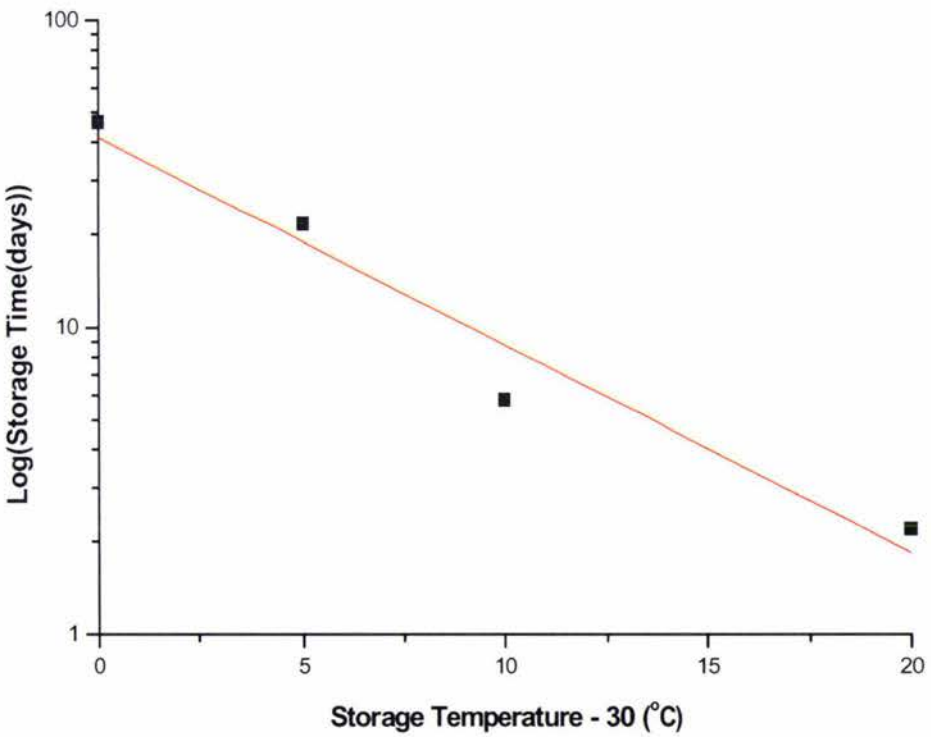


Figure 4.3 Least squares fit of the storage time at which approximately 39% of the soluble material has become insoluble, as a function of storage temperature - 30. The plotted line has the equation: y=41.539exp(-0.1557x), where y is the storage time, and x is the storage temperature -30°C.

4.3 Acidification of MPC85

As stated by Schmidt (1982), and Walstra and Jenness (1984b), caseins are a group of phosphate containing milk specific proteins that precipitate upon acidification to pH 4.6 and represent approximately 80% of total milk proteins. It was thought that the decrease in solubility of samples stored from 30°C-50°C could be caused by casein precipitation. This theory was tested by acidifying a 5% MPC85 solution (using an MPC85 powder that had not being exposed to high temperatures) to pH 4.6 using 6M HCl prior to centrifuging using the same sample preparation for solubility testing as stated in chapter 3. The supernatant was found to contain 25.5% soluble material. This result is significant and will be discussed in section 4.4.

4.4 Comparison between Storage Temperatures

The range of storage temperatures were chosen as these represented the extremes that may be experienced commercially. As high as a 50°C storage temperature may sound, it could be possible for a poorly insulated cargo ship crossing the equator to reach temperatures above 40°C. Results in figure 4.1 show that even short times above 40°C cause marked reduction in solubility.

The earlier sections presented and discussed aspects of the soluble materials curve with respect to the storage temperatures at which they were kept. Figure 4.4 plots the concentration of soluble MPC85 as a function of storage time for all the MPC85 powders under investigation, at all of the storage temperatures.

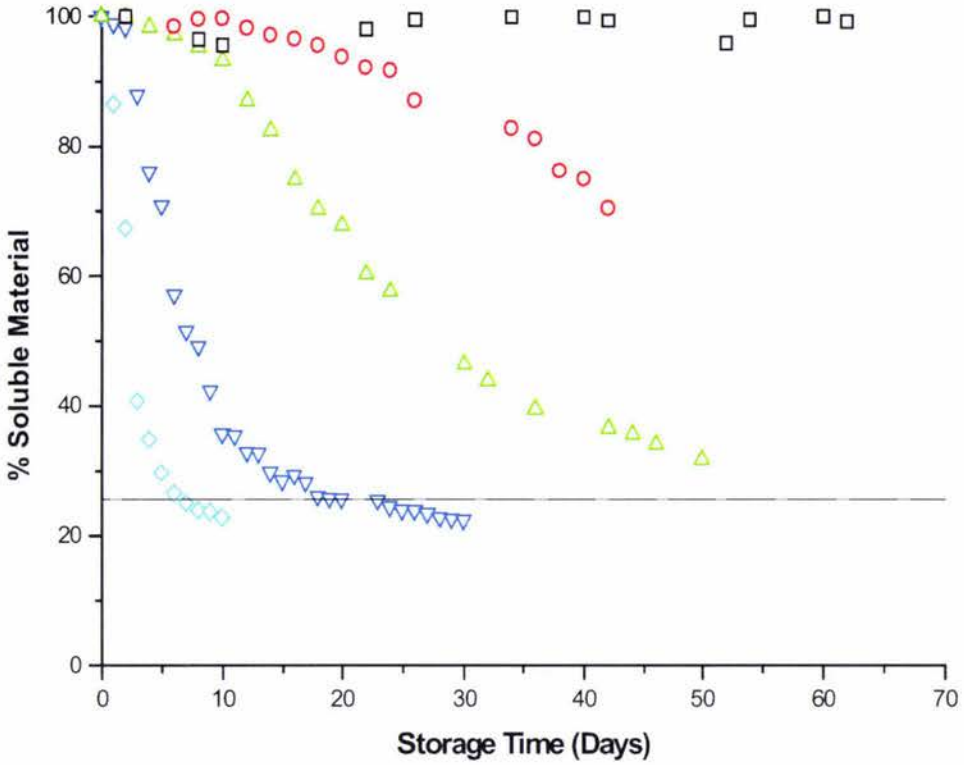


Figure 4.4 Concentration of total soluble material in MPC85 stored at (□) 20°C, (○) 30°C, (△) 35°C, (▽) 40°C, and (◇) 50°C as a function of storage time. The dashed line represents 25.5% soluble material found after MPC85 acidification.

Figure 4.4 shows that the lower the storage temperature, the longer an MPC85 sample takes to decrease in solubility. The solubility curves for MPC85 stored at 40°C, 35°C, and 30°C display the shoulders associated with region "1", as discussed in section 4.2. The shoulder observed in the solubility curve for the sample stored at 40°C was less pronounced and of shorter duration than those from the samples stored at lower temperatures (30 and 35°C). This is due to the rapid decline in solubility after a short storage time of only 2 days at 40°C.

Another feature of figure 4.4 is the dashed line which represents the concentration of soluble MPC85 left in solution upon acidification to pH 4.6 (ie the isoelectic pH known to cause casein precipitation). This amount (approximately 25.5%) is higher than the 22% plateau that samples

stored at 40°C and 50°C reduce to. One can conclude that more than just the caseins are becoming insoluble when exposed to storage temperatures greater than 20°C. This can be accounted for by considering the milk salts associated with the casein micelle. When acid precipitation occurs then these salts become solubilised and dissolve into the whey, as opposed to renneting where they stay associated with the micelle. Therefore the difference in solubility between the two methods could be largely accounted for by the colloidal minerals, although it should be noted that experiments shown in chapter 7 display some of the whey proteins precipitating after being stored at high temperatures.

Another point of interest found in figure 4.4 is that the solubility of MPC85 stored at 20°C does not change over the period of testing. This could possibly mean that 20°C is insufficient to reduce the solubility of MPC85, and as such an adequate storage temperature (for short term storage at least).

4.5 Equation for Solubility Curves

The solubility of MPC85 samples that had undergone storage at different temperatures for different amounts of time was one of the major focal points of this research. This is due to a hypothetical situation that could arise in the future where a company/researcher/etc. could need to know the properties of a similar MPC85 that for whatever reason had been stored at elevated temperatures. Hence it would be useful to obtain a set of kinetic equations to describe the variation of solubility. The best comparison between the samples stored at different temperatures for different amounts of time with regard to solubility can be seen in figure 4.5. The data have been fitted to a master curve with the equation:

$$\%\ \text{soluble material} = (3.56 \times (1 + 0.85\tau) \times \exp(-\tau \times 0.8) + 1) * 22.11 \qquad \textbf{(4.1)}$$

(Notice the exponential variation). In equation 4.1, τ represents normalised time, whereby sample times for storage temperatures 40°C, 35°C, 30°C, and 20°C were divided by factors of

2.7, 10, 24, and 100 respectively. Equation 4.1 can be modified to calculate % solubility of MPC85 without the normalisation of data, and to act as a function of both storage time and temperature. To do this, the normalised time, τ, is made into the function:

$$\frac{1}{\tau} = \frac{\left(-0.00585T^3 + 0.79754T^2 - 36.313T + 553.99\right)}{t} \qquad \textbf{(4.2)}$$

Where t is equal to storage time and T is the storage temperature.

The solubility of an MPC85 stored at a temperature between 20°C and 50°C may be calculated by substituting equation 4.2 in equation 4.1. These equations were created by using the curve fitting functions of computer based math programs.

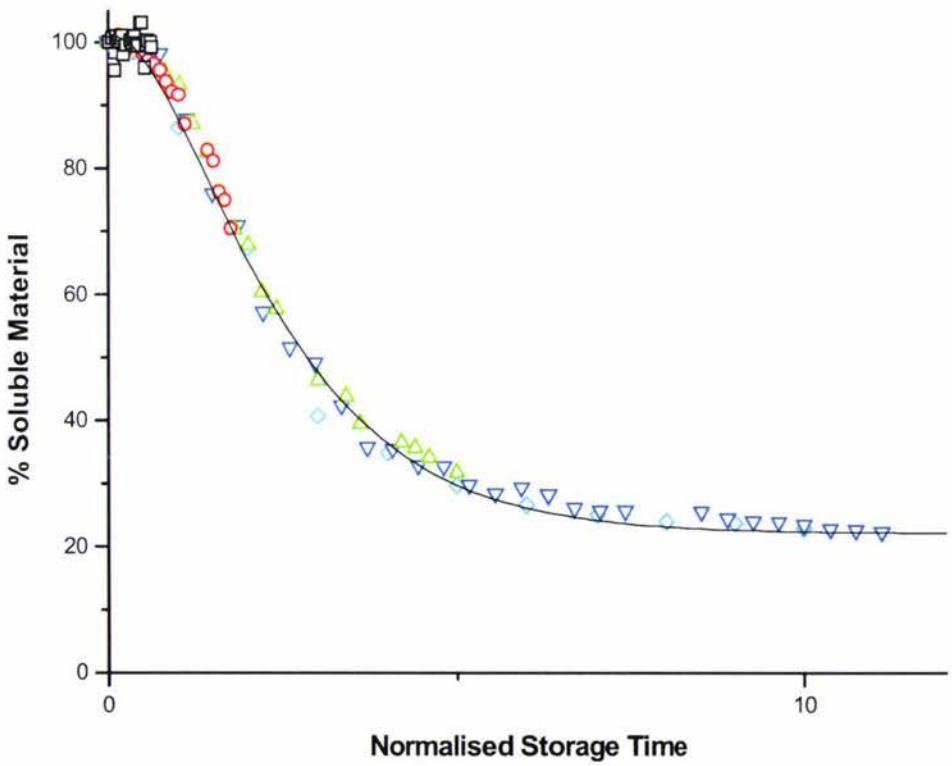


Figure 4.5 Concentration of soluble material remaining in MPC85 for 20°C (□), 30°C (○), 35°C (△), 40°C (▽), and 50°C (◇) storage temperature, as well as equation (4.1) (solid line), as a function of normalised storage time.

4.6 Summary

In this chapter, the influence of storage temperature on the solubility of MPC85 powder was studied. It was found that once the storage temperature was raised above 20°C the solubility of an MPC85 powder decreased with time. Moreover, at elevated storage temperatures the solubility decreased at a faster rate.

Samples stored at 20°C displayed no change in solubility over a two month period. Samples stored at 30°C, 35°C, and 40°C displayed delay times before a decrease in solubility could be noticed. However this delay time was not observed for samples stored at 50°C.

The remaining soluble material observed when all the casein was removed from MPC85 sample by acidification to pH 4.6 was greater than the minimum solubility observed for the stored MPC85 samples (Figure 4.4). However when the colloidal salts were taken into consideration, the minimum solubility observed could be accounted for almost entirely by the casein micelles, although as previously mentioned, chapter 7 shows whey proteins becoming involved in the precipitation of reconstituted powders that were stored at high temperatures.

A set of kinetic equations were presented that accurately modelled the % soluble material remaining in solution after heated storage of MPC85 powders. Considering figure 4.5, the solubility of MPC85 samples stored at all temperatures were able to be placed on one master curve. This implies that the same mechanism was occurring at all storage temperatures. Further discussion about this process can be found in chapter 9.

5 Rheological Properties of Renneted Skim Milk Supplemented with MPC85 Powder

5.1 Data Analysis

The operation of the rheometer is to apply a harmonic, low amplitude shear strain γ, of angular frequency ω as follows:

$$\gamma = \gamma_0 \cos \omega t \tag{5.1}$$

Note: γ_0 is the strain amplitude, and t is time. The strain applied to the sample will result in a stress σ, which will resist the motion of flow. The rheometer measures this stress to classify the material, but it may be found mathematically by:

$$\sigma = \sigma_0 \cos(\omega t + \delta) \tag{5.2}$$

Note: σ_0 is the stress amplitude. In general, the stress will have a phase difference from the strain δ ($0<\delta<90^\circ$), where δ (or $\tan\delta$), is the viscous/elastic ratio for the material at frequency ω. The elastic (in-phase) and viscous (out-of-phase) components of the stress can be separated, and determine:

G' ≡ the in-phase shear storage modulus

$$= (\sigma_0/\gamma_0)\cos\delta \tag{5.3}$$

G'' ≡ the out-of-phase shear loss modulus

$$= (\sigma_0/\gamma_0)\sin\delta \tag{5.4}$$

One of the most common ways to present viscoelastic measurements is by combing both G' and G'' to form the complex shear modulus: G^*. This value is a measure of the energy dissipated per cycle of deformation per unit volume, and is given by:

$$|G^*| = \left[(G')^2 + (G'')^2\right]^{1/2} \quad \textbf{(5.5)}$$

Experimental details of the sample preparation for rheological tests can be found in chapter 3. Three sequential rheological tests were performed on each sample. Firstly a time sweep was performed to monitor the action of the rennet that has been added to the sample, and as such the eventual gelation. Secondly a frequency sweep is completed so that the viscoelastic properties of the sample may be classified. Lastly a strain sweep is completed to discover the strength of the gelled sample. Typical experimental results are shown in figures 5.1A, B, and C respectively.

Figure 5.1A is a plot of the magnitude of the complex modulus of a reconstituted skim milk sample supplemented with MPC85 as a function of time after the rennet addition. Two parameters of interest may be extracted from this graph. Firstly the gelation time (where the aggregating micelles begin to form a network), defined as when G^* is greater than 1 Pa (shown as point 1). Secondly the gel strength at three hours is found from the last measurement taken from the time sweep (point 2). For convenience, the final gel strength will be defined as the symbol G_F^*.

Figure 5.1B displays a typical plot obtained from a frequency sweep. Values shown are the storage (G') and loss (G'') modulus of skim milk supplemented with MPC85 as a function of frequency.

Figure 5.1C is a typical plot of stress as a function of the applied strain of the renneted skim milk/MPC85 sample obtained from a strain sweep. This breaking point of the network, also known as the fracture stress/strain, may be found by considering point 1 in figure 5.1C.

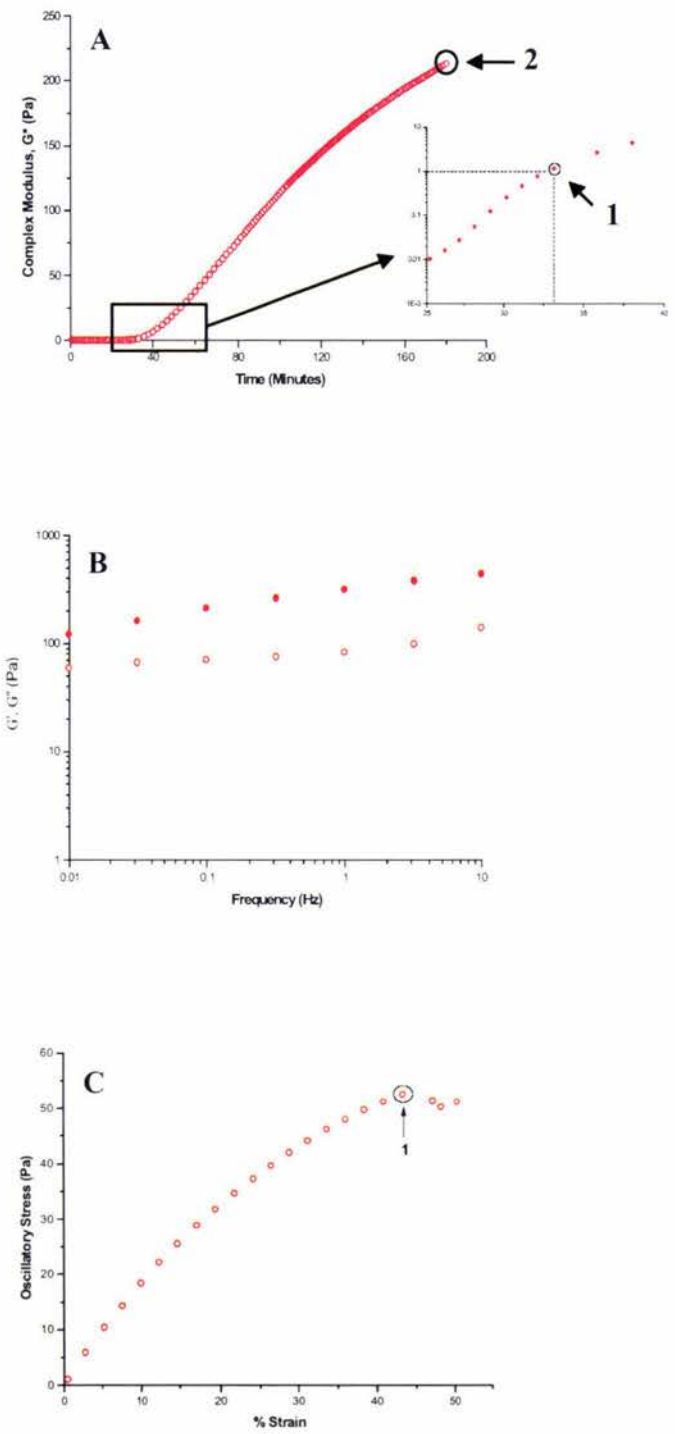


Figure 5.1 Typical results from rheology tests, showing A) the time sweep, B) frequency sweep, and C) the strain sweep. Point 1 in graph A) displays the gelation time, and point 2 displays the final gel strength. Point 1 in graph C) shows the fracture stress.

5.2 Results

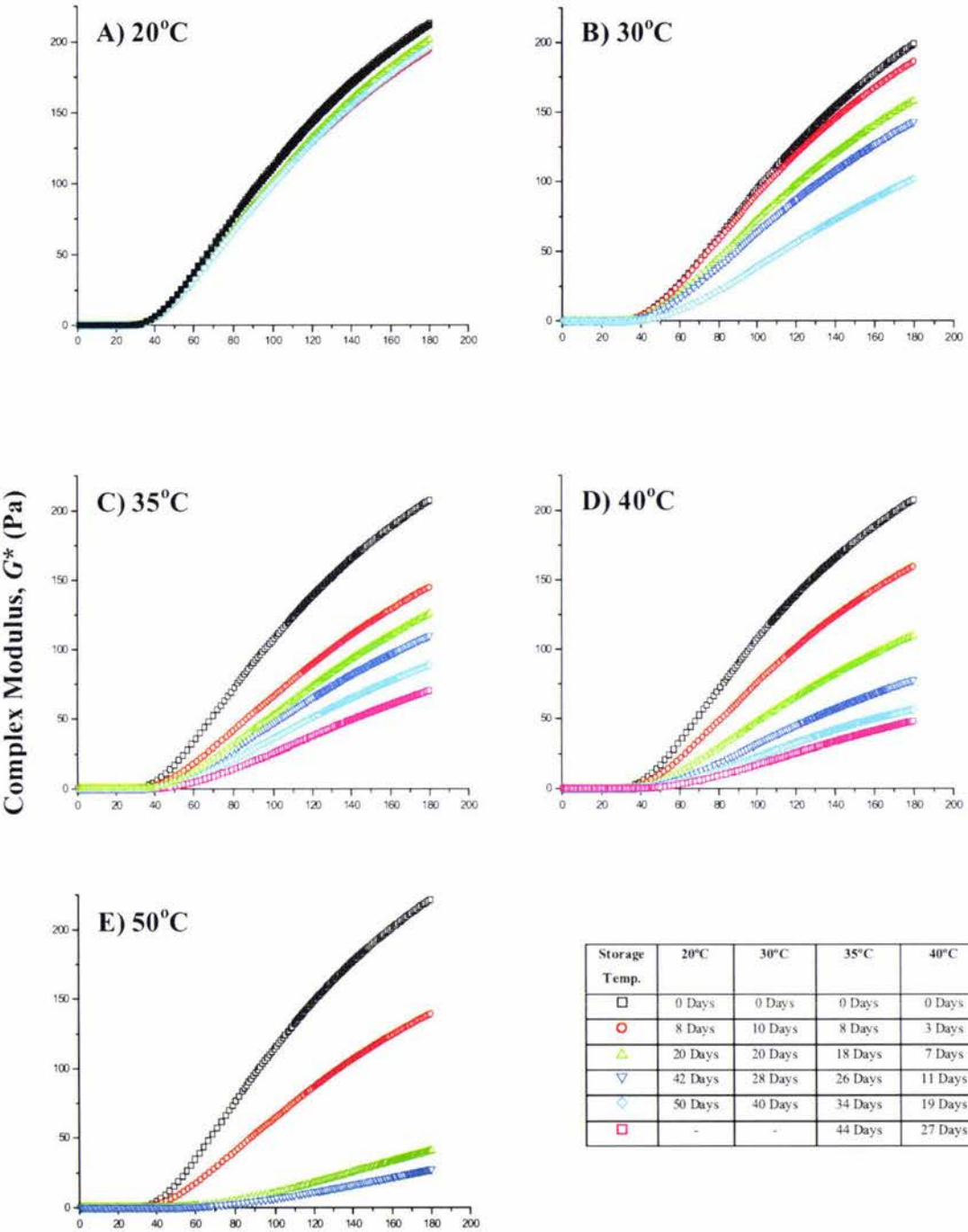
Note that the individual graphs that make up figures 5.2, 5.4, and 5.5 represent different storage times from different temperatures. Many measurements were performed, but few are reported for the clarity of the figures.

5.2.1 Time Sweep

Figure 5.2A shows the complex modulus (G^*) as a function of time after rennet had been added to the skim milk/MPC85 solutions. As can be seen, prolonged storage of MPC85 at 20°C did not affect the rheological properties of skim milk/MPC85 samples. For example the sample that had been stored for 50 days yielded almost exactly the same gel strength after 3 hours, G_F^*, as a MPC85 sample that had not been exposed to excess temperatures at all. This is reported in Figure 5.3A.

Figure 5.2B depicts G^* as a function of time after rennet addition to skim milk/MPC85 solutions for samples that had been stored at 30°C. As storage time of the MPC85 was increased, G^* increased at a lower rate resulting in a smaller final gel strength at three hours. This decrease in G_F^* did not commence until approximately 15 days as shown in Figure 5.3B. As a result the final gel strength decreases from an initial value of approximately 200 to 120 Pa.

Figure 5.2C shows G^* for skim milk/MPC85 solutions for MPC85 stored at 35°C as a function of time after rennet addition. Samples that are prepared using MPC85 stored at 35°C have a final gel strength that decreases from the control without delay in respect to storage time. This can be seen more clearly in Figure 5.3C, where the final gel strength, G_F^*, is plotted as a function of storage time. It can be seen that a near linear decrease exists caused by storage for this amount of time at 30°C.



Storage Temp.	20°C	30°C	35°C	40°C	50°C
□	0 Days	0 Days	0 Days	0 Days	0 Days
○	8 Days	10 Days	8 Days	3 Days	2 Days
△	20 Days	20 Days	18 Days	7 Days	5 Days
▽	42 Days	28 Days	26 Days	11 Days	10 Days
◇	50 Days	40 Days	34 Days	19 Days	-
□	-	-	44 Days	27 Days	-

Figure 5.2 Complex modulus as a function of time for MPC85 stored at different temperatures. Also shown is the key for each graph.

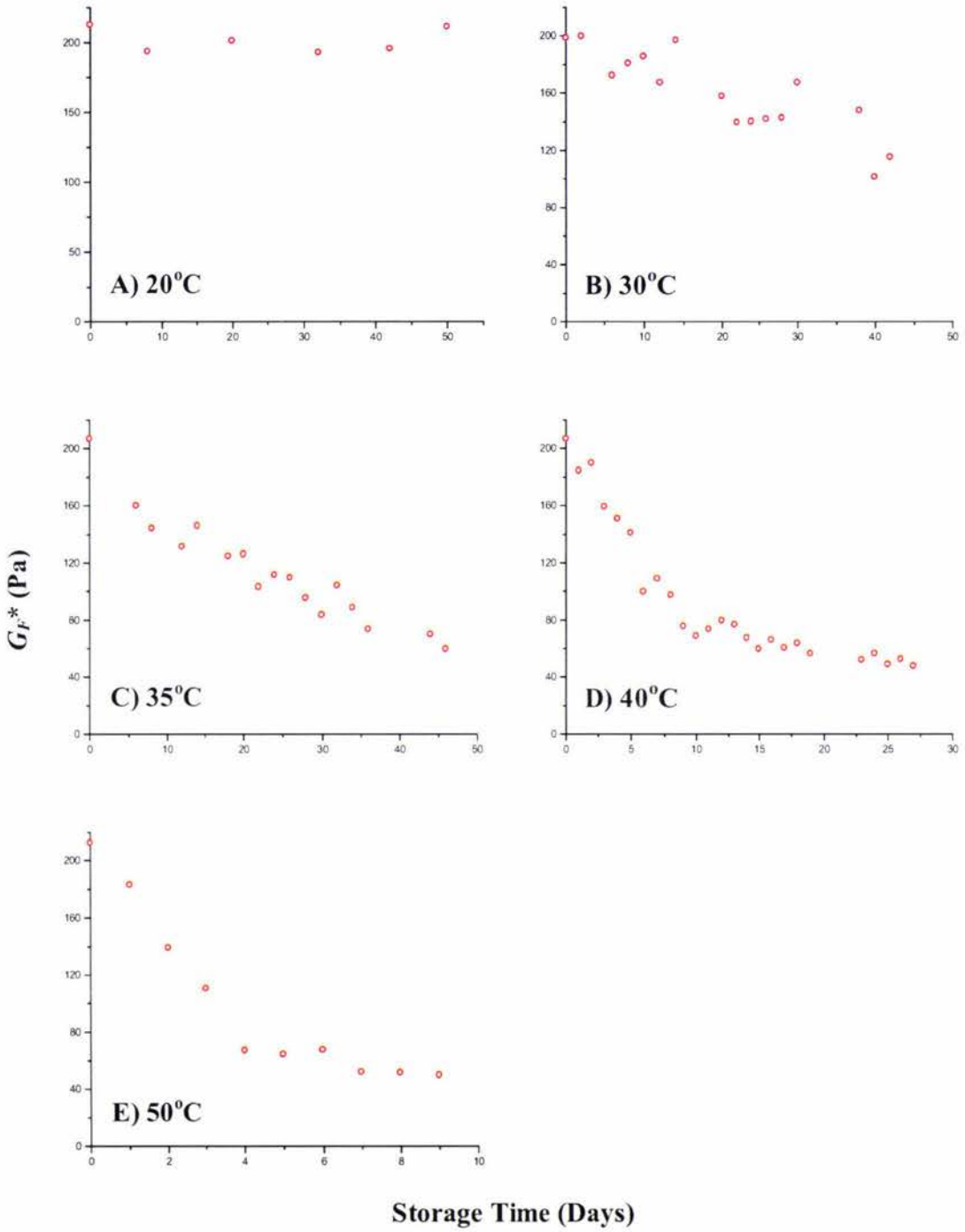


Figure 5.3 Final gel strength of rheology experiments using MPC85 stored at different temperatures as a function of storage time. Note the differing abscissa scales.

The magnitude of the complex modulus of samples prepared using MPC85 stored at 40°C as a function of time after rennet addition is shown in figure 5.2D. In this set of data the firming rate decreases with storage time without delay. Figure 5.3D displays the final gel strength, G_F*, at three hours as a function of time. The plot decreases to what would seem to be a plateau value.

Skim milk/MPC85 made from samples stored at 50°C, produced rennet induced gels which have the gel strengths decrease very quickly with storage time, as can be seen in Figure 5.2E. The gel strength for this set of data can be seen to reach a plateau value after which no further decrease of the sample's properties occur with time. This is reflected by Figure 5.3E, the final gel strength at 3 hours as a function of storage time.

5.2.2 Frequency Sweep

At the conclusion of the 3 hour time sweep, a frequency sweep was performed to quantify the viscoelastic properties of the rennet induced gel. These results are shown in Figures 5.5A-5.5E. The general trend for all samples was that as storage temperature increased, the storage, G', and loss, G'', moduli decreased. For MPC85 stored at 20°C this decrease is very small, whereas for 50°C storage it was far more noticeable. All samples tested at all frequencies had G' greater than G'', but by less than a decade, so all samples can be considered as weak gels (Lapasin and Pricl, 1995). This can be seen more clearly be considering Figure 5.4. This figure plots the dynamic moduli (i.e. the storage and loss modulus) of a viscoelastic material as a function of rotational frequency. When compared with the results in Figure 5.5A-E, then gel like behaviour can indeed be observed.

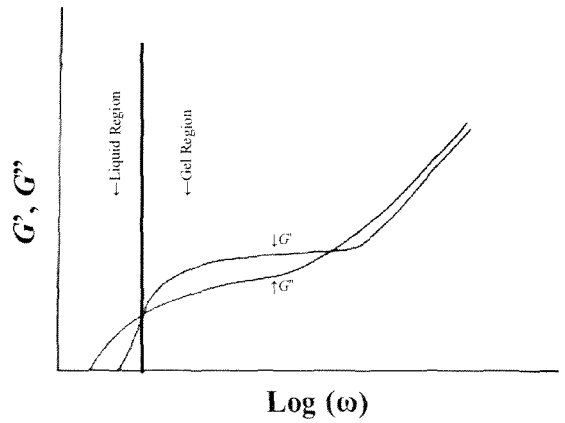
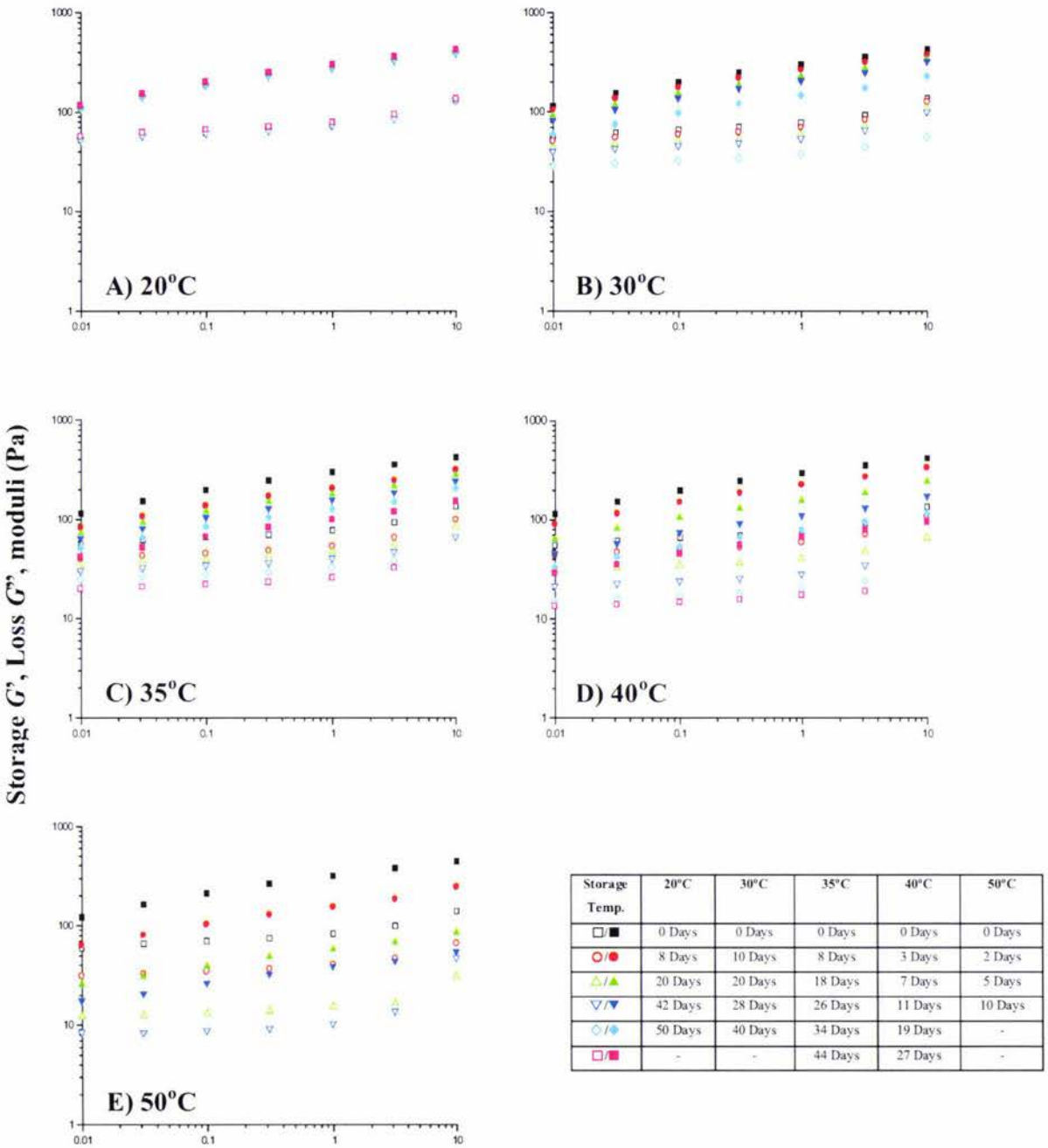


Figure 5.4 Dynamic moduli of a viscoelastic material as a function of rotational frequency.

5.2.3 Strain Sweep

Figure 5.6A is a plot of the applied strain, as a function of the oscillatory stress for samples made using MPC85 stored at 20°C. The data are mostly constant as can be seen from Figure 5.7A, the plot of breaking oscillatory stress as a function of storage time.

The stress of skim milk/MPC85 made from samples stored at 30°C, during the strain sweep stage, as a function of the strain applied by the rheometer is shown in Figure 5.6B. As storage time for MPC85 samples increased, the gel strength of samples decreased. This is also shown in Figure 5.7B, the fracture point of the gels measured as a function of the storage time.



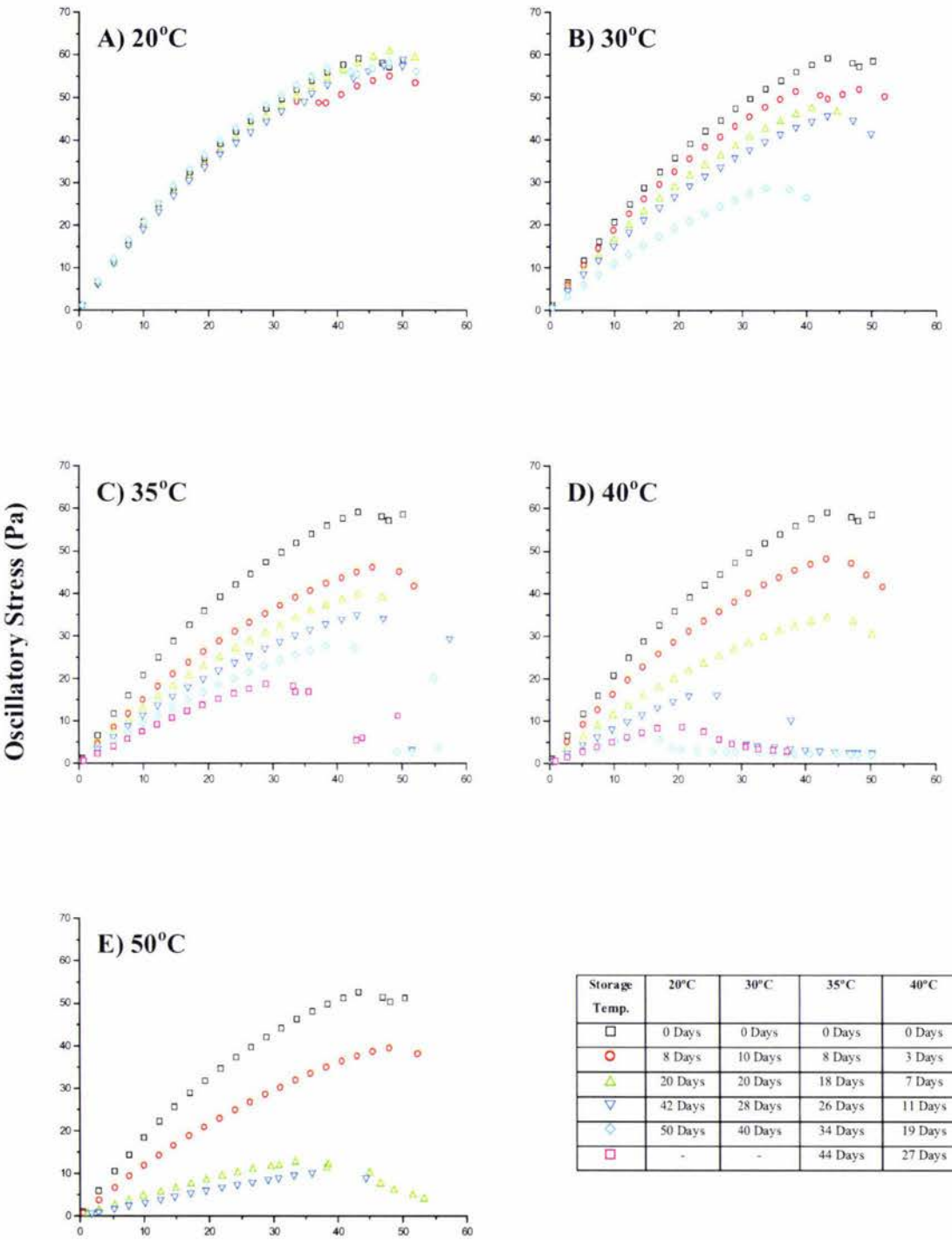
Storage Temp.	20°C	30°C	35°C	40°C	50°C
□/■	0 Days	0 Days	0 Days	0 Days	0 Days
○/●	8 Days	10 Days	8 Days	3 Days	2 Days
△/▲	20 Days	20 Days	18 Days	7 Days	5 Days
▽/▼	42 Days	28 Days	26 Days	11 Days	10 Days
◇/◆	50 Days	40 Days	34 Days	19 Days	-
□/■	-	-	44 Days	27 Days	-

Figure 5.5 Viscoelastic properties of gels made from MPC85 stored at different temperatures. *G'* are solid symbols, *G"* are open symbols. Also shown is the key for each graph.

Figure 5.6C relates the oscillatory stress of samples, prepared using MPC85 stored at 35°C, as a function of strain. As storage time is increased, a dramatic reduction in the breaking stress occurred. This is reflected by Figure 5.7C which plots the breaking stress of samples as a function of storage time. Figure 5.7C shows a linear decrease of the fracture stress over the storage time.

The oscillatory stress of samples made from MPC85 stored at 40°C, in response to the applied strain is shown in Figure 5.6D. The oscillatory stress required to fracture the gel, shown by Figure 5.7D, decreased with storage time. Figure 5.7D also clearly displays a breaking stress plateau value reached at approximately 15 days. After this time prolonged storage did not decrease the already low stress required to fracture the renneted gel.

Figure 5.6E depicts the oscillatory stress produced by the rennet induced sample containing MPC85 stored at 50°C, as a function of the applied strain. The data decreases from that of the control sample (MPC85 not exposed to heat), and a plateau is reached after 7 days. Figure 5.7E also shows this occurrence by comparing the gel fracture stress with storage time at 50°C.



Storage Temp.	20°C	30°C	35°C	40°C	50°C
□	0 Days	0 Days	0 Days	0 Days	0 Days
○	8 Days	10 Days	8 Days	3 Days	2 Days
△	20 Days	20 Days	18 Days	7 Days	5 Days
▽	42 Days	28 Days	26 Days	11 Days	10 Days
◇	50 Days	40 Days	34 Days	19 Days	-
□	-	-	44 Days	27 Days	-

Figure 5.6 Stress-strain curves for gels made from MPC85 stored at different temperatures. Also shown is the key for each graph.

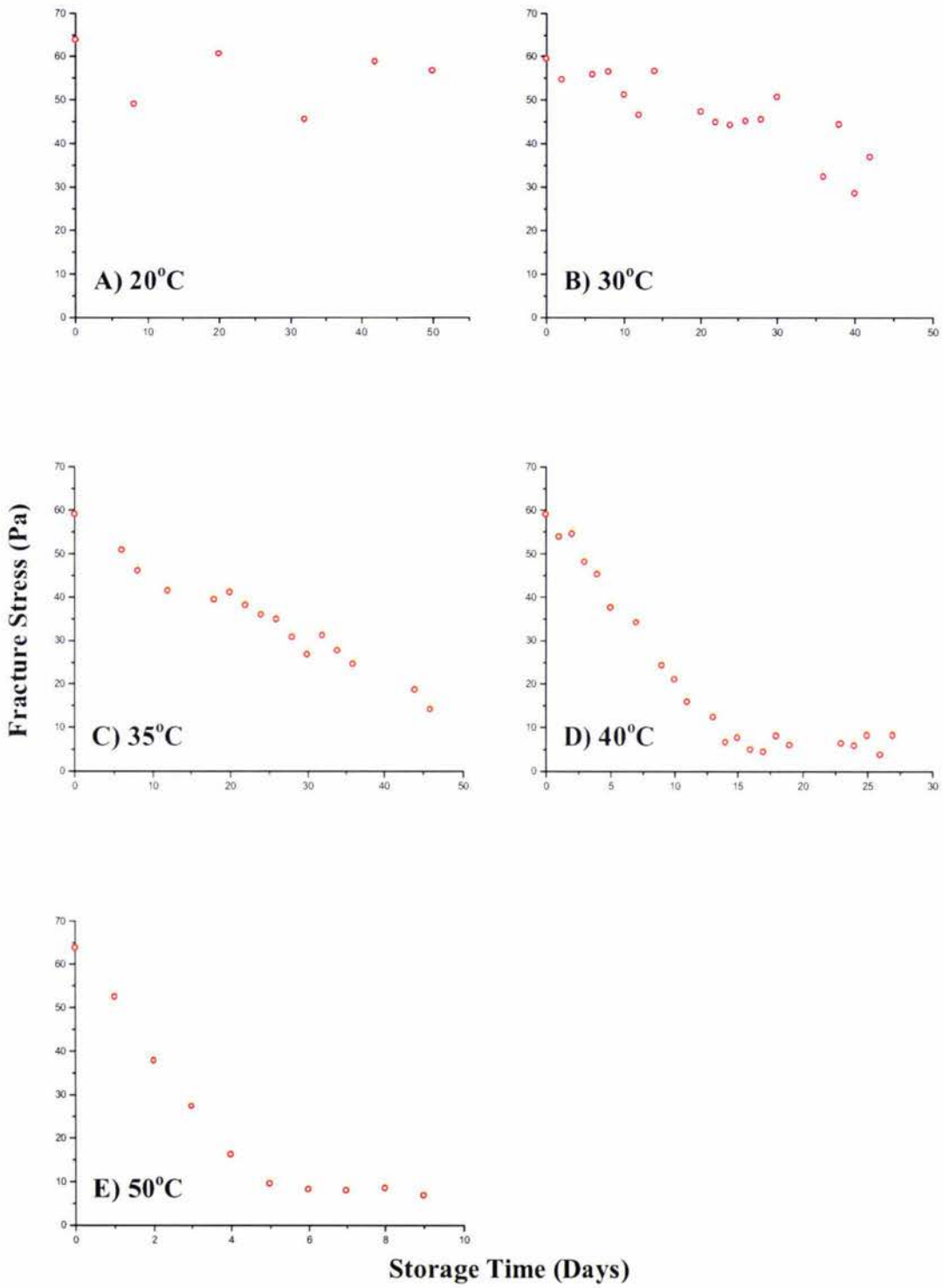


Figure 5.7 Breaking stresses of samples prepared from MPC85 stored at different temperatures as a function of storage time. Note the differing abscissa scales.

5.3 Comparison between Storage Temperatures

The previous sections in this chapter presented and discussed separately the addition of MPC85 that had been stored at 20°C, 30°C, 35°C, 40°C, and 50°C, to skim milk prior to rennet addition. Figure 5.8 plots the final gel strength at the end of the time sweep for all five storage temperatures as a function of storage time. All samples, except those prepared using MPC85 stored at 20°C, show a tendency for G_F* to decrease with time. Note that the lines are meant only as a guide.

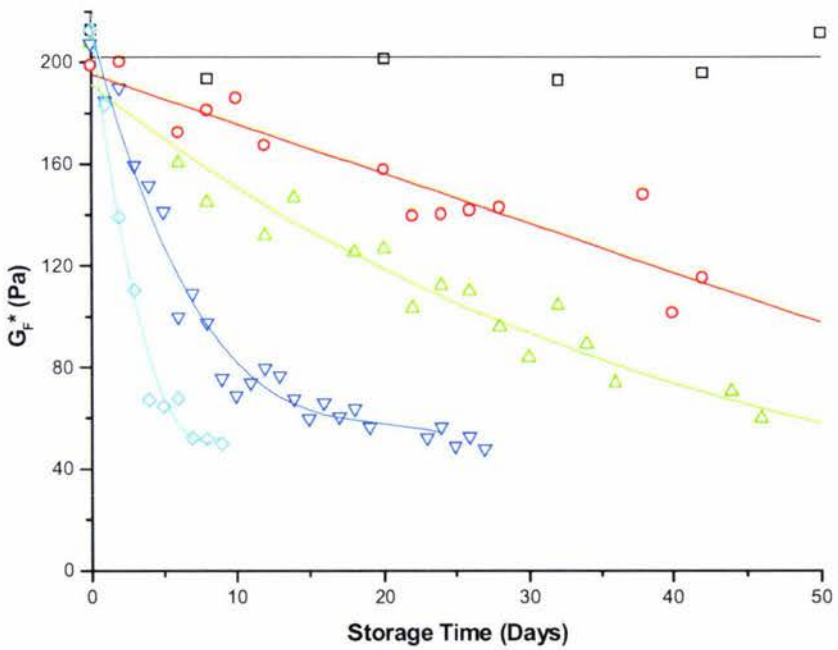


Figure 5.8 Final gel strength as a function of storage time for skim milk supplemented with MPC85 stored at 50°C (□), 40°C (○), 35°C (△), 30°C (▽), and 20°C (◇).

Just as a master curve was found for the solubility of MPC85 samples with varying storage time and temperatures, so was one produced for rheology results that are shown in figure 5.8. These can be seen in figure 5.9.

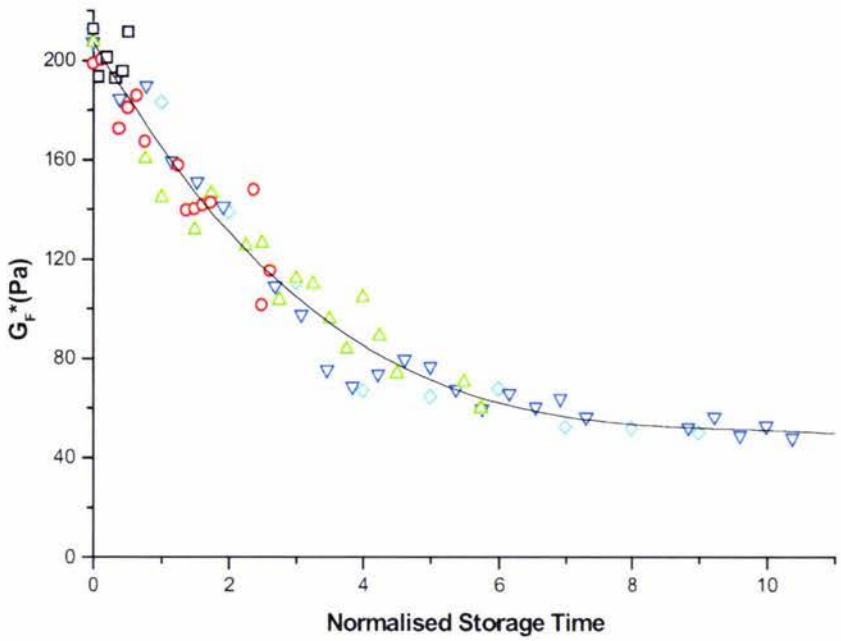


Figure 5.9 Final gel strength of skim milk/MPC85 samples prepared from MPC85 stored at 20°C (□), 30°C (○), 35°C (△), 40°C (▽), and 50°C (◇), as a function of normalised storage time.

The data on this curve have been fitted using the equation:

$$G_F^* = -0.1688\tau^3 + 4.844\,\tau^2 - 47.19\,\tau + 207.32 \qquad \textbf{(5.6)}$$

In equation 5.6 τ represents normalized time, whereby stored times for storage temperatures 40°C, 35°C, 30°C, and 20°C were divided by factors of 2.6, 8, 16, and 100 respectively. Equation 5.6 can be modified to calculate G_F^* without the normalisation of data, and to act as a function of both storage time and temperature. To do this, the normalisation time is manipulated using equation 5.7.

$$\frac{1}{\tau} = \frac{3608.6\exp(-0.1788T)}{t} \quad \textbf{(5.7)}$$

Where t is equal to the storage time and T is the storage temperature. To calculate the final gel strength (G_F*) of an MPC85 stored at a temperature between 20°C and 50°C, equation 4.2 needs to be substituted into equation 4.1. These equations were created by using the curve fitting functions of computer based math programs.

It can be noticed that Figure 5.9 does not have a 'shoulder' or delay time in its curve, and the final gel strength of renneted skim milk/MPC85 samples begins to decrease with minimal storage time. This reason that this occurs may be that although only small amounts of MPC initially become insoluble, when they are incorporated into a gel, the network as a whole becomes noticeably weaker. The mechanisms that occur in the MPC powders during heated storage are discussed in detail in chapter 9.

The gelation times of skim milk/MPC85 samples prepared from MPC85 stored at all the different storage temperatures are shown in Figure 5.10. As can be seen the gelation time increases for all samples except those prepared using MPC85 stored at 20°C

The frequency sweep tests reiterated the results from the time sweep. As storage time of most MPC85 samples increased, the viscoelastic properties (G' and G'') of the rennet induced skim milk/MPC85 gels decreased. No change occurred when MPC85 powders were stored at 20°C. Furthermore all skim milk/MPC85 gels behaved as weak gels, denoted by a storage modulus (G') greater than the loss modulus (G''), by no more than one decade (Lapasin and Pricl, 1995).

The strain sweeps showed that, excluding MPC85 stored at 20°C, the fracture point of gels (measured as the oscillatory stress responding to the applied strain) decreased with storage time. This is another indication of the weakening of the gels.

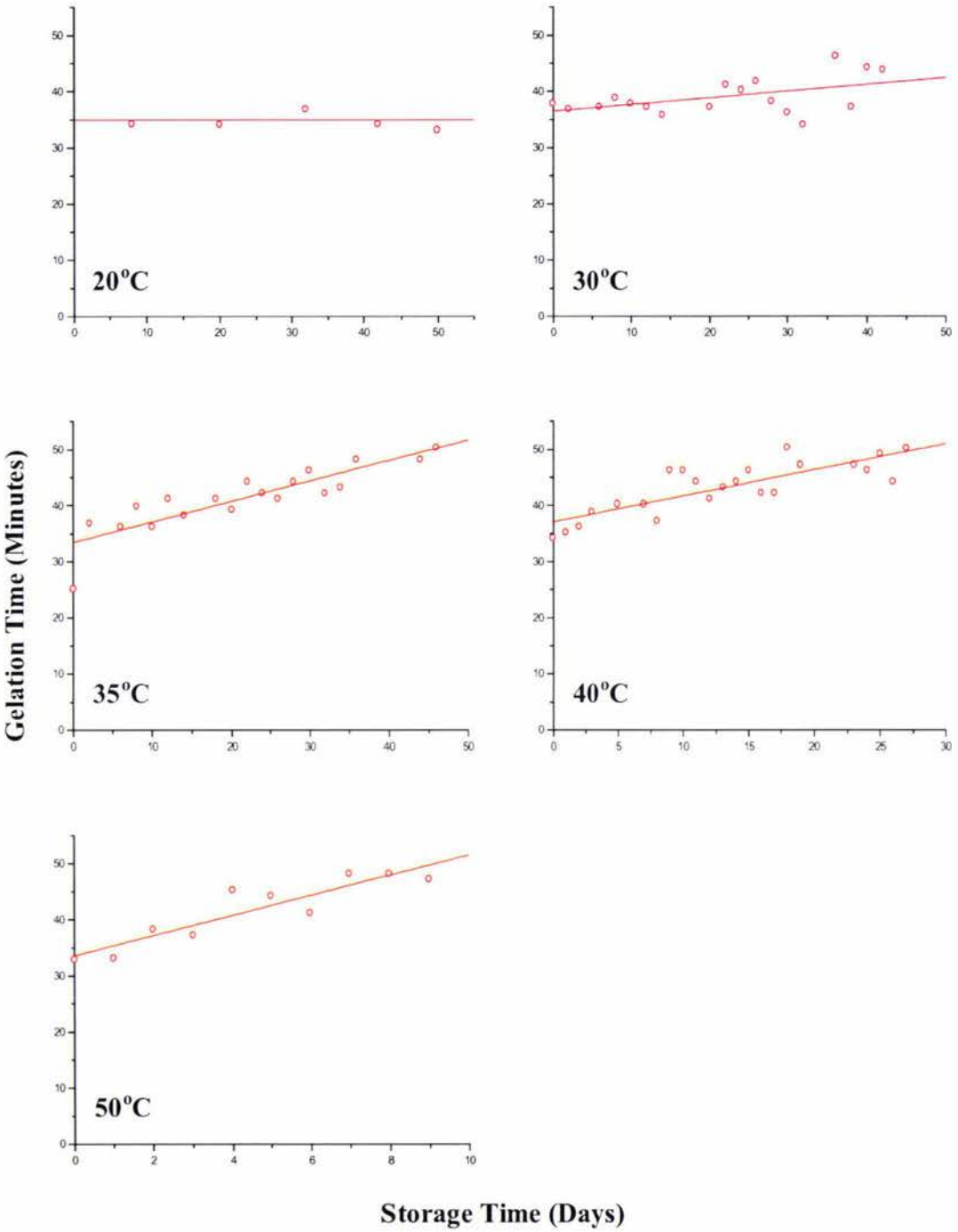


Figure 5.10 Gelation times of samples prepared from MPC85 stored at different temperatures as a function of storage time. Note the differing abscissa scales.

5.4 Summary

In this chapter the influence of different storage temperatures of MPC85 on the rheological properties of rennet induced skim milk supplemented with MPC85 was investigated. It was found that results did not change when MPC85 was stored at 20°C, but if stored at higher temperatures (30°C, 35°C, 40°C, or 50°C), the gels were weaker as storage time increased. In addition to this, both the viscoelastic properties (G' and G''), and the stress required to fracture the formed gels decreased with storage time. Another effect was that the gelation time increased with storage time. Note that all these effects occurred faster as the storage temperatures were increased. These effects and their relation to the physical processes taking place within the samples will be discussed in detail in chapter 9.

6 Effect of Hydration Time on the Rheological Properties of Renneted Skim Milk/MPC85 Solutions

6.1 Introduction

Many different food products are produced by the reconstitution of dry ingredients into a solution, and this process often requires an hydration time before further processing can occur. In many cases this hydration time can determine several properties of the manufactured product (ie gel structure), and the ability to minimise this time would be hugely beneficial. For this reason renneted skim milk/MPC85 solutions were prepared with different hydration times to quantitatively test the effect of hydration time on the solutions' physical properties.

The samples were prepared according to the protocol described in Section 3.4.2.1, and the experimental methods were the same as those used in chapter 5. Three sets of samples are investigated in this chapter, a fresh MPC85 powder (100% soluble) and the same MPC85 powder after storage of 7 and 30 days at 40°C (88% and 22% soluble respectively). These are termed MPC samples of high, medium and low solubility, respectively.

6.2 Results

6.2.1 Time Sweep

A plot of the complex modulus (G^*) as a function of time after rennet addition to skim milk samples supplemented with high solubility MPC85, with varying hydration times is shown in figure 6.1A. The effect of increasing the hydration time for this set of samples was an increase in G^*, and an increase in gel strength. This is reflected by figure 6.2 which displays the G_F^* (ie

G^* at three hours) as a function of the hydration time, showing that the final gel strength increased with hydration time.

The variation of the complex modulus (G^*) for skim milk samples supplemented with medium solubility MPC85, with different hydration times is shown in figure 6.1B. There was no change during the first 7 hours hydration time; however, as the hydration time was increased from 7 to 24 hours, the gel strength increased. The final gel strength (figure 6.2) as a function of hydration time shows this effect more clearly.

Figure 6.1C depicts the complex modulus (G^*) as a function of time for a low solubility MPC85 powder. There was no discernable change in G^* as the hydration time was varied between 1 and 24 hours. Figure 6.2 shows there is no significant change in the final gel strength for samples prepared with low solubility MPC85.

When high solubility MPC85 was reconstituted with skim milk, a greater hydration time resulted in a higher gel strength. The gelation time for samples prepared using a high solubility MPC85 remained constant at approximately 38 minutes.

Samples prepared using medium solubility MPC85 powder produced a gelation time that remained approximately constant as hydration time increased, yielding a value of 48 minutes.

The gelation time obtained for rennet induced samples prepared using a low solubility MPC85 also showed minimal change. The gelation times remained constant at approximately 59 minutes.

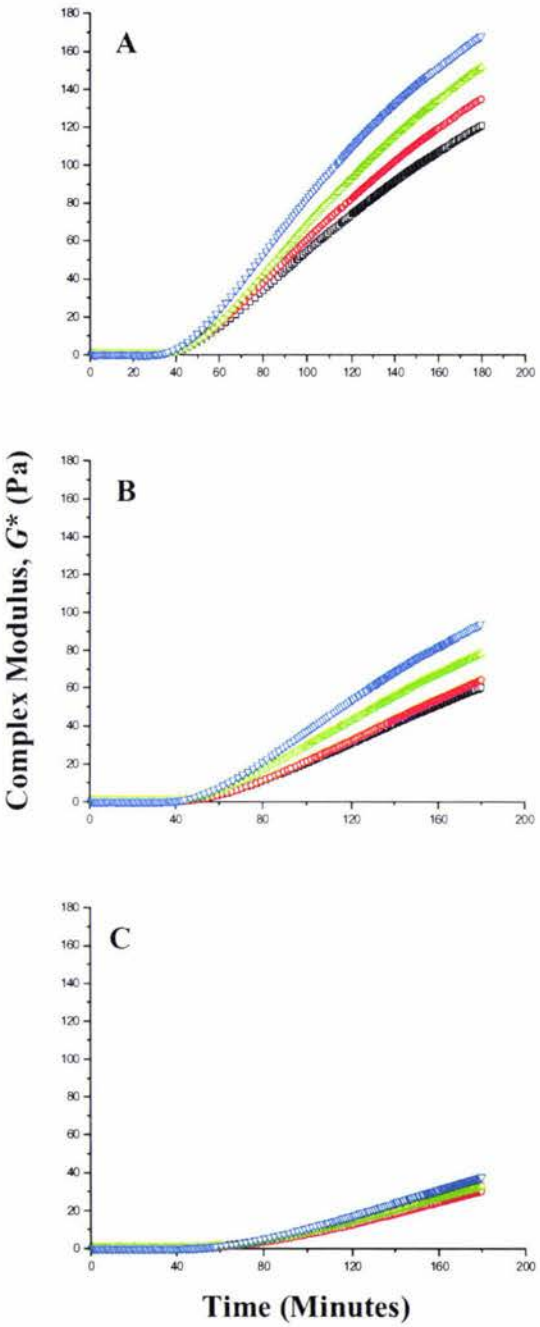


Figure 6.1 Complex modulus (G^*) of renneted skim milk/MPC85 after 1 hour (□), 7 hours (○), 16 hours (△), and 24 hours (▽) hydration, as a function of time after rennet addition for samples prepared with A) high solubility, B) medium solubility, and C) low solubility MPC85.

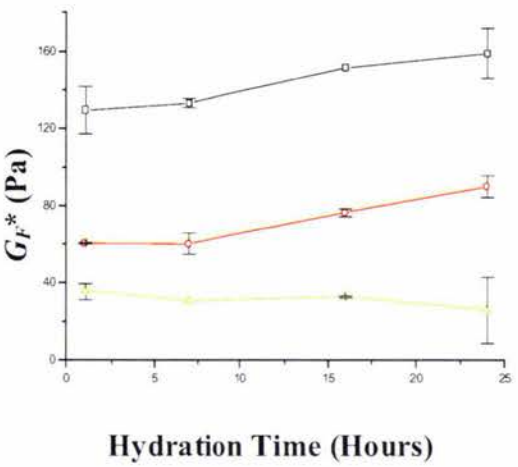


Figure 6.2 Final Gel Strength of skim milk supplemented with (□) high solubility, (○) medium solubility, and (△) low solubility MPC85 powder, as a function of hydration time. Note that this figure contains average values, while figure 6.1 contains examples of each test.

6.2.2 Frequency Sweep

Solutions prepared with high, medium, and low MPC85 powders all yielded similar results. As the hydration time increased, both the storage modulus (G') and the loss modulus (G'') increased in magnitude. The overall behaviour, of all samples at all frequencies was that of a weak gel, as G' is greater then G'' by less than 1 decade at all frequencies. Results from these experiments are shown in figures 6.3A, B, and C.

6.2.3 Strain Sweep

The stress suffered by samples prepared using high solubility MPC85 during the strain sweep experiment is shown in figure 6.4A, as a function of the applied strain. As the hydration time increased, the fracture strain also increased. This is more clearly shown in figure 6.5, which displays a plot of the fracture stress against hydration time for all samples.

Figure 6.4B depicts the stress of the skim milk plus medium solubility MPC85 samples produced as a function of the strain applied. Extending the hydration time of a medium solubility MPC85 powder increased the stress that a sample could withstand before fracturing.

Figure 6.4C displays the stress response of skim milk plus low solubility MPC85 samples to applied strain. It was found that fracture stress showed minimal change with hydration time.

These observations will be related to what is occurring within samples in detail in chapter 9.

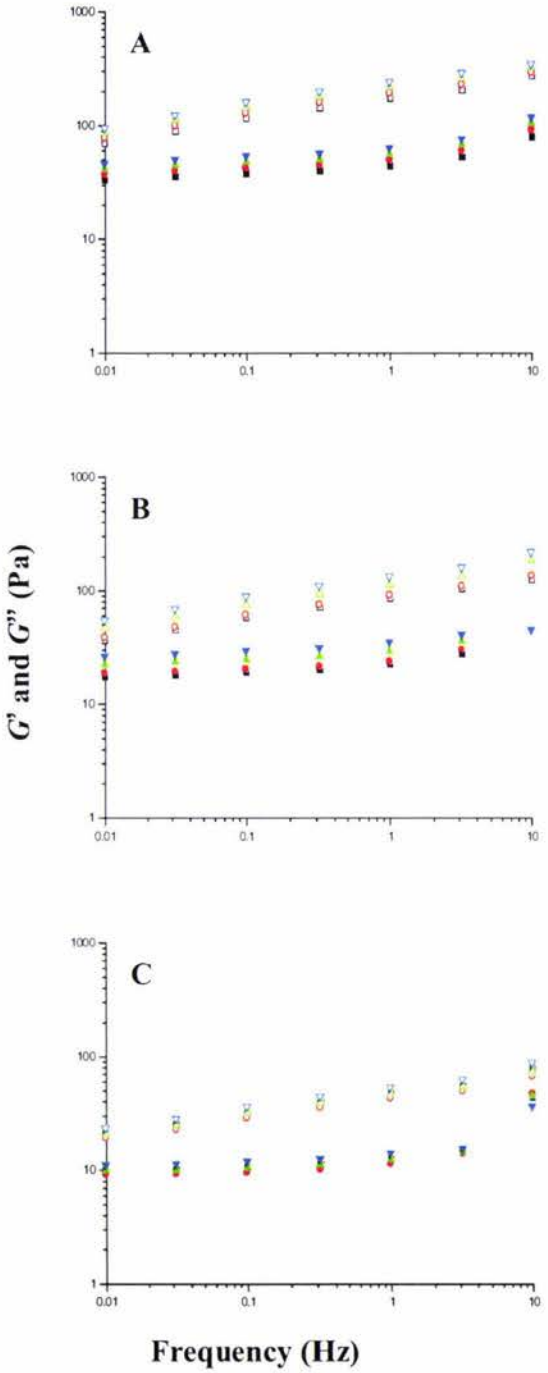


Figure 6.3 Storage (G') and loss (G'') moduli of skim milk/MPC85 solutions after 1 hour (G'-■, G''-□), 7 hours (G'-●, G''-○), 16 hours (G'-▲, G''-△), and 24 hours (G'-▼, G''-▽) hydration, as a function of frequency using A) high solubility, B) medium solubility, and C) Low solubility MPC85.

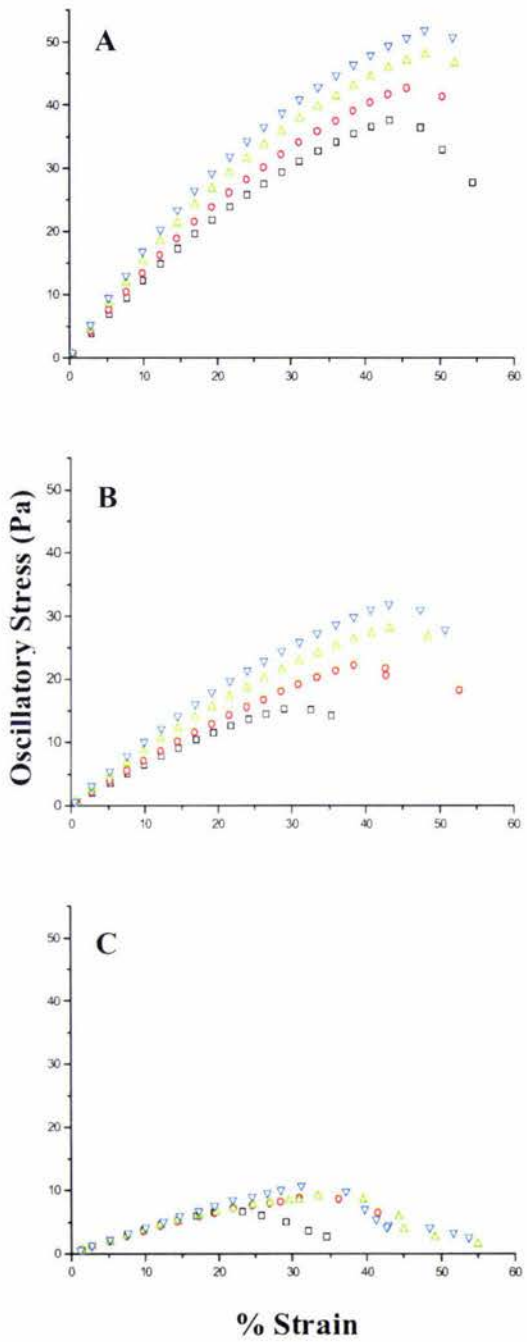


Figure 6.4 Oscillatory stress of skim milk supplemented with MPC85 after 1 hour (□), 7 hours (○), 16 hours (△), and 24 hours (▽) hydration, as a function of strain for A) high solubility , B) medium solubility, and C) low solubility MPC85.

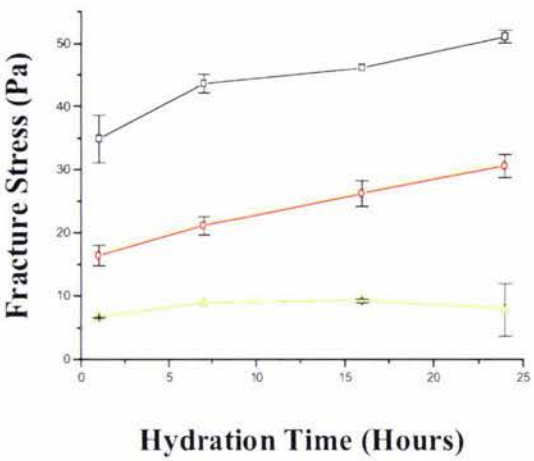


Figure 6.5 Fracture stresses of samples resisting applied strain as a function of hydration time for samples with (□) high solubility, (○) medium solubility, and (△) low solubility MPC85.

6.3 Comparison between Samples

The previous sections in this chapter investigated the individual effects of samples hydrated for different times, using MPC85s of different solubility. Figure 6.6 shows the variation of the final gel strength (G_F*) at three hours for the rennet induced skim milk/MPC85 samples, as a function of time for all three data sets. The samples prepared with high solubility MPC85 form the strongest gels. The gel strength increases with hydration time for a high and medium solubility MPC85. Samples formed with low solubility MPC85 changed very little with hydration time.

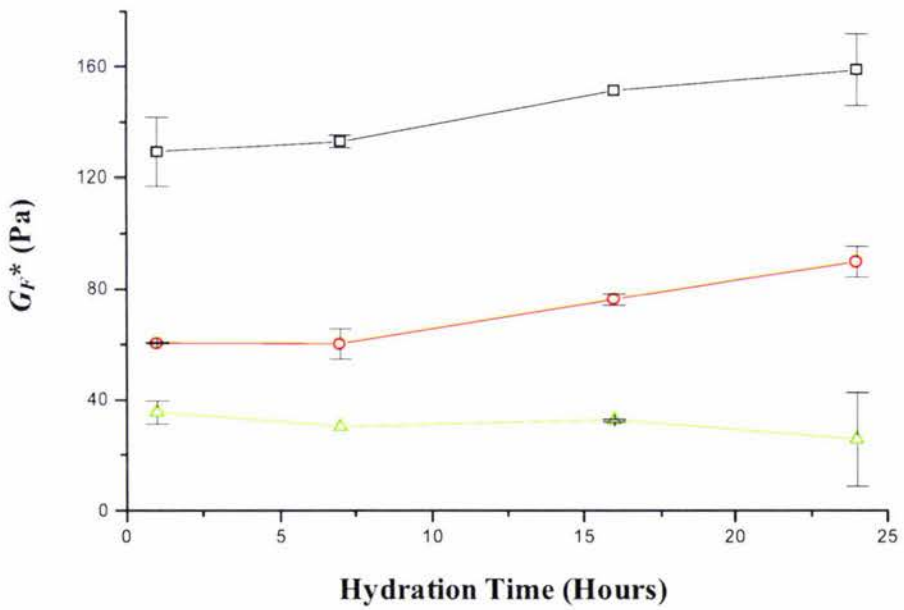


Figure 6.6 Final gel strength (G_F*) of skim milk supplemented with a high (□), medium (○), and low (△) solubility MPC85, as a function of hydration time.

A very interesting feature of the hydration testing experiments is the insensitivity of the gelation time to the hydration time (Table 6.1). This is interesting because most other sample properties (eg final gel strength, fracture stress, etc), especially for high and medium solubility MPC85, were affected by hydration time. These results however, indicate that the hydration time has no effect on the aggregation or gelation times.

Table 6.1 Effect of varying hydration time and MPC85 solubility on gelation times for skim milk/MPC85 samples. Note that hydration times with letters indicate repeat tests.

	Gelation Time (Hours)		
Hydration Time (Hours)	High Solubility MPC85	Medium Solubility MPC85	Low Solubility MPC85
1	0.59	0.82	0.92
1a	0.64	0.82	0.99
7	0.64	0.84	1.04
7a	0.67	0.87	-
16	0.66	0.77	1.01
16a	-	0.79	1.01
24	0.61	0.74	0.96
24a	0.62	0.77	1.19
Average	0.63	0.80	1.02
Standard Deviation	0.03	0.04	0.09

The frequency sweeps performed as part of the investigation into hydration times revealed that for samples containing medium or high solubility MPC85 an increase in hydration time produced an increase in both G' and G''. Samples prepared with low solubility MPC85 showed only a small increase in G' and G'' with frequency. All samples had values of G' greater than G'', at frequencies from 0.01 to 10Hz, but by less than one decade. Hence all samples behaved as weak gels (Lapasin and Pricl, 1995).

Figure 6.7 reveals that the sample fracture stress increased as the hydration time of a sample supplemented with high and medium solubility MPC85 was increased. Skim milk supplemented with high solubility MPC85 formed the strongest gels (shown by the highest fracture stress), at all hydration times. Samples prepared using medium solubility MPC85 formed gels that were weaker than those made using high solubility MPC85, but stronger

than samples made using low solubility MPC85. Longer hydration times did not change the fracture stress of samples supplemented with low solubility MPC85.

Also shown for comparison is the fracture strain as a function of storage time (figure 6.8). This graph shows that the general trend is an increase in fracture strain as the hydration time increased. This is interesting, especially in the case of samples prepared using low solubility MPC85, since no change in the fracture stress was observed with increasing hydration time (figure 6.7). The significance of this will be discussed later in chapter 9.

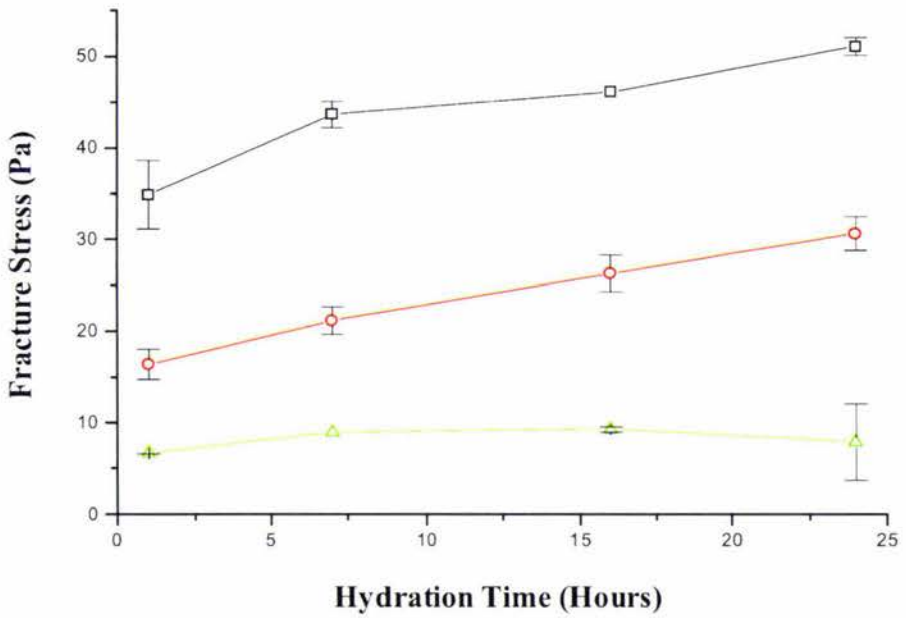


Figure 6.7 Fracture stresses of samples prepared using high (□), medium (○), and low (△) solubility MPC85 powders resisting applied strain as a function of hydration time.

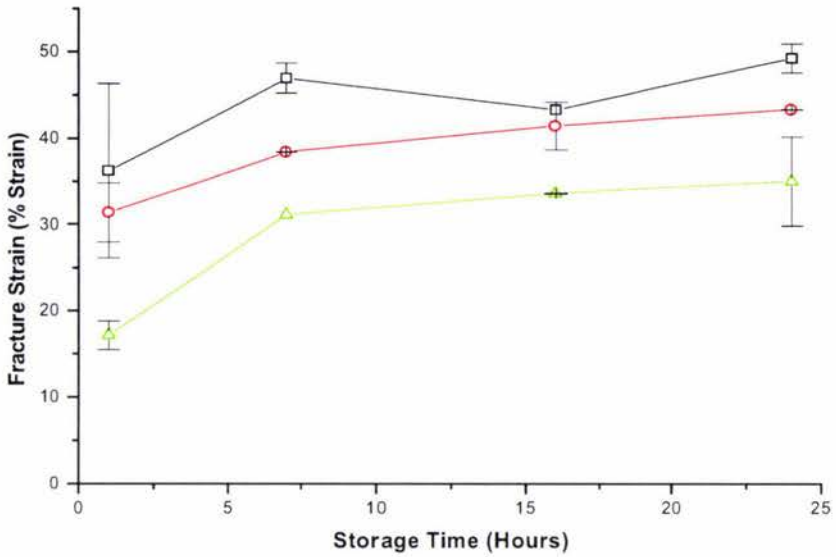


Figure 6.8 Fracture strain of samples prepared using high (□), medium (○), and low (△) solubility MPC85 powders resisting applied strain as a function of hydration time.

6.4 Summary

This chapter investigated the effects of the variation of hydration time on the rheological properties of samples, which had been prepared using a high, medium, or low solubility MPC85. Results showed an increase in gel firming rate, and final gel strength with hydration time, for high and medium solubility MPC85, whilst samples formed with low solubility MPC85 varied little. The viscoelastic moduli (G' and G'') and the fracture stress increased with hydration time for all samples except those made with low solubility MPC85. The fracture strain increased with increasing hydration time for all samples including those supplemented with low solubility MPC85. However, gelation times were not affected by hydration time for any of the prepared samples.

7 Composition of MPC85 as found by Polyacrylamide Gel Electrophoresis (PAGE)

7.1 Introduction

Several techniques (ie solubility testing, rheology, variation in hydration time), have been used to determine the effects of storage and hydration of MPC85 on the renneting properties of skim milk fortified with MPC85. Although these techniques will prove to be extremely useful as a means of determining physical properties as a function of storage time and temperature of MPC85, the need arises to investigate the composition of the powders under study in an effort to link the observed functional changes to physical alterations occurring in the powders. This chapter uses sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) to study the composition of MPC powder. This method was chosen because of its well known ability to separate dairy proteins (Anema and Klostermeyer, 1997).

The methods used to prepare samples for PAGE analysis were described in detail in chapter 3. The samples tested comprised of MPC85 powders that were stored at storage temperatures (20°C, 35°C, and 50°C), and further to this, samples from each storage temperature were examined using two different testing regimes. These were reduced samples of reconstituted MPC85 solutions, and supernatants of centrifuged MPC85 solutions. In addition to this some samples were also tested using non-reduced samples of reconstituted MPC85 and the supernatants of centrifuged MPC85 solutions. The data are presented as plots of the protein levels found in the stored MPC85 compared with a control MPC85 powder (ie a sample not stored at high temperatures). Examples of each type of gel run can be seen in figures: 7.1, 7.3, 7.5, and 7.7.

7.2 Results

7.2.1 Reduced Samples of Reconstituted MPC85

Figure 7.1 displays a typical reduced SDS-PAGE gel for MPC85 samples stored at 50°C. Note that the protein bands become less defined as storage time increases, indicating a change in the proteins that alters their charge and/or size. The intensity does not diminish significantly.

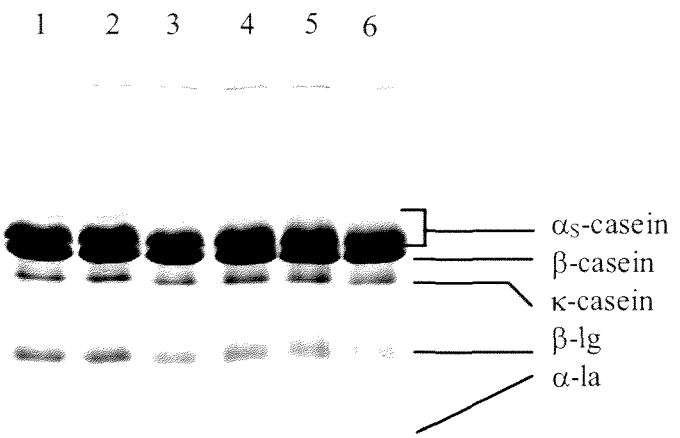


Figure 7.1 Reduced SDS-PAGE sample for MPC85 stored at 50°C for: lanes 1 and 2: 0 days; lane 3: 1 day; lane 4: 2 days; lane 5: 5 days; lane 6: 10 days. Abbreviations used are: α-La = α-Lactalbumin, and β-Lg = β-Lactoglobulin.

Figure 7.2 displays the amount of protein detectable using a reduced, SDS-PAGE experiment for reconstituted MPC85. MPC85 that was stored at 20°C and 35°C showed little change in detectable protein in regard to storage time. However when stored at 50°C a small decrease was noticed. When considering the total amount of protein detectable in reduced MPC85 by SDS-PAGE, samples stored at 50°C reduce to approximately 80%, while samples stored at 20°C and 35°C do not change significantly.

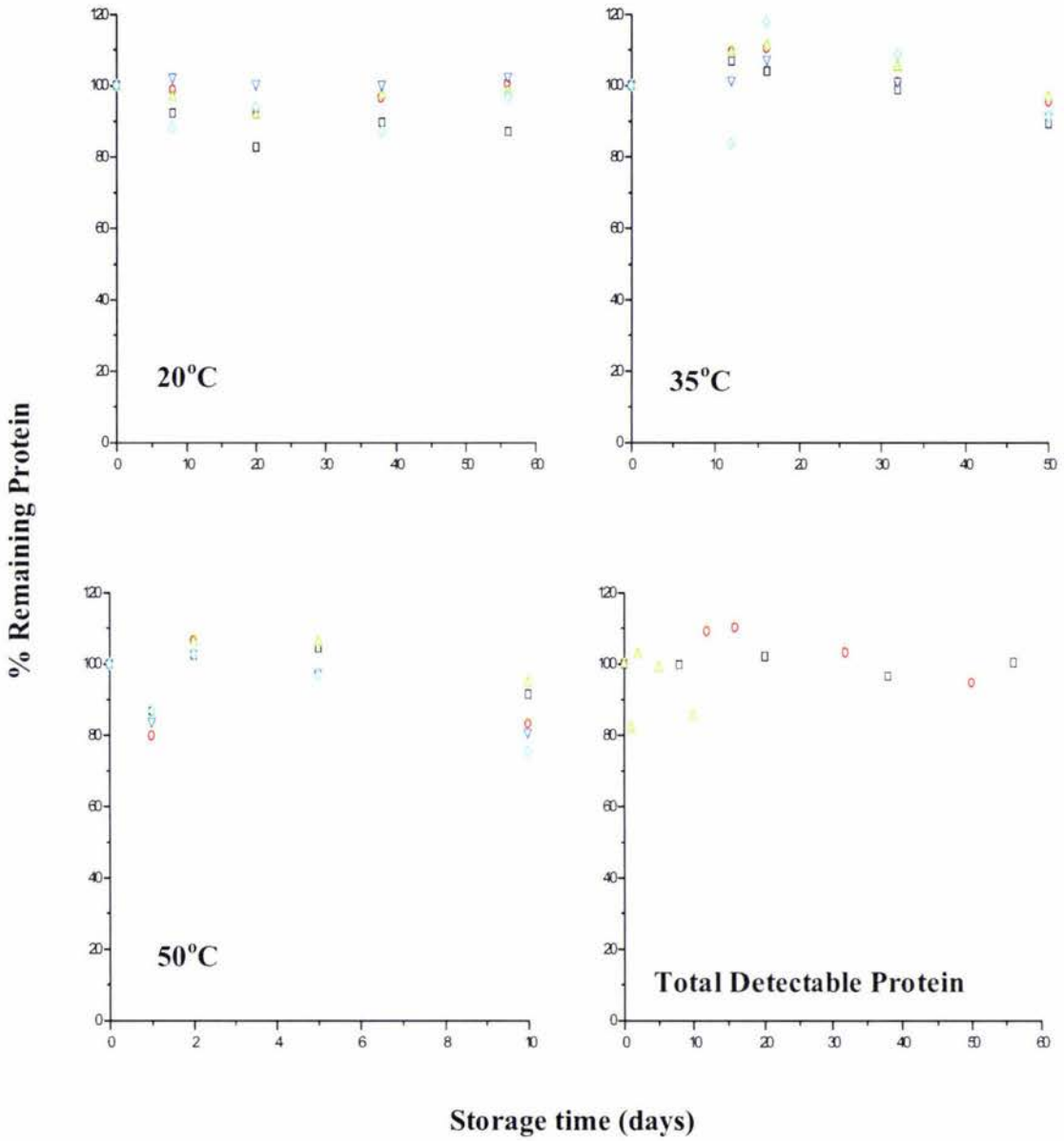


Figure 7.2 Remaining protein as a function of storage time for reduced solution of MPC85 stored at 20°C, 35°C, and 50°C storage, showing the detectable proteins (□) α_S-casein, (○) β-casein, (△) κ-casein, (▽) β-Lg, and (◇) α-La. The total detectable protein is also shown for MPC85 stored at (□) 20°C, (○) 35°C, and (△) 50°C storage. Note the different storage time scales. Method for quantifying PAGE can be found in section 3.4.4.2.

7.2.2 Supernatant of Reduced MPC85 Samples

Figure 7.3 displays a typical gel of the reduced supernatant from centrifuged MPC85that was stored at 50°C. As storage time increased, the protein bands became very diffuse, indicating a change in the proteins that altered their charge and/or size. A reduction in intensity also occurred.

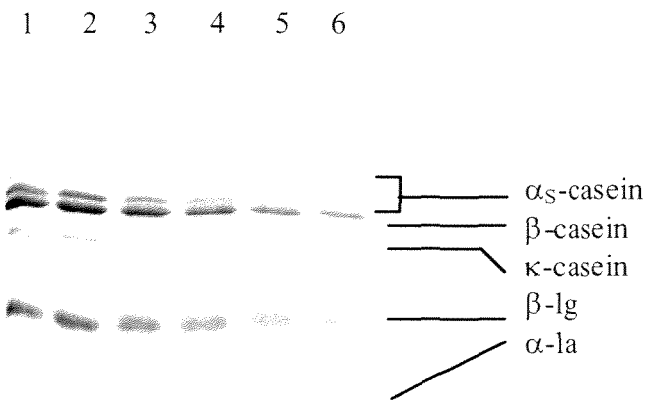


Figure 7.3 Reduced SDS-PAGE sample for the supernatant of centrifuged MPC85 stored at 50°C for: lanes 1 and 2: 0 days; lane 3: 1 day; lane 4: 2 days; lanes 5: 5 days; lane 6: 10 days.

The detectable protein in MPC85 stored at 20°C and tested by this method, did not change markedly with storage time as shown by figure 7.4. Figures 7.4 also exhibits the detectable proteins in MPC85 stored at 35°C and 50°C. The same general trends are observed in each graph that is a decrease in the amount of detectable protein as storage temperature increases. The α_S-casein, and κ-casein are the most affected proteins decreasing to approximately 20% and 40% respectively of their original amounts. β-casein is also affected by storage at high temperatures halving in the original amount from a storage temperature of 50°C.

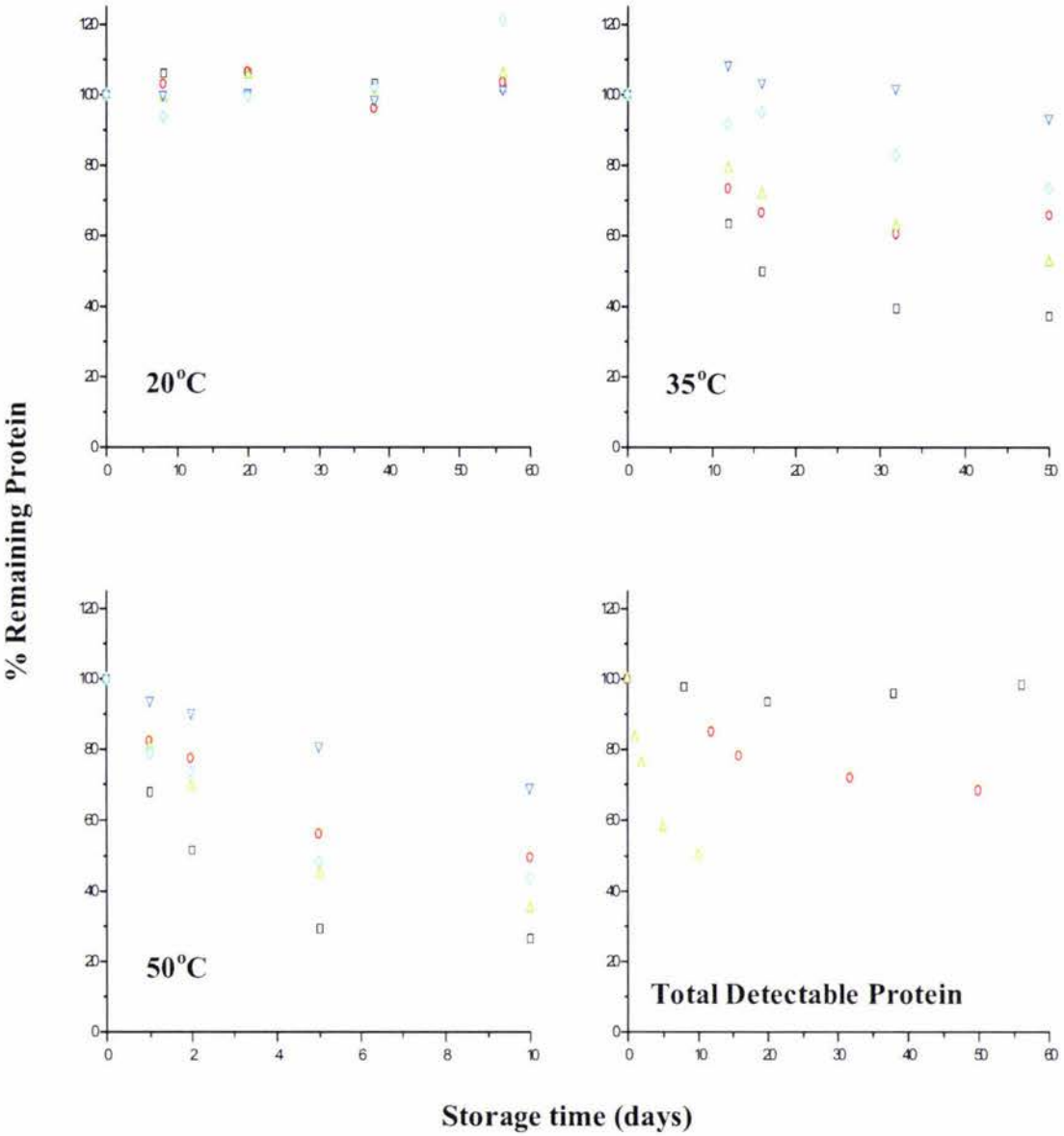


Figure 7.4 Remaining protein as a function of storage time for supernatant of reduced solution of MPC85 stored at 20°C, 35°C, and 50°C storage, showing the detectable proteins (□) α_S-casein, (○) β-casein, (△) κ-casein, (▽) β-Lg, and (◇) α-La. The total detectable protein is also shown for MPC85 stored at (□) 20°C, (○) 35°C, and (△) 50°C storage. Note the different storage times.

At temperatures up to 35°C the detectable whey proteins were minimally affected by storage, decreasing by only 20% for α-lactalbumin, and not at all for β-lactoglobulin. However, α-lactalbumin detectable in MPC85 stored at 50°C decreases to approximately 50%, and β-lactoglobulin decreases to 80%.

The total protein found in the reduced supernatant of MPC85 is shown in figure 7.4. As storage temperature increased the amount of total protein detected by this test decreased from 100% remaining in MPC85 stored at 20°C, down to approximately 50% for MPC85 stored at 50°C.

7.2.3 Non-Reduced Samples of Reconstituted MPC85

Figure 7.5 displays a typical gel for non-reduced MPC85 stored at 50°C. As storage time increased, the protein bands became much less defined indicating a change in the proteins that altered their charge and/or size. This test resulted in minimal change in intensity.

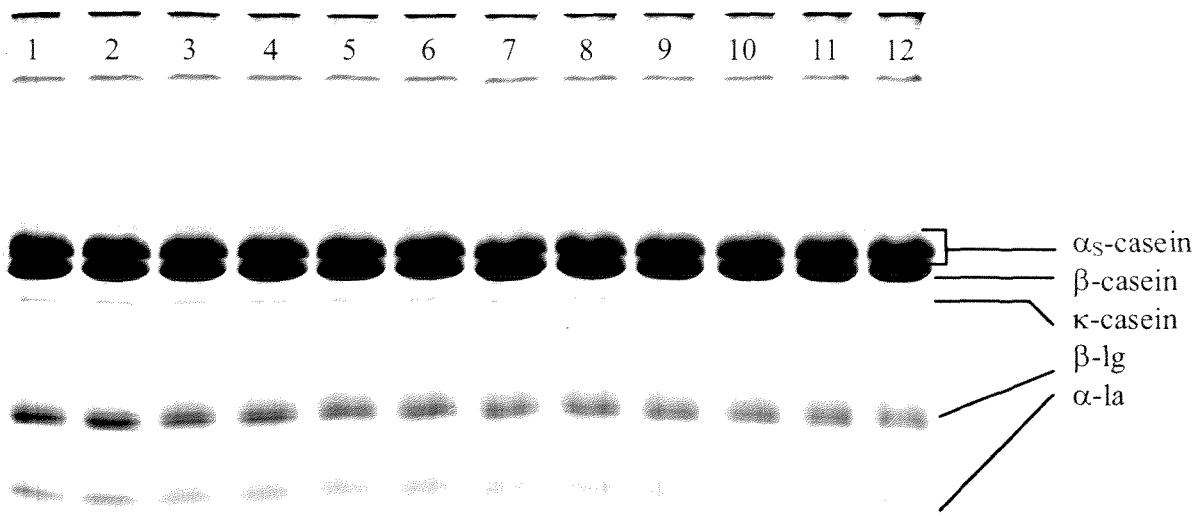


Figure 7.5 Non-reduced SDS-PAGE sample for MPC85 stored at 50°C for: lanes 1 and 2: 0 days; lanes 3 and 4: 1 day; lanes 5 and 6: 2 days; lanes 7 and 8: 3 days; lanes 9 and 10: 4 days; lanes 11 and 12: 5 days.

MPC85 stored at 20°C and 50°C were tested using a non-reduced SDS-PAGE method, yielding the results shown in figure 7.6. Samples stored at 20°C showed no change over the testing period. Samples stored at 50°C show a decrease in data towards the centre of the graph. Although data appears to be decreasing it is more likely that as the protein band became blurred (as shown in figure 7.5), then the proteins (especially the less abundant proteins (ie κ-casein, β-Lg, and α-La)) are merging with the background, as opposed to decreasing in quantity. With this effect in mind, the general trend is that this method shows negligible change in the amount of detectable protein in MPC85 when stored at different temperatures.

The fact that this method of testing produced similar levels of protein regardless of solubility is an important result. What this means is that SDS dissociates most, if not all, of the aggregated proteins. This suggests that most or all of the aggregated proteins responsible for the formation of the insoluble material in MPC85 are formed predominantly by non-covalent linkages that are dissolved under SDS conditions.

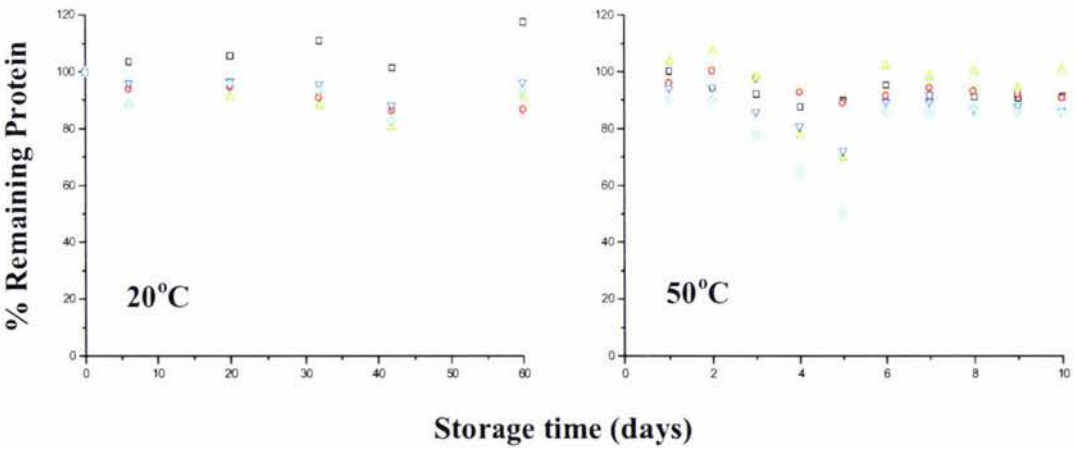


Figure 7.6 Non-reduced solution of MPC85 stored at 20°C and 50°C storage, showing the detectable proteins (□) α-casein, (○) β-casein, (△) κ-casein, (▽) β-Lg, and (◇) α-La. Note the different storage times.

7.2.4 Non-Reduced Supernatant of Reduced MPC85 Samples

Figure 7.7 displays a typical gel of the non-reduced supernatant for centrifuged MPC85 stored at 50°C. The protein bands in this type of experiment become more diffuse as storage time increases indicating a change in the proteins that altered their charge and/or size.

The amount of different proteins remaining in solution for samples of MPC85 stored at 50°C were also analysed by testing the supernatant of MPC85 samples that had been stored from 0 to 10 days. It was found that the detectable α-casein and β-casein reduced to about 20% and 60% of their original values respectively. The amounts of κ-casein, β-Lactoglobulin, and α-Lactalbumin detectable reduced to about 80%-90%. The total protein detectable by this test reduced to approximately 60% of its original value. These results can be seen in Figure 7.8.

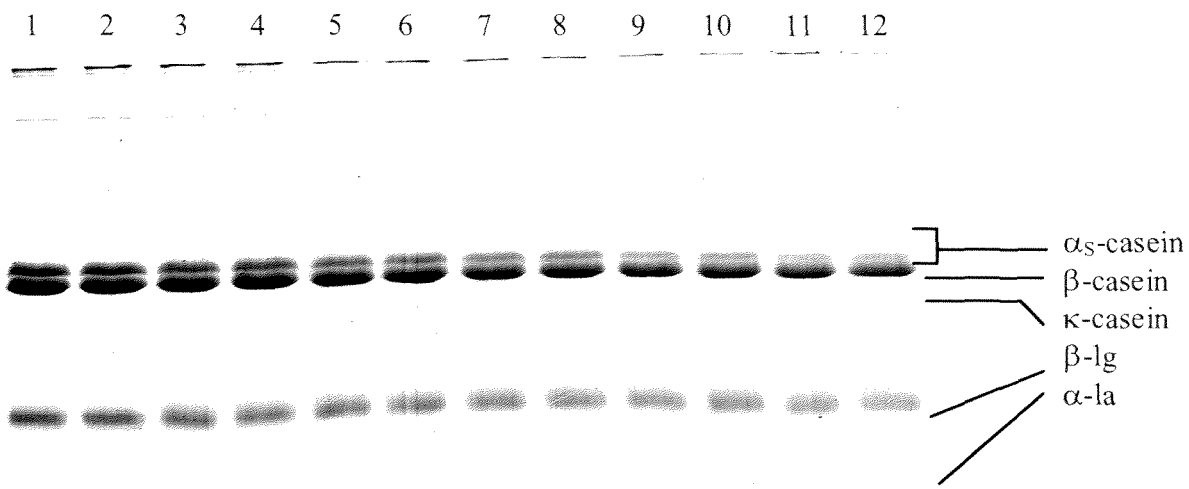


Figure 7.7 Non-reduced SDS-PAGE sample using the supernatant of centrifuged MPC85 stored at 50°C for: lanes 1 and 2: 0 days; lanes 3 and 4: 1 day; lanes 5 and 6: 2 days; lanes 7 and 8: 3 days; lanes 9 and 10: 4 days; lanes 11 and 12: 5 days.

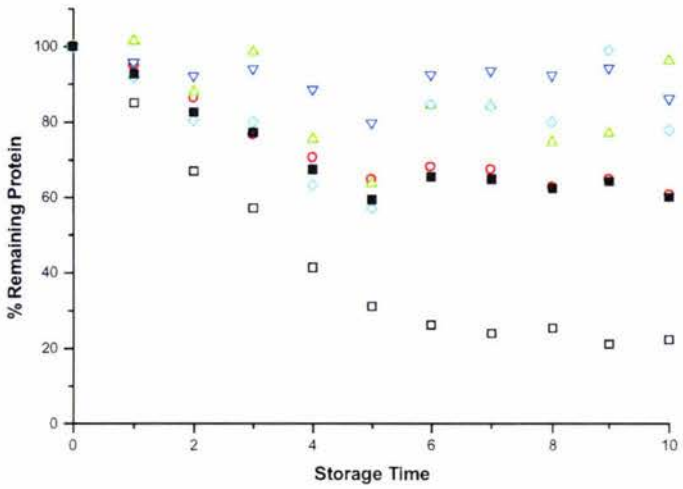


Figure 7.8 Supernatant of non-reduced MPC85 solution showing the remaining proteins α-casein (□), β-casein (○), κ-casein (△), β-Lg (▽), α-La (◇), Total Protein (■) for 50°C storage.

7.3 Comparison between Results

The analytical method of polyacrylamide gel electrophoresis indicated modification to the proteins (evidence by blurring of bands), which made quantification difficult. Despite this, SDS-PAGE was still able to render many useful results. The loss in definition can be seen on all the gels (eg figures 7.1, 7.3, 7.5, and 7.7).

A far greater decrease in detectable protein with storage time was found from results of the supernatants of MPC85, both reduced and non-reduced, compared with samples that were not centrifuged. This suggests that the proteins were modified by heated storage in a way that reduced solubility.

7.4 Summary

In this chapter the composition of MPC85 powders were examined in an effort to rationalise the behaviour of the skim milk/MPC85 solutions. The decrease in detectable protein found by the PAGE experiments in this chapter will be discussed further in relation to other experiments in the general discussion chapter. PAGE showed that as storage time and temperature increased, the solubility decreased. This effect occurred far more with the caseins, than with the whey proteins. Also as storage time increased, protein bands seen on the gels became more diffuse and less defined. What this indicates is that storage of MPC85 at high temperatures modified the proteins in a way that altered their charge and/or size.

8 Storage of MPC85 as Analysed by Mass Spectroscopy

8.1 Introduction

Thus far, a number of different techniques have been used to study the effects on MPC85 powder of storage at temperatures above 20°C. Mass spectroscopy was performed on MPC85 samples stored at 50°C to investigate possible causes of the changes.

8.2 Data Analysis

Experimental details are described in detail in chapter 3. The spectrum of proteins (separated by the HPLC) found in high solubility MPC85 is shown in figure 8.1A. This figure can be considered a record of what 'order' (i.e. time of occurrence) the proteins were injected into the mass spectrometer. This can be compared with MPC85 that had been stored for 10 days at 50°C as shown in figure 8.1B. It can be seen that the proteins displayed in figure 8.1B appear to be less defined as a result of storage, than those seen in figure 8.1A. One theory of the decrease in MPC85 properties, such as solubility, was that glycation of the proteins present in the original powder (i.e. lactose groups becoming attached to the proteins) was occurring. To analyse this occurrence, software associated with the mass spectrometer allows the area of interest where a particular protein is located was selected from the full spectrum of the powder. Peaks can then be located as specified by the particular molecular weight for the protein being analysed, as well as subsequent peaks which indicate the lactose groups attached to the protein. An example of this can be seen in figures 8.2A and B. By this method the amount of protein that is glycated may be analysed quantitatively. The basic method of determining this quantification is to observe the protein peaks present, and determine the relative amount of each. For example, figure 8.2 B shows the relative abundance of κ-casein A with 0, 1, and 2 lactose groups as 100,

40, and 20 respectively. As such the amount of κ-casein A after 7 days storage at 50°C with 0, 1, and 2 lactose groups taken as a percentage is 62.5, 25, and 12.5%. This chapter analyses α_{s1}-casein, α_{s2}-casein, two genetic variants of β-casein denoted β-A1 casein and β-A2 casein, and κ-casein in two forms: on is own, and with a carbohydrate group attached to it (both can be seen in figure 8.2A, and with glycation in figure 8.2B).

8.3 Results

A plot of the relative amount of α_{s1}-casein with 0, 1, and 2 lactose groups is shown in figure 8.3A. Even when the MPC85 powder had not been exposed to high temperatures, some of this protein has 1 lactose group attached. As fresh milk is not glycated, this indicates that glycation is occurring during either the MPC85 manufacturing process or when stored at -18°C for long periods. As storage time increased, equal amounts of this protein were found with either 1 or 0 lactose groups attached. The powder was stored for 3 days at 50°C before any α_{s1}-caseins with two lactose groups were detected, and after 5 days the relative amount reached a plateau of 20%.

Figure 8.3B represents the relative amount of α_{s2}-casein present in MPC85 with 0, 1, and 2 lactose groups attached, as a function of storage time. Protein with 2 lactose groups became detectable after 3 days, after this time the amount of α_{s2}-casein with 0, 1, and 2 lactose groups remained constant.

The relative amounts of β-A1 casein, κ-casein, and κ-casein with a carbohydrate group attached (figures 4C, E, and F respectively) all showed the same trends. Initially 80% had no lactose group and 20% has one lactose group. Over a 10 day period their proportions change to 55% and 35% respectively. Proteins with two lactose groups are detected after 4 days. The amount with 2 lactose groups changed from 10 to 15% during the rest of the storage trial.

Figure 4D depicts β-A2 casein as a function of storage time. Protein with no lactose groups and 1 lactose group change from approximately 75-50%, and 20-40% respectively, over a period of 4 days. Protein with 2 lactose groups was always present and slowly increased from 10-15% over storage period.

The whey proteins were not analysed using mass spectroscopy as they required further processing to separate them for study.

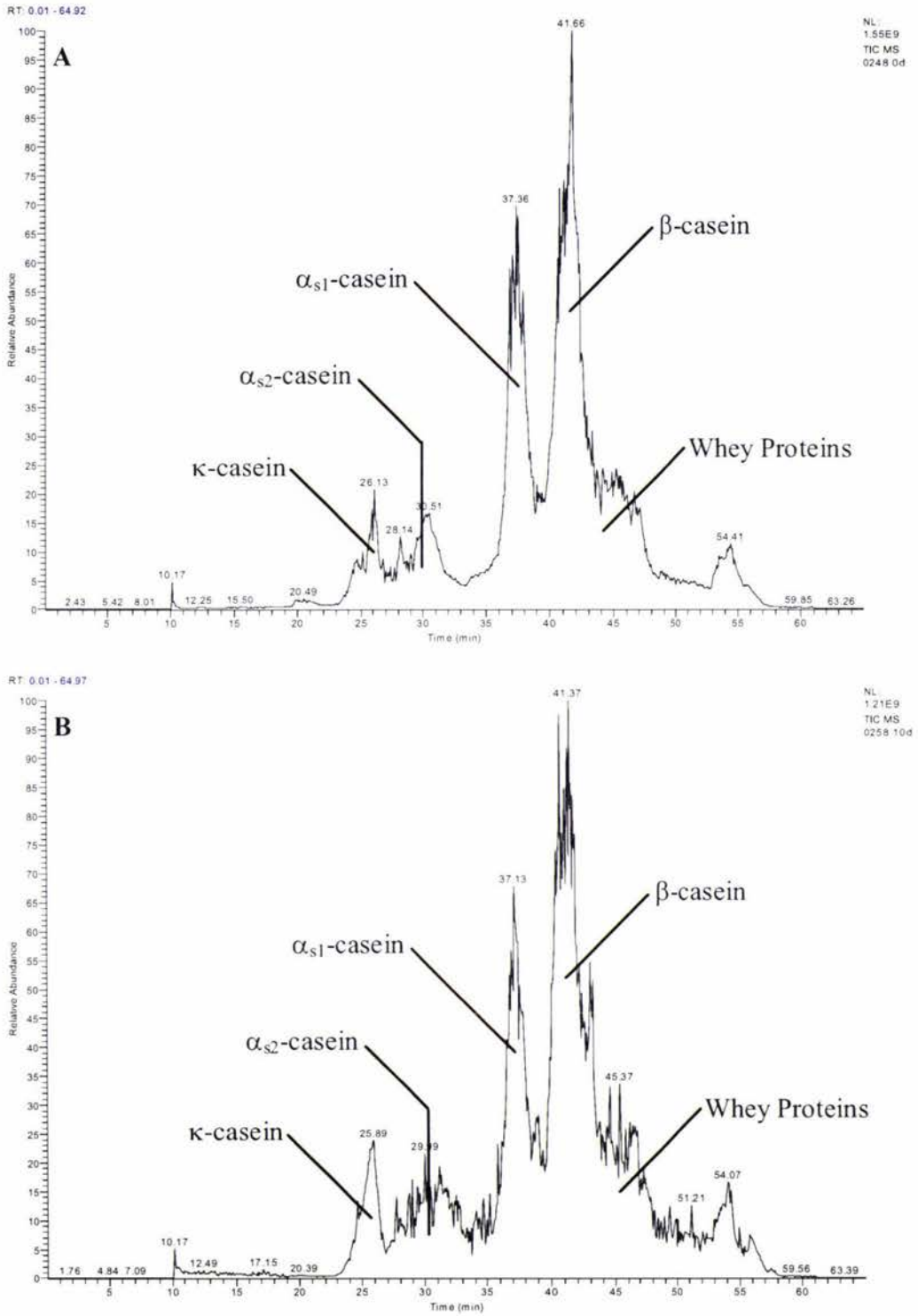


Figure 8.1 Full spectrum of proteins in MPC85 powder after A) No heat exposure, and B) 10 days storage at 50°C, as separated by HPLC.

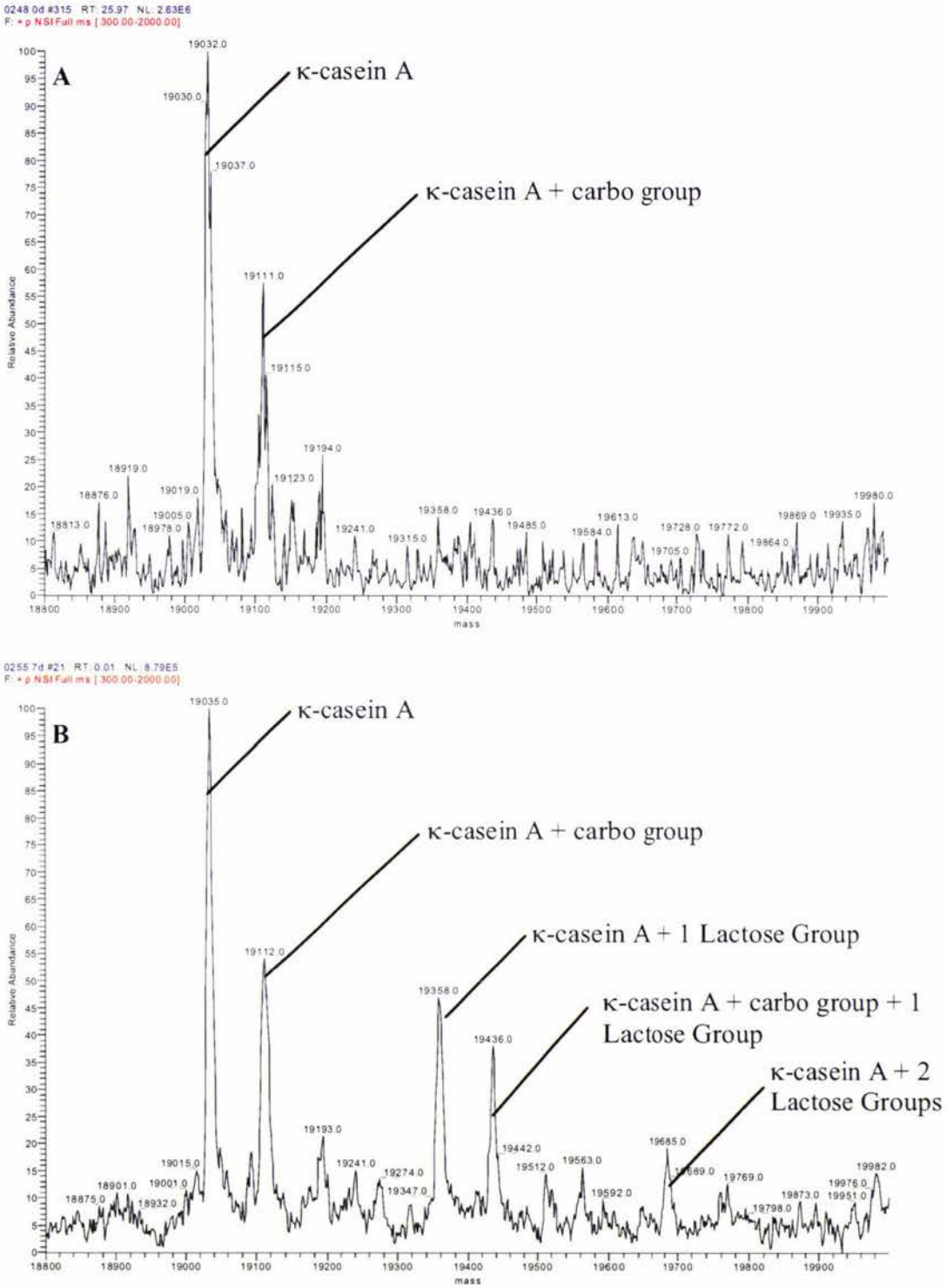


Figure 8.2 Mass spectrum of κ-casein A after A) no day's storage, and B) seven days storage at 50°C.

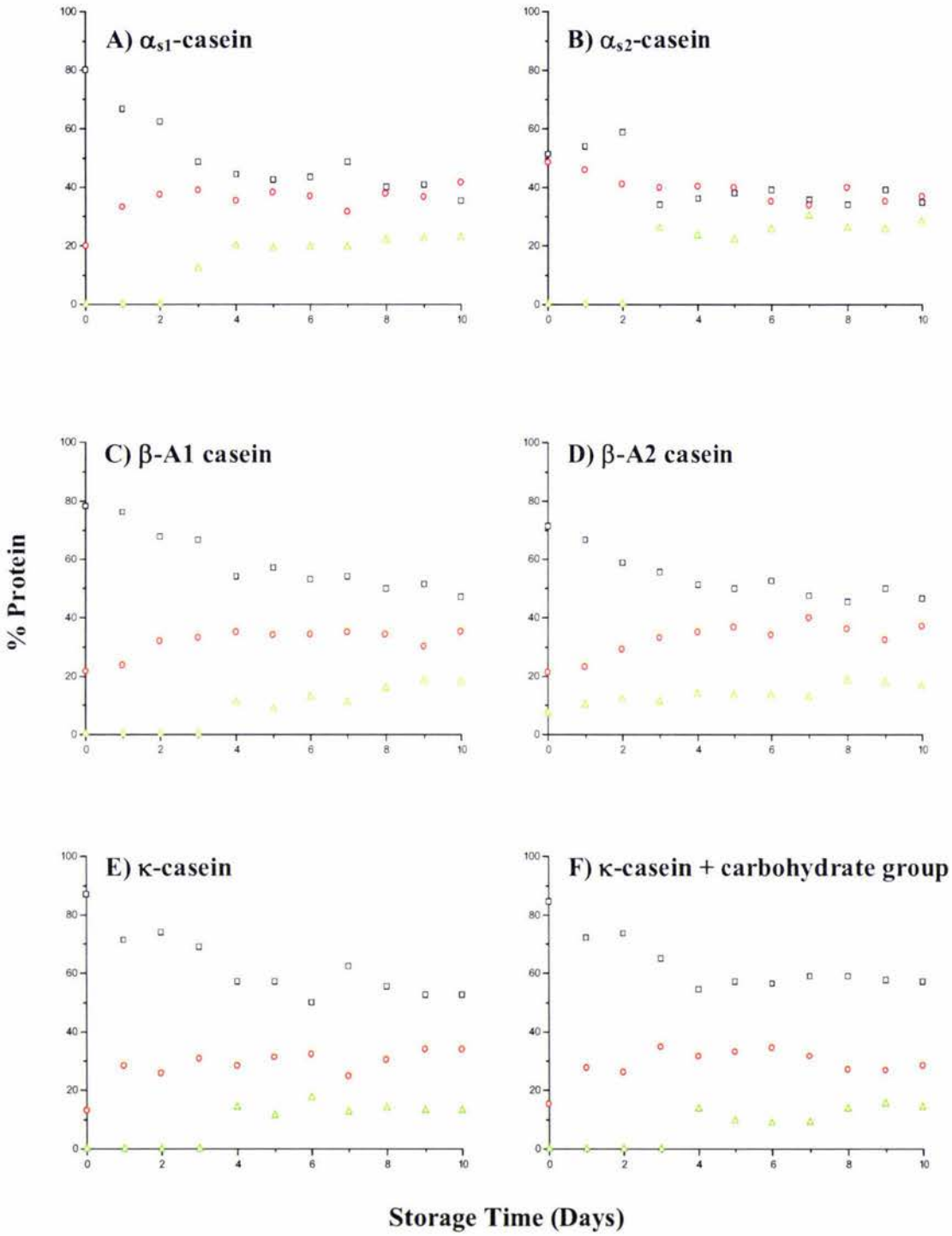


Figure 8.3 Relative amount of the proteins in MPC85 powder with no lactose group (□), 1 lactose group (○), and 2 lactose groups (△), as a function of storage time.

8.4 Summary

Mass spectroscopy has shown that most glycation and/or changes of proportions of proteins with lactose groups attached occurred during the first 4 days of MPC85 storage at 50°C. As previously noted, a proportion of each protein was present in the original powder with a lactose group attached. This could likely have been caused during the manufacture of the MPC85 powder. As storage time increased, the proportion of each protein with one or two lactose groups attached increased from their original values before reaching a plateau. None of the proteins under study totally glycated. Only caseins were studied using mass spectroscopy, as additional sample processing would be required to use mass spectroscopy to monitor the glycation of whey proteins.

9 General Discussion

The purpose of this thesis was the study of the effects of storage and hydration of milk protein concentrates (MPC) on the rheological properties of skim milk supplemented with MPC. In addition to this, the effects of storage time and temperature on the solubility of MPC were also investigated. Different experiments were carried out to quantify the changes that occurred in the samples under investigation, and many results were produced. This chapter analyses and discusses the correlations between results from different experimental methods.

9.1 The Decrease in Solubility

Chapter 4 documents the decrease in solubility of MPC85 with storage at temperatures greater than 20°C. Following are some of the reasons why this decrease in solubility could be taking place.

A possible mechanism for this is the formation of a network of cross-linked proteins over the surface of the MPC85 powder particles forming a barrier to water absorption. The mass spectrometry results provide some evidence for this because they indicate that MPC85 proteins became glycated during storage at 50°C. This is significant because glycation is one of the first steps in Maillard reactions, which can be accompanied by reactions that result in the cross linking of proteins (Jimenez-Flores and Kosikowski, 1986; Mistry and Pulgar, 1996). Furthermore, Maillard reactions produce 5-hydroxymethylfurfural (HMF) as an intermediate step and Jimenez-Flores and Kosikowski (1986) have shown that HMF production increases exponentially with storage temperature suggesting that protein cross-linking does too. This would account for the current observations that MPC85 solubility decreases exponentially with storage temperature (as seen in figure 4.3). It is important to understand that Maillard reactions may not directly cause cross-linking; rather they

promote changes in the milk properties that also favor cross-linking to occur. For instance, upon heating of milk, side chains of some amino acids become reactive and can combine with lactose or other functional groups to form inter and intramolecular bonded species. One indication that these types of reactions are occurring is the Maillard reaction, the first step of which is the reaction between ε-amino groups of lysine and lactose. These Maillard reactions are often accompanied by cross-linked products such as lysinoalanine (LAL) or histidinoalanine (HAL). So a measure of cross-linking can be obtained from the Maillard reaction products such as HMF, or a direct measure of the cross-linked products such as HAL or LAL could be made. It should be noted that small amounts of browning were noticed in samples that decreased in solubility. This is important as browning of milk powders can be taken as an indication of Maillard reactions taking place.

Results found from PAGE testing (reported in chapter 7.3.1 and 7.3.3) showed that reconstituted MPC85 solutions did not display a large decrease in detectable protein as would be expected if large amounts of cross-linking of protein had occurred. This does not reject the notion of cross-linking reactions, rather it suggests that only a small amount of cross-linking may be required to reduce solubility. One way that this theory could be true is if the cross-linking occurred on the surface of the MPC85 powder particles formed during manufacture since this would involve only a small amount of the total protein. This could possibly be verified using electron microscopy. Note that such small amounts of cross-linking may not be able to be detected by PAGE as the resolution of the bands decreases with storage time of MPC85.

Possible supporting evidence for the above theory is shown in figure 9.1 (McKenna, 2000). These micrographs display reconstituted MPC92 (ie milk protein concentrate ultra/diafiltered to 92% protein). The powder particles, displayed in figure 9.1A have a skin covering their surface. Figure 9.1B shows a closer view of the surface of the powder particle, and shows that this skin consists of fused or cross-linked casein micelles. Note that this figure displays a low solubility MPC85.

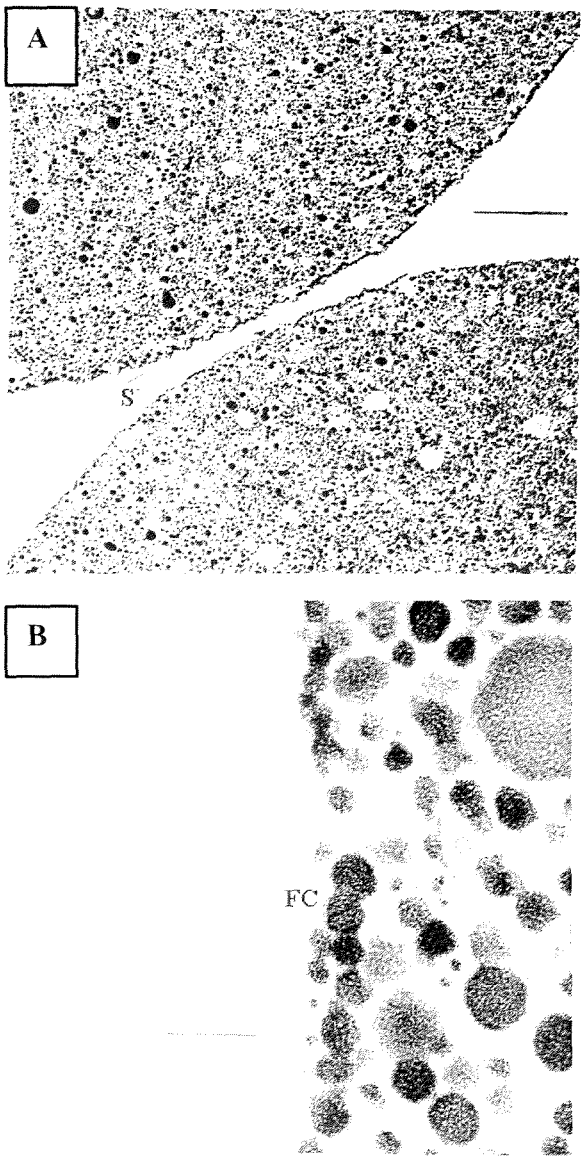


Figure 9.1 Transmission electron micrographs of reconstituted MPC; S = skin on the surface of the particle, FC = fused casein micelles (A) bar = 2μm; B) bar = 0.2μm) (McKenna, 2000).

The reason that cross-linking could lead to a decrease in solubility could be related to the hydration of the MPC85. As the amount of cross-linking on a powder surface increases (ie with increased storage), then the rate at which powder particles become hydrated, and disperse into solution as individual casein micelles, may be reduced. For this reason, when rheology experiments were performed involving the variation of the hydration time, then it

is expected that the MPC85 with a greater hydration time will have more water permeate through the powder particles, so releasing more casein micelles to join the network. Increased gel strength was found with increased hydration time for samples prepared with MPC85 of high and medium solubility. This effect was not noticed with samples containing low solubility MPC85, where it must be assumed cross-linking has occurred to such an extent that an increase in hydration time cannot enable more casein micelles to join the network. Also, the amount of cross-linking is unlikely to change with hydration time, and consequently the action of rennet on κ-casein is unlikely to change with hydration time. For this reason the gelation time being independent of hydration time (shown in table 6.1) can be explained. Also note that gelation causes a 3-dimensional network to be formed which can be independent of the strength of the bonds. This is reflected by figure 6.2 which displays samples with the same hydration times having different final gel strengths.

A pictorial representation of the argument given above is shown in figure 9.2. Initially there is no cross-linking and the solubility is at a maximum. Cross-linking increases with storage time, but the solubility does not decrease until significant surface area is impermeable as the particles interior can still be solubilised. For this reason, a delay time or "shoulder" can be seen in some of the solubility curves. A major decrease in solubility will occur when a greater deal of cross linking has occurred and the solubility curve will move into region 2. Because there is a wide range of particle sizes, some smaller powder particles may be totally cross-linked, and as such decreasing the solubility, while other larger particles will only be partially cross-linked, as shown in figure 9.2. When all protein capable of cross-linking has done so, then the curve can be seen to be in region 3, and solubility has reached a minimum.

It should be noted that the solubility does not reach 0%. PAGE results (figure 7.8) showed that whey proteins remained soluble, and as such would make up a large amount of the minimum soluble material. In addition to this, soluble salts and minerals (ie lactose) will remain in the soluble phase. It is also possible that some of the cross-linked powder particles fracture on reconstitution releasing soluble casein into solution.

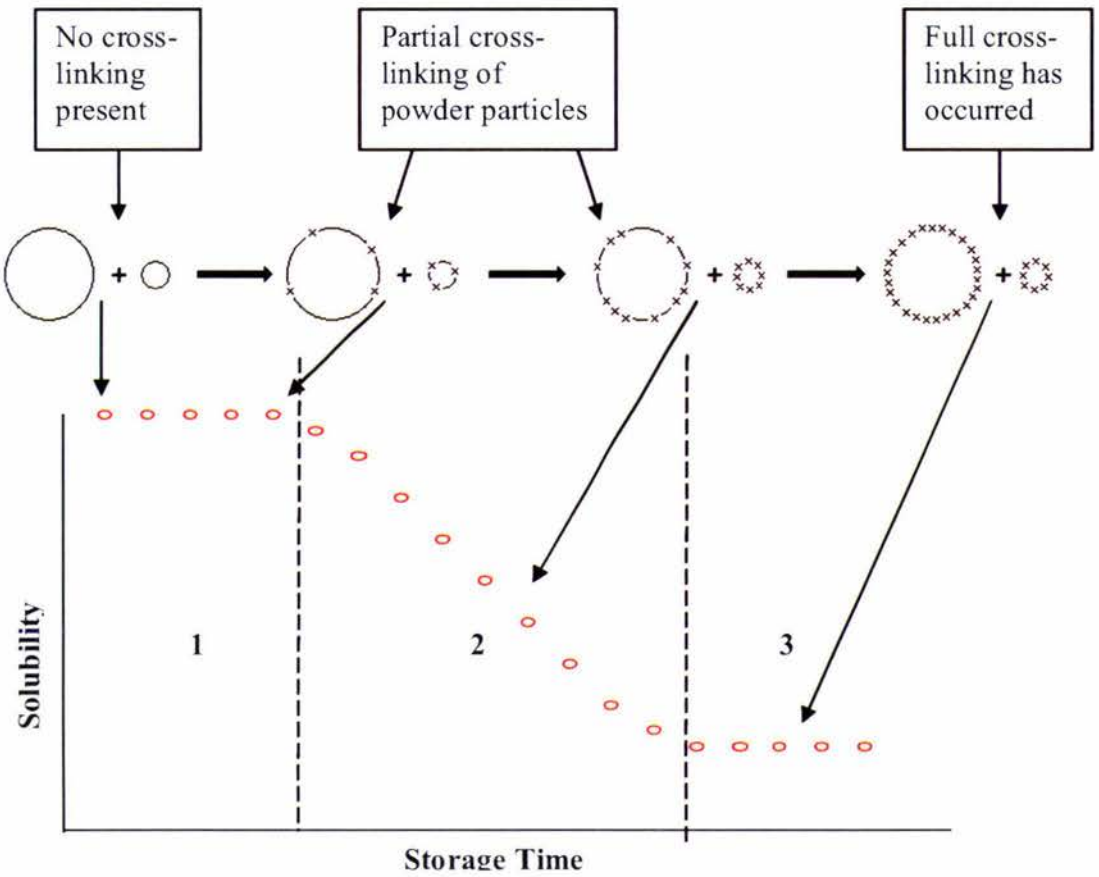


Figure 9.2 Simplified view of cross linking and its effect on solubility. Region 1 shows no cross-linking occurring, hence maximum solubility. In region 2 some cross linking has occurred and solubility is decreasing. Region 3 has MPC85 proteins totally cross-linked and solubility has reached a minimum. Spheres represent MPC85 powder particles after various stages of cross linking.

9.2 Correlation between Solubility and Rheology

Figure 9.3 shows a plot of the final gel strength from the rheology experiments (figure 5.3) against the solubility of the respective MPC85 samples (figure 4.1). When all data were considered, a strong positive correlation was observed, indicating that the modification occurring to MPC85 which reduces its solubility also has an effect on the structure of gels formed by renneted skim milk/MPC85 samples. The correlation coefficients between final gel strength and solubility for each storage temperature are shown in table 9.1. All storage temperatures resulted in a strong correlation except 20°C, and in this case a low significance is important and expected, as data were not changing as storage time increased.

Table 9.1: The correlation that occurred between samples tested by rheology and solubility testing for MPC85 stored at 50°C, 40°C, 35°C, 30°C, and all data. Note data for 20°C not shown as neither solubility nor G_F* varied with time.

Storage Temperature	R value	Significance (p)
50°C	0.9547	<0.0001
40°C	0.9907	<0.0001
35°C	0.9267	<0.0001
30°C	0.8607	0.00016
All Data	0.9492	<0.0001

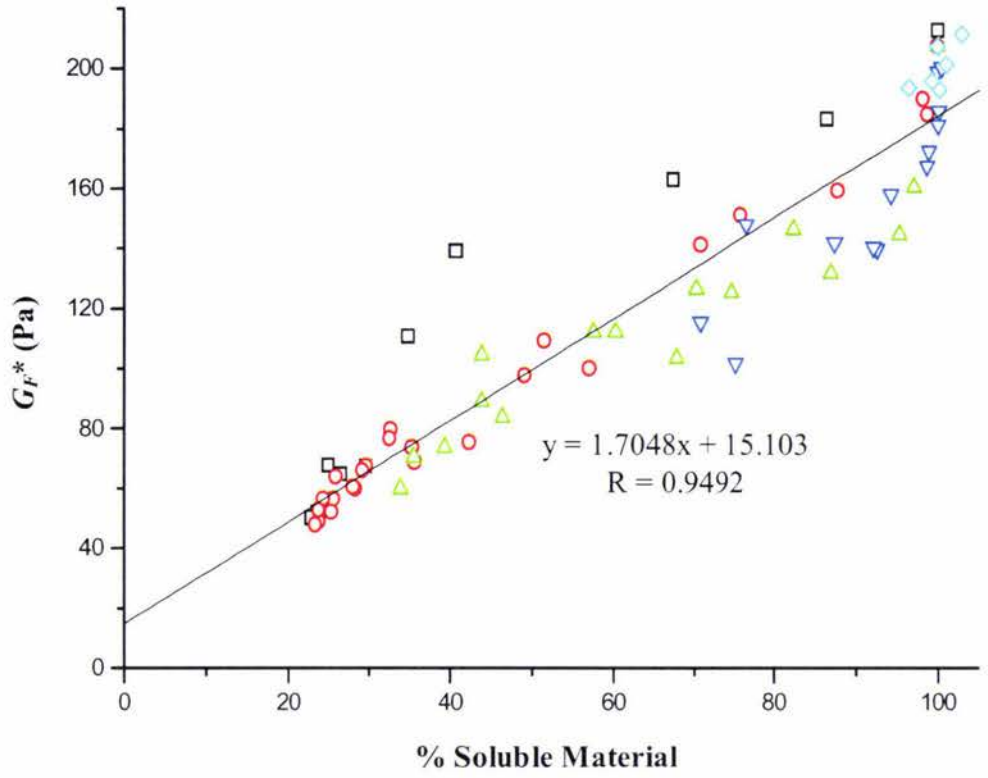


Figure 9.3 Final gel strength (G_F*) from rheology tests as a function of solubility of the same MPC85 samples stored at 50°C (□), 40°C (○), 35°C (△), 30°C (▽), and 20°C (◇).

The correlation between the solubility of MPC85 and the rheological properties of renneting skim milk/MPC85 solutions is very important because this can give insight into the reactions taking place. As discussed in the literature review, two main reactions occur; a primary (or enzymatic cleavage) phase, and a secondary (or coagulating) phase. Weaker gels can be produced if either of these two phases is dramatically retarded.

A large range of factors can affect the primary phase (eg heating, pH, salt concentrations, etc). An excellent review is offered by Zoon *et al.* (1988a, b and c, and 1989a and b). The change in gelation times for skim milk/MPC85 samples made using MPC85 stored at 50°C from 0 to 10 days, was an increase of approximately 7 minutes, which could be the result of

cross-linking between proteins. Cross-linking could increase the gelation time because it reduces the accessibility of κ-casein, and hence the rennet must diffuse through the skim/MPC85 solution for a longer time before there are enough renneted casein micelles to form a network. Vasbinder *et al.* (2003b) showed that although pre-heated skim milk solutions when acidified produce a weaker gel due to whey protein denaturation, the initial aggregation is independent of the heat treatment. It would seem that although the gelation time is increasing with storage time, and as such a retardation of the primary renneting phase occurs, that the secondary phase is also affected.

Waungana *et al.* (1998) showed that by increasing the protein content of renneted milk, a greater firming rate and higher final gel strength may be obtained, due to increased bonds/bridges in the gel network. However, inclusion of inert/non-interacting material (for example, fat globules coated with an inert membrane) can sometimes cause no change to gel strengths or even weaken them (Cho *et al.*, 1999). If the insoluble portion of MPC85 were behaving as a non-interacting or weakly interacting particle, then this could be used to explain the reduction in gel strength.

Figure 9.5 is a pictorial representation of what could be happening in the renneted skim milk/MPC85 samples. In the case of skim milk/MPC85 prepared with high solubility MPC85, initially a concentrated but uniform solution of protein will be added to the rheometer. Renneting commences quickly and cleavage of κ-casein occurs and a network will form, detectable by the increase in gel strength. At the end of the three hour time sweep, a uniform gel will have formed. When a skim milk/MPC85 sample reconstituted with low solubility MPC85 is added to the rheometer, the solution will contain some protein capable of proteolysis along with weak or non-interacting particles. The casein micelles that are still accessible to rennet have the κ-casein cleaved, but the gelation time is longer when compared with that from the high solubility MPC85. The eventual gel has a lower gel strength than a sample prepared with high solubility MPC85 due to a network forming with the addition of weak or non-interacting particles. For comparison a sample consisting of reconstituted skim milk (10% w/w) is also shown in figure 9.4. Initially soluble proteins are added to the rheometer, with all of the casein micelles capable of renneting. This occurs with a gelation time longer than either of the two skim milk/MPC85

samples due to the lower protein levels (Waungana *et al.*,1998). The eventual network that forms is uniform, but has a lower concentration, hence lower gel strength.

One other possibility that could occur with regard to rheology experiments is that as the solubility of MPC85 decreases, sedimentation occurs within the sample placed into the cone and plate geometry. A pictorial example of this is shown in figure 9.4. If this was occurring, then the eventual gel may be forming on top of the insoluble material, yielding lower gel strengths due to decreased casein concentration in the region of measurement below the cone surface.

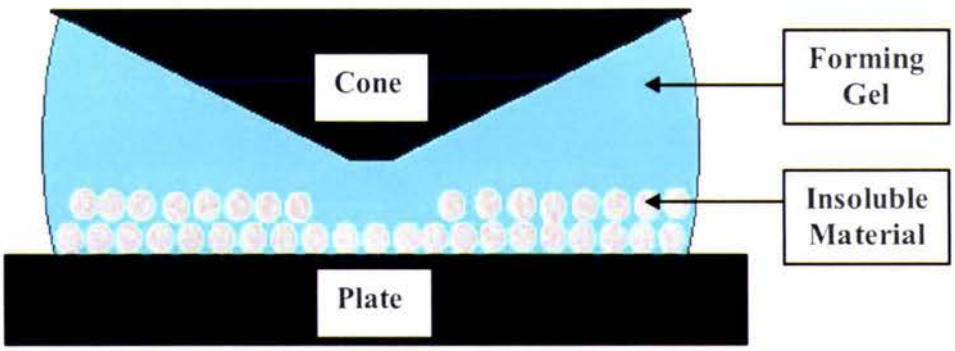


Figure 9.4 Skim milk/MPC85 sample between cone and plate geometry of a rheometer showing a gel network forming on top of insoluble material. Note that the cone angle is greatly exaggerated.

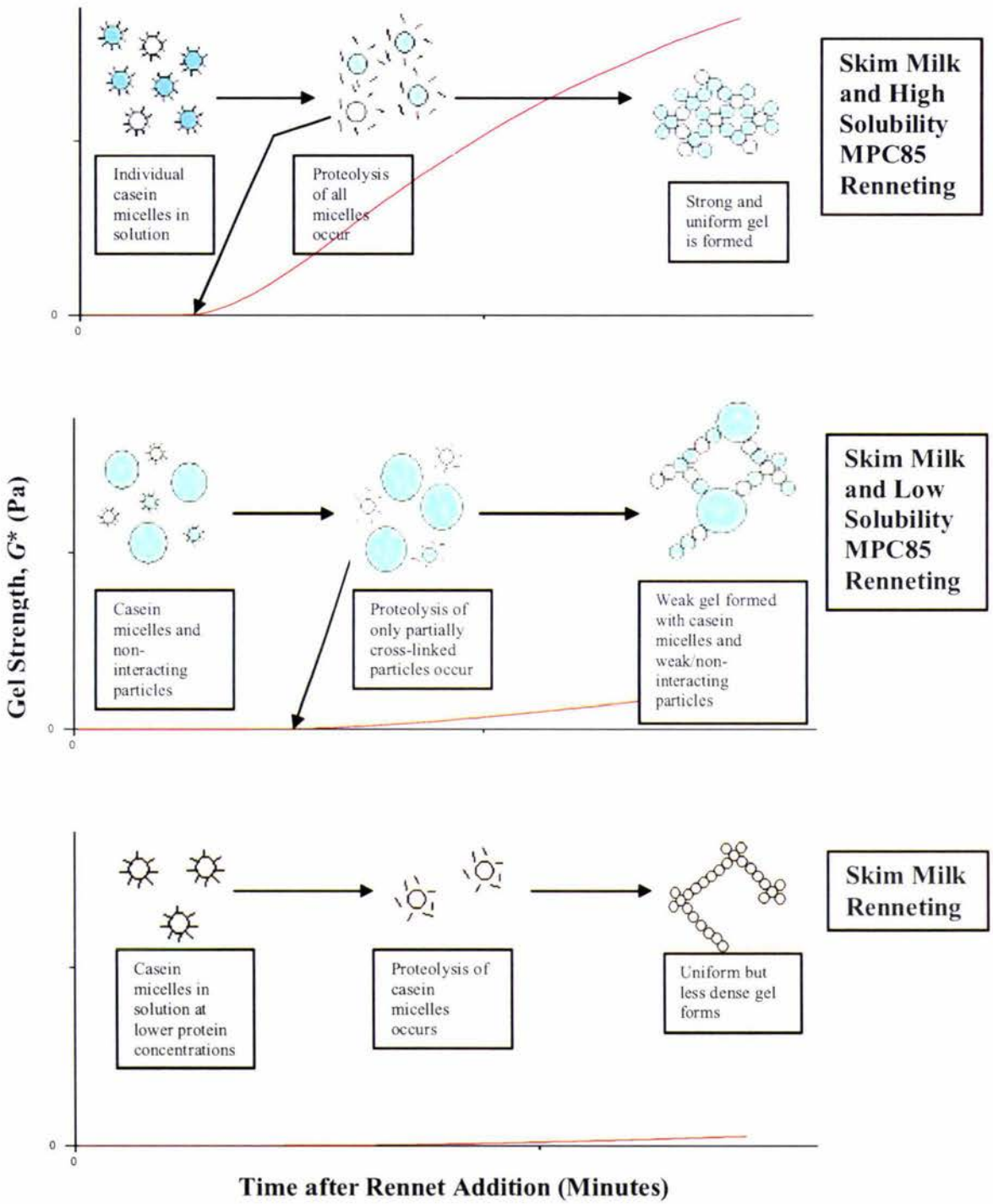


Figure 9.5 Schematic representation of gel strength of skim milk/MPC85 solutions as a function of time after rennet addition, with high solubility (top) and medium solubility (middle) MPC85. Also shown is a skim milk solution with no MPC85 addition (bottom). Coloured particles indicate MPC85 as a source.

9.3 Comparison between PAGE and Solubility

A strong correlation was also found between the detectable proteins found from reduced samples of centrifuged MPC85 solutions using PAGE (found in figure 7.4), and the concentration of soluble material (found in figure 4.1). The data shown in figure 9.6 are plotted as normalised protein level against solubility. This normalised protein level is defined as the relative % of detectable protein in MPC85 stored at one of the five temperatures compared with the detectable protein in a MPC85 powder that had not been exposed to high temperatures. Figure 9.6 shows that as solubility decreased so did the detectable protein.

The specific PAGE results used to form the correlation in figure 9.6 could be regarded as another method of performing a solubility test and therefore a correlation between these results would be expected. The difference between the two tests is that PAGE has more information about which of the proteins found in MPC85 became insoluble and/or by how much. As such, a simple solubility test may be used as an indication of the detectable total protein found from a PAGE test of this type.

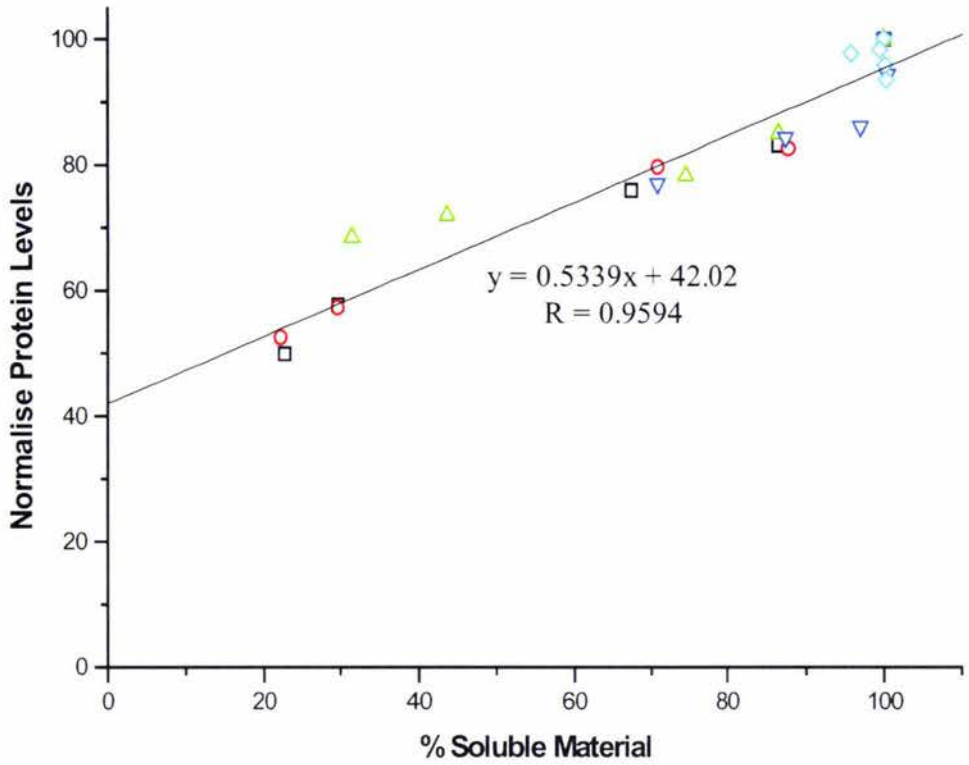


Figure 9.6 Normalised protein levels found in MPC85 stored for different amounts of time at 50°C (□), 40°C (○), 35°C (△), 30°C (▽), and 20°C (◇) as found using PAGE, as a function of % soluble material of the MPC85 powder.

9.4 Comparison between PAGE and Rheology

Figure 9.7 displays the correlation between the detectable proteins found from reduced samples of centrifuged MPC85 solutions using PAGE (found in figure 7.4), and the final gel strength from a rheology test of a skim milk/MPC85 sample containing the corresponding MPC85 (found in figure 5.3). As can be seen, the final gel strength from figure 9.7 decreases with the total detectable protein. As discussed in section 9.3, this type of PAGE test may be analogous to a solubility test and as such is a further indication that rheology results of renneted skim milk/MPC85 samples can be estimated through solubility testing.

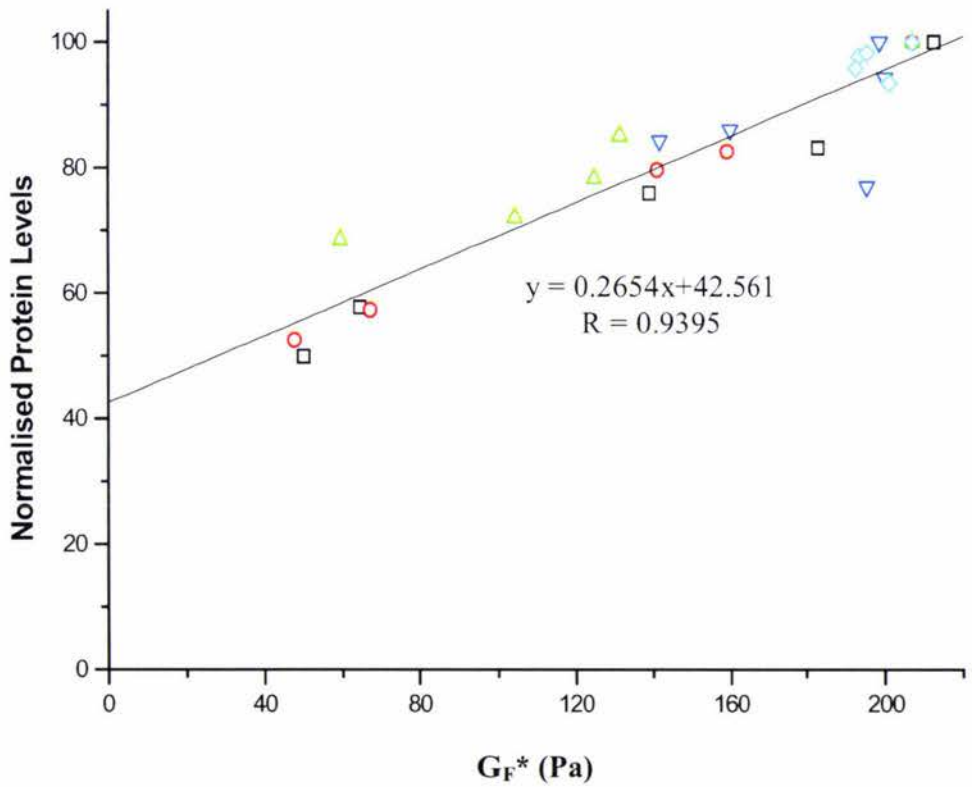


Figure 9.7 Normalised protein levels found in MPC85 stored for different amounts of time at 50°C (□), 40°C (○), 35°C (△), 30°C (▽), and 20°C (◇) as found using PAGE, as a function of final gel strength.

9.5 Comparison between Mass Spectrometry and Solubility

The correlations between each non-glycated protein (found in figure 8.3) and solubility (found in figure 4.1) are shown in figure 9.8, and the significance levels are shown in table 9.2. As can be seen, the correlations that exist between the amount of non-glycated proteins and solubility are all highly significant. The clearly discernable trend supports the theory of cross-linking reactions, indirectly measured by the Maillard reaction, taking place in the powder and as such the reduction in solubility of the MPC85 powder (Jimenez-Flores and Kosikowski, 1986; Mistry and Pulgar, 1996).

Results found using PAGE (figures 7.1, 7.3, 7.5, and 7.7) had shown blurring of protein bands indicating that the proteins found in MPC85 were being modified in a way that altered their charge and/or size. From the results found from mass spectroscopy (from chapter 8) and the correlation shown in this chapter, this modification can indeed be seen to be the glycation of proteins. As mentioned earlier, glycation is one of the initial reactions of a Maillard reaction that indicate cross-linking taking place. This reinforces the suggestion to perform tests to detect levels of HMF, LAL, or HAL within the stored MPCs, as a method to gauge any cross-linking reactions that may be occurring (Jimenez-Flores and Kosikowski, 1986).

Table 9.2: The correlation that occurred between the solubility and non-glycated proteins

Protein	R value	Significance (p)
α_{S1}-casein	0.9698	<0.0001
α_{S2}-casein	0.8485	0.00096
β-A1-casein	0.9491	<0.0001
β-A2-casein	0.9723	<0.0001
κ-casein	0.9095	0.00011
κ-casein+ carbo group	0.9455	<0.0001

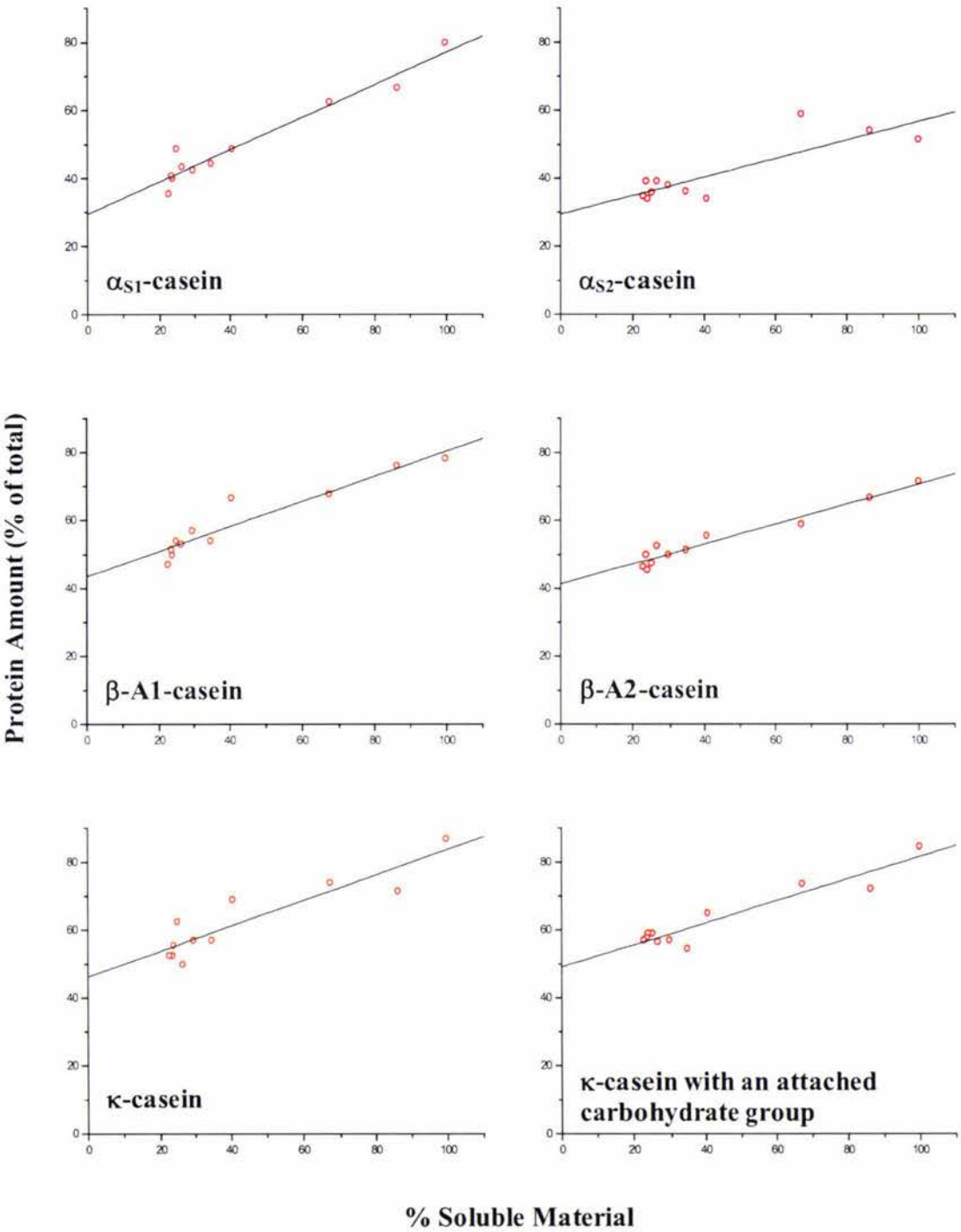


Figure 9.8 Non-glycated proteins (% of total protein) found in MPC85 stored at 50°C as a function of % soluble material.

9.6 Summary

In this chapter correlations have been discussed between solubility and various other methods used to determine the properties of MPC85 stored at different conditions. It was found that solubility can be taken as a measure of the properties found using other tests and as such, is an extremely useful quantity. The concept of cross-linking of micelles measured indirectly via a Maillard reaction was introduced. This theory was supported by the glycation of proteins found from mass spectroscopy, and the modification indicated by gels prepared via PAGE methods.

10 Conclusions and Recommendations

10.1 Conclusions

The following conclusions can be drawn from the present study:

1. The solubility of MPC85 stored at temperatures greater than 20°C decreased with storage time.

A) The rate at which the solubility of MPC85 decreased was dependent on the temperature at which it was stored. The higher the temperature, the faster the rate at which solubility decreased.

B) It was shown that the solubility could be taken as a measure of the condition of MPC85 powders. This was shown by the correlation with other tests.

2. Rheological investigations have shown that the properties of milk protein concentrate affected the physical properties of rennet induced skim milk/MPC85 gels, at storage temperatures greater than 20°C.

A) The complex modulus, G^*, of renneted skim milk/MPC85, decreased as storage time increased.

B) The gelation time increased with storage time.

C) The force required to break the formed gels decreased with storage time.

3. Hydration testing revealed important information regarding sample preparation of skim milk/MPC85 solutions.

A) Hydration time did not change the gelation time, however the gelation time increased as the solubility of the powder decreased.

B) Gels made from high and medium solubility MPC85 had the gel strength at three hours increase with hydration time. No increase in gel strength occurred for gels made with low solubility MPC85.

4. Polyacrylamide Gel Electrophoresis gave a valuable insight into the composition of the samples stored at different temperatures.

A) PAGE was used to monitor the rate at which different MPC85 proteins became insoluble.

5. Mass Spectroscopy was used to monitor the glycation of MPC85 caseins after storage at 50°C.

A) All caseins became glycated at 50°C as storage time increased.

B) Some caseins were glycated without storage at temperatures higher than -18°C. This suggested that MPC85 manufacture could cause glycation, or alternatively storage at -18°C over very long periods did not entirely inhibit glycation.

10.2 Future Work

The following areas are recommended for further investigation:

1. Moisture content of the sample powders was not part of the experimental design, as the samples were sealed in airtight bags during storage trials. Further work could be conducted to investigate the effect of dry versus humid storage conditions.

2. The addition of salts to the systems under study could be useful. For example, it is known that the addition of Ca^{2+} to a milk solution promotes aggregation. With this in mind, could the addition of salts such as Ca^{2+}, lactose or other naturally occurring salts compensate for the loss in solubility of MPC85 from storage?

3. Research into the possibility of Maillard reactions or cross-linked reaction products could be performed to confirm the results of this work, such as testing MPC85 samples for HMF, LAL or HAL levels.

4. An electron microscopy investigation of MPC85 powder stored at the temperatures used in this research to assess the validity of cross-linking of proteins.

References

Anema, S.G. and Klostermeyer, H. (1997). Heat-induced, pH dependent dissociation of casein micelles on heating reconstituted skim milk at temperatures below 100°C. *Journal of Agricultural and Food Chemistry*, Volume **45(4)**, 1108-1115

Babella, G. (1989). Scientific and practical results with use of ultrafiltration in Hungary. *Bulletin of the International Dairy Federation*. 244: 7-25

Caric, M. and Kalab, M. (1987). Effects of drying techniques on milk powders quality and microstructure: A review. *Food Microstructure*, Volume **6**, 171-180

Cheryan, M., van Wyk, P.J., Olson, N.F. and Richardson, T. (1974). Secondary phase and mechanism of enzymatic milk coagulation. *Journal of Dairy Science*, Volume **58**, 477-481

Cho, Y.H., Lucey, J.A. and Singh, H. (1999). Rheological properties of acid milk gels as affected by the nature of the fat globule surface material and heat treatment of milk. *International Dairy Journal*, Volume **9**, 537-545

Dalgleish, D.G. (1983). Coagulation of renneted bovine casein micelles: dependence on temperature, calcium ion concentration and ionic strength. *Journal of Dairy Research, Volume* **50**, 331-340

Dalgleish, D.G. (1992). The enzymatic coagulation of milk. In: *Advanced Dairy Chemistry Volume 1: Protein*. Fox, P.F. (Ed.). Elsevier Science Publishers Ltd, Essex, London, 579-619

Davenel, A., Schuck, P., Marchal, P. (1997) A NMR relaxometry method for determining the reconstitutability and the water-holding capacity of protein-rich milk powders. *Milchwissenschaft*, Volume **52(1)**, 35-39

de Castro-Morel, M. and Harper, W.J. (2002). Basic functionality of commercial milk protein concentrates. *Milchwissenschaft*, Volume **57(7)**, 367-370

de Kruif, C.G. (1999). Casein micelle interactions. *International Dairy Journal*, Volume **9**, 183-188

de Kruif, C.G., Jeurnink, T.J.M. and Zoon, P. (1992). The viscosity of milk during the initial stages of renneting. *Netherlands Milk and Dairy Journal*, Volume **46**, 123-137

Doug Durian research group at UCLA. http://www.physics.ucla.edu/~dws/light.html

Foltmann, B. (1981). Mammalian milk-clotting protease: Structure, function, evolution and development. *Netherlands Milk and Dairy Journal*, Volume **35**, 223-231

Fox, P.F. (1987). Cheese Manufacture: Chemical, biochemical and physical aspects. *Dairy Industries International*, Volume **57(7)**, 11-13

Green, M.L. (1982). Effect of the composition and properties of casein micelles of interaction with ionic materials. *Journal of Dairy Research*, Volume **49**, 87-98

Hill, J.P. and Lowe, R. (1997). Dye binding characteristics of β-Lactoglobulin variants. *Bulletin of the International Dairy Federation*, III.14: 195-199

Holt, C. and Horne, D.S. (1996). The hairy casein micelle: evolution of the concept and its implications for dairy technology. *Netherlands Milk and Dairy Journal*, Volume **50**, 85-111

Holt, C. (1997). The milk salts and their interactions with casein. In: *Advanced Dairy Chemistry Volume 3: Lactose, water, salts and vitamins, 2nd edn.* Fox, P.F. (ed.), Chapman & Hall, London, 233-256

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

Jimenez-Flores, R. and Kosikowski, F.V. (1986). Properties of ultrafiltered skim milk retentate powders. *Journal of Dairy Science*, Volume **69**, 329-339

Kameswaren, S. and Smith, D.E. (1999). Rennet clotting times of skim milk based gels supplemented with an ultrafiltered milk protein concentrate. *Milchwissenschaft*, Volume **54(10)**, 546-550

Kuo, C.J. and Harper, W.J. (2003a). Rennet gel properties of milk protein concentrates (MPC). *Milchwissenschaft*, Volume **58(3/4)**, 181-184

Kuo, C.J. and Harper, W.J. (2003b). Effect of hydration time of milk protein concentrate on cast feta cheese texture. *Milchwissenschaft*, Volume **58(5/6)**, 283-286

Laemelli, U.K. (1970). Changes of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685

Lapasin, R. and Pricl, S. (1995). Rheology of industrial polysaccharides: theory and applications. Blackie Academic and Professional, Chapman and Hall, Glasgow, UK, London

Lomholt, S.B. and Qvist, K.B. (1999). Gel firming rate of rennet curd as a function of rennet concentration. *International Dairy Journal*, Volume **9**, 417-418

McKenna, A.B. (2000). Effect of processing and storage on the reconstitution properties of whole milk and ultrafiltered skim milk powders. Doctor of Philosophy in Food Technology Thesis, Massey University, Palmerston North New Zealand

McMahon, D.J. and McManus, W.R. (1998). Rethinking casein micelle structure using electron microscopy. *Journal of Dairy Science*, Volume **81**, 2985-2993

Mistry, V.V. and Hassan, H.N. (1991). Delactosed, high milk protein powder. 2. Physical and functional properties. *Journal of Dairy Science*, Volume **74**, 3716-3723

Mistry V.V. and Pulgar, J.B. (1996). Physical and storage properties of high milk protein powder. *International Dairy Journal*, Volume **6**, 195-203

Novak, A. (1991). Milk Protein Concentrate. In: *New Applications of Membrane Processes*. International Dairy Federation, Brussels, Belgium pp 51-66

Pomprasirt, V., Singh, H. and Lucey, J.A. (1998). Effect of heat treatment on the rennet coagulation properties of recombined high total solids milk made from milk protein concentrate powder. *International Journal of Dairy Technology*, Volume **51(3)**, 65-71

Ruettimann, K.W. and Ladisch, M.R. (1987). Structure, properties and enzymatic coagulation. *Enzymatic Microbiology Technology*, Volume **9**, 578-589

Saito, Z. (1985). Particle structure in spray-dried whole milk and in an instant skim milk powder as related to lactose crystallization. *Food Microstructure*, Volume **4(2)**, 333-340

Shimmin, P.D. and Hill, R.D. (1964). An electron microscope study of the internal structure of the casein micelle. *Journal of Dairy Research*, Volume **(31)**, 121-123

Schmidt, D.G. (1982). Association of caseins and casein micelle structure. In: *Developments in Dairy Chemistry Volume 1: Proteins*. Fox P.F. (ed.), Applied Science Publishers, London, UK, 61-86

Swaisgood, H.E. (1982). Chemistry of milk protein. In *Developments in Dairy Chemistry Volume 1*. Fox, P.F. (ed.), Applied Science Publishers, London, Chapter **1**

Swaisgood, H.E. (1996). Characteristics of milk. In *Food Chemistry*, Fennema, O.R. (ed.) 3rd Edition, Marcel Dekker, New York, 841-878

van Hooydonk, A.C.M. and Walstra, P. (1987). Interpretation of the kinetics of the renneting reaction in milk. *Netherlands Milk and Dairy Journal*, Volume **41**, 19-47

Vasbinder, A.J., Alting, A.C., Visschers, R.W. and de Kruif, C.G. (2003). Texture of acid milk gels: formation of disulphide cross-links during acidification. *International Dairy Journal*, Volume **13**, 29-38

Vasbinder, A.J. and de Kruif, C.G. (2003). Casein-whey protein interactions in heated milk: the influence of pH. *International Dairy Journal*, Volume **13**, 669-677

Vasbinder, A.J., Romella, H.S. and de Kruif, C.G. (2003). Impaired rennetability of heated milk; Study of enzymatic hydrolysis and gelation kinetics. *Journal of Dairy Science*, Volume **86**, 1548-1555

Walstra, P. and Jenness, R. (1984a). Proteins. In: *Dairy Chemistry and Physics*. John Wiley, New York, NY, Chapter 6, 98-122

Walstra, P. and Jenness, R. (1984b). Casein Micelles. In: *Dairy Chemistry and Physics*. John Wiley, New York, NY, Chapter 13, 229-253

Walstra, P. (1990). On the stability of casein micelles. *Journal of Dairy Science*. Volume **(73)**, 1965-1979

Walstra, P. (1999). Casein sub-micelles: do they exist? *International Dairy Journal*, Volume **9**, 189-192

Waungana, A. (1995). Rennet coagulation properties of heated milk. Master of Technology Thesis, Massey University, Palmerston North, NZ.

Waungana, A., Singh, H. and Bennett, R.J. (1998). Rennet coagulation properties of skim milk concentrated by ultrafiltration: effects of heat treatment and pH adjustment. *Food Research International*, Volume **31(9)**, 645-651

Warburton, S. and Pixton, S.W. (1978). The significance of moisture in dried milk. *Dairy Industries International*, April 23, pp 26-27

Zoon, P., van Vliet, T. and Walstra, P. (1988a). Rheological properties of rennet-induced skim milk gels. 1. Introduction. *Netherlands Milk and Dairy Journal*, Volume **42**, 249-269

Zoon, P., van Vliet, T. and Walstra, P. (1988b). Rheological properties of rennet-induced skim milk gels. 2. The effect of temperature. *Netherlands Milk and Dairy Journal*, Volume **42**, 271-294

Zoon, P., van Vliet, T. and Walstra, P. (1988c). Rheological properties of rennet-induced skim milk gels. 3. The effect of calcium and phosphate. *Netherlands Milk and Dairy Journal*, Volume **42**,295-312

Zoon, P., van Vliet, T. and Walstra, P. (1989a). Rheological properties of rennet-induced skim milk gels. 4. The effect of pH and NaCl. *Netherlands Milk and Dairy Journal*, Volume **43**, 17-34

Zoon, P., van Vliet, T. and Walstra, P. (1989b). Rheological properties of rennet-induced skim milk gels. 5. Behaviour at large deformation. *Netherlands Milk and Dairy Journal*, Volume **42**, 35-52

Zwiggers, A. (1992). Outline of milk protein concentate. *International Food Ingredients*. Volume **3**, 18-23

Appendix 1: Preliminary Experiments

A-1 Introduction

Preliminary investigations were required to identify appropriate experimental conditions, and to assess the suitability of the various experimental techniques since initially there was minimal literature available concerning the renneting properties of milk protein concentrates. These preliminary results will be used to identify the reconstitution, hydration, and storage conditions required for a full study. The methods being investigated for suitability are rheology and diffusing wave spectroscopy (DWS). Note: the methods quoted in this section were used to determine suitable methods for the full study, and should not be confused with those mentioned in chapter 3.

A-2 Materials and Methods

A-2-1 Sample Preparation

Three different groups of samples are analyzed in this section, all of which use a solution of SMP (Skim Milk Powder), as a base mix. The production details of this SMP are:

Reference Number : LH0056
Factory Registration Number : X5204
Cypher : EK29

This SMP is the same as that used in the full experimental investigation. The base solutions (which will be defined as SMP_{aq}) were made up to 10% (w/w) of the above specified SMP, in milliQ water (with 0.02% (w/w) Sodium Azide). The SMP was stirred into the milliQ water for a minimum of two hours, before being refrigerated for several hours ahead of further preparation to ensure both preservation and adequate hydration. Sample groups one and two were then prepared individually by adding concentrations of 1%, 2.5%, or 5% (w/w), of two different types of MPC85 powders (Milk Protein Concentrate which has 85% protein by weight).

Group one used an MPC that is known as a "high solubility powder", which was of a high grade, as it has been stored under optimum conditions (<-18°C). The production details of this powder are:

NZ DRI Sample	: 010023534
Batch Number	: 20225
Submitter	: Alan Baldwin

Group two was prepared in the same manner as group one, except that a "low solubility powder" was used, which had not been stored under optimum conditions. The production details of this powder are:

Alapro	: 4580
Hautapu	: 12/12/2001

For each of the six samples that make up groups one and two, the powder was added to the base mix SMP_{aq} at the allocated concentrations making six individual 100 gram samples. The powders were added over one minute to the SMP_{aq} which was under a high stir rate, and then mixed for a further ten minutes to totally dissolve. The solutions were left over night (at 4°C) before being used in a test to ensure adequate hydration of the sample.

The third group of samples is that of the base SMP_{aq}, but mixed to higher concentrations. These concentrations were made up on the assumption that the protein content of MPC85 is approximately twice that of the SMP. So to compare with groups one and two, samples in group three were made up to concentrations of: 12%, 15%, and 20% (w/w). Due to the similarities between group three and the SMP_{aq} base mix of groups one and two, it was prepared by the same method of stirring for two hours, and as before, leaving over night before testing. Again this is done for hydration purposes.

All experiments involved the use of rennet to induce gelation. The production details of the rennet are:

Pfizzer Chy-Max
100% Chymosin
80 ru/ml
From NZ Coop. Rennet co. Ltd.

Rennet is a very potent agent, so it was used in a diluted form to avoid large errors from transference inaccuracies. The rennet used in all tests was diluted to 10% (w/w) in milliQ water (with 0.02% (w/w) Sodium Azide). Note that the diluted samples of rennet were considered to be of good quality for use in experiments for a period of two days. After this time they were discarded due to assumed degraded quality. To ensure that the rennet samples did not expire prematurely they were kept refrigerated at all times and removed only when they were to be added to the test samples. Once this was done they were immediately returned to the refrigerator.

A-2-2 Experimental Methods

Sample preparation for both the rheology and DWS experiments was identical. All experiments were conducted at 30°C. The previously prepared samples (as described above) were preheated in a water bath to a temperature of 30°C, for one hour before testing began. Once this temperature had been reached, 200µl of the diluted rennet was added to each 50g sample and the sample was then inserted into the apparatus. Care was taken at this point not to introduce air bubbles, as air bubbles in a rheology experiment increase the elasticity of the system, while in a DWS setup the laser beam would be scattered by the air bubbles. Both these effects would be detrimental to results. Note that air bubbles can also denature milk proteins, which would also have large unwanted effect on the results.

The rheometer used was a rheometrics SR-5000, which tested each sample by means of a couette flow double gap geometry. A water bath controlled the sample temperature at 30°C. Each sample was examined for 3 hours under the regime of a 'Dynamic Time Sweep Test'. The rheometer, operating in a controlled strain mode, had the settings: Frequency = 0.1 Hz and Strain = 0.5%.

The DWS setup has many more individual components than the rheometer, and as such involved an extensive alignment process. The laser beam, launched from a Spectra Physics 125A laser (operating at 632nm and delivering 50 mW), was expanded to approximately 1cm at the sample cell. This cell was a 4ml plastic cuvette with a 1cm optical path length. For consistency with the rheometer tests, the sample cell was in constant thermal contact with a heat reservoir of 30°C, controlled by a water bath. The light scattered by the sample was collected in backscattering geometry using a single mode fiber optic cable (P1-3224-PC-5, Thorlabs Inc. Germany), fitted with a GRIN lens (F230FC-B FC, Thorlabs Inc. Germany). The fiber optics were connected to a Malvern photomultiplier tube (Malvern Instruments Ltd, Malvern, Worcester, UK), and the analysis was performed using a Malvern 7132 correlator. As before, testing occurred over a period of three hours, with one correlogram being formed every two minutes. In each of these tests the fast sample time

was $\tau = 1\mu s$, and the slow sample time was $\tau = 10\mu s$. A basic DWS setup is shown in figure A-1.

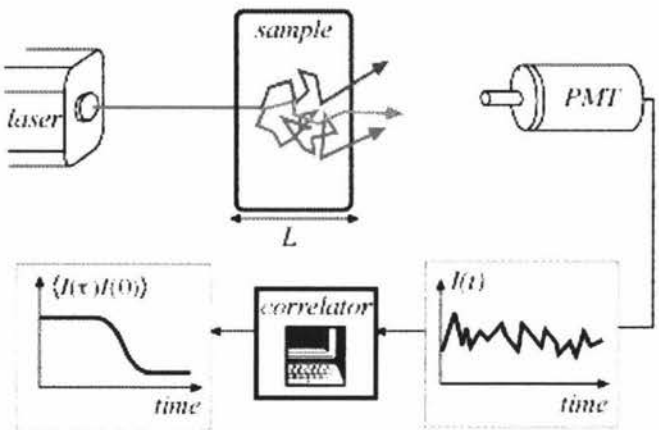


Figure A-1 Representation of a basic DWS setup. A laser beam is multiply scattered by a sample and then collected by a photo-multiplier tube (PMT), which creates a time-intensity curve. This information is interpreted by a correlator to produce a correlation curve, from which data can be found. (Doug Durian Web Site).

A-3 Data Analysis

The aggregation and gelation times of each sample was measured after the addition of rennet. The methods used were all standardised, and as such all samples were analysed the same way.

A-3-1 Rheology

Figure A-2 represents a typical set of results obtained for a sample being measured by the rheometer operated as described in the experimental methods section. The value plotted, G^*, is known as the Complex Elastic Modulus, and it is from this quantity that the values of

interest may be extracted. The addition of rennet to a milk solution causes the breakdown of κ-casein, and destabilisation of the casein micelle. Initially this has no effect on G*, until sufficient κ-casein have been cleaved for a network to begin to form. The time at which this occurs is defined as the aggregation time and is represented by point 1 in figure A-2.

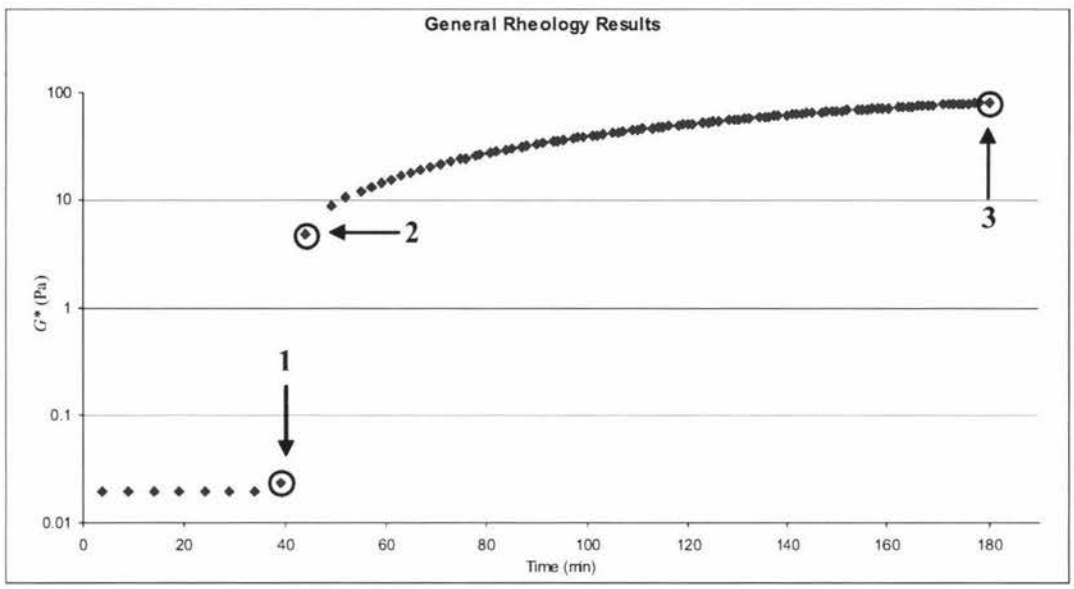


Figure A-2: Results from rheology test using 1% (w/w) high solubility MPC in the base mix SMP_{aq}.

The initial aggregation process occurred very quickly and a network was formed. When this happened G^* began to level off as the network/gel strengthened. The time at which G* started to level off was defined as the gelation time and can be seen as point 2. Note that there are other methods to define a gel point (such as when the dynamic moduli of a system equal one another (ie $G' = G''$), when G^* reaches a certain value, etc.). The current method was chosen, as this is the time at which the rheometer starts to measure an elasticity.

Another useful comparison between data sets is the final gel strength. This property was evaluated for these sets of experiments by recording the final value of G^* for each 3 hour experiment. An example of this can be seen as point 3 in figure A-2.

A-3-2 DWS

Typical results from a DWS test can be seen in figure A-3. The aggregation time, as with rheology tests, can be seen as the last point before deviation away from the initial group of points occurs (point 1). Note that figure A-3 plots values of τ which represents the mobility of particles in solution. As τ increases, mobility decreases, and hence a firmer gel is formed.

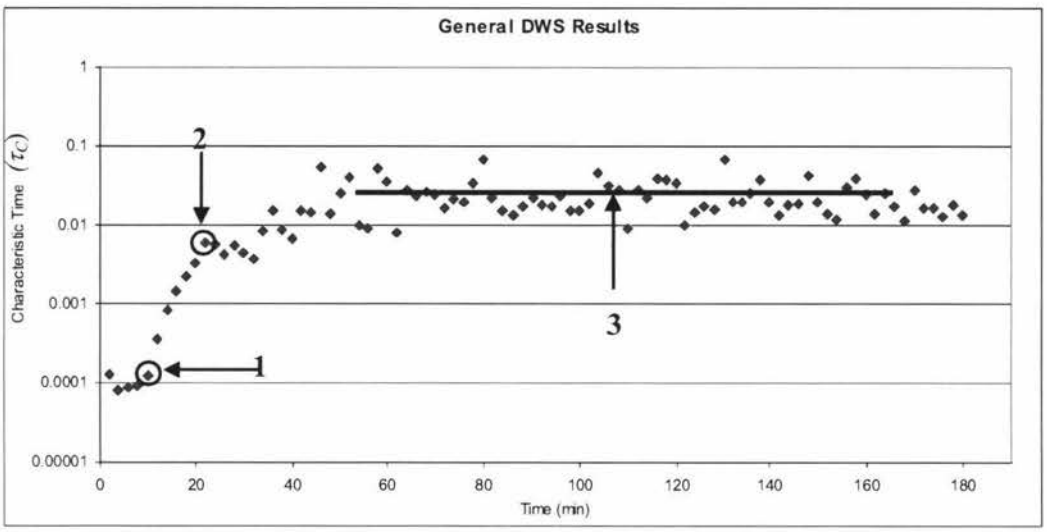


Figure A-3: Results from DWS test using 1% (w/w) high solubility MPC in the base mix SMP_{aq}.

The gelation point is clearly distinguished in the rheology results. This is not the case with DWS measurements and the last point on the smooth curve, shown by point 2 on figure A-3 was taken as the gelation time.

As the signal level became noisy once the gelation time had been reached, the "strength" of the gel due to the concentration of the sample became quite difficult to evaluate. It was taken as the average value of this noisy signal. This may be seen as the line through the data identified as value 3 in figure A-3.

A-4 Results

A-4-1 Rheology

Figure A-4 shows a plot of the aggregation time as defined in the data analysis sections for sample groups one, two, and three as a function of the added MPC to the base mix SMP_{aq}. As previously stated, the assumed protein content of SMP was half that of the MPC85 powders.

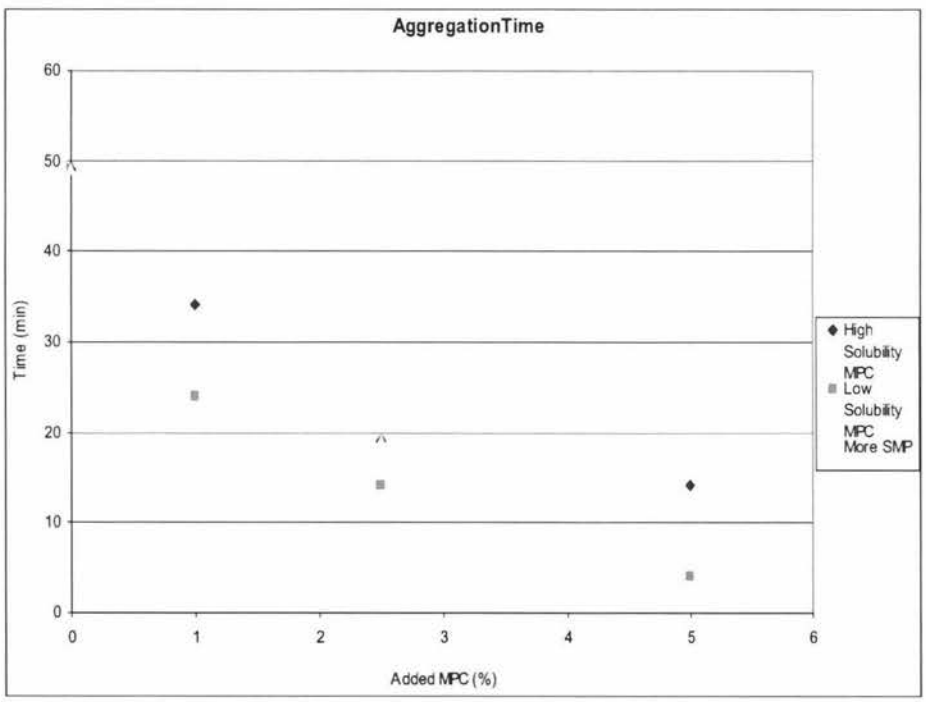


Figure A-4: Aggregation time against added MPC for samples found using the rheometer.

Note that the results for the gelation times found by the rheometer are not displayed since they are equal to the aggregation times plus 10 minutes.

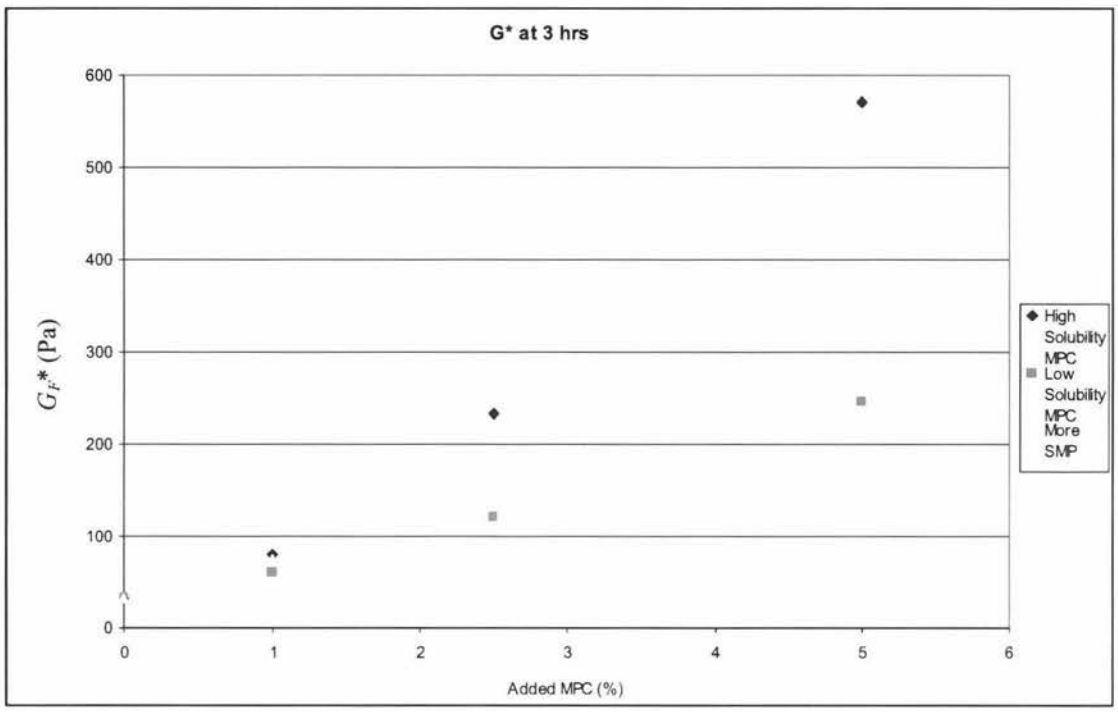


Figure A-5: The strength of the gel formed by the samples at the end of three hours plotted as a function of added MPC.

Figure A-5 is a plot of the gel strength of each of the samples at the end of the three-hour tests. The use of G_F* to gauge the gel strength of a sample is universally recognised as a way to quantify such a rheological constant.

A-4-2 DWS

Figure A-6 is the aggregation times of the samples in each of the three experimental groups found using DWS.

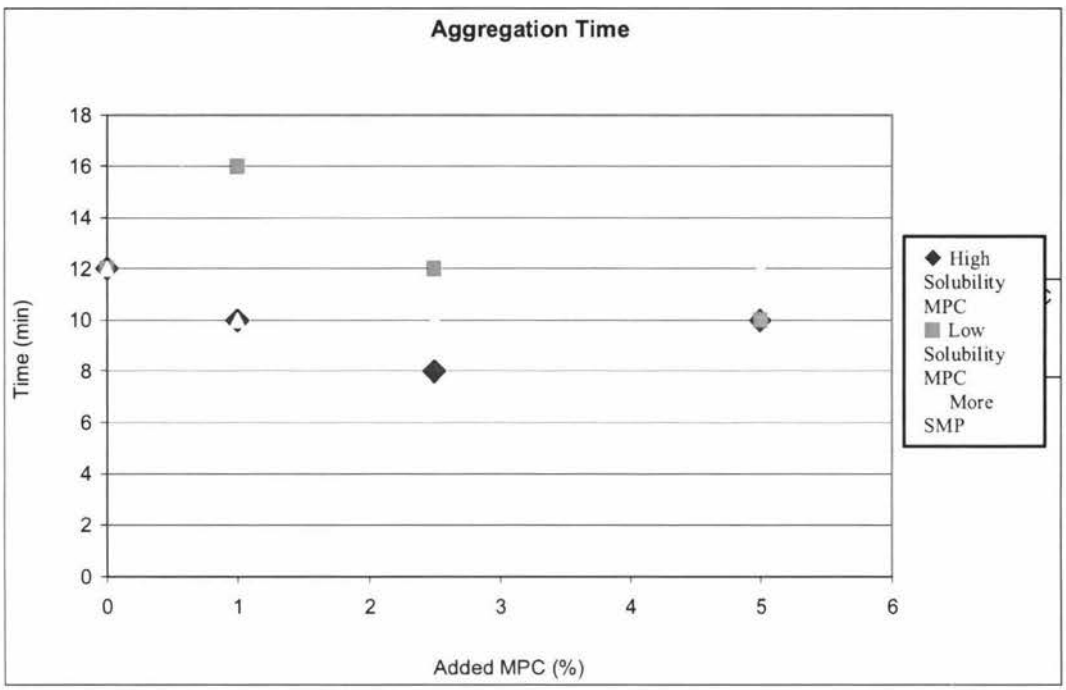


Figure A-6: Aggregation time against added MPC for samples found using DWS.

Figure A-7 illustrates the Gelation times found for all the samples tested using DWS. Figure A-8 depicts the average plateau values measured using DWS. Due to the wide spread of data points these values should be regarded as approximate magnitudes.

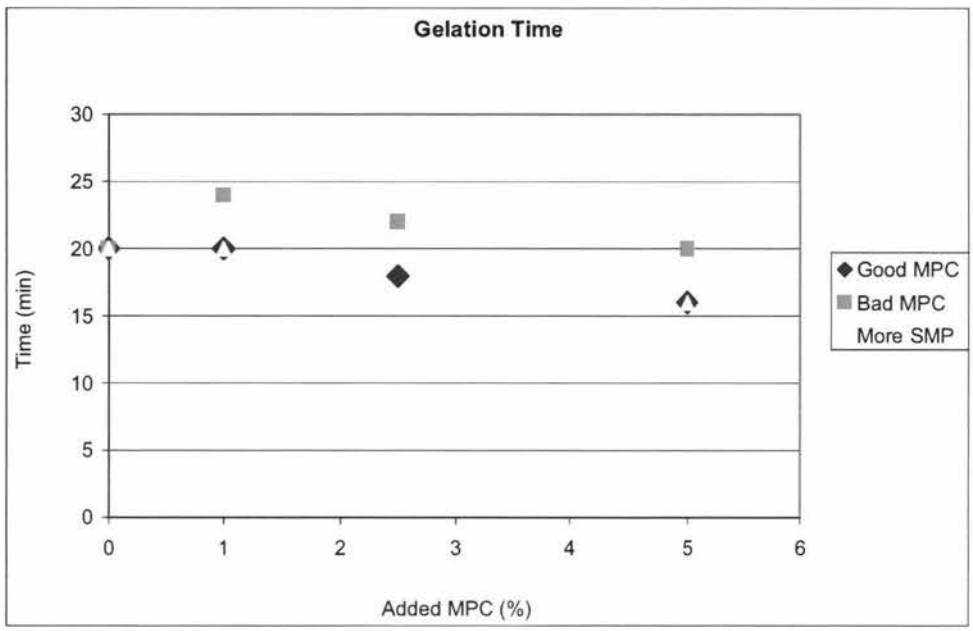


Figure A-7: Gelation time against added MPC for samples found using DWS.

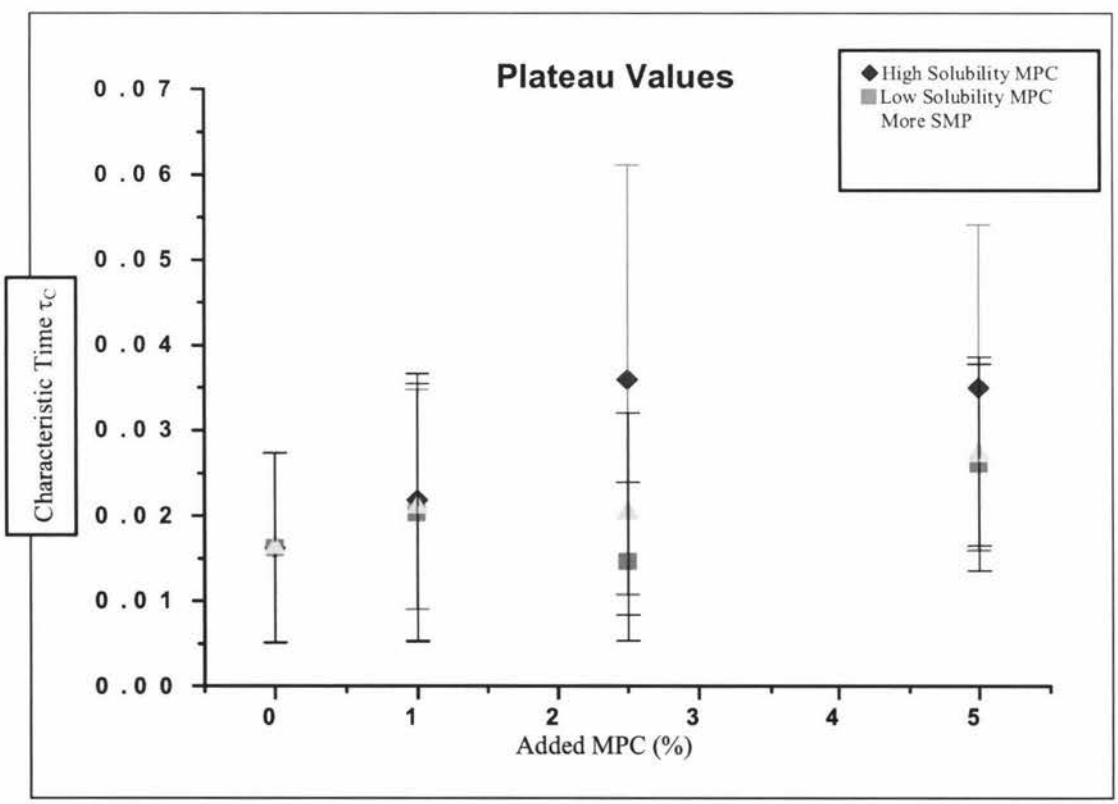


Figure A-8: The characteristic time of the gel formed by the samples at the end of three hours found from DWS measurements.

A-5 Discussion

There are many differences between the results obtained from the rheology and DWS experiments. The following are some of the possible causes of these discrepancies.

As mentioned in the experimental methods section of this report, both experiments employed temperature control through the use of water baths. Although the control of temperature is a desirable trait for any experiment, the problem arises specifically in this project due to the fact that there is not a single water bath available that is able to control the temperature of both experiments. So the rheology and DWS experiments may have been ran at slightly different temperatures and hence some of the differences noticed between the two data sets may be due to temperature variation. This could be tested by performing some rheology tests at 25°C to measure effect of temperature.

Another source of discrepancy between the results is the physical operation of the rheometer. Shear must be applied to the sample to make measurements with a rheometer. Keeping the amount of shear applied to samples to a minimum was one of the objectives for this phase of experimentation, and to a point it can be said that this was achieved. But should the sample be considered at a microscopic level, where the eventual gel network forms, an apparent shear would be noticeable and this process will be delayed. This is not the case with DWS as no shear is applied, and as such, the samples tested are able to form networks unhindered. So it is feasible that the results obtained from rheology measurements may be delayed when compared with those obtained from DWS methods.

DWS is very sensitive so even the smallest change in particle size in a sample may become noticeable through the use of this technique, whereas for a rheology experiment, these changes may go unnoticed. This will no doubt account for many of the differences noticed between the results obtained above. Possibly the most obvious difference between the outputs of the two types of tests can be seen in the first two points in figure A-3. This is due to the action of the rennet. As stated earlier, rennet is a very powerful additive and will begin the proteolysis of micelles immediately upon addition to the system. The initial drop

in particle size noticeable in figure A-3 does not appear in the rheology results due to a reduced amount of sample resolution.

Now that many of the differences between the two data sets can be accounted for, a closer look can be taken at the results individually produced to assess the quality of the observations.

Firstly consider the results shown in figure A-4. It is expected that as the particle concentration of a sample was increased, the associated kinetics involved with the gelation would be accelerated. This is exactly what happens, but it should now be understood why the three groups of samples have their observed distribution. The fact that the high solubility MPC samples aggregate faster than those made up with a higher SMP concentration to the same protein content is no surprise. This happens because the micelles are of a higher quality and so react faster with the added rennet. What is initially unexpected is that the low solubility MPC samples aggregate faster than the high solubility MPC samples. The reason behind this is that many of the micelles that make up the low solubility MPC powder did not react with the chymosin present in the added rennet. This in turn means that the same amount of rennet used in other tests has to react with fewer micelles and in a smaller volume, due to the space being taken up by lower quality micelles. Hence aggregation will occur at a faster rate.

The results presented in figure A-5 are valid as the faster a reaction occurs, then the stronger the gel will be at the end of the three hour testing period. The reason that the low solubility MPC samples form the weakest gels even though they have the fastest reaction is due to the quality of the components. Because not all of the micelles from the low solubility MPC powder react with the rennet, gelation has to occur over the same volume as with the other two groups of samples, but with fewer components. So the gel strength created by the high solubility MPC samples is higher than that of the SMP_{aq} samples, simply due to the fact that it will react faster, and the quality of all its components is higher.

Once gelation has occurred, results obtained from DWS become erratic. This is possibly due to the sample no longer being ergodic (a pre-requisite for DWS testing). This is unfortunate as both the aggregation times (shown in figure A-6), and the gelation times (shown in figure A-7), can be seen to decrease in a fashion that mimics the rheology experiments. The reason for this lack in resolution is most likely due to the small times that are encountered in classifying these two quantities. One solution to this problem could be to reduce the amount of rennet used in the tests, so as to slow the reaction process, and gain a better insight into the dynamics of the experimental system.

Lastly, consider the plateau values obtained using DWS (figure A-8). These values, although not as smooth when shown in graph form, can be seen to mimic the results obtained through rheological methods (figure A-5).

A-6 Summary

The important points about the preceding MPC tests are:

- The samples containing "Low Solubility MPC Powders" reacted with the rennet far quicker than all other samples, but the resulting gels were weaker.
- The results produced by samples containing "High Solubility MPC Powders" are approximately equal to those found using samples containing equivalent amounts of Skim Milk Powder.

However:

- A better quality rheology process needs to be established.
- The rennet concentration used in testing could be reduced to increase the gelation time to better record the gelation process.

- Due to inconsistencies in results DWS will no longer be used to test samples. These experiments established the methods used in the investigation of MPC powders described in this thesis.