Acid Coagulation Properties

of

Milk Powder

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Abstract

The relationship between heat treatment classification and the acid coagulation properties of reconstituted skim milk powders was investigated.

Fifteen samples of commercial skim milk powder, acidified with glucono-δ-lactone (GDL), were analysed using a Bohlin C-VOR rheometer. Powders varied in stated heat treatment classification (based on WPNI) and country of manufacture.

It was shown that low heat powders (WPNI ≥ 6.0) and powders without heat classification coagulated more slowly than medium heat or high heat treated powders. Gel strength (storage modulus, G') of the acidified milks was also influenced by heat treatment classification. Low heat and unclassified powders produced weaker gels (40-50 Pa) than medium and high heat milk powders (>228 Pa), however low heat powders from Sweden did not follow the same trend.

Using reversed-phase high performance liquid chromatography (RP-HPLC), concentrations of major proteins were determined. It was shown that powders with lower levels of native whey protein had better coagulation characteristics (faster coagulation times and higher gel strengths) than milk powders with higher native whey protein concentration. This correlated to increasing heat treatment classification and WPNI. These findings suggest that the heat induced modifications of milk proteins enhance coagulation properties, and that low heat milk powders are less suitable for acid milk gel applications.

Keywords: Skim milk powder, milk proteins, acid coagulation, gelation, rheology, heat treatment, high performance liquid chromatography

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Table of Contents

List of Tables

List of Figures

Chapter 1
Introduction

1.1 Milk and dairy products

Milk has been a part of the human diet for thousands of years. It consists of a large number of substances, including protein, fat, lactose, water and minerals, and is often regarded as a complete food as it is capable of sustaining life on its own.

In a commercial sense, milk has a major advantage over other foods because of its versatility and flexibility. Milk can be processed into a vast number of products, many of which can be used as ingredients in a wide range of foods, which have both functional and nutritional considerations in mind. The financial significance of milk utilisation through the development of new and value-added dairy products is well recognised.

The properties and yield of dairy products are greatly influenced by:

- the amounts and relative proportions of each of the milk constituents
- processing conditions

These two points are of major significance to this thesis and will be discussed shortly.

1.1.1 Milk powder

Milk powder is a major dairy commodity made by evaporating milk to dryness. Large scale manufacture is accomplished through spray drying; a process in which pasteurised milk is concentrated by evaporation, then sprayed into a heated chamber where moisture immediately evaporates leaving fine particles of powdered milk solids.

The production of milk powder from milk facilitates transportation and storage, and prolongs shelf-life. Milk powder is more versatile than liquid milk, and can be used in a large number of applications. It is used widely as an industrial ingredient in value added products, incorporated into foodstuffs such as confectionary, ice cream, bakery goods, infant formulae, fortified nutritional blends, convenience foods, and fermented dairy products.

1.1.2 Yoghurt

Yoghurt is a well-known fermented dairy product, popular world over for its textural and sensory attributes. Technically speaking, yoghurt is an acidified milk gel. It is commonly produced by adding a starter culture of lactic acid bacteria (*Streptococcus thermophilus* and *Lactobacillus delbruekii* subspecies *bulgaricus*) to milk. These bacteria convert the milk sugar lactose to lactic acid and acetaldehyde, which are responsible for the distinctive sour taste and aroma of yoghurt, respectively. Moreover, the acid produced too, gives rise to the characteristic semi-solid gel texture of yoghurt, acting on the milk proteins causing a viscous milk gel and prolonging the shelf life of the product.

1.1.3 Yoghurt powder

One recent application of milk powder is its use in yoghurt powder. This product is a preparation of milk powder and freeze-dried starter culture, sold to consumers for personal yoghurt manufacture. The powder is reconstituted with cold water, and incubated between 30 and 40ºC in a vacuum flask for 8-10 hours, resulting in freshly set yoghurt.

Customer complaints have been received however, regarding the coagulation ability of yoghurt powders for yoghurt making at home. Specifically, concerns have been raised about the lack of curd firmness after the recommended incubation time. While it is possible that such problems could be linked to the resuscitation and growth ability of the culture, in-depth investigations looking at the contribution of the milk powder have not been carried out.

1.2 Factors affecting acid coagulation

Milk is made up of many components, with one of the main constituents being protein. As stated earlier, the properties and yield of dairy products are influenced to a great extent by the amounts and relative proportions of each of the milk constituents. Milk proteins (caseins and whey proteins) play an enormous role in the acid gelation process of milk, and for this reason will be focussed on throughout this thesis.

Liquid milk for fermented milk products is generally subjected to a reasonably severe heat treatment (e.g. 90ºC for 10 minutes) with a marked effect on the end product. At such temperatures, whey proteins denature and aggregate with casein micelles and dissociated casein molecules via disulphide bonding. These protein interactions form a highly cross-linked gel network. A comprehensive description of these phenomena is given in chapter 2.

1.3 Objectives of the study

A large amount of research has been conducted into the heat-induced modifications of milk proteins and the effects on gel formation. In spite of this, little has been published on the effect of heat treatment received during the manufacture of milk powder in regards to gelling ability.

With the issues outlined above in mind, the objective of this project was therefore to examine the effect of processing heat treatments, dry matter, incubation temperature and protein profiles on the acid coagulation properties of reconsituted milk powder.

The outline of the report of this project is as follows:

- Literature review of milk protein chemistry and its significance in relation to the functional properties of milk powder and fermented products (Chapter 2)
- Rheological analysis (coagulation time and curd strength) at different incubation temperatures of acid gels made from reconstituted milk powder of different dry matter concentrations and processing heat treatments (Chapter 3)
- Analysis of the individual protein composition (protein profile) of the milk powders used in the project (Chapter 4)
- General discussion and conclusions of the results and implications for future research (Chapter 5)

Chapter 2
Review of the Literature

2.1 Milk and milk proteins

Milk is a complex and variable mixture of water, lactose, fat, protein, organic acids and minerals, as well as a vast number of other substances. The focus of this project is on the protein fraction of milk. Bovine milk contains about 3.5% total protein. Of this, approximately 80% are caseins, chemically defined as the milk proteins that precipitate at pH 4.6, and the other 20% are those that remain in solution; known as whey or serum proteins. Neither the casein nor the whey protein fractions are homogeneous in their composition. Typical concentrations of the major casein and whey proteins are shown in Table 2.1

Table 2.1 Typical concentrations of the proteins in milk (Ng-Kwai-Hang, 2002)

Protein		**Typical concentration in milk (g/L)**
Caseins:		*25.2*
α_{s1}-casein	α_{s1}-CN	10
α_{s2}-casein	αs_2-CN	2.6
β-casein	β-CN	9.3
κ-casein	κ-CN	3.3
Whey proteins:		*5.6*
β-lactoglobulin	β-LG	3.2
α-lactalbumin	α-LA	1.2
Serum albumin	BSA	0.4
Immunoglobulin	Ig	0.8

2.1.1 Caseins

The first evidence of different components in the casein fraction in milk was provided by Mellander in 1939. Using electrophoresis, three different caseins were separated; α-casein, β-casein and γ-casein, named in order of decreasing migration ability. Waugh & von Hippel (1956) divided α-casein into α_s-(calcium-sensitive) and κ-casein (calcium-insensitive), with Annan and Manson (1969) showing further subdivision of α_s- casein into α_{s1}- and α_{s2}-casein. Groves (1969) showed that γ-casein represented the C-terminal segment of β-casein after proteolytic cleavage by plasmin. Consequently, the casein fraction of milk is made up of α_{s1}-, α_{s2}-, β- and κ-caseins in proportions of approximately 4:1:4:1.

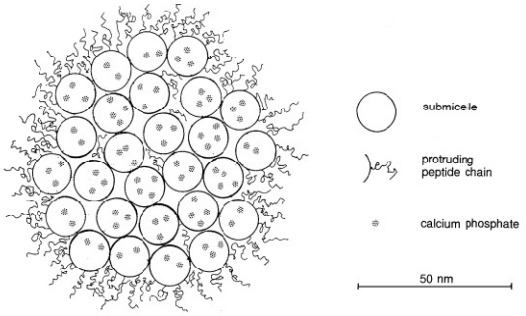
Due to high proline content, caseins show little tertiary or organised secondary structure (Fox & McSweeney, 1998). This accounts for the stability of caseins against heat denaturation as there is very little structure to unfold. The casein peptide chains contain polar and apolar regions giving them an amphiphilic structure. This, as well as their proline and phosphate content, is the basis for the ability of caseins to form micelles. Phosphate groups are esterified to the caseins via the hydroxyl groups of serine. These phosphoserine residues bind calcium, which binds colloidal calcium phosphate (CCP). It is these bonds that contribute to the linking of caseins together, and the formation of micelles. Milk has a very high calcium concentration, approx. 1200 mg/L, and about half is bound to casein via CCP. The concentration of protein and calcium in milk would cause precipitation of α_{s1}-, α_{s2}- and β-casein (by binding to their phosphoserine residues), however the κ-casein, which is soluble in calcium, interacts with and stabilises the other caseins to initiate micelle formation and a stable colloidal state (Farrel *et al*., 2006). Around 95% of caseins in milk are aggregated in micelles, functioning to fluidise the casein molecules and soluble calcium and phosphate (Farrel *et al*., 2006).

Several theories about the casein micelle structure have been proposed (Fig. 2.1). The sub-micelle model (Schmidt, 1982) evolved after evidence from electron microscopy and light scattering suggested that the casein micelle was an assembly of small sub-units. In this model, the casein proteins are said to be hydrophobically aggregated to form the sub-micelle units, and these units are linked by CCP to form the micelle. The distribution of κ-casein between sub-micelles is heterogeneous, and the sub-micelles with high levels of κ-casein are located at the surface whereas those with low levels are located in the interior. On the surface of the micelle, the hydrophilic and negatively charged C-terminal ends of the κ-casein protrude out and form a hairy layer that prevents micelle-micelle aggregation by steric and electrostatic repulsion (Walstra, 1999).

In recent years, new models that do not rely on the formation of sub-micelles have been proposed (Holt and Horne, 1996; Horne, 1998). The dual-binding model of Horne (Fig 2.2a) proposes that the individual casein molecules interact via hydrophobic regions in their primary structure.

Although there is no unanimously accepted model, there are some general properties that have been agreed on. These include the notion that a) partly-hydrophobic caseins are stabilised by the hydrophilic tail of κ-casein located mainly near the micelle surface, and b) that CCP has an integral role in micelle structure.

a)



b)

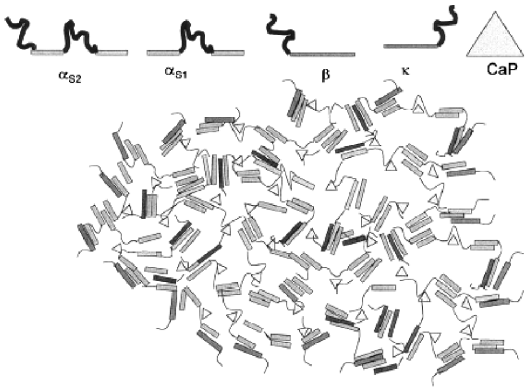


Figure 2.1 Models of the casein micelle;
a) The sub-micelle model (Walstra, 1999), **b**) The dual binding model (Horne, 1998).

2.1.2 Whey proteins

Whey proteins share few common features other than being soluble at pH 4.6. The three main whey proteins are β-lactoglobulin (β-LG), α-lactalbumin (α-LA) and blood serum albumin (BSA), which make up 50, 20 and 10% of total whey protein respectively. The remaining 20% consists of immunoglobulins, and trace amounts of several other proteins including enzymes.

Unlike caseins, whey proteins are globular with organised secondary and tertiary structures. This means they are susceptible to heat denaturation (DeWit & Klarenbeek, 1984). Whey proteins possess a number of cysteine residues as internal disulphide bonds (Fox & McSweeney, 1998). On denaturation, a reactive thiol group of β-LG is exposed, which forms disulphide-thiol bonds with other β-LG molecules as well as with κ-casein and to some extent with α_{s2}-casein (Sawyer, 1969; Creamer *et al*., 2004; Lowe *et al*., 2004).

2.1.3 The effect of heat on milk proteins

The effect of heat on the milk system is an important consideration in dairy chemistry, as heat treatments are involved in the manufacture of most milk products. When milk is heated, a number of reactions occur. The importance of each reaction is determined by the heating conditions, as well as milk composition and concentration products (Anema, 2009). In regards to milk proteins, reactions of particular significance are whey protein denaturation, the interactions of denatured whey proteins with other proteins, including caseins on the micelle surface and caseins dissociated from the micelles. These three reaction processes can alter the physico-chemical properties of milk and play a major role in determining the stability of milk and the functional performance of heated milk products; a key point, which will be of relevance later in this thesis.

When milk is heat-treated at temperatures of 60ºC and above, the whey proteins unfold and irreversibly denature. As well as this, caseins, particularly κ-casein, dissociate from the micelles. As previously mentioned, disulphide-thiol bonding can then occur, causing whey proteins to aggregate with themselves as well as with κ-casein. The formation of complexes between whey proteins and κ-casein during heat treatment markedly affects the protein organisation in both the colloidal and serum phases of milk, and consequently its technological applications. Because of this, much research has been conducted into this area, and this has been summarised in a recent review carried out by Donato and Guyomarc'h (2009). These authors discuss the proposed pathways for whey protein/κ-casein complex formation. Initially, primary aggregates of denatured β-lactoglobulin or β-lactoglobulin/α-

lactalbumin form. These then bind to the surface of the casein micelle. Various studies have shown a positive relationship between the denatured whey proteins and the dissociated κ-casein, both found in the serum phase of milk after heat treatment. This evidence suggests that formation of the serum whey protein/κ-casein complexes is related to dissociation of the κ-casein. However it remains unclear as to whether the dissociation of κ-casein occurs before or after its interaction with denatured β-lactoglobulin. Some studies have shown that the dissociation κ-casein is related to the concentration of β-lactoglobulin or of β-lactoglobulin's free thiol group (Donato *et al*., 2007; Renan *et al*., 2007), while other studies have shown that of κ-casein dissociation can occur in the absence of whey protein denaturation (Anema *et al*., 2007).

In any case these complexes, essentially contain denatured whey protein and κ-casein in a ratio of 1-5 whey proteins : 1 casein, assembled mainly through disulphide bonding and hydrophobic interactions (Guyomarc'h *et al*., 2003; Jean *et al*., 2006; Donato *et al*., 2007; Anema, 2007). Traces of other caseins may be found (Jean *et al*., 2006), especially in the micelle-bound complexes (Guyomarc'h *et al.*, 2003). It has been shown that the size of the complexes increases with heating (Anema & Li, 2003; Anema *et al*., 2004; Renan *et al*., 2007), but decreases (along with density) when more κ-casein is involved (Donato & Guyomarc'h, 2009). It has also been shown that the complexes have an increased surface hydrophobicity compared to casein micelles (Jean *et al*., 2006; Guyomarc'h *et al*., 2007), meaning the serum complexes should be prone to hydrophobic reactions.

2.1.4 Methods of milk protein analysis

2.1.4.1 Total protein determination

In milk, as well as in other foodstuffs, nitrogen is the element which essentially characterises proteins. Because of this, nitrogen determination has always been used as a standard method for the protein content in food. In dairy products, the nitrogen compounds are made up of 78% caseins and 17% whey proteins while urea, free amino acids and peptides make up the remaining 5% of the non-protein nitrogen fraction (NPN).

The Kjeldahl method (IDF standard 20-2: 2001) is internationally recognised as the reference method for measuring protein content in milk products, and is listed as such in the Codex Alimentarius. It was first published in 1883 by Kjeldahl in Denmark. The principle of the method is as follows: the sample is digested in concentrated sulphuric acid, converting the nitrogen into ammonium sulphate. After the digest, the ammonium sulphate is then converted into ammonia gas by heating with sodium hydroxide. The ammonia is then steam distilled into an excess of boric acid solution to trap the volatile ammonia by forming ammonium borate. The amount of ammonia trapped in the borate is determined by titration with standard hydrochloric acid or sulphuric acid.

The Kjeldahl method has its advantages and disadvantages. Precision and reproducibility is good and because of the method's wide use, it has become the standard method for comparison against other methods. However, the method assumes that all the nitrogen present in a sample is protein, and, because NPN is included in this total nitrogen result given, the protein content is often overestimated.

2.1.4.2 Denatured whey protein determination

A lot of research has been directed towards determining and understanding the denaturation processes of the major whey proteins when milk is heated. Early studies (e.g. Rowland, 1933) precipitated the casein and denatured whey protein by adjustment of pH to the isoelectric point of the casein (4.6) and analysed the supernatant using the Kjeldahl method. This was then compared to the Kjeldahl results for the original milk, giving estimates of initial native whey protein levels and levels after heat treatment. However, because of the need for more rapid analysis, the whey protein nitrogen index (WPNI) method was developed. In the WPNI method, the casein and denatured whey protein are co-precipitated from milk using a saturated solution of sodium chloride. The co-precipitate is removed by filtration, and the remaining native whey proteins are reacted with a known excess of protein binding dye (amido black). The protein-dye complex is removed by centrifugation and the remaining dye is measured colormetrically.

Although the WPNI method is fairly reproducible and can give an estimate of the level of whey protein denaturation, research into the denaturation and interactions of the individual whey proteins requires more accurate separation and analysis procedures. There are several quantitative methods for separating and determining the level of individual whey proteins in milk. Methods that have been used include polyacrylamide gel electrophoresis (e.g. Hillier & Lyster, 1979), capillary electrophoresis (e.g. Fairise and Cayot, 1998) high performance liquid chromatography (e.g. Kessler & Beyer, 1991) and various immuno-based assays (e.g. Lyster, 1970). While these may be more appropriate, they can be more technically difficult and time consuming.

2.1.4.3 High performance liquid chromatography

High performance liquid chromatography (HPLC) allows rapid and automated analysis of milk proteins which is characterised by good separations, high resolutions and accuracy, and reproducible results.

Milk proteins may be separated and quantified by HPLC on the basis of their differing hydrophobicities. Reversed phase HPLC (RP-HPLC) is one such technique and will be used in this project. Recent applications of RP-HPLC in milk protein quantification include studies conducted by Bordin *et al*. (2001), Veloso *et al*. (2002) and Bonfatti *et al*. (2009).

2.1.4.4 Capillary electrophoresis

Capillary electrophoresis is another rapid method of protein separation and quantification. This technique separates ionic species by their charge and frictional forces and was first used to separate whey and casein proteins simultaneously (de Jong *et al*., 1993). Recently, it has been developed to analyse not only concentration, but phosphorylation level and genetic polymorphisms of milk proteins (Heck *et al*., 2008).

2.2 Milk powder

Milk powder is dehydrated milk solids. Its manufacture involves the gentle removal of water from liquid milk at the lowest possible cost under stringent hygiene conditions while still retaining all the desirable properties of liquid milk; colour, flavour and nutritional value (Pearce, 1995). Whole (full cream) milk contains typically about 87% water, while skim (defatted) milk contains about 91%. During milk powder manufacture, this water is removed by boiling at a low temperature under reduced pressure in a process known as evaporation. The resulting concentrated milk is then sprayed as a fine mist into hot air to further remove moisture and give a powder.

The main reasons for converting milk into a powder form are to reduce bulk for storage and transport, to extend shelf-life by reducing water activity and thus preventing or slowing spoilage reactions and to change the product chemically, physically and/or functionally for particular end uses (Pearce, 1995).

2.2.1 Spray dried milk powder processing

Milk powder manufacture (Fig. 2.2) starts with raw milk received at the factory. This is pasteurised (held 72ºC for 15 seconds), and then separated into skim milk and cream fractions using a centrifugal separator. In whole milk powder manufacture, cream is added back into the skim milk to produce milk with a standardised fat content of 26-30% (dry basis). Surplus cream is used to make other products such as butter or anhydrous milk fat.

Preheating is the next step in milk powder production and is critical to end product functionality and shelf life. This process involves heating the standardised milk to temperatures between 75 and 120ºC and holding for a specific length of time. The exact heating and holding time depends on the type of product and its intended end use. Preheating causes controlled denaturation of whey proteins, destroys bacteria, inactivates enzymes, generates natural antioxidants (which in turn prevent the oxidation of fat in whole milk powder), and imparts heat stability. Preheating may be done with indirect heat via heat exchangers or with direct heat via steam injection or infusion, or by a combination of the two.

Evaporation follows the preheating step. Skim milk is concentrated from 9 to 50% w/w total solids in multiple stages (effects) in a falling film evaporator. The evaporation is carried out under a vacuum at temperatures between 40 and 70ºC. The flow of concentrated milk (concentrate) and vapour leaving the evaporator is essentially co-current, with the concentrate leaving at a temperature between 40 and 58ºC. The water vapour driven off of the milk is normally used to heat the next effect of the evaporator, which is operated at a lower pressure and temperature than the preceding effect. This vapour may be compressed mechanically or

thermally, for use as heating steam in the same or the prior effect. More than 85% of the water in milk can be removed in the evaporator.

The concentrate from the evaporator is heated immediately (70-80ºC) before spray drying. This reduces concentrate viscosity and the drier load. Whole milk concentrate is homogenised at this step to reduce fat globule size.

Spray drying involves atomizing the milk concentrate from the evaporator into very fine droplets. This is done at the top of a large drying chamber, a drier, in a flow of hot air (180-245ºC) using either a spinning disk atomizer or a series of high pressure nozzles. As the atomized droplets fall through the hot air, water is removed, reducing the moisture content to around 6%. A fine powder results, with a mean particle size of typically <0.1 mm diameter. Final or secondary drying takes place in a fluid bed, or series of such beds, in which hot air is blown though a layer of 'fluidised' powder, removing water to give the product a moisture content of 2-4%. Cyclones are then used for primary separation of fine powder from the exhaust air. Bag filters are used for final treatment of exhaust air.

From the fluid bed, the powder is stored in hoppers until it is ready for export to the packing line.

Once a powder has been manufactured, proximate analysis, as well as physical, microbiological, sensory and functional tests are carried out to ensure the product is suitable for its intended application. Tests that are routinely used for checking quality and grading milk powder are listed in Appendix A.

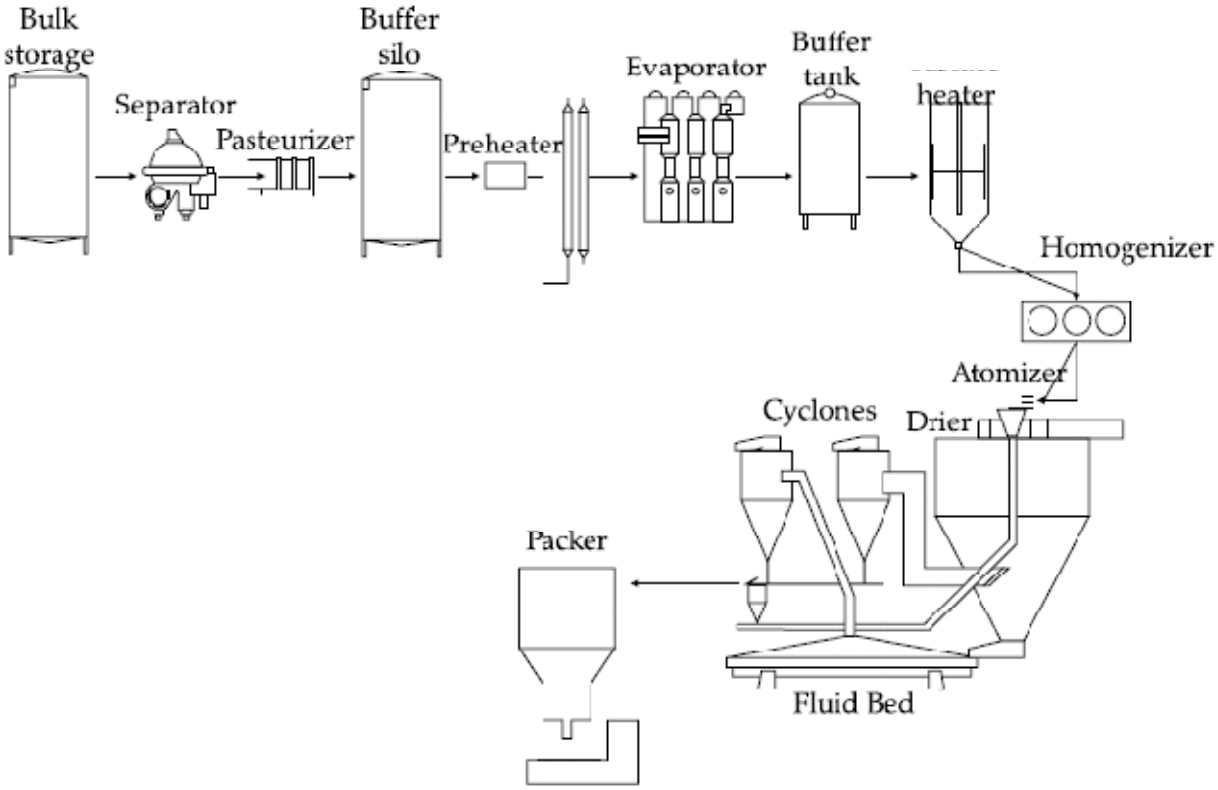


Figure 2.2 Flow diagram of the milk powder manufacturing process (Pearce, 1995)

2.2.2 Milk powder applications and functionality

As previously mentioned, the main purposes for producing milk powder from milk are to 1) reduce bulk for storage and transport, 2) extend shelf-life and 3) change the product chemically, physically and/or functionally for particular end uses. The first two points are highly significant from an export point of view; e.g. dried milk is a cost effective and convenient method of meeting the needs of people in countries that produce less milk than is consumed. However it is the final point which is of most relevance to this study and will be discussed in detail here.

Milk powder is used widely as an industrial ingredient in value-added products. It is incorporated into foodstuffs such as confectionary, ice cream, bakery goods, infant formulae, fortified nutritional blends, convenience foods and fermented dairy products such as yoghurt. Depending on the desired end product, plant conditions can be modified to produce powder to meet a customer's specification. An example of this is powder agglomeration. Standard powders, because of their fine dusty nature, do not reconstitute well in water. To counter this, agglomerated "instant" powders were developed. During processing, the small particles of powder ("fines") leaving the drier are recovered in cyclones and returned to the drying chamber in close proximity of the atomiser. The wet concentrate droplets collide with the fines and stick together, forming larger, irregular shaped agglomerates. The result is a less dusty product that dissolves in water more easily.

2.2.3 Influence of preheat treatment on milk powder functionality

Another processing consideration, highly important to powder functionality, is the level of preheat treatment received. As previously mentioned in section 2.1.3 of this review, heating of milk to temperatures above 60ºC induces chemical changes, namely the modification (denaturation) of whey proteins. Because of this, the extent of whey protein denaturation is commonly used as an index to estimate the total heat treatment applied to a milk powder during manufacture. This in turn gives an approximation of the powder's expected functional properties.

In the early 1960s, the American Dry Milk Institute (now the Amercian Dairy Products Institute), published standards of dry milk. Employing WPNI, the widely accepted heat classification of milk powder was put in place. Low, medium and high heat categories were established to reflect the application of increasing preheat temperatures before milk evaporation. As shown in table 2.2 values are reported as "milligrams of undenatured whey

protein nitrogen per gram of (skim) milk powder", with a working range of >6 for low heat treatments, to <2 for high heat treatment.

Table 2.2 Heat classification of skim milk powders based on the WPNI, the typical heat treatments used in their manufacture, their functional properties and suggested applications (adapted from Kelly *et al*., 2003)

Heat Treatment Classification	Typical preheat combination of temperature/time	WPNI (mg of N / g powder)	Functional properties	Applications or end uses
Low heat	70ºC/15 s	≥ 6.0	Solubility Lack of cooked flavour	Recombined milk Cheese making Standardisation
Medium heat	95ºC/35 s	6.0 - 1.5	Emulsification Foaming Water absorption Viscosity	Ice cream Confectionary
High heat	120ºC/120 s	≤ 1.5	Heat stability Water binding Gelation	Recombined evaporated milk Sweetened condensed milk Bakery products

The heat classification of milk powders was originally introduced in order to supply skim milk powder suitable for incorporation in bread. The addition of milk solids improves the eating quality and shelf life of bread, however milk powder of low or medium heat treatment depresses loaf structure. Because of this, a simple system for high heat powder classification was required. Other applications of high heat powder include recombined evaporated milk which withstands in-tin heat sterilisation (ie. is heat stable).

While high heat treatments are related to improved keeping quality of powder, other functionality factors must be taken into consideration. High preheat temperatures are generally associated with greatly increased concentrate viscosity, plant fouling and decreased solubility of the final product. Also, high heat powders are not as suitable as low heat powders for typical beverage use (instant milk powder) or for the manufacture of recombined hard cheeses. Minimal levels of whey protein denaturation are necessary in these applications to retain similar sensory properties to fresh milk on reconstitution, and to reduce interference with syneresis (moisture expulsion), respectively.

2.3 Acid coagulation of milk (fermentation)

In acid coagulation of milk, as opposed to enzyme-induced coagulation used for cheese-making, the casein micelle properties are altered by lowered milk pH (Lucey & Singh, 1997). This causes CCP to dissociate from the micelles, meaning the negative charges of the micelles are neutralised. Aggregation of the micelles then occurs at pH 4.6, the isoelectric point of the casein micelle. A porous network of loosely linked aggregates is formed and this is known as a milk gel.

Acidified milk gels are one of the oldest and most popular foodstuffs produced throughout the entire world. The popularity of fermented milk products such as yoghurt and fresh acid-coagulated cheese varieties (e.g. cream cheese, cottage cheese, quarg), is due to favourable sensory properties (texture and taste) as well as health claims and curative benefits.

2.3.1 Acidification approaches

The acidification of milk to form gels can be achieved several ways. Cultures of lactic acid bacteria (LAB), which ferment lactose to lactic acid, are used for fermentation products as yoghurt and cottage cheese. For research approaches, where a precise amount of acid addition is needed, direct addition of acids is another means of acidification and these may be inorganic (HCl) or organic (gluconic acid). During the last 20 years, milk acidification with glucono-δ-lactone (GDL), which hydrolyses to gluconic acid, has been commonly used for investigating changes that occur in milk and on casein micelles during gel formation.

2.3.2 Mechanisms of gel formation

At normal milk pH (6.70), most proteins have a net negative charge causing electrostatic repulsion which stabilises the casein micelles. When the pH is dropped to 6.00, the net negative charge on the casein micelles is lowered, resulting in a reduction of the electrostatic repulsion. The structural size of the micelle remains the same, as little CCP is dissolved above pH 6.0 (Lucey, 2004). From pH 6.0 to ~5.0, deionisation of the κ-casein tails occurs, resulting in an increase in micelle surface potential. Also deionised are the acidic residues of α- and β-caseins (aspartic, glutamic, phosphoserine), which causes progressive transfer of CCP into the aqueous phase (CCP in milk is dissolved entirely by around pH 5.0). With the charges of casein molecules neutralised and electrostatic repulsion reduced, hydrophobic interactions between the caseins then occurs.

Milk for fermented milk products is generally subjected to a reasonably severe heat treatment, e.g. 90°C for 5-10 minutes, which results in a) denatured whey proteins and b) κ-casein dissociation from the micelles. Through disulphide bonding of these proteins, a cross- linked

gel network is built up between whey protein–κ-casein-complexes, and whey protein–κ-casein-micelle-complexes.

2.3.3 Factors affecting acid coagulation

The process of acid coagulation of milk is influenced by many different factors, mainly pH (during heat-treatment), temperature (heat-treatment and during incubation) and whey protein concentration as outlined below.

The effect of temperature prior to acidification has been widely documented and will be focussed on in this study. Compared with unheated milk, milk heated at high temperatures (up to 100ºC) for several minutes results in acid milk gels with greatly improved characteristics. Some of the first to report heat induced changes were Dannenberg and Kessler (1988) who showed that gel firmness was increased as a consequence of denatured whey protein and κ-casein interactions. Since then, numerous studies of gel viscoelasticity and microstructure have been carried out, revealing that heat-treated milk gels have more homogeneous networks with higher connectivity, lower porosity, and a higher whey retention capacity (reviewed in Donato & Guyomarc'h, 2009). It has also been shown that the pH of gelation is lowered and that such heat-induced changes have been positively correlated with the denaturation rate of whey proteins and the formation of the denatured whey protein/ κ-casein complexes (Donato & Guyomarc'h, 2009).

Recent studies have demonstrated that the pH of milk during heat treatment is another factor affecting coagulation properties (Anema & Li, 2003; Donato *et al*., 2006). This can markedly influence the level of denatured whey protein/casein micelle interaction and κ-casein dissociation and subsequently the gels formed on acidification. For example, gels produced from milk that had been adjusted to pH 7.1 prior to heat treatment were approximately twice as firm as those heated at pH 6.5 (Anema *et al*., 2004). This increase in firmness has been explained by denatured whey protein distribution, with more serum complexes (as opposed to casein bound complexes) observed in the firmer gels (Anema *et al*., 2004).

Because of its responsibility in the formation of gel structure, the protein fraction of milk is an obvious factor influencing acid coagulation properties. Increasing milk concentration increases protein concentration. An increased number of protein components means more contact points within a given unit area and greater structural connectivity; subsequently gel firmness is increased (Anema, 2008). The composition of the protein fraction of milk is also significant, with the importance of whey proteins and κ-casein and denaturation level already discussed.

Genetic polymorphisms in both caseins and whey proteins have shown to influence protein expression and physico-chemical properties. For example, the B allele of κ-casein has been associated with higher κ-casein concentration in milk compared to A and also with higher total protein and casein ratio (Hallen, 2008). This author also showed that AA and AB genotypes of β-lactoglobulin are associated with increased total β-lactoglobulin concentration in milk compared to cows carrying BB alleles.

2.4 Acid coagulation properties of reconstituted milk powder

Many studies have used reconstituted skim milk powder for studying the acid gelation of milk (e.g. Lucey *et al.,* 2000; Lee & Lucey, 2004; Anema, 2008a,b). This allows variables such as milk concentration and composition to be controlled easily, as milk powder is generally well characterised by routine proximate analysis. However, proximate analysis does not provide detail (concentration, denaturation level, or genotype) on individual caseins or whey proteins. This is unfortunate when specific protein interactions are the key to functional properties, such as gel formation, in milk.

A vast amount of research has been devoted to the heat-induced modifications of milk proteins and the unequivocal effects on gel formation. In spite of this, little has been published on the effect of heat treatment received during the manufacture of milk powder in regards to fermented product applications.

With the issues outlined above in mind, the objective of this project therefore was to examine the protein profiles and the effect of preheat treatment on the acid coagulation properties of reconsituted milk powder.

Chapter 3
Rheological Characteristics of Reconstituted Milk Powder

3.1 Introduction

Acid coagulated dairy products are popular due to their semi-solid (viscoelastic) textural properties. In products such as yoghurt, failure to coagulate and form a sufficiently firm gel means a less acceptable product for consumers. Because of this, monitoring the coagulation process and analysing textural properties is of important commercial interest.

The rheological and textural properties of viscoelastic materials can be determined from several test techniques including small amplitude oscillatory rheology (SAOR), large amplitude oscillatory shear, penetration, and texture profile analysis. The ideal method should be able to observe the gel formation process, making dynamic and non-destructive measurement and techniques such as SAOR the most useful.

A structured system, such as the cross-linked network of a milk gel, will gain energy from oscillatory motion as long as the motion does not disrupt the structure. This energy is stored in the sample and is described by storage modulus (G'). The magnitude of the storage modulus depends on the number of interactions between the components in a sample and the strength of each interaction; it is a measure of the structure of a sample. Storage modulus is therefore a very useful means of monitoring structural changes in a sample, such as those during milk coagulation.

Many studies have used SOAR for examining the coagulation properties of milk and milk powder under various conditions (e.g. Vasbinder *et al.,* 2003; Lee & Lucey, 2004; Lakemond & van Vliet, 2008; Anema, 2008a,b). Few however, with the exception of Augustin *et al*. (1999) and Lucey *et al*. (1997), have investigated the differences in coagulation properties of different milk powders.

This chapter describes the coagulation properties of different reconstituted skim milk powders as determined by SAOR. Coagulation times and final gel strengths of the samples, determined on the basis of storage modulus, were studied.

The effects of experimental factors on coagulation properties were also investigated. Incubation temperature was varied with the expectation that it would influence coagulation time and gel strength. To examine the effect of milk solids concentration on these parameters,

solutions of 11% and 14% total solids were used. These concentrations were chosen to simulate fresh skim milk and yoghurt dry matter concentrations respectively.

Strength of the set gels after cooling was also studied, as commercial acidified milk products (e.g. yoghurt) are usually stored and often consumed at refrigerator temperatures (approximately 5ºC).

3.2 Methods and materials

3.2.1 Milk powder samples

15 samples of commercial skim milk powder were obtained for survey. Of these 7 were produced in NZ, while the other eight were manufactured in Europe. Powders ranged in stated heat treatment classification; low, medium, high. Two samples did not have heat treatment defined. WPNI was specified for 12 of the 15 samples.

Full proximate analysis reports of the powder samples were not available. Information on manufacture and processing conditions from dairy companies could not be obtained due to their confidentiality concerns, and little other information was unfortunately provided on each sample.

3.2.2 Chemicals and reagents

Glucono-δ-lactone (GDL), with a stated purity of 99-100%, was purchased from Sigma-Aldrich Pty. Ltd, Stockholm, Sweden. Distilled water was used throughout.

3.2.3 Preparation of reconstituted milk powder

Milk powder samples were prepared to 11 and 14% total solids (w/v). Powder was weighed and mixed with a small amount of water to form a homogeneous paste. This was made up to volume in a measuring cylinder and transferred into a beaker. The solution was mixed for 30 minutes with a magnetic stirrer, and left to stand for a further 30 minutes at room temperature. The milk was then refrigerated overnight before analysis the following day.

3.2.4 Rheological analysis

A standardised amount of GDL (0.18 g per g of milk powder) was added to each reconstituted milk. This equated to 2 and 2.5% GDL (w/v) for 11% and 14% TS milks, respectively. Each solution was mixed vigorously with a spatula for 30 seconds. The solution was then transferred immediately to the C25 measuring cup of a Bohlin VOR rheometer (Malvern Instruments Nordic AB, Uppsala, Sweden) fitted with a 2 g cm torsion bar. Oscillation mode

was used with a frequency of 1 Hz and a constant strain of 0.0412. Photographic documentation of the rheometer set up may be seen in Appendix B.1.

Each measurement was carried out for 5 hours at a set incubation temperature (20, 30 and for some samples also at 40ºC). After 5 hours, the test temperature was ramped down over a 30 minute period, before being held at 5ºC for 15 minutes. Curd firmness (storage modulus; G') of the developing gel was plotted against time. Coagulation time (CT) was recorded as the time from GDL addition until a 0.5 Pa increase in elastic modulus was detected by the instrument. Curd firmness was recorded after 5 hours and again at the end of the 5ºC period.

Rheological measurements of samples were carried out only once at the selected testing conditions (varied temperature or milk concentration). This was due to the highly time consuming nature of the test; nearly 6 hours per sample. However, before sample testing commenced, the reproducibility of the method was studied.

3.3 Results

3.3.1 Repeatability of the rheological measurements

A powder was reconstituted a total of 10 separate times, each analysed on the rheometer (see appendix B.2 for an illustration of this). Mean coagulation time, gel strength at 5 hours, and gel strength at 5ºC was 1.18 ± 0.04 hours, 203.6 ± 14.6 Pa, and 435.7 ± 25.7 Pa respectively. The results verified the reliability of the method, and the decision to place confidence in unduplicated runs was made.

3.3.2 Coagulation time (CT)

Milk concentration (11 vs 14% TS) did not greatly affect CT (Table 3.1). The greatest difference between treatments was 0.25 hrs (15 mins), while the mean difference was just 0.11 ± 0.02 hrs or (6.6 ± 1.2 mins).

Table 3.1 Coagulation times of different reconstituted skim milk powders at different total solids concentrations (11 vs 14%)

Sample	Origin	Heat treatment classification	WPNI (mgN/g)	11% (hrs)	14% (hrs)	ΔCT 11 vs 14% (hrs)
1	NZ	Medium	2.9	1.08	1.09	0.01
2	NZ	Medium	2.9	0.94	0.94	0.00
3	NZ	Medium	2.6	1.01	0.91	0.10
4	NZ	Medium	1.7	0.96	0.77	0.19
5	NZ	Medium	3	1.12	1.14	0.02
6	NZ	Low	7.6	1.68	1.89	0.21
7	NZ	High	0.5	1.04	0.93	0.11
8	Sweden	Medium	3.9	0.87	0.80	0.07
9	Sweden	Low	6	0.92	0.94	0.02
10	Sweden	Low	6	1.09	1.16	0.07
11	Sweden	Medium	5.3	0.81	0.78	0.03
12	Sweden	High	0.9	0.81	0.81	0.00
13	Sweden	Unspecified	Unspecified	1.71	1.96	0.25
14	Sweden	Unspecified	Unspecified	1.59	1.54	0.05
15	Netherlands	Low	Unspecified	2.01	1.93	0.08
						Std Dev = 0.11

The CT of milk powder samples reconstituted to 14% total solids are shown in Table 3.2. From this data it can be seen that coagulation time decreased with increased incubation temperature.

With regards to the different powders, lengthened coagulation times were generally seen in the low heat samples and those with no heat classification specified (samples 6,10,13,14,15). This trend was observed at all test temperatures, but was more pronounced at 20°C.

Table 3.2 Coagulation times of different reconstituted skim milk powders (14% TS) at varied run temperatures

Sample	Origin	Heat treatment classification	WPNI (mgN/g)	CT, 40°C (hours)	CT, 30°C (hours)	CT, 20°C (hours)
1	NZ	Medium	2.9	0.33	1.09	3.15
2	NZ	Medium	2.9	ND	0.94	2.60
3	NZ	Medium	2.6	ND	0.91	2.64
4	NZ	Medium	1.7	ND	0.77	2.58
5	NZ	Medium	3	ND	1.14	2.80
6	NZ	Low	7.6	0.76	1.89	4.09
7	NZ	High	0.5	0.29	0.93	2.75
8	Sweden	Medium	3.9	ND	0.80	2.19
9	Sweden	Low	6	ND	0.94	2.36
10	Sweden	Low	6	ND	1.16	3.54
11	Sweden	Medium	5.3	0.26	0.78	2.44
12	Sweden	High	0.9	0.21	0.81	2.47
13	Sweden	Unspecified	Unspecified	ND	1.96	3.36
14	Sweden	Unspecified	Unspecified	ND	1.54	3.07
15	Netherlands	Low	Unspecified	0.77	1.93	4.28

ND = Not determined

An example of a typical rheometer run result, following the coagulation of different reconstituted milk powders, may be seen in Figure 3.1. Notice the small difference of the CT between medium and high heat treated powder milk and the much slower CT of the low heat powder milk.

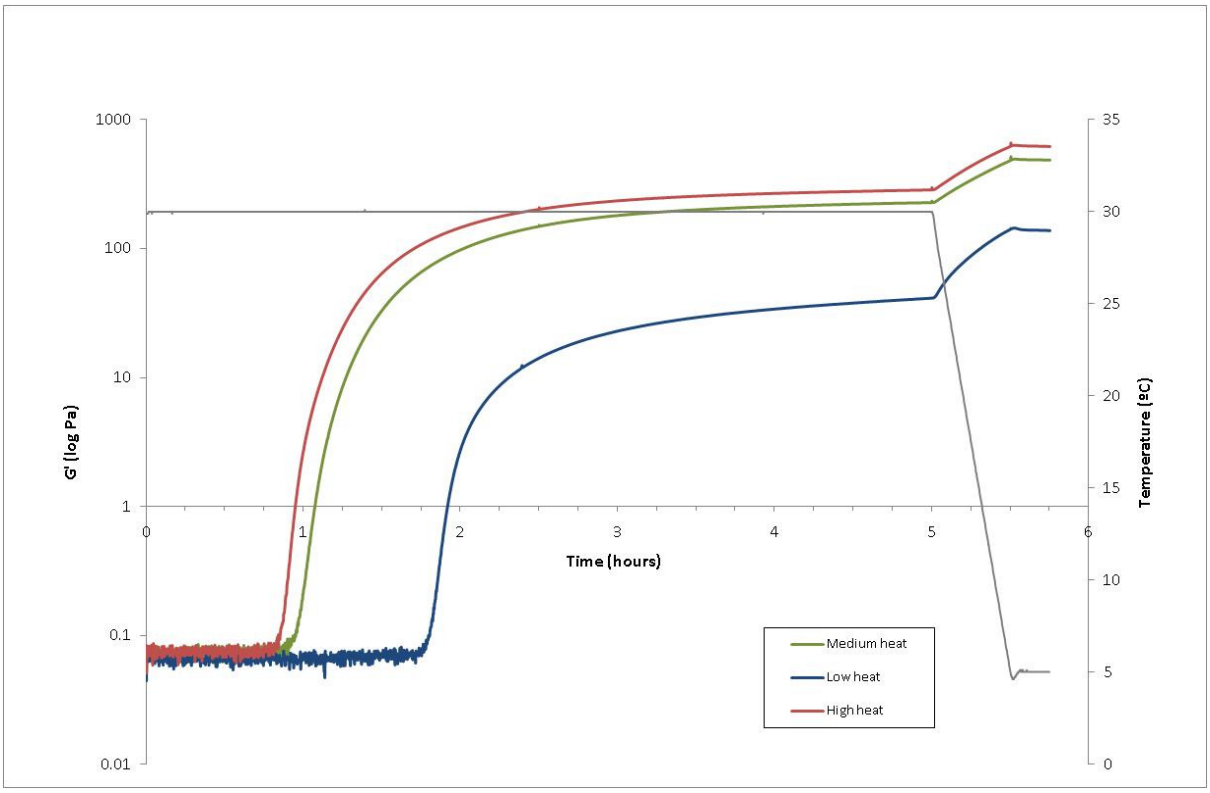


Figure 3.1 Example of rheological behaviour of different (low, medium and high heat) reconstituted milk powders acidified with GDL. Graph produced from Bohlin VOR rheometer data output.

3.3.3 Gel strength

Gel firmness of samples, expressed as G' after 5 hours at the given incubation temperature, are shown in Table 3.3. When tested at 20ºC compared to 30ºC, G' decreased at least 56% in all except two samples (samples 13 and 14). Of the samples tested at 40ºC, both G' increases and decreases were observed when compared to the 30ºC data.

At all tested temperatures, it appeared that the lowest G' values were generally produced by the low heat samples and those with unspecified heat class (samples 6,13,14,15). However, the Swedish low heat samples (9 and 10) did not behave like the NZ low heat (sample 6). These displayed much higher G' values at both 30ºC (274 and 231 Pa compared to 41) and 20ºC (99 and 33 Pa compared to 13) respectively.

Table 3.3 Gel strength of different reconstituted skim milk powders (14% TS) with varied run temperatures

Sample	Origin	Heat treatment classification	WPNI (mg N / g)	G' 40ºC (Pa)	G' 30ºC (Pa)	G' 20ºC (Pa)	ΔG' between 30 and 20ºC (%)
1	NZ	Medium	2.9	323	229	47	-79
2	NZ	Medium	2.9	ND	239	77	-68
3	NZ	Medium	2.6	ND	255	81	-68
4	NZ	Medium	1.7	ND	310	98	-69
5	NZ	Medium	3	ND	238	61	-74
6	NZ	Low	7.6	27	41	13	-68
7	NZ	High	0.5	416	284	69	-76
8	Sweden	Medium	3.9	ND	357	130	-64
9	Sweden	Low	6	ND	274	99	-64
10	Sweden	Low	6	ND	231	33	-86
11	Sweden	Medium	5.3	374	386	121	-69
12	Sweden	High	0.9	192	298	131	-56
13	Sweden	Unspecified	Unspecified	ND	40	33	-17
14	Sweden	Unspecified	Unspecified	ND	46	39	-15
15	Netherlands	Low	Unspecified	66	50	14	-72

ND = not determined

Figure 3.2 shows the effect of milk concentration on gel strength. Samples prepared at a lower total solids concentration (11%) had 41% lower G' values on average after 5 hours at 30ºC, than 14%.

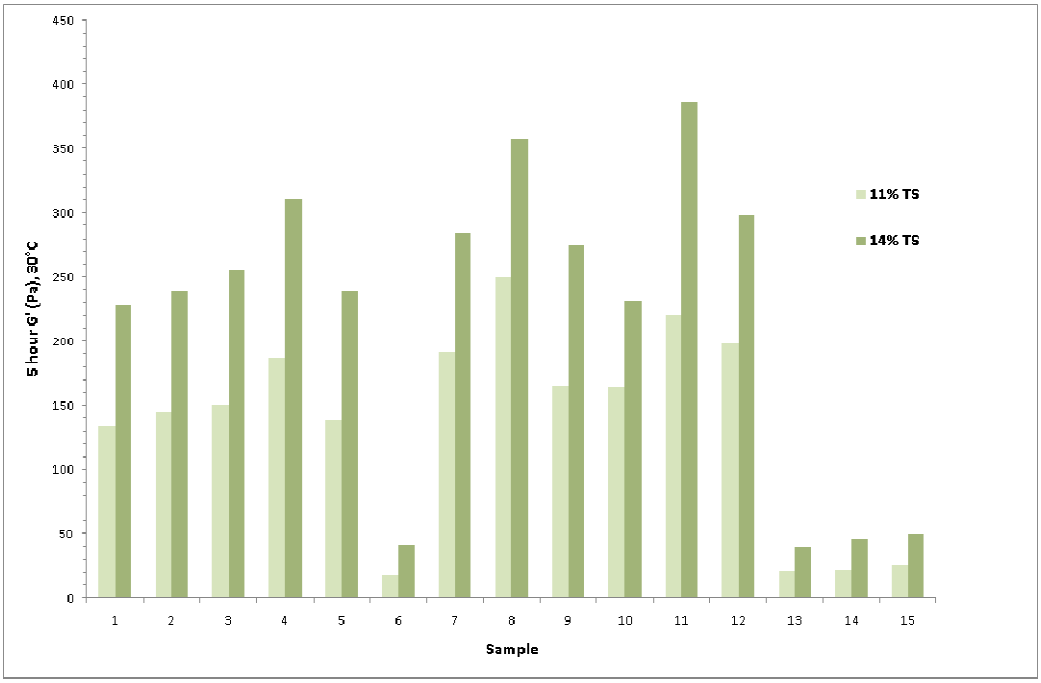


Figure 3.2 Comparison of gel strength with varied milk concentration. Milks prepared at lower TS concentration (11% versus 14%) produced gels with lower G' values after 5 hours.

Cooling the gels had a marked effect on gel strength. G' values at the end of the 15 minutes at 5ºC period were at least double that recorded at the end of the 5 hour incubation at 30ºC (Fig. 3.3)

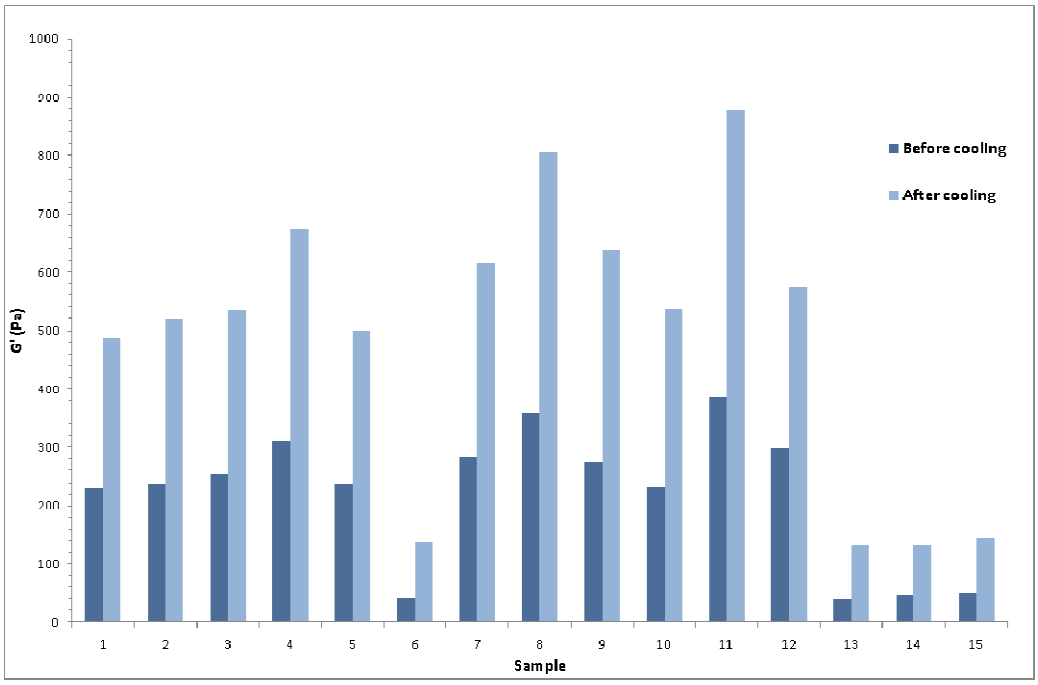


Figure 3.3 Comparison of gel strength before and after cooling at 5ºC.

3.4 Discussion

The use of small amplitude oscillatory rheology (SAOR) to investigate the coagulation properties of milk is a powerful tool that has been used by a number of researchers(e.g. Vasbinder *et al.,* 2003; Lee & Lucey, 2004; Lakemond & van Vliet, 2008; Anema, 2008a,b). In this study SAOR technology, utilising the latest Bohlin C-VOR system, showed that coagulation times of reconstituted skim milk powders was decreased with increased run temperature, but not affected by dry matter concentration. Gel strength was increased with increased milk concentration , and was also influenced by incubation temperature. Cooling of the developed gels increased gel strength. With regards to the heat classification of samples, the general trends observed were longer coagulation times and lower final G' values in the classified low heat and unclassified heat treatment samples compared to the medium and high heat classified samples.

Milk concentration did not affect coagulation time because GDL addition was standardised, with equivalent amounts of acid used per gram milk powder. Subsequently the acidification rate was standardised.

Decreased coagulation time with increased incubation temperature could be explained by reaction kinetics; a higher incubation temperature meant that a higher rate of GDL hydrolysis to gluconic acid took place. In all likelihood, it was faster acid production at the higher temperature that lead to shorter gelation times (pH of gelation reached more quickly), and this has been documented in several other studies that also used GDL for milk acidification (e.g. Fetahagic *et al*., 2002; Anema, 2008b).

Milks incubated at 30ºC compared to 20ºC had higher G' values after 5 hours. This is a trend that has been observed by others (Anema, 2008b), and may be put down to the higher GDL hydrolysis and acidification rate. However, when samples were incubated at 40ºC, G' did not increase further as expected. In samples 6, 11 and 12, G' was lower at 40ºC compared to gels produced at 30ºC. One possible reason for this may be the loosening of intermolecular forces in casein particles caused by solubilisation of colloidal calcium phosphate. Work by Lucey *et al.* (2000) and Anema (2008b) both suggested this when incubation at 40ºC resulted in faster acidification but weaker gels.

The observations of increased gel strength with increased milk concentration were expected. Increasing milk concentration increases protein concentration, and consequently an increase the number of protein interactions in the gel network. G' directly measured the increase. Similar work conducted by Anema (2008a) showed the same trend; higher final G' values in milk gels with higher total solids concentration.

All gels showed marked increases in G' after being cooled at 5ºC in this study. This trend has been described in other studies (Lucey *et al*., l997; Anema, 2008b), and may be explained by hydrophobic interactions. Hydrophobic reactions are strongly dependent on temperature and play an important role in the assembly of casein micelles (Horne, 1998). It has been suggested that the decreased strength of hydrophobic interaction when the temperature is reduced may lead to a less compact conformation of the casein molecules, subsequently increasing their size within the acid gel network. This is thought to alter the balance between inter-particle and intra-particle bonds, so that there are more inter-particle bonds between casein molecules as the temperature is reduced, resulting in notable increases in G' (van Vliet *et al*., 1989).

The slower coagulation times and low gel strengths observed in the low heat and unclassified heat treatment samples (particularly obvious in samples 6, 13, 14 and 15) may be related to protein composition and processing conditions, namely a lower level of thermally denatured whey protein. As already described in the literature review, denatured whey proteins are critical in the gelation of acidified milk. While samples 13 and 14 had no WPNI or heat classification stated, it could be assumed that they contained low levels of denatured whey protein and were low heat powders on the basis of their poor coagulation properties. This assumption can be supported by work conducted by Augustin *et al*. (1999). These authors examined the effects of varied preheat treatments of milk powder (specific time and temperature combinations), looking at the coagulation properties of yoghurt prepared from each powder. Yoghurt gel strength and syneresis level was analysed, as was denatured whey protein concentration. Denatured whey protein level increased with increased preheat treatment as expected. Trends between higher denatured whey protein concentration and increased gel firmness were noted, as well as decreased whey drainage (syneresis). It is possible that similar underlying whey protein and heat treatment trends were being witnessed in this study with regards to the different coagulation abilities of different powders.

Another relevant study, examining the effects of preheat treatment on the rheological properties reconstituted milk powder gels, was that of Lucey *et al*. (1997). Acid gels made from high, medium, low and ultra low heat skim milk powder were investigated. The results of this project could be described as similar to study conducted by Lucey *et al.* (1997), where the rheological results of high and medium heat milk powders produced gels with higher G' values than gels made from low heat milk powders. Other trends, such as reduced coagulation time with increasing heat treatment, may also relate to observations in this study.

Lucey *et al*. (1997) found that gel strength and coagulation time only differed slightly between high and medium heat powders, but a large difference existed between these and the low heat powders. It is possible that this trend was also being seen in this study. In the New

Zealand powders for example, the high heat sample had a similar G' to the medium heats (284 Pa compared to an average of 254 Pa), yet the low heat sample was much lower (41 Pa). When considering the low heat European samples however, samples 9 and 10 did not follow the same trend. The gel strength of these samples, compared to all other low heat or unclassified samples (6, 13, 14, 15), was markedly higher (274 and 231 Pa compared to 41, 40, 46 and 50 respectively). One possible reason for this contrast may be differences in WPNI. The New Zealand low heat sample had a reported WPNI value of 7.6 mg N/g, while samples 9 and 10 were 6.0 mg N/g. This meant that the New Zealand sample contained higher amounts of undenatured whey protein, indicating a lower level of heat treatment. It is possible that, at 6.0 mg N/g compared to 7.6 mg N/g, enough whey protein was thermally denatured by preheat treatment to form a sufficiently firm gel network.

However, because of low sample numbers and missing information (heat classification and/or WPNI values) this is purely speculation. Protein analysis of all samples was therefore required to confirm any such threshold effects, and/or relationships between protein levels and coagulation properties. This is investigated in the following two chapters.

It was observed that Swedish medium heat powders produced stronger gels compared to the New Zealand counterparts. Samples 8 and 11 (Swedish) had an average final G' of 372 Pa, while the average of the New Zealand medium heats (samples 1-5) was 254 Pa. While there were not enough samples/results to confirm statistical differences, these dissimilarities may be explained by differences in milk composition due to different cow breeds; Swedish Holstein and Swedish Red & White, versus New Zealand Holstein Fresian and Jersey. Differences in levels of individual milk proteins became of even more interest for this reason.

Chapter 4
Protein Profiling on Milk Powders

4.1 Introduction

Milk protein is divided into two major fractions; caseins and whey proteins. Unlike caseins, whey proteins are susceptible to thermal denaturation owing to their globular tertiary structure (Fox & McSweeney, 1998). The unfolding of this structure largely determines the behaviour of milk in subsequent applications (Anema, 2009).

During the manufacture of milk powder, milk undergoes several heat treatments; pasteurisation, preheating and drying. The level of native whey protein remaining in final product is measured by the WPNI method. WPNI analysis is the industry standard for indicating the thermal history of the milk, particularly preheat treatment (Anema, 2009). Total protein in the final product is routinely measured by Kjeldahl analysis (IDF Standard, 2001).

Unfortunately, Kjeldahl and WPNI analysis do not provide any information on individual caseins or individual whey protein levels. Because of the interest in the denaturation level and interaction of the individual proteins in relation to coagulation ability, particularly the associations between β-lactoglobulin and κ-casein (Dannenberg & Kessler, 1988) this study required methods of more accurate milk protein separation and analysis. Capillary electrophoresis (CE) was used to examine the various protein fractions in milk under denaturing conditions. Upon examination of these results, it was decided that another method of protein quantification was required. Subsequently, two HPLC methods were employed; one to quantify total protein levels regardless of state (analysed after denaturation) and one to analyse whey proteins in only their native (undenatured) state.

4.2 Methods and materials

4.2.1 Capillary electrophoresis quantification of milk proteins

4.2.1.1 Chemicals

Hydroxymethyl-aminomethane (TRIS), 3-morpholinopropanesulphonic acid (MOPS), ethylene-diamine-tetraacetic acid disodium salt dihydrate (EDTA), dithiothreitol (DTT) methylhydroxyethylcellulose 30000 (MHEC), citric acid, urea, and sodium citrate were all of analytical grade, purchased from Sigma Aldrich (Stockholm, Sweden).

4.2.1.2 Buffer preparation

The sample buffer (pH 8.6) consisted of 167 mM TRIS, 42 mM MOPS, 67 mM EDTA, 17 mM DDT, 6M urea and 0.05% (w/w) MHEC. The run buffer (pH 3.0) consisted of 0.19 M citric acid, 20 mM sodium citrate, 6 M urea and 0.05% (w/w) MHEC.

4.2.1.3 Sample preparation

Aliquots of the 15 reconstituted milk powder samples remaining from rheological analysis (see section 3.2.3), were saved and frozen at -15ºC until capillary electrophoresis analysis was carried out. A thawed sample (335 µL) was taken and mixed with 500 µL sample buffer. After mixing, the sample solution was left at room temperature for 1 h and subsequently centrifuged for 5 minutes at 5000 *g*.

4.2.1.4 Capillary electrophoresis

Capillary electrophoresis of reconstituted milk powder samples was carried out on a Beckman P/ACE MDQ Capillary Electrophoresis system controlled by 32 Karat™ Software, version 7.0 (Beckman Instruments, CA, USA). The method of separation used was as described by Heck *et al*. (2008). Separation was carried out in fused silica capillaries with dimensions 40 cm x 50 µm I.D. Samples were injected by pressure at 0.5 psi for 20 seconds. Detection was achieved with a UV detector set at 214 nm. Data was processed with the P/ACE System Series Software. Standard curves were constructed from a protein mixture (α-LA, β-LG, α_{S2}-CN, α_{S1}-CN, κ-CN, β-CN A1, β-CN A2) injected at various volumes.

4.2.2 High performance liquid chromatography determination of total milk proteins

4.2.2.1 Chemicals

Acetonitrile (CH_3CN), hydrochloric acid (HCl), trifluoroacetic acid (TFA), urea, tris-HCl, tri-sodium citrate and dithiothreitol (DTT) were used. All chemicals were of HPLC analytical grade and purchased from VWR International (Lutterwoth, United Kingdom). Demineralised ultrapure water was used throughout.

4.2.2.2 Buffer preparation

Buffer A (0.1% TFA in water) and buffer B (0.1% TFA in acetonitrile) were prepared by adding 1 mL of TFA into 999 mL of water or acetonitrile respectively.

Sample buffer for protein denaturation was prepared by dissolving 24 g urea, 0.78 g Tris-HCl, 0.65 g tri-sodium citrate and 38 mg DDT in 42 mL water. Dilution buffer was prepared by dissolving 18 g urea in 45 ml 0.1% TFA (buffer A).

4.2.2.3 Sample preparation

Unfortunately, because of difficulty in transferring samples internationally, not all 15 powders analysed for CE in Sweden could be obtained for HPLC testing in New Zealand. However, three of the same New Zealand powders (high, medium and low heat; samples 1, 6 and 7 respectively) were available for HPLC analysis. These were reconstituted to 10% total solids using the same procedure described in section 3.2.3. Skim milk (200 µL) was directly added into 800 µL sample buffer and mixed well. After standing for 1 hour at room temperature, the sample was centrifuged at 14,000 *g* for 10 min. The sample was then diluted 1:3 in dilution buffer and centrifuged at 14,000 *g* for 10 min.

4.2.2.4 Reversed phase high performance liquid chromatography

The HPLC equipment consisted of a Shimadzu High Performance Liquid Chromatography system, equipped with a binary pump gradient unit and autosampler. A photo diode array detector was used. The equipment was controlled by Class V.P. 7.0 software. Separations were performed on a reversed-phase analytical column C8 (Zorbax 300SB-C8 RP, Agilent Technologies) with a silica-based packing (3.5 µm, 300 Å, 150 x 4.6 I.D.). The chromatographic conditions used were as described by Bonfatti *et al*. (2008). The flow rate was 0.48 mL/min, the column temperature was kept at 45ºC, and detection was made at wavelengths of 214 and 280 nm. The injection volume was 10 µL. Results were calculated from extinction coefficients at 280 nm, α-LA = 20.06, β-LG = 9.41, κ-casein = 9.5, α_{s1}-casein = 10.5, α_{s2}-casein = 1, and β-casein = 4.6 (S. Chen, personal communication September 29, 2009; R&D Scientist at Westland Milk Products Ltd).

4.2.3 High performance liquid chromatography of native whey proteins

4.2.3.1 Chemicals

Methanol (CH_3OH), hydrochloric acid (HCl) and trifluoroacetic acid (TFA) were used. All were of HPLC analytical grade and purchased from VWR International (Lutterworth, United Kingdom). Demineralised ultrapure water was used throughout.

4.2.3.2 Buffer preparation

Buffer A (0.03% TFA in water) and buffer B (0.022% TFA in methanol) were prepared by making 0.30 mL or 0.22 mL TFA up to 1 L with water or methanol respectively.

4.2.3.3 Sample preparation

Remaining reconstituted powder samples from HPLC analysis of major proteins (section 4.2.2.3) were adjusted to pH 4.6 with a known amount of 5% HCl. After filtration from the curd, the whey was centrifuged at 14,000 *g* for 10 minutes and pipetted into autosampler vials for analysis.

4.2.3.4 Reversed phase high performance liquid chromatography

The HPLC equipment was the same as that used in section 4.2.2. Separations were performed on a reversed-phase analytical column C8 (Zorbax 300SB-C8 RP, Agilent Technologies) with a silica-based packing (3.5 µm, 300 Å, 150 x 4.6 I.D.). The chromatographic conditions used were as follows: isocratic elution for 8.5 minutes at 55% buffer B, then a linear gradient from 55-79% buffer B over 1 minute, followed by an isocratic elution at 79% buffer B for 9.5 minutes, then a linear gradient return to starting conditions of 55% buffer B over 1 minute, remaining here for further 3 minutes. The flow rate was 1.00 mL/min, the column temperature was kept at 42ºC, and the detection was made at a wavelength of 280 nm. The injection volume was 50 µL. Results were calculated from α-LA and β-LG standard curves.

4.3 Results

4.3.1 Capillary electrophoresis quantification of milk proteins

Concentrations of the major protein fractions as determined by capillary electrophoresis (electropherogram example shown in Figure 4.1) are presented in Table 4.1. Sample 4, a New Zealand medium heat powder, had the highest concentration of major proteins; 33.6 mg/mL. Apart from this sample, all other milks had low major protein concentrations compared to the reference values e.g. 14 out of 15 samples were outside the expected range.

There was difficulty in detecting α-LA and α_{S2}-CN. This may have contributed to the low concentrations of major proteins; however, it is likely that the low levels of β-LG and β-CN found had a greater influence. The concentrations of these proteins were significantly lower than those reported in the literature; less than half in most cases.

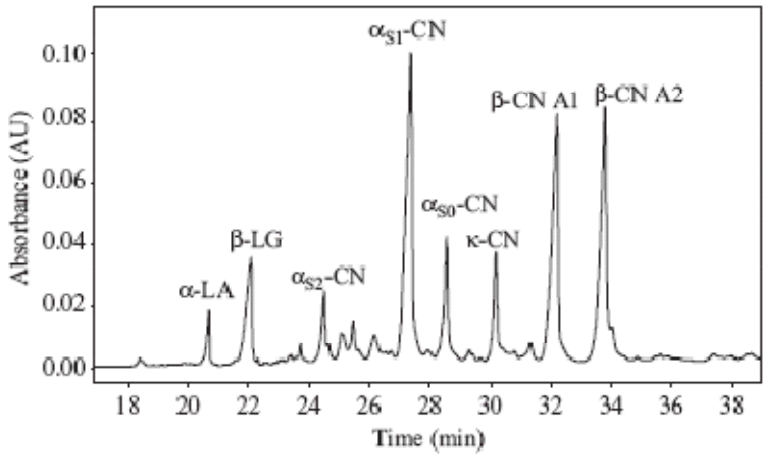


Figure 4.1 Example of an electropherogram of milk proteins.
α-LA: alpha-lactalbumin; β-LG: beta-lactoglobulin; α_{S2}-CN: alpha S2-casein; α_{S1}-CN: α S1-casein; α_{S0}-CN: α S0-casein; κ-CN: kappa casein; β-CN A1: beta-casein A1; β -CN A2: beta-casein A2.

Table 4.1 Concentration of milk proteins in reconstituted milk powder samples as determined by capillary electrophoresis. There was difficulty in α_{S2}-casein detection.

Sample	Heat treatment	WPNI	α-LA	β-LG	α_{S2}-CN	α_{S1}-CN	κ-CN	β-CN	Total
		mg N/g	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml
1	Medium	2.9	1.1	1.4	1.2	12.2	5.5	6.5	27.9
2	Medium	2.9	1.0	1.2	1.4	8.7	4.8	6.3	23.4
3	Medium	2.6	1.1	1.2	1.2	10.2	6.1	7.0	26.8
4	Medium	1.7	1.2	1.3	1.5	13.2	6.9	9.5	33.6
5	Medium	3	1.1	1.2	ND	10.6	6.1	9.8	28.8
6	Low	7.6	1.0	1.2	ND	9.6	5.6	6.3	23.7
7	High	0.5	0.8	0.9	ND	5.0	4.6	5.2	16.5
8	Medium	3.9	1.2	1.4	ND	9.5	4.5	4.7	21.3
9	Low	6	1.2	0.6	ND	5.2	3.0	4.5	14.5
10	Low	6	1.3	0.7	ND	5.8	3.0	5.5	16.3
11	Medium	5.3	1.2	0.6	ND	5.4	3.2	4.5	14.9
12	High	0.9	ND	0.6	ND	4.1	3.0	4.5	12.2
13	Unknown	Unknown	1.2	0.5	ND	4.9	2.9	4.1	13.6
14	Unknown	Unknown	1.2	0.8	ND	5.3	2.9	4.7	14.9
15	Low	Unknown	1.1	0.7	ND	5.9	2.9	4.9	15.5
Reference values (Ng-Kwai-Hang, 2002)			1.2	3.2	2.6	10	3.3	9.3	29.6

ND = not detectable

4.3.2 High performance liquid chromatography

Concentration totals of milk proteins after denaturation, as determined by the RP-HPLC, are shown in Table 4.2. This established that the samples had typical overall protein concentrations (approximately 3.5%) and that there were no abnormalities in detection. A typical chromatogram is shown in Figure 4.2.

Table 4.2 Concentration (g/ 100 mL) of major milk proteins in reconstituted milk powder as determined by RP-HPLC

Sample	Caseins (%)					Whey Proteins (%)			Total protein
	α_{S1}-CN	α_{s2}-CN	β-CN	κ-CN	Total CN	α-LA	β-LG	Total WP	CN +WP (%)
Low heat	1.13	0.36	1.17	0.32	2.98	0.11	0.39	0.50	3.48
Medium heat	1.18	0.43	1.28	0.30	3.19	0.12	0.39	0.51	3.70
High heat	1.15	0.36	1.36	0.31	3.18	0.11	0.38	0.49	3.67

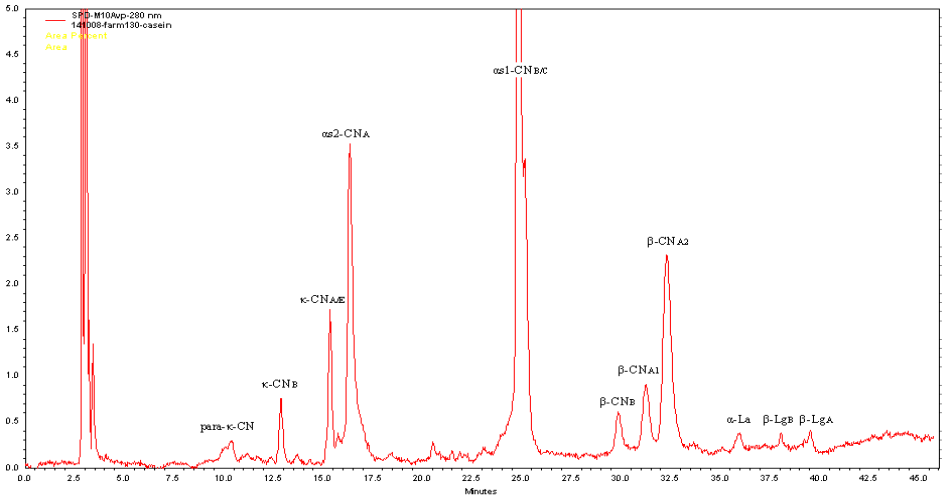


Figure 4.2 Typical chromatogram of milk sample analysed by the RP-HPLC method outlined in section 4.2.2. Peaks of major proteins and genotype are labelled: α-LA: alpha-lactalbumin; β-LG: beta-lactoglobulin; α_{S2}-CN: alpha S2-casein; α_{S1}-CN: α S1-casein; κ-CN: kappa casein; β-CN A1: beta-casein A1; β -CN A2: beta-casein A2.

Table 4.3 shows the concentrations of native (undenatured) whey proteins, quantified by the second method of RP-HPLC described in section 4.2.3. Decreased native whey protein concentrations were observed with increasing heat treatment.

Table 4.3 Concentration of native major whey proteins in reconsituted milk powder as determined by RP-HPLC

Sample	**Native Whey Proteins (%)**		
	α-LA	**β-LG**	**Total WP**
Low heat	0.065	0.261	0.326
Medium heat	0.047	0.099	0.146
High heat	0.012	0.007	0.019

4.4 Discussion

It is difficult to draw conclusions from the capillary electrophoresis data. When compared to reference values (Ng-Kwai-Hang, 2002), it is apparent that protein losses have occurred in most samples. The sample preparation or CE analysis may have caused low recoveries. It is possible that samples were not adequately reconstituted, resulting in low protein concentration. Another possibility may be that the 6M urea sample buffer was not concentrated enough to solubilise all of the protein in the reconstituted sample. In raw milk, for which Heck *et al*. (2008) developed the method, no difficulties in protein separation by CE were experienced. Because of time constraints in this study, the validity of this method for testing reconstituted milk powder was assumed and not fully established before use. However, the process of drying alters milk chemistry, making milk powder vastly different from fresh milk. Thermal denaturation of whey proteins takes place and disulphide bonding with κ-casein occurs. It is more than likely that these enhanced protein-protein interactions interfered with separation and that higher amounts of urea are required for complete protein solubilisation and determination in milk powder.Consequently the results of CE in this study were were inconclusive and uninformative.

In contrast to the CE results, the total protein concentration determined by HPLC was as expected; approximately 3.5%. This placed confidence in the reconstitution method, and it was presumed that errors in protein detection had arisen during CE analysis.

The first HPLC method described analysed the concentration of each individual protein fraction using denaturing conditions. The results showed little difference between the different powders. The second HPLC method however, analysed whey proteins in their native state, and differences between the different powders were apparent. Levels of native whey protein decreased with increasing heat treatment category, consistent with the concept of thermally induced whey protein denaturation (Anema, 2009).

Chapter 5
General Discussion, Conclusions and Recommendations

5.1 Relationships between rheology and protein

Milk proteins have a significant role in the acid coagulation of milk. The association of caseins and denatured whey proteins, especially κ-casein and β-lactoglobulin, results in the formation of a cross-linked gel network. Heating milk at high temperatures prior to acidification denatures whey proteins, and the positive effects of this on coagulation properties are well established. Because of this, it is common practice in the food industry to subject milk to relatively severe heat treatment when it is intended for use in gelled dairy products.

Rheological characteristics and protein profiles of different reconstituted skim milk powders were compared. After acidification of each sample with GDL, small amplitude oscillatory rheology was used to follow gel formation and gel firmness. CE was used to quantify the individual protein fractions in the milk. Unfortunately the CE data appeared to be unsatisfactory; proteins did not separate sufficiently and only half of the expected total protein concentration could be accounted for. Because of this, the CE results were not considered further, and RP-HPLC was used instead.

Native whey protein levels (α-LA and β-LG) and the rheological features (CT and G') of three of the New Zealand powders (a low, medium and high heat) were examined. Unfortunately, relationships between rheology and protein analysis cannot be confirmed in this study due to low sample numbers, varying amounts of information about samples (manufacturing conditions, proximate composition), and variation in sample size between rheological and protein testing. The results do conform to the well established concept however, that increased levels of thermally induced whey protein denaturation improves coagulation ability, as decreased CTs and increased G' values were observed.

Much research has been done on protein interactions and the rheological properties during the acidification and gelation of milk. Several studies in particular (Anema, 2008b; Lucey *et al*., 1997; Lucey & Singh, 1997; van Marle & Zoon, 1995; van Vliet & Keetels, 1995) have discussed the effects of processing variables, especially the preheating of milk on the formation and properties of gels prepared by acidification with GDL. Such studies showed that the increased heat treatment of milk prior to acidification results in reduced gelation time

and increased gel firmness. This was explained by the increased associations between denatured whey proteins and caseins. It is reasonable to apply the same explanation to observations made in this study. The reconstituted milks analysed had recieved heat treatment during powder manufacture, with heat treatment category and/or WPNI indicating the severity. Powders that had recieved greater heat treatments had improved coagulation characteristics, and native whey protein analysis showed that higher heat treatments resulted in greater levels of whey protein denaturation.

5.2 Conclusion and Recommendations

Milk powder is more versatile than liquid milk and can be used in a large number of applications. Acid coagulated dairy products, such as yoghurt, is an example of a very important application.

In this study, the acid coagulation properties of different reconstituted skim milk powders were investigated. The aim was to understand factors that may influence undesirable textural properties, as consumer acceptability of acid coagulated dairy products relies largely on this.

Texture is a physical property that derives from the structure of food and the way its constituent ingredients interact. In this study, the structural properties of acid coagulated milk gels were examined by rheological analysis. Proteins, the milk constituents of main significance to coagulation, were quantified.

Milk powders classified as low heat coagulated more slowly than powders which were categorised as medium or high heat. Furthermore, low heat powders produced weaker gels compared to medium and high heat powders. Milk protein analysis by HPLC showed that native whey protein concentration decreased in powders with increasing heat category (low to high).

Although the associations seen between coagulation characteristics and the protein profiles in this study are not statistically confirmed, many studies support the notion that increased heat treatment of milk improves coagulation properties due to more complexes between caseins and denatured whey proteins. It may be suggested that low heat skim milk powder is not as suitable as medium or high heat powders in applications where firm texture is an important characteristic of product quality.

The influence of milk powder characteristics on coagulation ability needs to be studied further, as its thermal history greatly influences functionality.

A large study with numerous samples of powders varying in heat treatment classification is required to statistically confirm the effects of processing conditions on milk proteins and acid coagulation ability. This could be extended further than the generalised low, medium or high heat classifications, and investigate the effect of specific temperatures during manufacture, e.g. preheat, spray drier, and fluid bed temperature.

Full protein profile analysis of the raw milk and final product, by way of the HPLC assays used in this study, could be utilised to look at the effects of thermisation on milk proteins. Following this, the acid coagulation characteristics of each reconstituted milk could be analysed rheologically, and any relationships between protein profiles and thermal history could be stasticially deduced.

To test the relevance of the results to real life application, the milks could be tested using microbial fermentation, rather than just standardised acidification with GDL. Sensory analysis could follow, and this would ultimately decide which type of powder is best for the consumer in the final product.

A comprehensive study of this type would be important in predicting and improving the textural qualities of value-added milk powder applications, such as reconstituted skim set yoghurts.

References

American Dry Milk Institute (1971). *Standards for grading of dry milks including methods of analysis*. ADMI Bulletin 916, American Dry Milk Institute, Chicago, Illinois,USA.

Anema, S. & Li, Y. (2003). Effect of pH on the association of denatured whey proteins with casein micelles in heated reconstituted skim milk. *Journal of Agricultural and Food Chemistry* 51, 1640–1646.

Anema, S., Lowe, E., & Li Y. (2004). Effect of pH on the viscosity of heated reconstituted skim milk. *International Dairy Journal* 14, 541-548.

Anema, S. (2007). Role of κ-casein in the association of denatured whey proteins with casein micelles in heated reconstituted milk. *Journal of Agriculutral and Food Chemistry* 55 (9), 3635-3642

Anema, S. (2008a). Effect of milk solids concentration on the gels formed by the acidification of heated pH-adjusted skim milk. *Food Chemistry* 108, 110-118.

Anema S. (2008b). Effect of temperature and rate of acidification on the rheological properties of acid skim milk gels. *Journal of Food Processing and Preservation* 32, 1016-1033

Anema, S. (2009). Chapter 8 - The whey proteins in milk: thermal denaturation, physical interactions and effects on the functional properties of milk. *Milk Proteins: From Expression to Food*, (pp. 239-281).

Annan, W.D. & Manson, W. (1969). A fractionation of the αs-casein complex of bovine milk. *Journal of Dairy Research* 36, 259.

Augustin, M., Cheng, L., & Clarke, P. (1999). Effects of preheat treatment of milk powder on the properties of reconstituted set skim yoghurt. *International Dairy Journal* 9, 415-416.

Bordin, G., Cordeiro Raposo, F., de la Calle, B., & Rodriguez, A.R. (2001). Identification and quantification of major bovine milk protein by liquid chromatography. *Journal of Chromatography A* 928, 63-76.

Bonfatti, V., Grigoletto, L., Cecchinato, A., Gallo, L. & Carnier, P. (2009) Validation of a new reversed-phase high-performance liquid chromatography method for separation and quantification of bovine milk protein genetic variants. *Journal of Chromatography A* 1216(9), 1528.

Creamer, L.K., Bienvenue, A., Nilsson, H., Paulsson, M., van Wanroij, M., Lowe, E.K., Anema, S.G., Boland, M.J. & Jimenez-Flores, R. (2004). Heat-induced redistribution of disulfide bonds in milk proteins. 1. Bovine β-lactoglobulin. *Journal of Agricultural and Food Chemistry* 52(25), 7660-7668.

Dannenberg, F. & Kessler, H.G. (1988). Reaction kinetics of the denaturation of whey proteins in milk. *Journal of Food Science* 53(1), 258-263.

de Jong, N., Visser, S., Olieman., C. (1993). Determination of milk proteins by capillary electrophoresis *Journal of Chromatography A* 652 (1), 207-213.

DeWit, J.N. & Klarenbeek, G. (1984). Effects of various heat treatments on structure and solubility of whey proteins. *Journal of Dairy Science* 67(11), 2701-2710.

Donato, L. & Guyomarc'h, F. (2009). Formation and properties of the whey protein/ κ-casein complexes in heated skim milk – A review. *Dairy Science and Technology* 89, 3-29.

Donato, L., Guyomarc'h, F., Amiot, S. & Dalgleish, D.G. (2007). Formation of whey protein/*κ*-casein complexes in heated milk: Preferential reaction of whey protein with *κ*-casein in the casein micelles. *International Dairy Journal* 17(10), 1161-1167.

Fairise , J. F. & Cayot , P. (1998). New ultrarapid method for the separation of milk proteins by capillary electrophoresis. *Journal of Agricultural and Food Chemistry* 46, 2628-2633.

Farrell, H.M., Malin, E.L., Brown, E.M. & Qi, P.X. (2006). Casein micelle structure: What can be learned from milk synthesis and structural biology? *Current Opinion in Colloid & Interface Science* 11(2-3), 135-147.

Fetahagic S., Macej, O., Djurdedjevic, J. & Jovanovic S. (2002) the influence of FDL concentration on milk pH change during acid coagulation. *Journal of Agricultural Sciences* 47, 75-85.

Fox, P.F. & McSweeney, P.L.H. (1998). *Dairy chemistry and biochemistry* Thomson Science, London.

Groves, M.L. (1969). Some minor components of casein and other phosphoproteins in milk. A review. *Journal of Dairy Science* 52(8), 1155-1165.

Guyomarc'h, F., Law, A.J.R. & Dalgleish, D.G. (2003). Formation of soluble and micelle bound protein aggregates in heated milk. *Journal of Agricultural and Food Chemistry* 51(16), 4652-4660.

Hallén, E. (2008). *Coagulation properties of milk – Association with milk protein composition and genetic polymorphism*. PhD thesis, Swedish University of Agricultural Sciences, Uppsala, Sweden.

Heck, J., Olieman, C., Schennink, A., van Valenberg H., Visker, M., Meuldijk, R. & van Hooijdonk, A. (2008) Estimation of variation in concentration, phosphorylation and genetic polymorphism of milk proteins using capillary zone electrophoresis *International Dairy Journal* 18 (5), 548-555.

Hillier, R. M. & Lyster, R. L. J. (1979). Whey protein denaturation in heated milk and cheese whey. *Journal of Dairy Research* 46, 95-102.

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

Horne, D.S. (1998). Casein interactions: casting light on the black boxes, the structure in dairy products. *International Dairy Journal* 8(3), 171-177.

IDF Standard (2001). *Milk – Determination of Nitrogen content.* International Dairy Federation (IDF Standard 20-2)

Jean, K., Renan, M., Famelart, M., & Guyomarc'h, F. (2006). Structure and surface properties of the serum heat-induced protein aggregates isolated from heated skim milk. *International Dairy Journal* 16, 303–315.

Kelly, A., O'Connell, J. & Fox, P. (Ed.) (2003). Manufacture and properties of milk powder In *Advanced Dairy Chemistry Volume 1:* Proteins (3rd Ed.) (pp. 1027-1054) Kluwer Academic Publishers, New York, USA.

Kessler, H.G. & Beyer , H.J. (1991). Thermal denaturation of whey proteins and its effect in dairy technology. *International Journal of Biological Macromolecules* 13, 165–73

Ikonen, T., Ojala, M. & Syväoja, E.L. (1997). Effects of composite casein and β-lactoglobulin genotypes on renneting properties and composition of bovine milk by assuming an animal model. *Agricultural and Food Science in Finland* 6, 283-294.

Lakemond, C. & van Vliet T. (2008). Rheological properties of acid skim milk gels as affected by the spatial distribution of the structural elements and the interaction forces between them. *International Dairy Journal* 18 (5), 585-593.

Lee, W. & Lucey, J. (2004). Structure and physical properties of yogurt gels: effect of inoculation rate and incubation temperature. *International Dairy Journal* 87, 3153-3164.

Lowe, E.K., Anema, S.G., Bienvenue, A., Boland, M.J., Creamer, L.K. & Jimenez-Flores, R. (2004). Heat-induced redistribution of disulfide bonds in milk proteins. 2. Disulfide bonding patterns between bovine β-lactoglobulin and κ-casein. *Journal of Agricultural and Food Chemistry* 52(25), 7669-7680.

Lucey, A., Tamehana, M., Singh, H. & Munro, P. (2000). Rheological properties of milk gels formed by a combination of rennet and glucono-δ-lactone. *Journal of Dairy Research* 67, 415-427

Lucey, J. (2004). *Formation, structural properties and rheology of acid coagulated milk gels.* Cheese: chemistry, physics and microbiology (3rd Ed.). Elsevier Applied Science, Barking, Essex, UK.

Lucey, J.A., Teo, C.T., Munro, P.A.& Singh H. (1997). Rheological properties at small (dynamic) and large (yield) deformations of acid gels made from heated milk. *Journal of Dairy Research* 67, 591-600.

Lucey, J.A. & Singh, H. (1997). Formation and physical properties of acid milk gels: A review. *Food Research International* 30(7), 529-542.

Lyster, L.J. (1970). The denaturation of α -lactalbumin and β -lactoglobulin in heated milk. *Journal of Dairy Research* 37, 233-243.

Manderson, G.A., Hardman, M.J. & Creamer, L.K. (1998). Effect of heat treatment on the conformation and aggregation of β-lactoglobulin A, B, and C. *Journal of Agricultural and Food Chemistry* 46(12), 5052-5061.

McKenzie, G.H., Norton, R.S., Sawyer, W.H., Marziali, A.S. & Ng-Kwai-Hang, K.F. (1971). Heat-induced interaction of β-lactoglobulin and κ-casein. *Journal of Dairy Research* 38(3), 343-351.

Mellander, O. (1939). Elektrophoretische untersuchungen von casein. *Biochemische Zeitschrift* 300, 240-245.

Ng-Kwai-Hang, K.F. (2002). Milk Proteins. Heterogeneity, Fractionation and Isolation. pp 1881-1894, Encyclopedia of Dairy Sciences. ed. H. Roginski, J.W. Fuquay & P.F. Fox Academic Press.

Pearce, K. (1995) "Milk Powder". Retrieved March 1, 2009 from: http://nzic.org.nz/ChemProcesses/dairy/3C.pdf

Renan, M., Guyomarc'h, F., Chatriot, M., Gamerre, V. & Famelart, M. (2007). Limited Enzymatic Treatment of Skim Milk Using Chymosin Affects the Micelle/Serum Distribution of the Heat-Induced Whey Protein/κ-Casein Aggregates. *Journal of Agricultural and Food Chemistry 55* (16), 6736–6745.

Rowland, S.J. (1933). The heat denaturation of albumin and globulin in milk. *Journal of Dairy Research* 5, 46-53.

Sawyer, W.H. (1969). Complex between β–lactoglobulin and κ–casein. A review. *Journal of Dairy Science* 52(9), 1347-1355.

Schmidt, D.G. (1982). Association of caseins and casein micelle structure. In P. F. Fox (Ed.), *Developments in dairy chemistry* (Vol. 1), *Proteins* (pp. 61–86). London, UK: Applied Science Publishers.

Vasbinder, A., van Mil, P. & Bot, de Kruif, C. (2003). Casein-whey protein interactions in heated milk: The influence of pH. *International Dairy Journal* 13, 669-677.

Veloso, A., Texeira, N. & Ferreira, I. (2002). Separation and quantification of major casein fraction by reverse-phase high-performance liquid chromatography and urea-polyacrylamide gel electrophoresis: Detection of milk adulterations. *Journal of Chromatography A* 967, 209-218.

van Marle, M & Zoon, P (1995). Permeability and rheological properties of microbially and chemically acidified skim-milk gels. *Netherlands Milk and Dairy Journal* 49, 47-65.

van Vliet, T. & Keetels, C. (1995). Effect of preheating milk on the structure of acidified milk gels. *Netherlands Milk and Dairy Journal* 49, 27-35

van Vliet, T., Roefs, S., Zoon, P. & Walstra, P. (1989). Rheological properties of casein gels. *Journal of Dairy Research* 56, 529-534.

Walstra, P. (1999). Casein sub-micelles: do they exist? *International Dairy Journal* 9(3-9), 189-192.

Waugh, D.F. & von Hippel, P.H. (1956). κ–casein and the stabilisation of casein micelles. *Journal of the American Chemical Society* 78, 4576-5582.

Appendix A

A.1 Milk powder proximate analysis

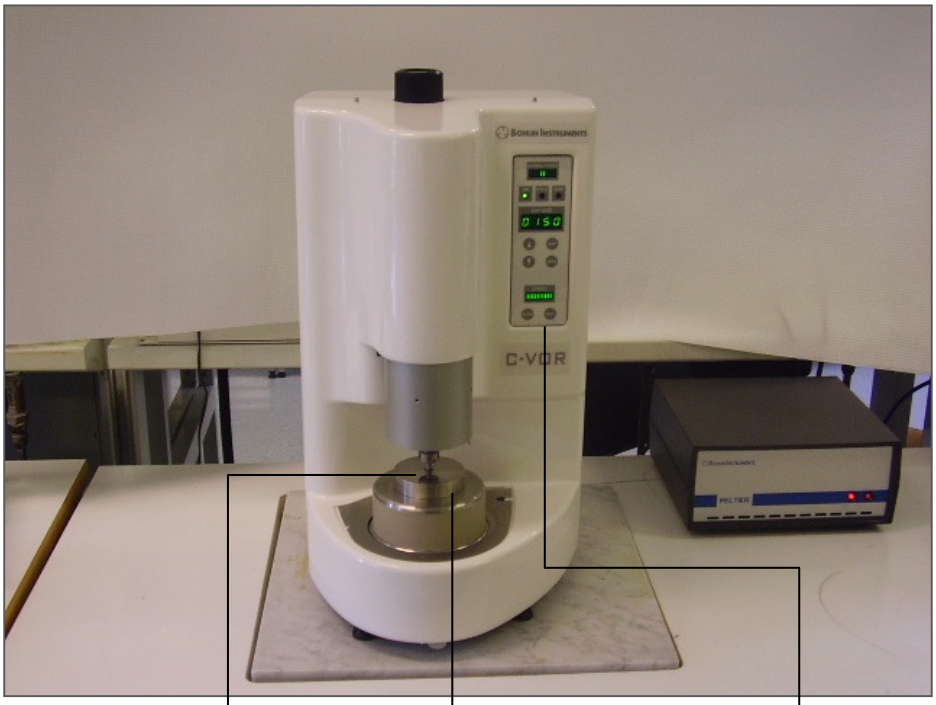
Standard common assay methods of milk powder analysis

Assay	Assay principle
Fat	Gerber, volumetric measurement Rose-Gottlieb, gravimetric measurement Near infrared spectroscopy
Moisture	108ºC air oven gravimetric Karl Fischer titration Toluene azeotropic distillation Near infrared spectroscopy
Titratable acidity	Reconstitute to standard total solids concentration and titrate with standard alkali to pH 8.35
pH	Reconstitute to standard total solids concentration, equilibrate at a standard temperature, glass electrode
Protein	Kjeldahl Kjelfoss Near infrared spectroscopy
WPNI	Reconstitute, precipitate casein and denatured whey proteins with NaCl, determine residual protein with amido black
Lactose	Reconstitute, deproteinate, add chloramine-T and potassium iodide, titrate liberated iodine with thiosulphate Near infrared spectroscopy
Ash	Char sample, then ash in furnace at 550ºC to constant weight
Free fat	Extract with ether and determine gravimetrically
Peroxide value	Fat extraction and colourmetric measurement of ferric thiocyanate formed by peroxide oxidation Fe^{2+} and Fe^{3+}
Insolubility index	Reconstitute, centrifuge and determine volume of insoluble material
Bulk density	Packed density Niro method, Stamph-volumeter Poured density

Appendix B

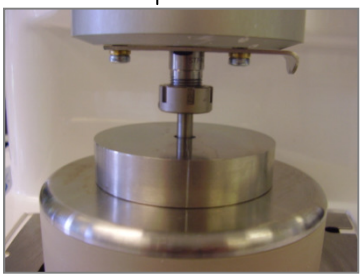
B.1 Rheometer and operation

The Bohlin C-VOR rheometer (Malvern instruments Nordic AB, Uppsala Sweden) used in this study



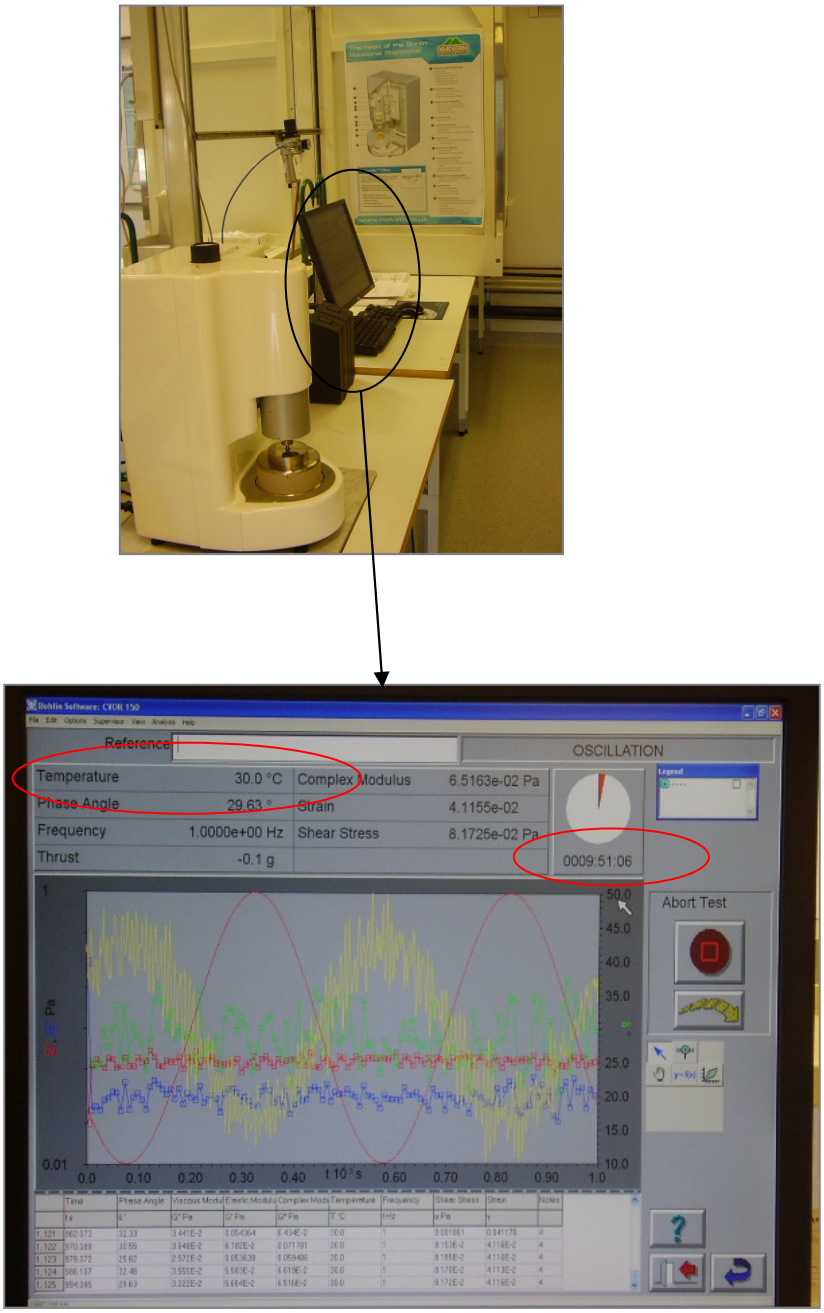
C25 sample cup and torsion bar

Approximately 12 mL of reconstituted milk powder sample transferred to here directly after GDL addition. Lid to prevent sample evaporation pictured to right



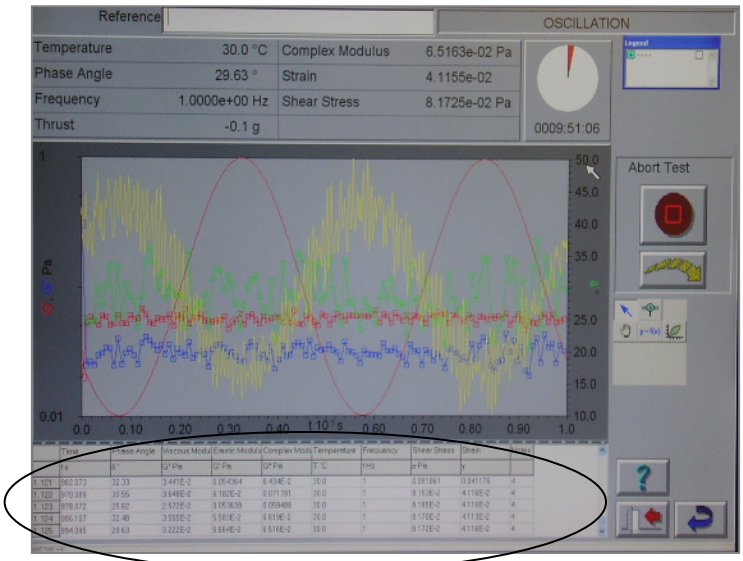
Manual control panel

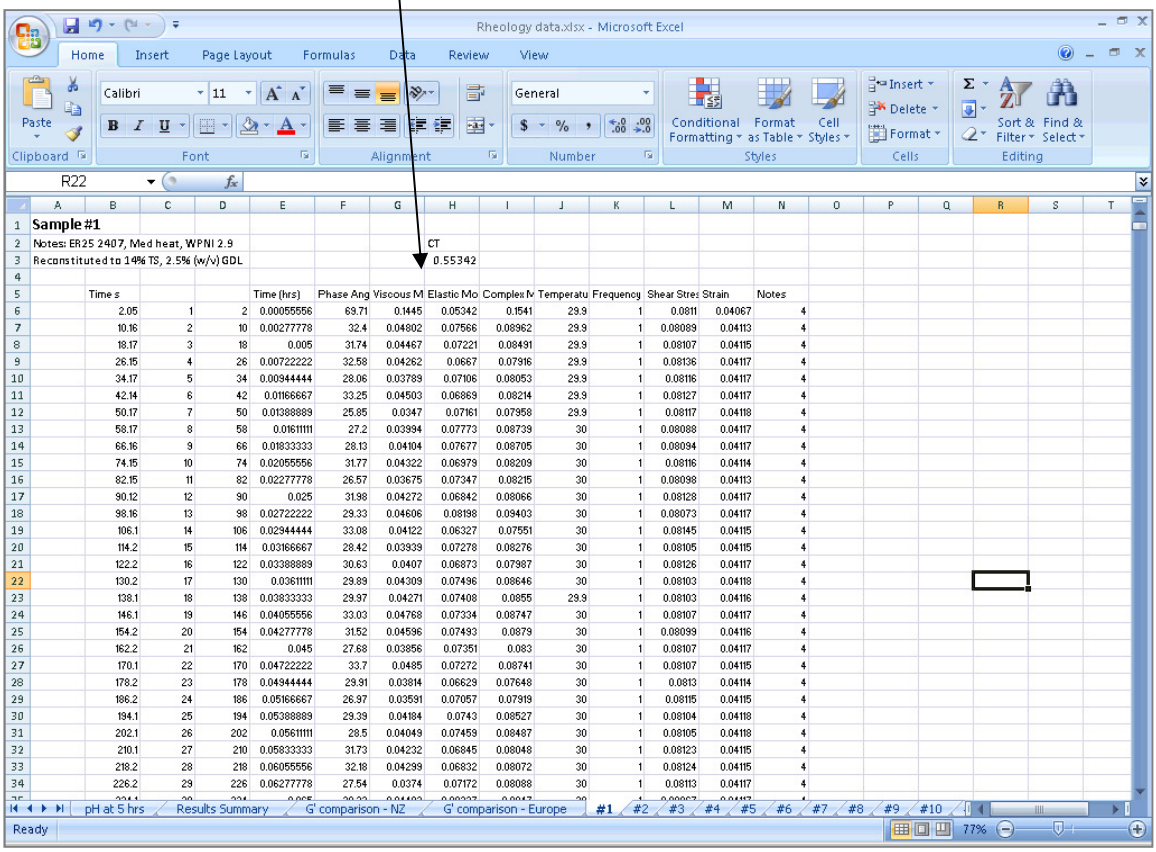
Rheometer set up with controlling PC



Controlling Bohlin software. Oscillation mode was used with a frequency of 1 Hz and a constant strain of 0.0412. Red trace = storage modulus (G'), blue trace = elastic modulus (G''), green trace = loss tangent (δ). In this instance it is 10 minutes into analysis time, incubation temperature 30ºC.

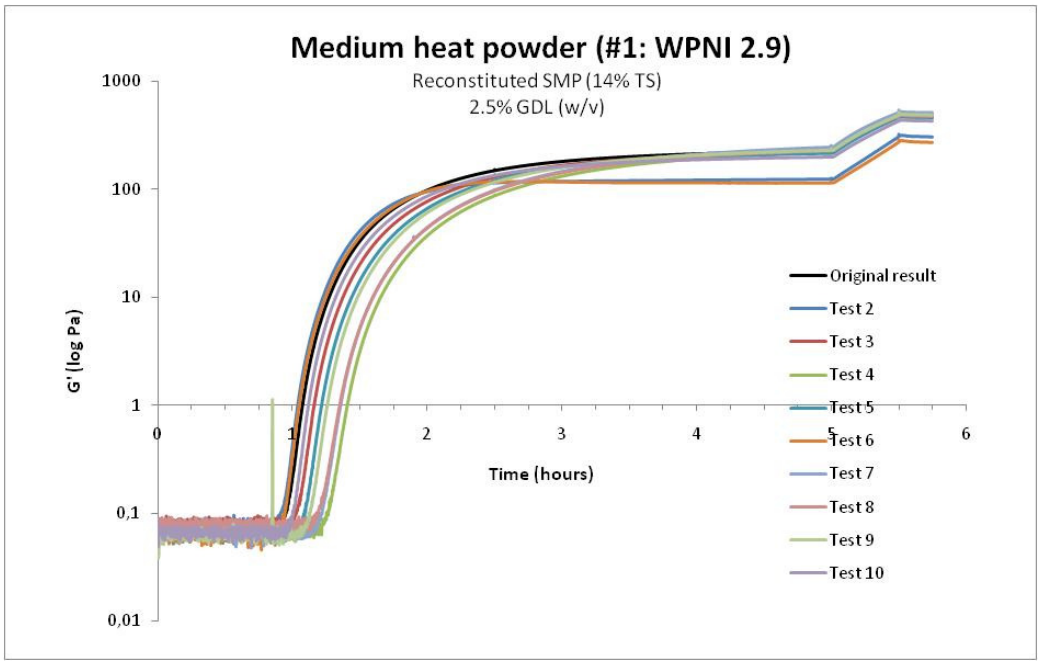
Example of data output from Bohlin software exported into Microsoft® Office Excel®2007 for manipulation. The variable of interest (G') could then be plotted against time (see over page)





B.2 Rheometer repeatability check

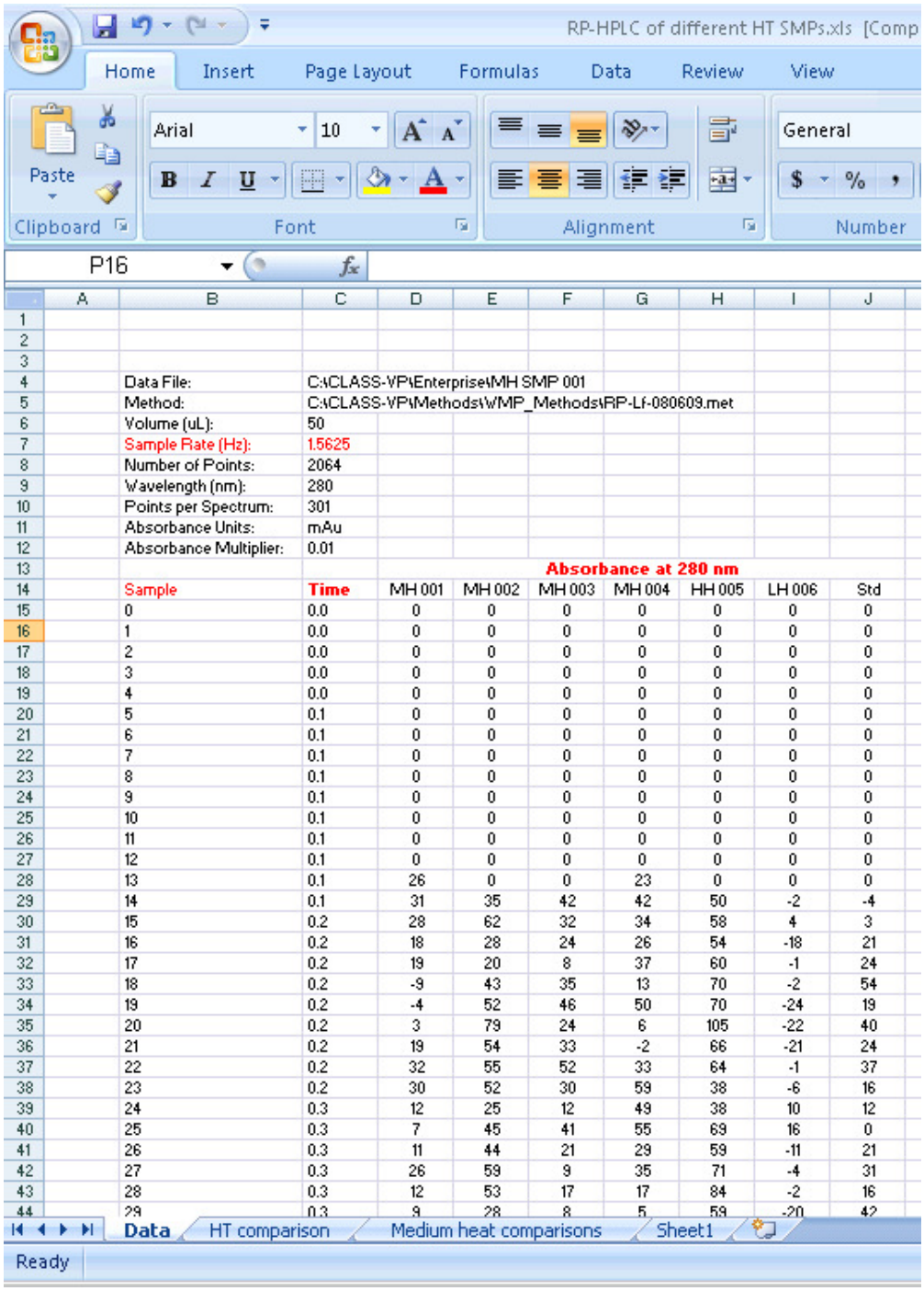
Repeatability of rheometer analysis, described in section 3.3.1, illustrated graphically. Log plot of G' against time.



Appendix C

C.1 HPLC analysis

Example of data from Shimadzu HPLC software (Class V.P. version 7.0) exported into Microsoft® Office Excel®2007 for manipulation.



RP-HPLC of different HT SMPs.xls [Comp

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P16

	A	B	C	D	E	F	G	H	I	J
1										
2										
3										
4		Data File:	C:\CLASS-VP\Enterprise\MH SMP 001							
5		Method:	C:\CLASS-VP\Methods\WMP_Methods\RP-Lf-080609.met							
6		Volume (uL):	50							
7		Sample Rate (Hz):	1.5625							
8		Number of Points:	2064							
9		Wavelength (nm):	280							
10		Points per Spectrum:	301							
11		Absorbance Units:	mAu							
12		Absorbance Multiplier:	0.01							
13				Absorbance at 280 nm						
14		Sample	Time	MH 001	MH 002	MH 003	MH 004	HH 005	LH 006	Std
15		0	0.0	0	0	0	0	0	0	0
16		1	0.0	0	0	0	0	0	0	0
17		2	0.0	0	0	0	0	0	0	0
18		3	0.0	0	0	0	0	0	0	0
19		4	0.0	0	0	0	0	0	0	0
20		5	0.1	0	0	0	0	0	0	0
21		6	0.1	0	0	0	0	0	0	0
22		7	0.1	0	0	0	0	0	0	0
23		8	0.1	0	0	0	0	0	0	0
24		9	0.1	0	0	0	0	0	0	0
25		10	0.1	0	0	0	0	0	0	0
26		11	0.1	0	0	0	0	0	0	0
27		12	0.1	0	0	0	0	0	0	0
28		13	0.1	26	0	0	23	0	0	0
29		14	0.1	31	35	42	42	50	-2	-4
30		15	0.2	28	62	32	34	58	4	3
31		16	0.2	18	28	24	26	54	-18	21
32		17	0.2	19	20	8	37	60	-1	24
33		18	0.2	-9	43	35	13	70	-2	54
34		19	0.2	-4	52	46	50	70	-24	19
35		20	0.2	3	79	24	6	105	-22	40
36		21	0.2	19	54	33	-2	66	-21	24
37		22	0.2	32	55	52	33	64	-1	37
38		23	0.2	30	52	30	59	38	-6	16
39		24	0.3	12	25	12	49	38	10	12
40		25	0.3	7	45	41	55	69	16	0
41		26	0.3	11	44	21	29	59	-11	21
42		27	0.3	26	59	9	35	71	-4	31
43		28	0.3	12	53	17	17	84	-2	16
44		29	0.3	9	28	8	5	59	-20	42

Data | HT comparison | Medium heat comparisons | Sheet1

Ready

Chromatogram of native whey proteins in different skim milk powders; α-lactalbumin peak on left, eluting between minutes 8.5 and 9.0, β-lactoglobulin peaks on right, eluting between 9.5 and 10.5 minutes.