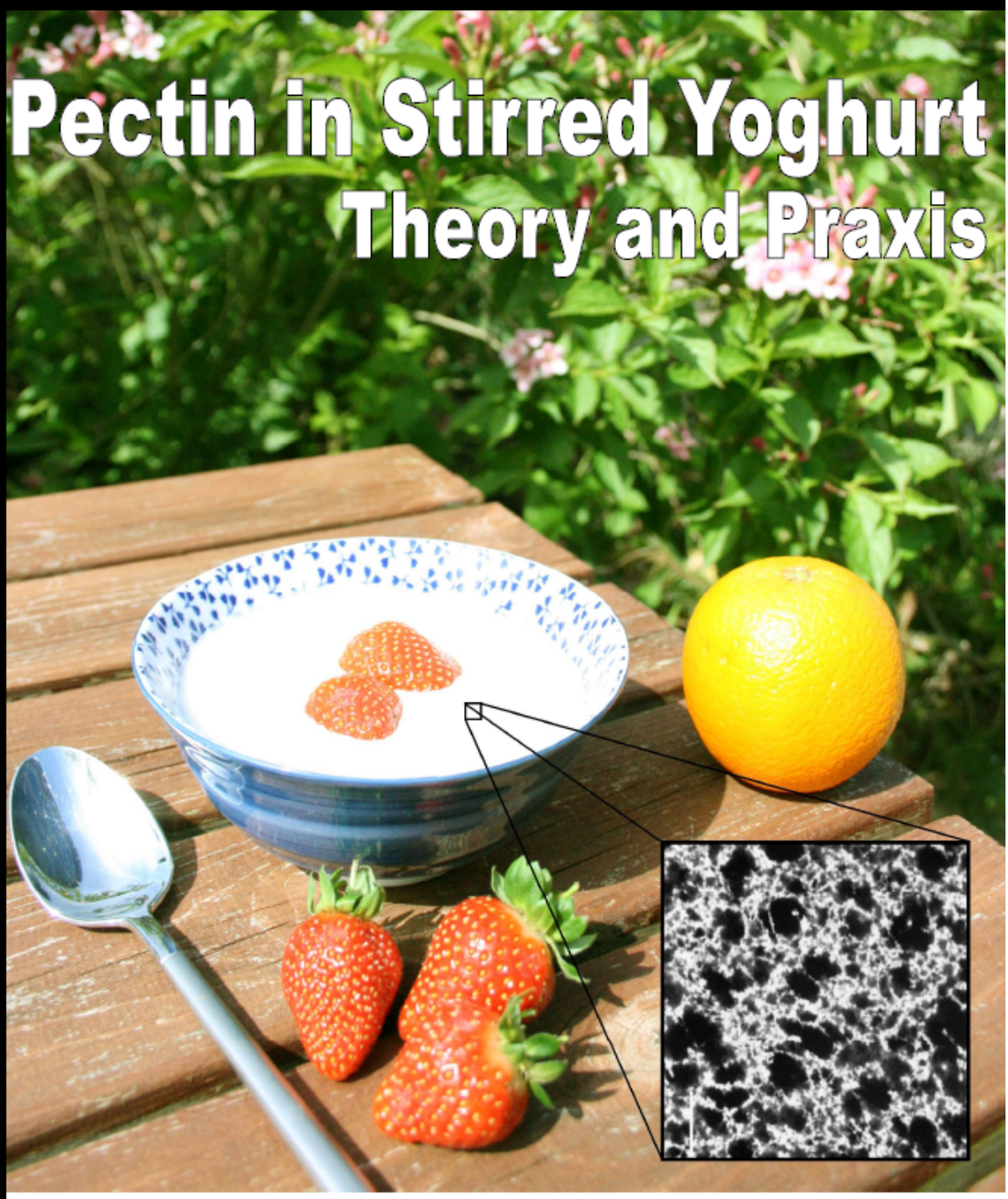
AALBORG UNIVERSITY
Department of Biotechnology, Chemistry and Environmental Engineering

Pectin in Stirred Yoghurt
Theory and Praxis



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Master Thesis

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Front page image: Photo by the author and CLSM image of yoghurt by [Lucey *et al.*, 1999]

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Preface

This report covers the 9th and 10th semester in chemical engineering at the Faculty of Engineering, Science and Medicine at Aalborg University. The project was done between 1st of September and 4th of June 2009 and covers 60 ECTS points as described in "Studievejledning for kemi, 9.-10. semester, 2008-2009, Tema: Langt afgangsprojekt".
The project is addressed mainly to people with a similar or higher educational level in chemistry, biotechnology or engineering. A DVD including all raw and analyzed data has been enclosed.
References have been done using the Harvard System described in [Holland, 2004]. Please note that British spelling have been used and the Danish notation of decimal separator "," is used in figures.

The project has been inspired by Danisco A/S who has also kindly supplied pectin and skim milk powder for the experimental work.

I would like to thank:
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Christian Fiil Nielsen
Aalborg, June 2009

Abstract

The present project has focussed on the application of low methylated (LM) pectin as stabiliser in the production of stirred yoghurt.

The production process of stirred yoghurt is described and combined with a review of recent literature on the subject of yoghurt stabilisation by pectin, this is used as the basis for a description of the interactions during fermentation and the stabilisation mechanism of LM pectin in stirred yoghurt.

The quality parameters of stirred yoghurt are described with the purpose of identifying the functional concentration range of LM pectin, and the limiting mechanisms. These mechanisms are used as the background for selecting and testing experimental methods for their applicability in determining the functional range of pectin and the effect of milk properties on the functional range of pectin.

The upper limit is due to depletion flocculation and is determined by analytical centrifugation. Determining the lower limit of the functional range of pectin requires a deeper understanding of the interactions between milk and pectin during acidification. To determine these, a line of experiments combining rheology and electrophoretic mobility have been carried out. These show that pectin adsorbs to the casein network after gelation without affecting the gelation process, and that the gelation depends strongly on heat treatment of the milk. Using this knowledge a method using electrophoretic mobility to determine the minimum in the functional range have been suggested.

Dielectric spectroscopy has been tested as a tool of monitoring yoghurt fermentation including particle-particle interaction and adsorption of pectin. This has helped identify the challenges and potential of this method. Intrinsic viscosity as a tool of predicting depletion flocculation has also been evaluated, showing some weaknesses of the application.

Resumé af projektet (Danish Abstract)

Dette projekt har fokuseret på anvendelse af lav methyleret (LM) pektin som stabilisator i produktionen af rørt yoghurt.

Produktionsprocessen for rørt yoghurt bliver beskrevet og kombineret med et review af den seneste litteratur om yoghurt stabilisering med pektin, bliver dette brugt som basis for en beskrivelse af interaktionerne under fermenteringen og den stabiliserende mekanisme af LM pektin i rørt yoghurt.

Kvalitetsparametrene for rørt yoghurt er beskrevet med det formål at identificere det funktionelle koncentrationsvindue af LM pektin, og de begrænsende mekanismer.

Disse mekanismer bruges som baggrund for valg og test af eksperimentelle metoder til bestemmelse af deres anvendelighed til at bestemme det funktionelle vindue af pektin og effekterne af mælkens egenskaber herpå.

Den øvre grænse skyldes depletion flokkulering og den bliver bestemt via analytisk centrifugering.

Bestemmelse af den nedre grænse af det funktionelle vindue af pektin kræver en dybere forståelse af interaktionerne mellem mælk og pektin under forsuring. For at bestemme disse blev en forsøgsrække som kombinerer rheologi og elektroforetisk mobilitet gennemført. Denne viste at pektin adsorberer til casein netværket efter gellering, uden at påvirke geleringsprocessen, og at geleringen afhænger kraftigt af varmebehandlingen af mælken. Ved at bruge denne viden er en metode til at bestemme den nedre grænse af det funktionelle vindue ved brug af elektroforetisk mobilitet blevet foreslået.

Dielektrisk spektroskopi er blevet testet som et redskab til at monitorere yoghurt fermentering inklusiv partikel-partikel interaktioner og adsorption af pektin. Dette har hjulpet til at identificere udfordringerne og potentialet af denne metode. Intrinsisk viskositet som et redskab til at bestemme depletion flokkulering er også blevet evalueret, og har vist nogle svagheder ved denne anvendelse.

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1 Project Introduction

The yoghurt production probably exceeds 20 million tonnes per year world wide [Lucey & Singh, 1998] and yoghurt is traditionally produced from whole milk [Tamime & Robinson, 2007]. The consumers in most western countries are becoming increasingly interested in low fat dairy products, e.g. skim milk stirred yoghurt [Valli & Traill, 2004 ; Sandoval-Castilla *et al.*, 2004].

The consumer's expectations to the appearance and sensory experience have not changed [Janhøj et al, 2008 ; Sandoval-Castilla *et al.*, 2004]. This presents a challenge as the milk fat contributes to the appearance and mouthfeel of the yoghurt [Lucey *et al.* 2008b ; Sandoval-Castilla *et al.*, 2004] and plays a crucial role in preventing yoghurt syneresis, which is unacceptable to the consumer [Tamime & Robinson, 2007]. To improve mouthfeel and prevent syneresis in low fat stirred yoghurt, the most often used solution is to add skim milk powder [Tamime & Robinson, 2007 ; Sandoval-Castilla *et al.*, 2004]. Adding skim milk powder adds considerably to the cost of the product, as the amount needed often increases the solids by 33-50 % [Tamime & Robinson, 2007]. An alternative solution is to replace the skim milk powder with a stabiliser [Tamime & Robinson, 2007]. Often pectin is used as pectin is effective in both stabilisation and improved mouthfeel[Tamime & Robinson, 2007] and has a reputation to be a natural, healthy product [Lundt *et al.* 2002 ; Marlett *et al.* 2002].

In order to achieve the desired properties of the stirred yoghurt, the type of pectin must be right and it must be dosed within a specific interval, as the quality of the stirred yoghurt is lowered both if too little or too much pectin is added [Maroziene & de Kruif, 2000].

The use of pectin to stabilise stirred yoghurt presents challenges as pectin, being a natural product, has varying properties, which greatly affects the final result, e.g. by shifting the optimum dose [Willats *et al.* 2006]. The properties are affected by the source of the pectin, which is most often citrus fruit peel or apple pomace. They are also affected by extraction method and following chemical treatments e.g. deesterification and amidation [Willats *et al.* 2006 ; Maroziene & de Kruif, 2000].

In order to produce pectin products with consistent properties these are produced on selected peel types and highly controlled extraction conditions and the final pectin products undergo a strict, analytical quality control.

The field of pectin properties versus applicability is an active field of research and much work have been done on pectin in fermented milk applications [Sejersen *et al.* 2007 ; Harte *et al.* 2007 ; Lucey & Singh, 1998 ; Sodini *et al.* 2004]

The area of stirred yoghurt has, to some degree, been neglected in this process and still leaves some questions to be answered [Everett & McLeod, 2005 ; Sodini *et al.* 2004].

In order to develop further specialised pectin products with new or improved functionalities there is a need for a deeper understanding of the functional properties of pectin in applications like stirred yoghurt.

This project focuses on two approaches to address this need. One is to work with the knowledge on the destabilising and stabilising mechanisms of pectin in stirred yoghurt, the other is to test the applicability of different methods to aid in the screening process of new pectin stabilisers.

In the long run knowledge of the stabilisation and destabilisation mechanisms of pectin in stirred yoghurt, and the relation between pectin properties and functional properties can aid in the design of pectin based stabilising ingredients with improved functional properties.
The following chapter will give an overview of milk and the yoghurt production process on a macroscopic and microscopic level, followed by a description of pectin and an attempt to sum up the knowledge on effect of pectin in the process. This understanding is summarised in a description of the effect of pectin in stirred yoghurt at the end of the chapter. Using this theoretical background the focus of the experimental part of this project will be found.

2 Theory

The purpose of this chapter is to give the theoretical background for the project and to attempt to sum up the knowledge on yoghurt production using pectin. The following sections will give a description of milk, the quality of yoghurt and the yoghurt production process on a macroscopic and microscopic level. The Danish legislation on the ingredients in yoghurt will be described shortly and this will be followed by a description of pectin and an attempt to sum up the knowledge on effect of pectin on the process and present this understanding in an illustrated summary at the end of the chapter. Using this theoretical background the focus of the experimental part of this project will be found.

2.1 Description of Milk

Milk is the secreted fluids from the mammary glands of female mammals. It contains all the necessary nutrients for the first phase of the life of every mammal's offspring. Throughout the world livestock are kept in order to collect their milk and milk is a part of the diet of humans around the world. The term "milk" is synonymous with cows' milk as cows' milk constitute most of the worlds milk production (85% in 1999) [Belitz *et al.*, 2004]. Raw milk contains water, solutes and suspended solids and consists on average of 4.6 % lactose, 3.9 % fat, 3.2 % protein and 0.7 % ash [Belitz *et al.*, 2004]. The composition changes according to breed, feed and season, and thus it are not possible to determine the exact compositions or properties of milk. The milk protein can be divided in two fractions, caseins, constituting 80% of the protein and whey proteins, accounting for the remaining 20 %. The whey proteins can be separated into β-lactaglobulin (9% of total skim milk protein), α-lactalbumin (4%), Immunoglobulin (2%) serum albumin (1%) and proteose peptone (4%) [Belitz *et al.*, 2004]. According to Andrews (1987) the latter is a mixture of peptides, including digested caseins, which according to Paquet (1988) is soluble after extensive heat treatment and acidification. The caseins are a mixture of α- (42% of total skim milk protein), β- (25%), γ- (4%) and κ-casein (9%) [Belitz *et al.*, 2004]. The caseins are insoluble in weakly acidic media. Both α-, β-, γ-, κ-caseins, α-lactalbumin and β-lactalglobulin have several genetic variants, resulting in alterations in the amino acid sequence of the proteins [Belitz *et al.*, 2004]. The implications of these substitutions are an active area of research. Milk fat occurs in the form of globules or droplets and is emulsified in the milk serum by a protein membrane. As the density of the fat globules, called cream, are less than that of the serum they tend to separate and float at the top of the serum phase. They are uneven in size, ranging from 100 nm to 10 μm.

The mineral contents of milk have been monitored for a year by Pouliot and Boulet (1995) and they have found that the concentration of sodium (21 mM), potassium (38-42 mM), magnesium (4-5 mM), citrate (18-20 mM), phosphate (15-20 mM) and chloride (28 mM) remains fairly stable, but the concentration of calcium(28-45 mM) is subject to considerable seasonal variation. The calcium level was shown to increase during the winter, from December to February. The observed increase in calcium content was found to be precipitated in the casein micelles and the serum concentration was fairly constant. The soluble calcium was 24 % of total calcium.

The whey proteins are a colloidal suspension, ranging from 3 nm to 6 nm in size. The caseins tend to form micelles ranging from 10 nm to 300 nm, with more than 90 mass percent in the range from 120 to 200 nm [Belitz *et al.*, 2004]. As the properties of the casein micelles are of great interest for this project these will be described in more detail.

Casein aggregates known as micelles constitutes the main fraction, at least 90 % of the caseins, and the remaining casein, called serum casein, is mostly β-caseins [Belitz *et al.*, 2004]. There exists equilibrium between dissolved caseins, casein complexes and casein micelles. The equilibrium is shifted towards micelles by lower pH, addition of calcium and increasing temperature. The equilibrium is shifted towards monomers by increasing citrate and phosphate concentrations [Belitz *et al.*, 2004]. The micelles consist of roughly spherical aggregates, called submicelles, that contains 15-25 casein molecules and are 10-15 nm in diameter. These are stabilised by hydrophobic interactions, hydrogen bonds and are connected and kept close together by calcium phosphate bridges between phosphoserine groups [Belitz *et al.*, 2004 ; Walstra 1990] See figure 1.

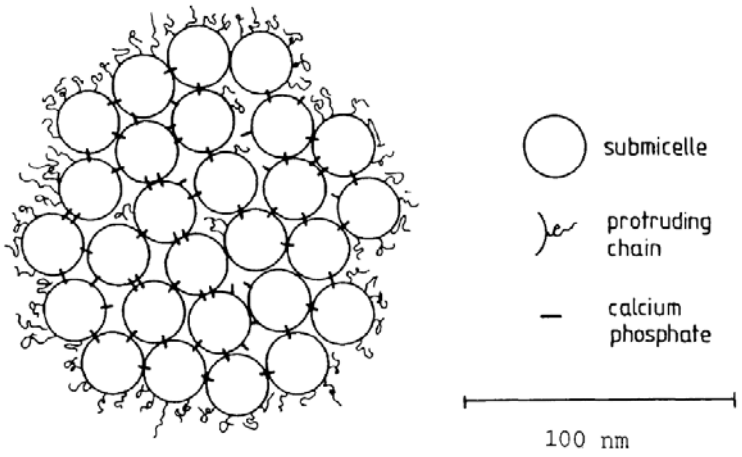


Figure 1: Model of the casein micelle. It consists of submicelles and is stabilised by κ-casein [Walstra, 1990]

Externally the micelles are sterically stabilised by the action of κ-casein. According to de Kruif (1999) the native casein micelle are sterically stabilised by a layer of κ-casein hairs, called a salted polyelectrolyte brush. The hairs consist of the C-terminal of κ-casein molecules protruding about 5 nm into solution [Dalgleish, 1998].

Its stability is related to the brush density, charge density along the chains, the concentration of positive divalent ions and the polarizability of the solvent, and can be destabilised by changing any one or more of these parameters [de Kruif, 1999].

The DLVO theory been applied to describe the stability of casein micelles (DLVO theory is described in appendix 2). The theory has its limitations as it can not describe the repulsion introduced by κ-casein, and hence cannot describe the remarkable stability of casein micelles [Walstra, 1990 ; Anema & Klostermeyer, 1996]. A surface layer of this type provides stabilisation in two ways, one is electrostatic repulsion and the other is steric stabilisation through entropic repulsions at close range [Anema & Klostermeyer, 1996]. The steric repulsion can be describe in two terms, the volume restriction term due to loss of freedom of motion of the hairs, this is always repulsive. The other is the mixing term and is determined by the

solvent quality, if it is good the effect is repulsive called osmotic repulsion if it is poor the term may be attractive [Walstra, 1990].

The calcium phosphate in the micelles are in the form of amorphous, colloidal calcium phosphate(CCP) (Ca:P ~1) and of the total content the micelles accounts for 76% of Calcium, 50% of Phosphate, 43% of Magnesium and 29% of Citrate[Pouliot & Boulet, 1995][Walstra 1990]. Casein micelle volume depends strongly on pH and temperature. At 10 degrees it may be close to 4 ml/g, at 40 degree it is only about 2.5 ml/g. the size have maxima at ph 6,7 and 5,3 and a minima at ph 6,0 (75% of maximum size)[Walstra, 1990].

2.2 The Quality of Stirred Yoghurt

The quality of stirred yoghurt can be considered as the yoghurts ability to meet the expectations of the consumers. The expectations of the consumers can be split into chemical composition, physical properties, and microbiology. The chemical composition is important to meet the expectations to nutritional value and pleasant flavour (smell and taste), the physical properties is a combination of appearance (smooth/shiny surface and colour) and mouth-feel/texture (consistency, viscosity) and expectations to microbiology is safety, e.g. no pathogens, no off flavours and sound appearance which means no yeast or moulds [Robinson *et al.*, 2006].

Problems in yoghurt quality may be caused by flaws in ingredients, errors in processing, problems related to bacterial fermentation and problems related to the formulation/recipe [Tamime & Robinson, 2007].

The texture is a very important property, it is the feeling of the yoghurt in the mouth, and it should be viscous/thick and smooth [Lucey & Singh, 1998]. Textural flaws can be lumpiness, granular or presence of nodules, which also affects the appearance [Lucey & Singh, 1998].

The appearance attribute consist of smoothness opposed by a coarse structure [Modler *et al.*, 1983]. Robinson and co-workers (2006) also considers the whiteness as an appearance attribute. The appearance of whey on the surface of the yoghurt decreases the consumer appeal [Modler *et al.*, 1983].

The focus of this project is on the formulation of yoghurt and the relation to appearance and texture. This means that microbiology, nutrition and flavour is not considered, likewise the flaws and errors introduced by ingredients or processing is not examined further.

As mentioned in the introduction, the addition of fat followed by homogenisation improves the texture of the end product [Robinson *et al.*, 2006]. To meet the sensory expectations of consumers, without adding milk fat, the method most often used is to increase the MSNF to improve the texture of yoghurt. According to Tamime & Robinson (2007) the milk solids non fat (MSNF) determines the experienced texture. When manufacturing yoghurt the separation of whey can be avoided by increasing the total solids [Lucey & Singh, 1998]. Increase of MSNF can be done by adding whey powder or skim milk powder, but also concentration of milk by evaporation or membrane filtration can be used [Tamime & Robinson, 2007]. No matter what method is used, the "bottom line" milk protein and fat is expensive constituents, and the fluctuations in the price of skim milk powder present a problem [Robinson *et al.*, 2006]. An alternative source of increasing viscosity to improve texture is addition of low methylated(LM) pectin [Robinson *et al.*, 2006]. In stirred yoghurt stabilisers are added to control textural defects and prevent whey separation [Lucey & Singh, 1998].

Understanding the formulation and production of stirred yoghurt with LM pectin and its importance to whey separation, texture and appearance is a necessary step on way to the goal of this project. The production of stirred yoghurt will be considered in both a macroscopic and a the microscopic scale in the "Description of the Production of Stirred Yoghurt" section below. Addition of pectin and its effect depending on dosage will be considered in the "Effect of Pectin in Stirred Yoghurt" section. Last a model of the microscopic scale interactions of pectin and milk during the production will be proposed.

2.3 Description of the Production of Stirred Yoghurt

The production of yoghurt can be separated in three different types, these are set yoghurt, which is the undisturbed yoghurt gel, drinking yoghurt, which is yoghurt gel that have been homogenised to give a drinkable product, and stirred yoghurt. The production method differs for these three products and only the production process of stirred yoghurt will be described here.

The production consist of several steps, the first is a preliminary treatment of the milk, followed by homogenisation, heat treatment, fermentation and final processing. The macroscopic process steps are described followed by a description of the microscopic processes behind these. The process is described for yoghurt without stabilisers.

2.3.1 Preliminary Treatment

Macroscopic

The first step in the process is standardisation of the fat content of the milk at the desired level, this is done by separation of the cream and skim milk by centrifugation followed by mixing of the necessary amounts of the two [Robinson *et al.*, 2006]. This is followed by a fortification of the milk solids non fat (MSNF) by evaporation, ultrafiltration, reverse osmosis, or addition of powders [Robinson *et al.*, 2006]. The powders may be skim- or whole milk powder, sodium caseinate, or whey powder. These are blended in to ensure dispersion of the powder. The level of MSNF should be fortified from the natural level of 82-86 g/kg to 120-140 g/kg for stirred fruit yoghurts [Robinson *et al.*, 2006]. If the product should have colours, sugar and/or stabilisers added, these are added at this point.

Microscopic

The preliminary treatment does not introduce changes on the microscopic level. It only serves the purpose of adjusting the amounts of milk constituents to make the milk suitable for yoghurt production.

The effect of temperature on the salt balance of milk have been studied by Schmitt and co-workers (1993) and it has been shown that cooling of milk increases the soluble calcium and phosphate concentration by 10%, and reheating to 37°C restored the initial equilibria, even though this took up to one hour. Heat treatment at 80-90 °C resulted in precipitation of calcium phosphate and calcium citrate resulting in a decrease of 25% in soluble amount [Schmitt *et al.*, 1993].

The micelles are kept together by CCP, hydrogen bonds and hydrophobic interactions. As the temperature decreases the hydrophobic interaction is weakened, resulting in an increasing volume of the casein micelle, and vice versa [Walstra, 1990]. At temperatures below 25 °C the solubility of β-casein is increased, and the proteins may extend from the micelle surface into solution, adding to the brush properties [Walstra, 1990].

2.3.2 Homogenisation

Macroscopic

The homogenisation is done by heating the milk to 60-70 °C and homogenise it by a high pressure homogeniser at 15-20 MPa [Robinson *et al.*, 2006]. This is done by pressing the liquid through an orifice or annulus and thereby exposing it to extreme shear.

Microscopic

The heating before homogenisation have an impact on the bacteria in the milk, but it is too weak to denature the milk proteins to a significant degree [Tamime & Robinson, 2007]. The extreme shear in the homogenisation process result in crushing of remaining powder particles and also the fat globules are reduced in size from an average of 3.5 µm to less than 2µm [Tamime & Robinson, 2007]. The smaller fat globules have a larger surface and these are coated with proteins and protein aggregates from solution [Robinson *et al.*, 2006].

The new fat globules have decreased chance of aggregation and creaming [Tamime & Robinson, 2007], and the whiteness of the stirred yoghurt can be increased by homogenisation due to smaller fat globules [Robinson *et al.*, 2006].

Homogenisation results in improved texture of the yoghurt due to interaction of fat globules with the casein micelle network during fermentation [Robinson *et al.*, 2006].

2.3.3 Heat Treatment

Macroscopic

Heat treatment of milk for yoghurt takes place at temperatures well above normal pasteurisation temperature. Two approaches are used, heat to 90-95 °C for 5-10 minutes in a plate heat exchanger or 80-85°C for 30 minutes in a process vessel [Robinson *et al.*, 2006]. The heat treatment is followed by cooling to incubation temperature, 42-43 °C [Robinson *et al.*, 2006].

Microscopic

Both methods remove the natural bacteria and causes denaturation of the whey proteins in the milk serum. The relation of temperature and remaining native part of protein have been described by Belitz and co-workers (2004), see figure 2.

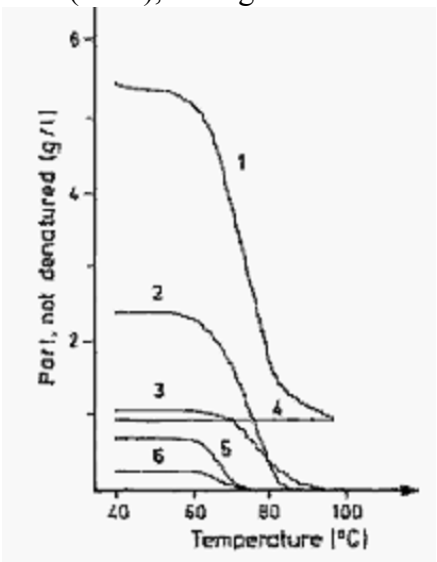


Figure 2: Denaturation of whey proteins by heating at various temperatures for 30 minutes. 1 Total whey protein, 2 β-lactoglobulin, 3 α-lactalbumin, 4 proteose peptone, 5 immunoglobulin, 6 serum albumin [Belitz *et al.*, 2004].

During heat treatment the two main whey proteins, β-lactoglobulin and α-lactalbumin are denatured and forms complexes [Tamime & Robinson, 2007]. The denatured whey proteins form complexes with casein micelles through hydrophobic interaction[Lucey *et al.*, 1998a] and the heat treatment results in covalent disulphide bonds between thermally denatured β-lactoglobulin and κ-casein on the surface on the casein micelles [Jang & Swaisgood, 1990]. This increases the size and volume fraction of the casein micelles [Tamime & Robinson, 2007]. The coating of the casein micelles with denatured whey proteins changes the pH of gelation of the milk, from pH 4.83 for the unheated sample to 5.17 by heat treating the milk at 80 °C for 30 minutes [Lucey *et al.*, 1999]. This is consistent with the isoelectric point of β-lactoglobulin(5.2) [Gao *et al.*, 1997] denatured whey proteins(5.2) [Remeuf *et al.*, 2003] and casein(4.6) [Remeuf *et al.*, 2003]. Little is known on the structure of the denatured whey proteins in solution or aggregated with casein micelles. The whey proteins are 20 % of the total mass of protein and this amount is enough to fully coat the casein particle surface.

Lefevre and Subirade (2000) have shown that the thermal denaturation of solutions of β-lactoglobulin at neutral pH results in a gel of thin strands of proteins, on the contrary close to the isoelectric point the gel is formed by protein particles. Roefs and De Kruif (1994) have suggested that the growth of denatured β-lactoglobulin aggregates during heat treatment progress like a chain polymerisation utilising disulphide bonds. According to Dalgleish (1990) no di- tri- and polymers of β-lactoglobulin is formed without these being bonded to κ-casein by disulphide bonds. This may be explained by the κ-casein sulphide being the initiator of the β-lactoglobulin chain polymerisation.

If these pieces of information is combined and applied to the heated milk system the structure of the denatured whey proteins will be strands of different lengths covalently attached at one or both ends to κ-caseins. The strand is negatively charged and may protrude into solution from the surface of the micelles. This would result in that close to the isoelectric point of the whey proteins these strands would start to aggregate and thus connecting the casein micelles to create a gel.

One effect of milk heat treatment on the yoghurt gel is increased cross linking within gels [Robinson *et al.*, 2006], this can be seen in figure 3.

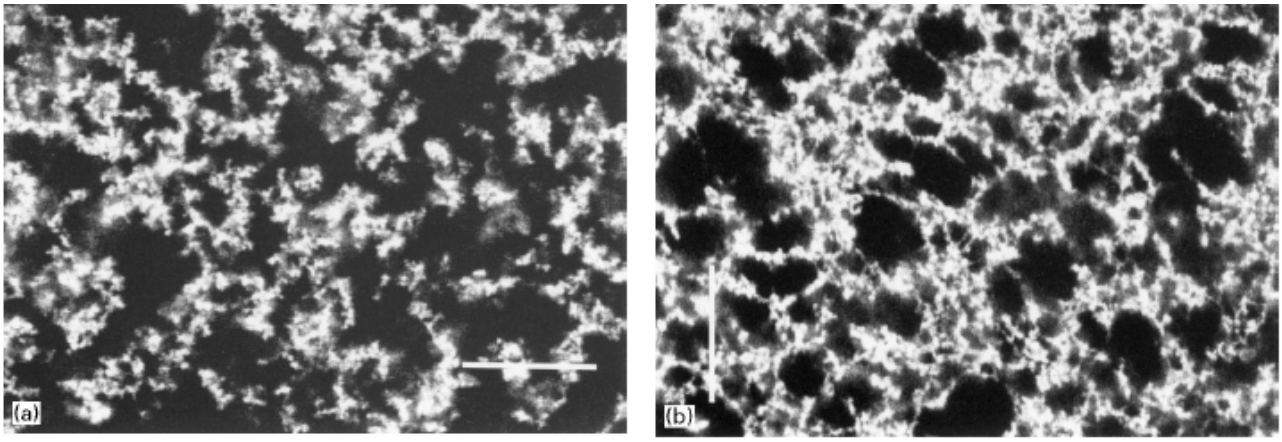


Figure 3: Fluorescence spectroscopy pictures showing difference in gel structure of unheated milk (a) and milk heated at 80 °C for 30 minutes (b) [Lucey *et al.*, 1999].

Also the heat treatment inactivates proteases and lipases from milk and bacteria, minimising rancid and bitter flavours [Tamime & Robinson, 2007].

2.3.4 Fermentation

Macroscopic

The milk is inoculated with a starter culture consisting of 50:50 *streptococcus thermophilus* and *lactobacillus delbrueckii* subsp. *bulgaricus* [Robinson *et al.*, 2006] and left to ferment in an anoxic and sterile environment. The vat used for the fermentation should be able to control the temperature precisely and cool the product down after the preset pH has been reached. The fermentation is done in 3-4 hours and is driven by the bacteria converting lactose to lactic acid, which results in a slow lowering of pH until the lactic acid content is 10-12 g/L. The bacteria symbiotically break down proteins to amino acids, and this is the reason for the fast growth of the two species [Robinson *et al.*, 2006]. The lowering of pH results in a gel and the fermentation is done when the end pH (around 4.3-4.2) is reached.

Microscopic

As described in the "Description of Milk" section, the casein micelle is stabilised by a brush of negatively charged κ-casein. The casein brush looses its charge during the lowering of pH during the fermentation and as pH approaches the isoelectric point of κ-casein the brush collapses and allows for particle interaction, resulting in aggregation of the unheated casein micelles [Lucey & Singh, 1998]. This model is only valid for unheated milk. The zeta potential of casein micelles, without heat treatment, during acidification have been described by Anema and Klostermeyer (1996), this can be seen in figure 4.

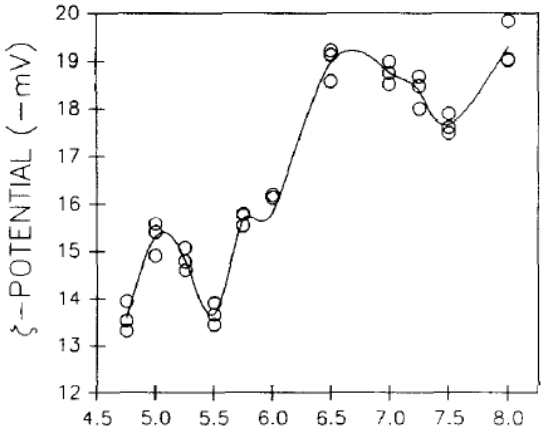


Figure 4: The zeta potential of casein micelles in unheated milk as a function of pH [Anema & Klostermeyer, 1996].

Figure 4 shows decreasing zeta potential with decreasing pH with a local minima at pH 5.5 and a local maxima at pH 5.0. At pH 4.8 gelation occurs due to loss of steric stabilisation by κ-casein.

As pH moves below the isoelectric point of the whey proteins, these will obtain positive charge resulting in electrostatic interaction with the κ-caseins. This, combined with further decrease in pH will make the κ-casein brush collapse and resulting in contraction of the network. This two-step mechanism can help explain the strands observed in figure 3.

The gel formation can be considered as a flocculation with increasing floc size[Walstra, 1990]. The particle fraction of the flocks decrease with increasing floc size and when the flocs span the full volume the gel is formed [Walstra, 1990].

The physical and rheological properties of gels made with GdL differs from those derived from fermentation, especially at high temperatures [Lucey & Singh, 1998].
Another effect that should be considered is the dissolution of CCP with lowering of pH. At pH 6.0-5.3 the CCP is dissolved leaving a more loose structure of the casein micelle [Matia-Merino & Singh, 2007], it becomes larger and absorbs serum [Walstra, 1990]. Below this pH electrostatic interactions of the caseins strengthens the micelle structure and it contracts again [Walstra, 1990].
At the critical pH the casein micelle can undergo rearrangements [Matia-Merino & Singh, 2007].

The appearance of the yoghurt is related to the microstructure of the yoghurt, and to achieve the desired appearance the network should be homogeneous [Sodini *et al.*, 2004].
Whey separation can occur if the gel undergoes substantial structural rearrangements [Lucey & Singh, 1998] and syneresis is defined as shrinkage of the gel combined with expulsion of whey. If the casein network is unstable it may undergo rearrangements resulting in the loss of the ability to entrap all the serum phase [Lucey & Singh, 1998]. This means that rearrangements of the network should be minimised. Different types of rearrangements threaten to change the fine gel structure and increase pore size, resulting in syneresis [Mellema *et al.*, 2002], illustrated in figure 5.

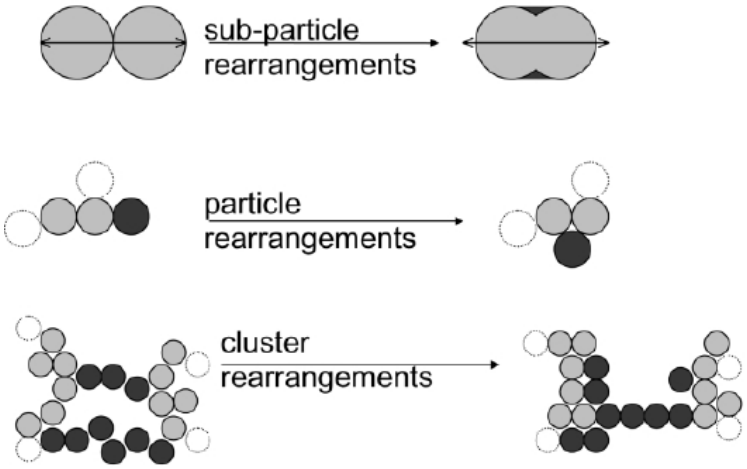


Figure 5: The three possible types of rearrangements, Sub-particle-, particle- and cluster rearrangements [Mellema *et al.*, 2002].

The sub-particle rearrangements are connected to the critical pH of 5.2, and as the gel is being formed at this pH it can participate in the formation of stronger bonds due to larger interaction surfaces. The particle rearrangement forms a more "stretched" or "smooth" network of strings and junctions and cluster rearrangements is related to loss of interconnectivity and a reduction in the number of strings and thereby larger pores leading to syneresis. Cluster rearrangements also lead to lower yield stress of the gel, it becomes more brittle [Mellema *et al.*, 2002].
The key to understanding rearrangements is the bond reversibility, the ability to break and form again. The more reversible the inter particle bonds are, the more prone the network is to syneresis [Mellema *et al.*, 2002].

2.3.5 Final Processing

Macroscopic

The gel is sensitive to shear before it is cooled so first the gel is cooled to around 20 °C, reducing the metabolic activity of the culture to a minimum. Then the gel is stirred carefully as the apparent viscosity is lowered with shear, increasing temperature at the shear and the shear time [Robinson *et al.*, 2006]. If it is fruit yoghurt, it is at this point the fruit preparation is added. The composition of the fruit preparation is made so that it matches the demands for the final product. This is done both in adding sufficient amounts of sugar, but also colouring, flavouring and stabiliser can be added. These additions help determine the colour, flavour and in the case of stabilisers, e.g. pectin, the viscosity of the final product, through a "carry-thru" effect [Robinson *et al.*, 2006]. After mixing the stirred yoghurt is packed in cartons and cooled to storage temperature.

To shortly summarise the macroscopic section, the production process can be altered in numerous ways, all of these can effect the quality of the final product, sometimes in surprising ways. To produce a stable quality of yoghurt requires a high degree of control over the process.

Microscopic

The fermentation temperature is typically 42 °C but if a lower temperature, e.g. 40 °C is chosen, it results in longer gelation times, but also a firmer and more viscous gel [Robinson *et al.*, 2006]. This is explained by the weaker hydrophobic forces holding the casein micelles together and an increase in size, resulting in a larger contact area. This trend continues as the temperature of the gel is lowered to storage temperature [Robinson *et al.*, 2006]. At high temperatures the casein network is more prone to rearrangements, resulting in whey separation.
The stirring of the gel is a crucial point in the production process. Stirred yoghurt consists of porous gel particles of aggregated casein micelles. These will sediment unless their volume fills the full volume or they form a volume spanning network. The rate of this sedimentation depends on the pore size, particle volume and stability of the casein network.
The effect of excessive stirring by a Brookfield viscometer at 25 °C of yoghurt have been shown using SEM by Özer (2004), the gel is broken up and the micelles are aggregated in small, dense particles, making the yoghurt prone to syneresis, see figure 6.

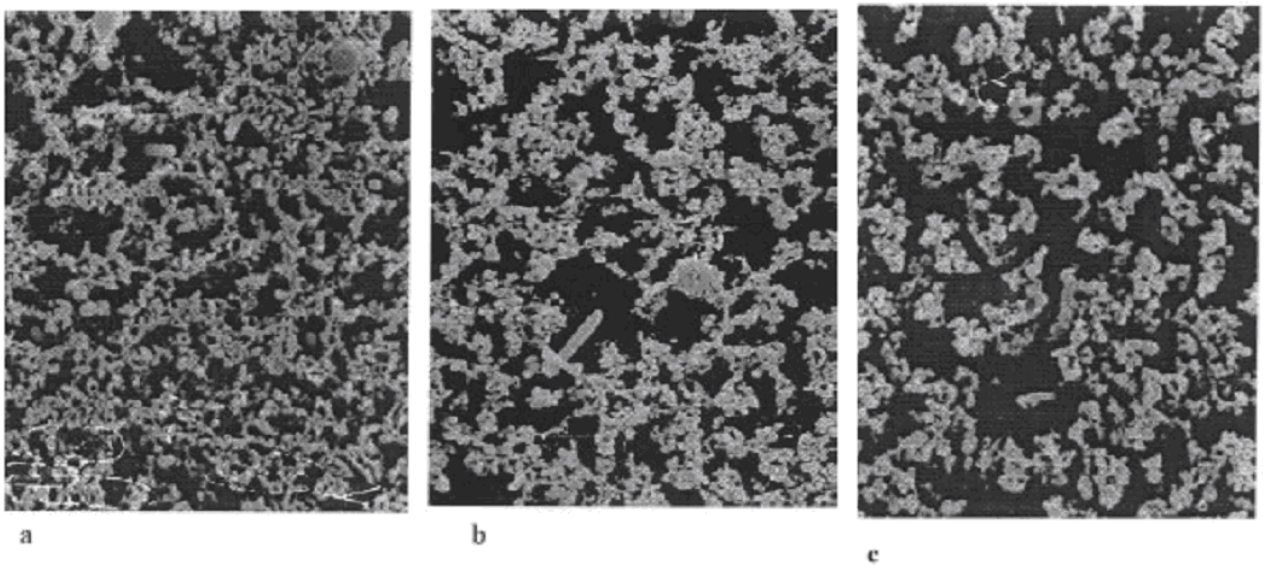


Figure 6: SEM images of yoghurt gel (a) stirred for 15 sek (b) and 30 sek (c) [Özer, 2004]

The measured viscosity of the samples seen in figure 6 was decreased by 16 % from 15 to 30 seconds of measurement.

The break down of the casein network by stirring depends on the shear, increasing temperature at the shear and the shear time [Robinson *et al.*, 2006]. This can be understood as rearrangement of the gel particles, resulting in more compact particles, and the strength of the interaction between particles is increased with lowering the temperature, resulting in a stronger gel, less prone to rearrangements. Hence, as the particle interactions is strengthened the gel, and hence the gel particles in stirred yoghurt, becomes stronger and this minimises the risk of damage resulting in syneresis. The gel strength can be considered both as the elasticity and the breaking strength of the gel.

Another effect is the size of the casein particles, when the temperature is lowered the particle size increases, this results in expansion of the gel and this can result in negative syneresis [Lucey *et al.*, 1997].

Texture flaws can be both an excessively firm gel by too high MSNF and excessive addition of stabilisers, on the contrary a thin texture can be due to low MSNF or low heat treatment [Lucey & Singh, 1998]. Whey separation is usually the result of improper formulation, culturing or processing [Modler *et al.*, 1983].

To shortly summarise the microscopic section, the properties of the stirred yoghurt are determined by the gel particle properties. These are affected by the heat treatment, temperature, rearrangements due to shear or bond reversibility, pH and fermentation rate and temperature.

2.4 Description of Pectin

Pectins are a diverse group of polysaccharides found in the cell walls of all land plants. It is normal in a western diet and around 4-5 g of pectin is consumed every day. Extracted pectin is used as a functional food ingredient in order to gel, thicken and stabilize numerous food products. It is also known as E440 and is classified "without a maximum dosage". The annual consumption is estimated to be around 45.000 tonnes [Willats *et al.*, 2006]. Even though pectin may be extracted from all plant material only a few sources are used commercially, namely citrus peel and apple pomace, As these pectins are the only ones that posses desirable functional properties and can be obtained at industrial quantities [Willats *et al.*, 2006].

Pectins structure is very complex and its elucidation is an active area of research [Willats *et al.*, 2006 ; Vincken *et al.*, 2003]. It may contain up to 17 different monosaccharides which are organised in a number of distinct patterns resulting in the polysaccharides that together build up the backbone and side chains of pectin [Vincken *et al.*, 2003]. The must abundant is α-D-Galacturonic acid which must be at least 65 % in order for the polysaccharide to be classified as pectin by the EU [Willats *et al.*, 2006]. The backbone of pectin consists of α-D-Galacturonic units linked with 1-4 glucosidic linkages. This is called homogalacturonan (HG). If every other Galacturonic unit is replaced by α-L-rhammnose the chain is called Rhamnogalacturonan I (RG I). A third type is called Rhamnogalacturonan II (RG II) and has a much more complex structure, with a number of different side chains. A few other chain types are also identified in pectin, but the main focus is on HG, as it is HG that defines the industrial applicability of pectin [Vincken *et al.*, 2003]. The structure of pectin is still a question of controversy [Vincken *et al.*, 2003] but it is clear that pectin is a branched macromolecule containing smooth regions (HG) and hairy regions (RG1, RG II). Two models for the pectin structure are presented in figure 7.

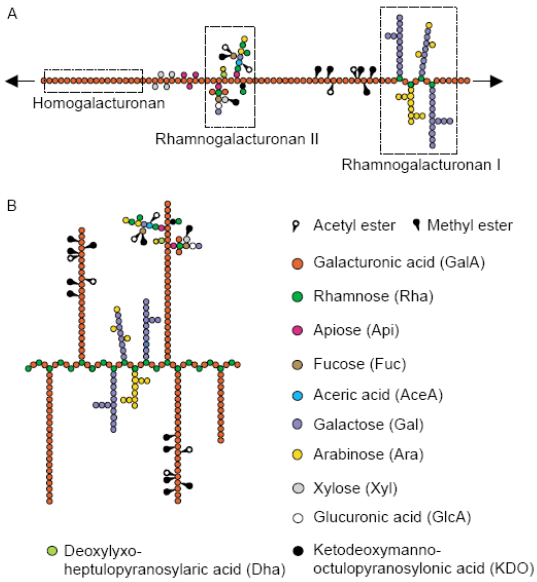


Figure 7: Two proposed models, A and B, of pectin structure [Willats *et al.*, 2006].

The acid groups of Galacturonic acid may be esterified with methanol. In poly-(α-D)-galacturonic acid the pK_a depends on the degree of protonation, the pK_a when fully protonated is around 3.3 and when 50 % protonated it is 4.35 and fully deprotonated around 5.4 [Rudan-

Tasic & Klofutar, 1996]. The pK_a of pectin depends on the charge distribution, polymer concentration, ionic strength and nature of the counterions [Rinaudo, 1996]. The degree of esterification(DE) and the distribution of non-esterified galacturonic acid groups have great impact on the functional properties of pectin, as blocks of 10 or more can form calcium cross links [Vincken *et al.*, 2003 ; Willats *et al.*, 2006].

The degree of blockiness can be characterised by calcium sensitivity, which is the increase in viscosity by addition of calcium [Laurent & Boulenguer, 2003].

The classification is based on DE, with high methylated (HM) pectin has at least 50 % methyl esters and low methylated (LM) pectin has less than 50 % methyl esters. Another important property of pectin is their molecular weight (M_w) or degree of polymerisation (DP). Pectin can be amidated by ammonia resulting in low esterified amidated pectin (LMA).

Pectins can form gels in solution by creating junction zones, and these can be made in two completely different ways. The classical picture is that LM pectin forms gels based on calcium bridging and HM pectin forms junction zones by hydrogen bonding and hydrophobic forces between methyl groups [Willats *et al.*, 2006]. HM pectin require high sucrose content and a low pH to gel [Tibbits *et al.*, 1998].

The limit of LM and HM pectin is arbitrary and the picture is more nuanced, and LM pectin (DE 31%) can form gels in absence of calcium at low pH(pH 3, 30 g/l) [Gilsenan *et al.*, 2000], and HM pectin have affinity for calcium and can form calcium induced gels [Tibbits *et al.*, 1998]. The apparent binding constant of calcium to pectin in gels is at least an order of magnitude higher than the binding constant of calcium to pectin in solution [Tibbits *et al.*, 1998].

From this it can be concluded that long chains of uncharged pectin can form junction zones at high concentrations, but more important, charged galacturonic acids interact with calcium, even when this does not lead to junction zones.

When pectin is in aqueous solution it can undergo two kinds of degeneration of the polymer, β-elimination and demethylation [Renard & Thibault, 1996]. β-elimination results in a break-up of the chain and demethylation reduces DE. These reactions are both base catalysed and does not occur at acid pH. When pectin is kept at neutral (the pH of natural milk) pH for extended periods of time or heated under these conditions, these reactions can change the properties of the pectin[Renard & Thibault, 1996].

The phase diagram of pectin with calcium ions is shown in figure 8.

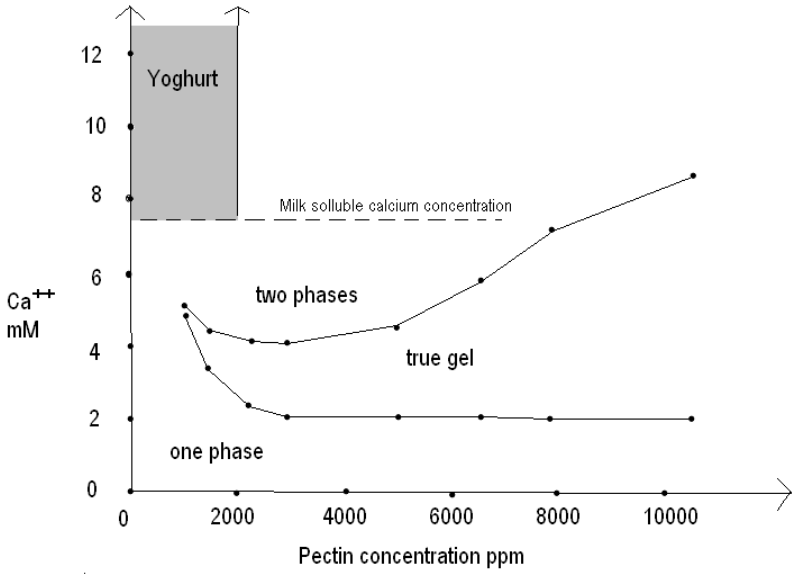


Figure 8: Phase diagram of LM pectin (DE=44, pH 7.0, 0.1 M NaCl, 20°C) drawn from [Axelos *et al.*, 1996]. The milk soluble calcium concentration is marked(---) and so is the polymer concentrations applied in yoghurt production.

Figure 8 shows a stable phase, a two phase system and a true gel. The structure of this diagram and the absolute values on the two axes is expected to depend strongly on pectin properties.

2.5 Effect of Pectin in Stirred Yoghurt

The effect of pectin in yoghurt preparations have been described by numerous authors. First a selection of findings from the recent literature will be presented, followed by a description of the mechanism of stabilisation of stirred yoghurt based on this and the previous sections.

2.5.1 Literature Description of Effects of Pectin in Yoghurt

Parker and co-workers (1994) added HM pectin (DE 73%) to yoghurt after fermentation followed by homogenisation, a method used for making drinking yoghurt. They found that the properties of the liquids without and with pectin changed from time dependent, shear thinning and with few particles below 0.2 μm to lowered viscosity and Newtonian rheology and a considerable particle fraction below 0.2 μm. This suggests a stabilising function of the pectin. With addition of pectin the zeta potential goes from +1 mV to -10 mV, Parker and co-workers (1994) claims that this is not enough to overcome van de Waals interactions, and hence the mechanism must be steric stabilisation due to adsorbed pectin protruding into solution. The value of the zeta potential is within -11±1 mV from 250-2000 mg/L pectin and hence does not vary with pectin concentration. This can be explained by surface saturation and that more particle surfaces being stabilised with increasing pectin concentration.
The interaction between pectin and casein is of electrostatic nature as the pectin goes from repulsion, resulting in phase separation, at pH 6, to adsorption and stabilisation at pH 4. Hence the interaction is not of hydrophobic character[Parker *et al*, 1994].
Parker and co-workers (1994) states that effective stabilising polymers is co-polymers with both areas of strong adsorption and areas weak to none adsorption.

Laurent and Boulenguer (2003) distinguishes between calcium sensitive (CS) and non calcium sensitive (NCS) HM pectin with the same DE (~70%) and intrinsic viscosity. This is based on the change in viscosity of a pectin solution upon adding calcium, where the viscosity of CS experiences an increase in viscosity.
Laurent and Boulenguer (2003) have made yoghurt by mixing various amounts of pectin into yoghurt by homogenisation, a method used for producing drinking yoghurt, and measured the viscosity of at different rotational speeds. This is seen in figure 9.

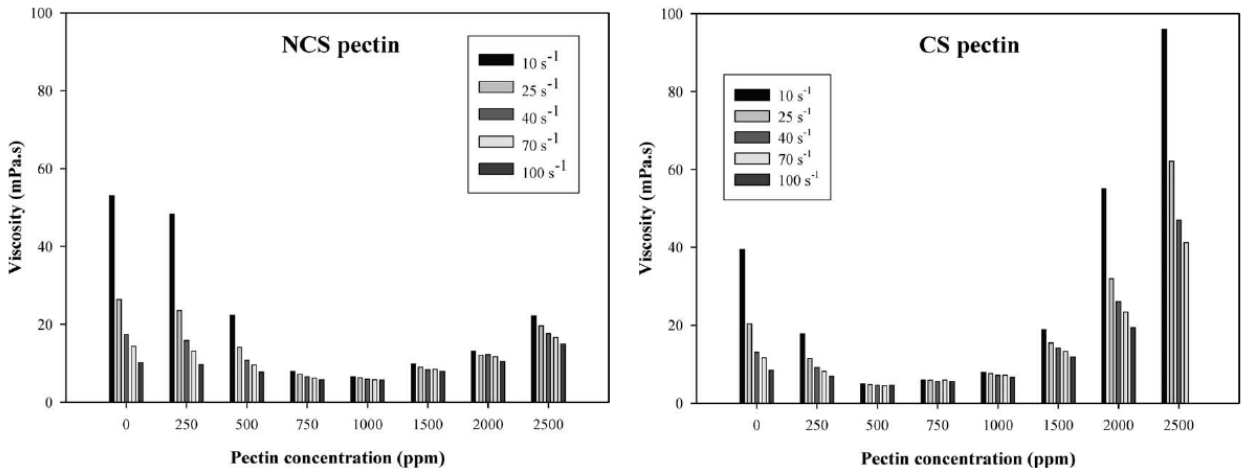


Figure 9: Viscosities of yoghurts at pH 4.2 with increasing amounts of pectin, at varying rotational speeds [Laurent & Boulenguer, 2003].

It is seen that the partially covered particles at low concentrations presents shear thinning rheology. Stabile particles present Newtonian rheology and this is followed by increased viscosity and shear-thinning. The authors assume that is due to increased volume fraction with increased pectin concentration due to thicker layers of pectin. Viscosity and volume fraction can be related by the Krieger-Dougherty equation(equation 1), showing that the relative viscosity (μ_{rel}) of drinking yoghurt increases with volume fraction of casein aggregates including adsorbed layer (Ψ) and intrinsic viscosity of the drinking yoghurt ($[\mu]_D$).

$$\eta_{rel} = \left(1 - \frac{\Psi}{p}\right)^{[\eta]_D}$$ Equation 1 [Laurent & Boulenguer, 2003]

At pH 4.2 addition of pectin up to 1000 mg/L (NCS) or 500-1000 mg/L (CS) results in little increase in volume after centrifugation, from this point the volume increases, this is assumed to be due to partial coverage of the micelle surfaces. The authors state that gel is due to diffuse particles filling the space. Increased volume fraction is less pronounced in NCS pectin. Gelling is introduced in CS pectin at concentrations above 1500 mg/L. NCS may have distribution of DE and hence contain a CS fraction [Laurent & Boulenguer, 2003].

Tromp and co-workers (2004) have centrifuged homogenised yoghurt with HM pectin (DE 72%), the method of producing drinking yoghurt, and determined serum pectin (non adsorbed) by viscosity. This constitutes from 50 % to 90 % of added pectin depending on added amount. The non adsorbed pectin creates a gel around the particles. The necessary amount of pectin stabilised casein micelles is 5 % when the serum is removed and these are resuspended in fresh serum, hence the pectin coated particles can form their own network [Tromp *et al.*, 2004]. The authors have shown that the percentage of pectin in serum is reduced with pressure of homogenisation, resulting from adsorption on the increasing area of casein micelles with increasing pressure. The authors have also shown by fluorescent marking of pectin and photo bleaching in confocal laser scanning microscopy that neither pectin adsorbed or in the serum phase has diffusive mobility, meaning that the serum pectin is involved in some form of binding that prevents it from moving through the solution [Tromp *et al.*, 2004]. The authors

conclude that the serum pectin is trapped in a permanent network with the pectin bound to casein and that this network is so weak that it does not affect the liquid character of the system.

Sejersen and co-workers (2006) have shown that the zeta potential of casein micelles changes in the range from negative to positive from pH 4,8 to 4,0 and at pH 4.0 the zeta potential changes from positive to negative by addition of HM pectin (DE 72%) from 0 to 0,5% pectin. The pectin induced negative zeta potential at pH 4.0 results in stable dispersion of particles [Sejersen *et al.*, 2006].

Ibanoglu (2005) have showed that a 1% HM pectin (DE 71%) solution has a stabilising effect on the denaturation of whey proteins by heat treatment.

Maroziene and de Kruif (2000) have observed the properties of milk and casein micelle size by DLS during acidification mixed with HM pectin (DE 73%), LM pectin (DE 35%) and LMA pectin (DE 35%, degree of amidation 20%). Depletion flocculation was observed at pH 6.7 (This will be described in detail in the "Determination of Functional Range" section). Depletion flocculation resulted in a transparent, liquid upper layer and a white liquid lower layer. The size of the micelles at pH 6.7 did not change upon addition of pectin, this confirms the absence of attractive interactions at this pH. The depletion flocculation was used to make a phase diagram depending on casein micelle volume fraction and pectin concentration, this can be seen in figure 10.

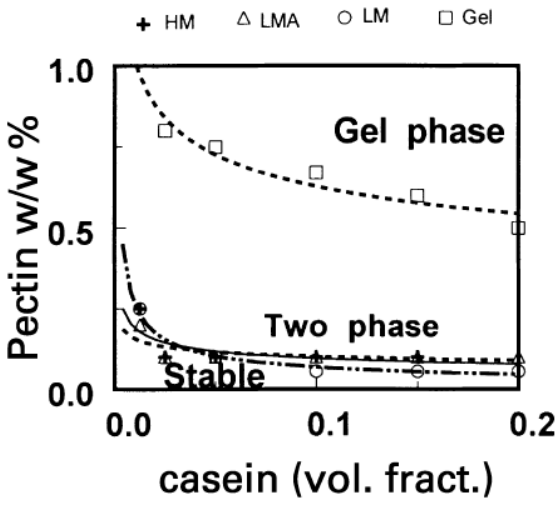


Figure 10: Phase diagram with casein micelle volume fraction on the abscissa and pectin concentration on the ordinate axis [Maroziene & de Kruif, 2000]

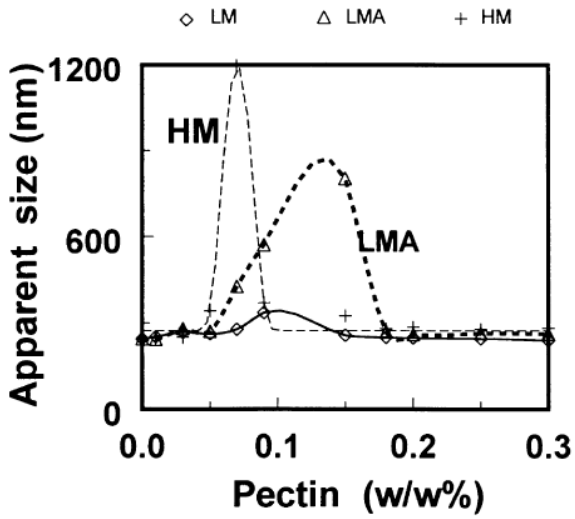


Figure 11: The apparent size of casein micelles with increasing amounts of pectin [Maroziene & de Kruif, 2000]

The function of pectin in the milk-pectin mixture with increasing concentration at pH 6.7 is stable, phase separation and gel phase at high polymer concentrations. The phase separation in skim milk occurs at concentrations of 2.0 g/L for HM and LMA pectin and at 1.0 g/L for LM pectin. The authors explain this by a larger effective volume occupied by LM pectin.

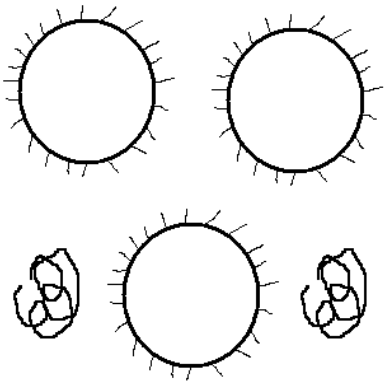
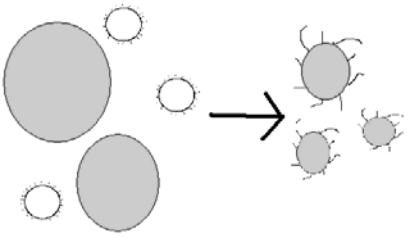
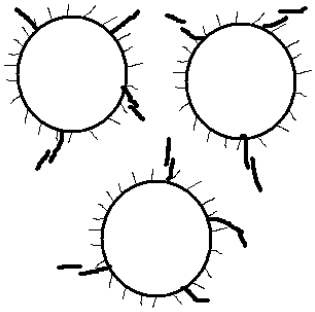
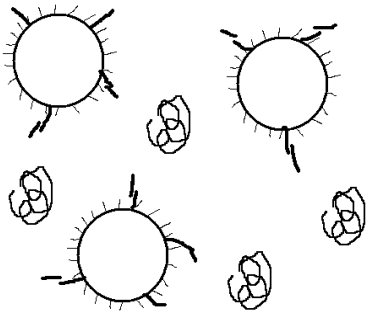
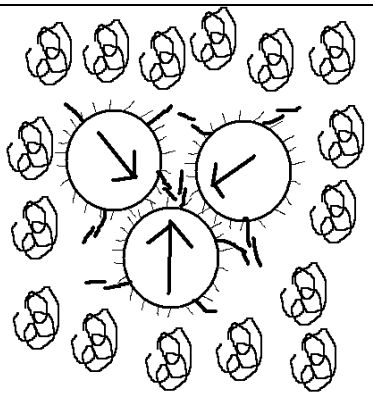
Following acidification to pH 5.3, the apparent size of the micelles were measured at increasing pectin concentration, this is seen in figure 11. The increase in size at intermediate concentrations is consistent with the pectin resulting in bridging flocculation, and as the pectin concentration increases further the micelles becomes fully coated and stabilised. The maximum apparent size is assumed to be the point of 50 % coverage of the micelle by pectin [Maroziene & de Kruif, 2000].
The function of pectin in the milk-pectin mixture with increasing concentration at pH 5.3 is stable, bridging flocculation, stable, depletion flocculation, gel phase [Maroziene & de Kruif, 2000].
The authors have also shown that pectin adsorbs rapidly at pH 5.3, but desorbs very slowly, in a matter of hours, after the pH is raised to 6.7 again.

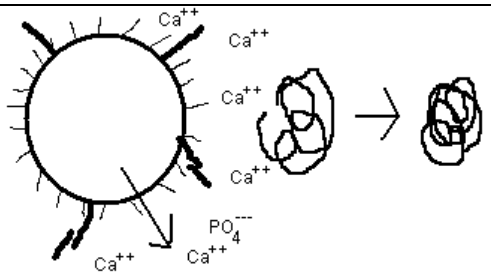
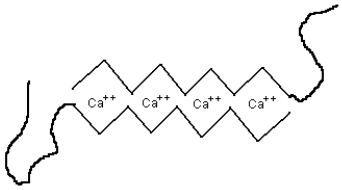
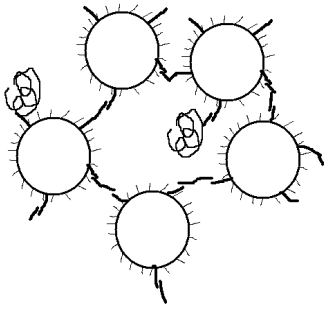
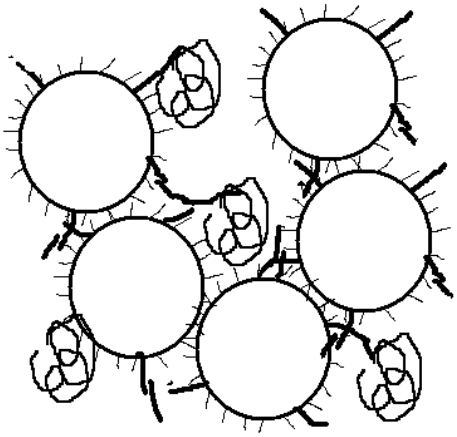
Tuinier and co-workers (2002) have shown by DLS that below pH 5.0 the layer thickness of HM pectin (DE 61%) continuously increases with decreasing pH to 100 nm at pH 3.0. The authors conclude that this is due to multilayer adsorption. The thickness of the pectin layer on latex particles is constant with change of pH and thus the increase in thickness of the pectin layer must be due to the change in positive surface charge of the casein micelle [Tuinier et al, 2002].
It is also confirmed that the adsorption of pectin on casein micelles is due to electrostatic interaction. The authors have shown that the upper limit of bridging is moved to higher concentration with lower pH and increase of charges on the casein micelle. The authors suggest that the pectin may have a varying affinity for casein micelles and this difference can contribute to the observations.

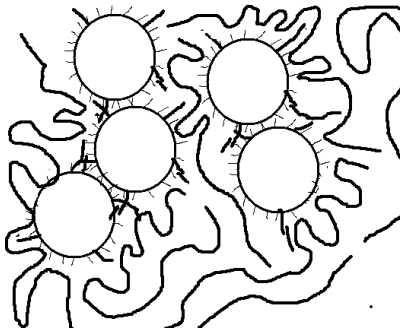
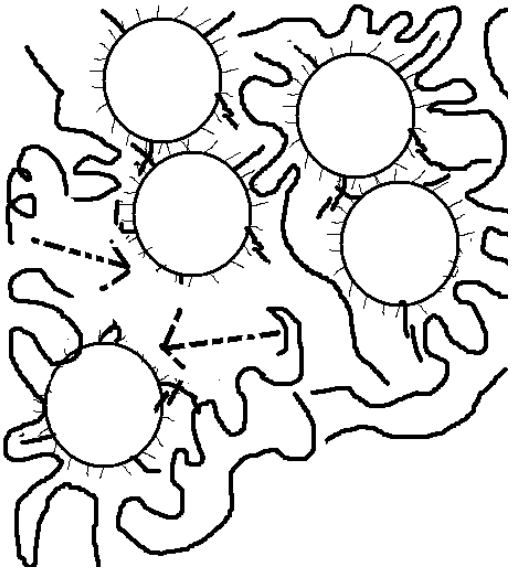
The stabilisation mechanism of LM pectin (DE 9%) has been investigated by Everett and McLeod (2005) by a combination of apparent viscosity, dynamic oscillatory rheology and measurements of water holding capacity (WHC). The results show that G’ and G’’ is unaffected from 0 until 1.5 g/L at this and higher concentrations these are highly reduced. This is consistent with the yoghurt gel being destroyed by depletion flocculation. It is noticed that low concentrations of LM pectin does not affect the gel properties. The same tendency is seen for apparent viscosity, but the water holding capacity shows a minimum at 1.0 g/L. followed by a strong increase. The reason for this is not gel formation, as this has been ruled out by rheology. A possible explanation is bridging flocculation followed by stabilisation and increasing volume fraction [Everett & McLeod, 2005].

2.5.2 Description of Pectin’s Functionality in Stirred Yoghurt

The purpose of this section is to sum up the previous sections in a summary of the important steps, on a microscopic scale, in the production of stirred yoghurt. This is done with the focus on identifying areas in the understanding that could be the subject of experimental study.
The process is assumed to be carried out using heat treatment and addition of a suitable amount of LM pectin.

In natural milk the pH is 6.7, the conductivity is around 5.0 mS/cm and initial processing temperature is around 10 °C. Under these conditions casein micelles are stabilised by a salted polyelectrolyte brush reaching 5 nm into solution. This brush consists of κ-caseins. At pH 6.7, when LM pectin is added, caseins, whey proteins and pectin are negatively charged, and hence only negative interactions occur.	
The milk is standardised in relation to fat and MSNF. The milk is then homogenised, resulting in that the size of the fat globules are reduced and they are coated with proteins making them able to participate in the gel network	
The milk is then heat treated at temperatures above 70 °C and β-lactoglobulin and α-lactalbumin is denatured, polymerised through disulphide bonds and bound to the surface of casein micelles by disulphide bonds with κ-casein. This results in strands of denatured whey proteins protruding, beyond the κ-casein hairs, into solution.	
The presence of pectin may result in depletion flocculation at concentrations above a critical concentration of pectin. The critical concentration depends on the volume fraction of casein micelles, on concentration and hydrodynamic radius of the pectin.	
Depletion flocculation of casein micelles is caused by the osmotic pressure and entropy loss from overlapping of the pectin, and result in a net attractive force between the casein micelles. These aggregate and precipitate, the aggregation is reversible.	

From pH 6.7, milks natural pH, to pH 5.4 not much happens to the interactions between pectin and casein micelles but the hydrodynamic radius of the pectin decrease due to charge neutralisation and ionic condensation of calcium. The reason is that at pH 6.5-5.25 the colloidal calcium phosphate, partly responsible for maintaining the structure of the casein micelles, dissolves increasing calcium concentration by 200%.	
As calcium concentration increases, the pectin's ability to create calcium bridges increases.	
At around pH 5.3 the denatured whey proteins loose their charge (pI = 5.2), making them able to form a casein-denatured whey protein- denatured whey protein-casein network. Besides that, the denatured whey proteins begin to achieve positive charge and hence, they may form ionic bridges with pectin.	
It is unknown whether the network actually forms in presence of pectins. At pH 5.0 the pectin is able to begin binding directly to the casein micelles increasing steric repulsion. As pH decrease to around 4.8 the polyelectrolyte brush around the casein micelles collapses allowing ionic bridges between caseins and pectin. This collapse also allows electrostatic and hydrophobic interaction between casein micelles, possibly resulting in a collapse of the denatured whey protein network. The denatured whey protein network may be replaced by a stronger, strained casein-casein and casein-pectin-casein network with higher G'. It is uncertain if the casein micelles are sufficiently sterically stabilised by pectin to inhibit new casein-casein interactions.	

After gelation the pectin protrudes from the surface of the micelle, making pectin-pectin bridges with both interacting and free pectin. They may also make bridges between micelles and thereby strengthen the bonds, minimising bond reversibility and thereby rearrangement. The pH drops further allowing more pectin to adsorb to the surface of the casein network.	
Free pectin still exists in the pores of the network when fermentation is ended by cooling. This interacts with the bound pectin, creating a weak pectin-pectin network with the stabilised casein network. The casein network is the stirred, resulting in breakage and exposing new surfaces. The bonds are not re-formed due to adsorption of free pectin on fresh casein surfaces. This result in pectin coated particles, stabilised by steric and electrostatic interactions, unable to aggregate. Stirring leads to smaller and smaller stabilised aggregates. When pectin adsorbs these aggregates increases the volume fraction of particles leading to an intermediate between a stable suspension and a gel.	

The LM pectin's functionality in stirred yoghurt can be separated in two functional areas:

Texture:

- To increase the effective volume fraction of the casein aggregates increasing viscosity of the product to improve mouthfeel.
- To create a weak, thixotropic casein-pectin and pectin-pectin gel increasing the thick mouthfeel.
- To sterically and electrostatically stabilise casein aggregates, preventing the formation of large aggregates and hence a grainy texture.

Appearance and whey separation:

- To prevent dense aggregates due to stirring, resulting in a smooth homogeneous product.
- To inhibit network rearrangements resulting in contraction of the gel particles and avoiding whey separation.
- To increase viscosity of the serum and slow down whey separation.
- To lower permeability due to the effectively immobilised serum pectin in a gel like state and slow down whey separation.

2.5.3 Effect of Dosage

The above explanation of the effect of LM pectin assumes that the dosage of pectin is within the functional range, meaning the range where the presence of pectin does not have a negative impact on the yoghurt quality.

As described in the introduction, wrong dosage, outside the functional range, results in a flawed product. This is shown in figure 12.

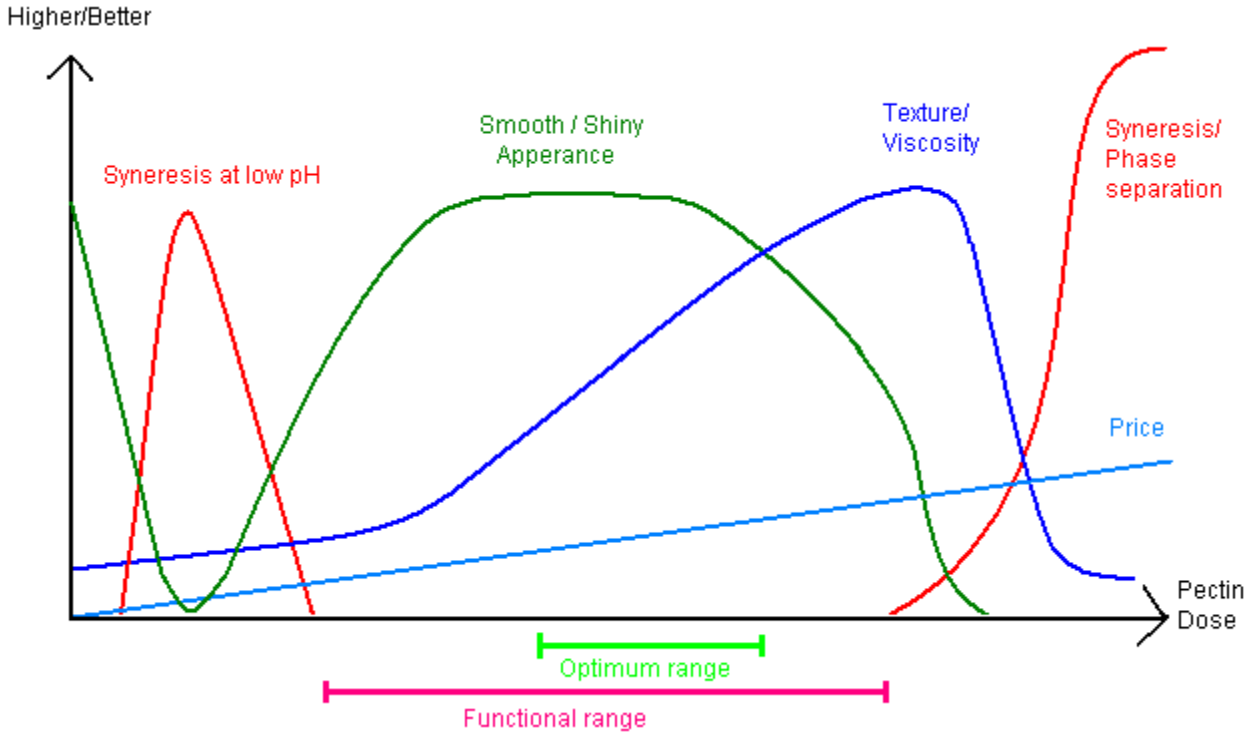


Figure 12: The quality parameters of yoghurt with increasing pectin dose. Shown below the graph are the functional range and the optimum range.

Figure 12 sums up the relation between dose and texture, syneresis and appearance. Syneresis occurs at low pH and low concentrations when the available pectinis not sufficient to cover the casein micelles, instead they are bridged by the pectin, resulting in compact flocs that sediment with time. Syneresis at high concentrations occurs due to depletion flocculation induced phase separation. This occurs at neutral pH and the effects persists through the production process resulting in a thin, inhomogeneous product. The viscosity of yoghurt increases with increasing pectin concentration. This adds positively to the experience of the texture of the yoghurt. When the stabilising effect reaches its maximum the gel can't be broken sufficiently apart, resulting in an uneven yoghurt surface. This reduces the smoothness and hence lowers the appearance quality. At low concentrations the bridging mechanism results in syneresis and this greatly reduces the appearance of the yoghurt.

Hence the functional range is limited by bridging flocculation at low concentrations and depletion flocculation at high concentrations of pectin. The optimum range is a compromise of the appearance, price and texture, and the optimum dose within these limits depends on the product, e.g. natural or fruit stirred yoghurt.

2.5.4 Legislation

According to Danish legislation, yoghurt must be produced only from "dairy milk", which is produced only from milk constituents, and bacteria. Yoghurt should also meet the criteria of many living bacteria throughout shelf life. Pectin in fruit compositions is allowed if it does not change yoghurt properties. All other additions result in that the product is no longer yoghurt and should be labelled as an "acidified product" [Bekendtgørelse om mælkeprodukter m.v., BEK nr. 335 af 10/05/2004]. "This means that LM pectin is mostly used in acidified milk based desserts". Even though applying LM pectin for stabilising yoghurt is not allowed in Denmark, it is used in the production of yoghurt worldwide [Tamime & Robinson, 2007].

2.6 Determination of the Functional Range

One particular area of great interest is the determination of the functional range of pectin.
There is a constant demand to determine the functional and optimal range of both new pectin products and new formulations using existing products. This can be done by making a line of yoghurts with increasing pectin content and then evaluate it by visual and sensory methods.
To minimise the requirement for these demanding experiments, other more simple screening methods can be applied to determine the functional range. The lower limit is determined by bridging of casein micelles by pectin due to incomplete surface coverage and the upper limit is determined by the depletion flocculation introduced by pectin.

To determine the functional and optimum ranges presents a challenge. These depend on the properties of the specific pectin, but also on the milk used. Pectin possesses varying properties depending on origin, extraction process and subsequent chemical modifications. Pectin is characterised by DE, M_w or [μ], and the methyl ester distribution. The properties of the milk vary just as much as the properties of pectin. Milk is characterised by protein content or MSNF, fat content, calcium content and heat treatment.
It is important not only to understand the effect of the pectin properties, but also to understand the effect of changes in milk properties in the application of pectin as a stabiliser. It has been shown above that MSNF, calcium content, and heat treatment of the milk is important to the quality of yoghurt. Therefore the effect of these on the interactions in the milk pectin system should also be considered.

The minimum functional concentration has been determined by Maroziene and de Kruif (2000) using DLS measurements of the apparent particle size as a function of pectin concentration. This is done by mixing milk and pectin followed by acidification to pH 5.3 by HCl. The results is seen in figure 11 in the "Literature Description of Effects of Pectin in Yoghurt" section. With varying milk properties, especially heat treatment, it is not likely that this method is robust enough to cover the full spectrum.
Parker and co-workers (1994) has measured the zeta potential and serum pectin after fermentation, mixing with pectin and homogenisation. They found that the amount of adsorbed pectin increased with increased amount added, due to an increasing stabilised surface area. The zeta potential remained almost constant. This method is only suitable for drinking yoghurts.
Laurent and Boulenguer (2003) have shown that stabilised particles made by a method similar to the one describe above show Newtonian rheology, while partially stabilised particles show shear thinning rheology, this is seen in figure 9. Applying this method to stirred yoghurt breaks the gel particles, and thus it does not represent the actual system.

The interactions before and after the fermentation is well described. The reason for the lack of a simple method to find the lower limit of the functional range may very well be that there is a gap in the present understanding concerning the interactions *during* the fermentation process. It is unclear when the pectin adsorbs to the micelles and if this affects or prevents the gel formation. It is also unclear how the heat treatment and thereby the adsorption of denatured

whey proteins affect the interactions with pectin. One way to visualise the adsorption of pectin to the casein micelles is to measure the surface charge, in the pH range of fermentation. The gel formation should also be monitored. By combining this information the interaction order may be uncovered.
If the order of interactions is known it may also be possible to determine a suitable method to determine what concentration of pectin is necessary to avoid bridging flocculation. Considering the surface charge could be useful as a full coverage should be evident in a stabilisation of surface charge when increasing pectin concentration. This experimental methods needed to do this will be considered in the next chapter.

The maximum functional concentration can be determined by observations of the natural milk-pectin mixture, as the depletion flocculation responsible is evident at this pH.
At pH 6.7 both the casein micelles and the pectin is negatively charged, and the interaction between pectin and casein micelles is repulsive [Maroziene & de Kruif, 2000].
Depletion flocculation requires a concentration of polymers high enough to occupy the phase volume between the casein micelles [Maroziene & de Kruif, 2000]. When the phase volume is occupied, a further increase of the pectin concentration results in increasing the osmotic pressure of the pectin [Maroziene & de Kruif, 2000]. As a pectin molecule can occupy a volume comparable to that of a casein micelle, there is a layer around the casein micelle that is depleted of pectin. Overlap of two such depletion layers makes the layers take up less volume, which then becomes available to the pectin molecules and this result in an attractive force between the casein micelles [Maroziene & de Kruif, 2000]. If this force is strong enough, the system phase separates. This is called depletion flocculation [Maroziene & de Kruif, 2000].
Tuinier and co-workers (1999) have applied the theory of coil overlap to a system of casein micelles and polymeric EPS and found a correlation between the theory and practical experiments of depletion flocculation. Hence the coil overlap parameter may be applicable to predict the critical concentration of pectin in skim milk (Appendix 6 contains a description of the coil overlap parameter).
Hansen (1994) have shown a negative relationship between intrinsic viscosity and the critical concentration due to depletion flocculation. The critical concentration can be determined as the concentration at 50 % serum volume after refrigeration and centrifugation of guar gum-skim milk mixtures. This is seen in figure 13.

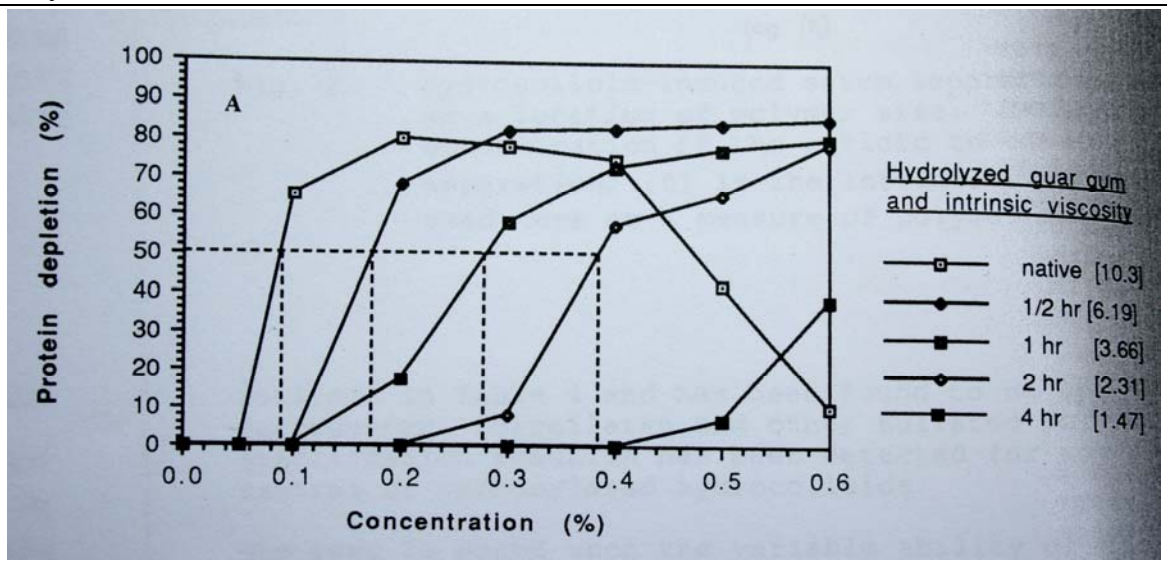


Figure 13: Shown is the percentage of serum separated from skim milk as a function of concentration of guar gum hydrolysed to different intrinsic viscosities [Hansen, 1994].

In figure 13 it is seen that as the intrinsic viscosity decreases the critical concentration increases.

The author has determined a linear correlation between log critical(concentration) and log(intrinsic viscosity), valid for guar gum and carboxymethyl cellulose this is given in equation 2.

$$critical\ concentration = 0.772 \cdot [\eta]^{-0.81}$$ Equation 2 [Hansen, 1994]

The findings of Hansen (1994) are not directly applicable in the case of determining the maximum in the functional range of pectin, as the important parameter is the onset of depletion flocculation.

To elucidate such a relationship for the depletion flocculation onset concentration requires a method for detecting the onset concentration. To detect the onset a method based on equilibrium measurements is a challenge because of the very small volume of serum at the top of the milk sample. To be able to test the influence milk properties like temperature and protein concentration means that many samples has to be run, hence the method should be simple and reproducible and the method should be able to show differences due to temperature. The selection of method will be discussed in the next chapter. Additionally, the effect of including the pectin during the heat treatment is not very well described. Will the pectin be broken down during the short time of treatment or will it affect the heat treatment of the milk proteins. An experiment should be designed to try to uncover the relative importance of these parameters. It is important to know if the heat treatment decreases the upper limit of the functional range.

When the functional range of a specific LM pectin has been determined the optimum concentration can be found by production of stirred yogurt within the functional range and analysing it by sensory characteristics to aid in the selection of the optimum concentration for the specific application.

3 Selection of Methods

The purpose of this chapter is to give reasoning and justification of the selected methods.
In this project the focus is on the changes introduced before, during and after production by adding pectin, and hence the focus is on the functional properties. As there is limited resources and time to do this project, some methods, such as expert panels and gravitational sedimentation, are out of the question. Available equipment at Section of Chemistry at Aalborg University makes it possible to determine a number of useful parameters, and the methods selected below are also based on this.
Hence the methods selected in this section must fulfil the purpose of helping to answer the questions asked in the previous chapter. Part of the purpose of this project is to apply new methods of characterisation of the functional properties of milk and yoghurt to add new tools to the toolbox of fermented milk product ingredient developers, so this is also considered in the selection of the methods used.

3.1 Milk, Pectin and Method of Acidification

Milk have properties that vary from day to day, and especially when the line of experiments progress over half a year there is a demand to keep milk parameters stable. This can be done using milk reconstituted from skim milk powder.
In special cases there may be a demand to use milk with little or non heat treatment, and in these cases the experiments should be done as quickly as possible using the same portion of fresh skim milk.
Fermentation presents a challenge as it is very hard to do two similar fermentations. Therefore addition of Glucono-d-Lactone(GdL) may be used as an alternative to fermentation when reproducibility is essential.
All milk preparations should be stabilised by keeping them at 1-2 °C and when possible by adding 0.02% NaN_3 to avoid bacteria introduced changes.
The pectin used should be pectin often applied to stabilise yoghurt. The nature of the project does not allow comparisons between different pectins.

3.2 Analyses before Fermentation

To determine if skim milk powder is rehydrated and the casein micelles have been separated sufficiently, particle size can be measured. This should be done using DLS, as this method is applicable in the size range of casein micelles and aggregates of these [Parker *et al.* 1994 ; Maroziene & de Kruif, 2000]. DLS is described in Appendix 3.

The determination of the phase diagram of pectin-calcium mixtures in water can be constructed from visual observation, as this system is transparent and phase separation results in phases with different refractive indexes.

To predict the onset of depletion flocculation, the intrinsic viscosity of pectin must be determined. This is done by measuring the viscosity of a concentration range of diluted solutions. To do this capillary viscometry can be applied, as it is a very sensitive method at

measuring relative viscosities in the range of 1-3. The determination of intrinsic viscosity is described in Appendix 6.

The stability and phase separation of milk pectin systems at neutral pH can be determined visually as done by Maroziene & de Kruif (2000). The authors use visual observation to create a phase diagram of pectin content against casein volume fraction. To make this process faster, get better control over parameters such as temperature and to detect the onset of phase separation more precisely, analytical centrifugation might be applied. The analytical centrifugation should be tested as a tool for determining stability depending on calcium content, protein content and the effects of heat treatment of neutral milk pectin mixtures. The theory of centrifugation and the analytical centrifuge is described in Appendix 5.

3.3 Analyses during Fermentation

To monitor fermentation the most used method is pH. Dielectric spectroscopy is tested as an alternative method by Kitamura and co-workers (2000), who found that it could be used to determine pH, acidity and gel hardness of the yoghurt during fermentation. This method may potentially supply even more information, e.g. changes in particle charge and aggregation during the acidification, in a single measurement. Hence the method should be tested on fermentation of yoghurt. This work is done in corporation with Ph.D Peter Vittrup Christensen, Section of Chemistry, Aalborg University. The theory and use of dielectric spectroscopy is described in Appendix 1.

The fermentation can be monitored using dynamic oscillating rheology to show gel point and build up of gel strength [Lucey & Singh, 1998]. The theory of rheology is given in Appendix 7.

The particle charge is an important parameter in understanding the aggregation of casein particles and adsorption processes during fermentation processes. To measure this Sejersen and co-workers (2007) have used the zeta potential, which is based on electrophoretic mobility. Parker and co-workers (1994) have also used the zeta potential to monitor particle stability of pectin coated casein aggregates.
According to Anema and Klostermeyer (1996) the quantitative use of zeta potential with casein micelles is problematic as the calculations are complicated by the fact that the nature of the double layer is unknown, the surface is diffuse and the structure is highly porous and highly hydrated. Therefore the electrophoretic mobility is used, this is a useful as an indicator of changes in chemical or physical properties of the surface, these should be apparent in changes in electrophoretic mobility [Anema & Klostermeyer, 1996]. This method can be applied to monitor changes in particle charge and pectin adsorption during fermentation. The theory of electrophoretic mobility is described in Appendix 4.

3.4 Analyses after Fermentation

To investigate the properties of yoghurt at the end of production processes a number of analyses are being used. The focus of this project is to investigate the relation between addition

of pectin and the physical properties of stirred yoghurt. The physical properties can be split in stability, appearance and texture/mouthfeel.

The appearance of yoghurt is a subjective property and the appearance should be smooth or shiny. This can be evaluated by a panel of skilled judges [Modler *et al.*, 1983]. In the present project this type of analysis is not an option.

The texture is sometimes evaluated by expert panels [Gallardo-Escamilla *et al.* 2007], but can also be evaluated as the apparent viscosity at a shear rate of 50-55 s-1 [Koksoy & Kilic, 2004] or by a Texturometer [Sandoval-Castilla *et al.*, 2004]. According to Tamime & Robinson (2007) the single interesting physical property of yoghurt is the flow properties, the viscosity. The purpose of this is to compare a new batch to previous batches with acceptable properties. This can be using many different techniques, from flow through a funnel to viscoelastic properties by a dynamic oscillating rheometer. According to the review by Sodini and co-workers (2004) the texture of yoghurt can be examined using sensoric panels, microscopic analyses and physical measurements, these being viscoelasticity, viscosity and WHC.
To obtain the most possible information of stirred yoghurt the viscoelastic properties of stirred yoghurt can be examined using dynamic oscillating rheology. This method is often applied, but it requires that the yoghurt gel can be broken in a reproducible way. This is very hard to do in laboratory scale, so instead another approach is used.
The flow properties of stirred yoghurt depend strongly on the volume fraction of the casein aggregates. The volume fraction is controlled by how much serum is trapped inside the particles, and this is determined by how much of the gel is broken and rearranged during the stirring process. This is controlled by the specific process, stirring time and equipment. This means that the strength of the gel is very important to the yoghurt properties.
The “gel strength” can be determined by the viscoelastic properties of the gel before stirring. One side is the elasticity, this can be measured continuously, and the other is the point where the gel is breaking. This can only be determined once, using a ramp of increasing shear strain [Girard & Schaffer-Lequart, 2007]. To monitor fermentation processes the gel elasticity can be measured throughout the process, showing the gel point and gel strength build-up. The strength of the gel determines the particle size, and hence both apparent viscosity and water holding capacity. Therefore the gel strength measured by G’ is a useful tool for evaluating the rheological properties of yoghurt.

The stability of yoghurt is actually the stability against whey separation of the yoghurt when the yoghurt is being stored. Stability can be determined by centrifugation and determination of the sediment volume, this parameter is called “water holding capacity” (WHC) [Everett & McLeod, 2005], by gravitational sedimentation and observation of serum separation after storage for 15 days [Koksoy & Kilic, 2004].
To do analyses of WHC centrifugation in an ordinary laboratory centrifuge can be useful. It does not provide any information of the kinetics of the whey separation, only the end point. To do analyses giving more detailed information of the whey separation analytical centrifugation

can be used. This technology can be used in a wide range of applications, and using this method may aid in the development of new ingredient products.

3.5 Statistics

When constructing an experiment it is often done to determine which input variables affect a given output variable. This is often done by varying the input variables one at a time. This approach is not clever as the input variables may affect each other. To fully eliminate this effect all combinations of input variable values must be tested and the data should be the basis of analyses of variance. This is not always possible due to time or money issues, but the way of thinking and the treatment of data is used where applicable. The purpose of using statistics in this project is to raise the data analysis from a subjective to an objective level. Using statistics gives the opportunity to prove if a trend is significant or just a mind trick or a result of the way the data is presented. It also presents a challenge, when used one must understand the background to use it correctly, avoiding over interpretation of the results. Therefore the method of statistical analysis used is described in Appendix 8.

3.6 Summary

In the process of working with the understanding of yoghurt stability and instability, a number of methods are applied.

The methods selected are DLS, capillary viscometry, analytical centrifugation and traditional centrifugation, electrophoretic mobility, dielectric spectroscopy and rheology.

Some of the methods are not often used with the milk-pectin system, and knowledge of the applicability of the used methods is gathered. If the methods prove useful, they may be applied to contribute to connecting the pectin properties with the functional properties of the pectin to stabilise stirred yoghurt. Hence the methods applied should be evaluated to see if these are suitable to aid in the screening process of new pectin based stabilising ingredients.

4 Problem Statement:

- *How can the functional range of LM pectin in stirred yoghurt be determined, and how do the milk properties affect the functional range?*
- *Is intrinsic viscosity of pectin a useful parameter to determine the onset of depletion flocculation in pectin milk mixtures?*
- *Does addition of pectin before heat treatment affect the functional range of LM pectin in yoghurt production?*
- *What is the influence of pectin addition during the gelation process, when does pectin adsorb to casein micelles and what is the effect of heat treatment on the adsorption?*
- *Can dielectric spectroscopy be used to monitor the yoghurt fermentation process in terms of gelation and pectin adsorption?*

4.1 Problem Definition

To answer the problem statement, a number of methods have been selected. The purpose of the problem definition is to combine problems and methods and reduce this to a line of experiments.

- Rehydration of milk is an important perquisite for making good experiments. The milk should be rehydrated and homogenized and to verify that the casein micelles have been separated the size distribution should be measured using DLS.
- To determine the intrinsic viscosity of pectin and pectin calcium mixtures capillary viscometry should be used. The conductivity and calcium concentration should be similar to the one in skim milk at neutral pH.
- The phase diagram of calcium pectin mixtures can be determined visually. This should be done at the conductivity of neutral skim milk.
- The onset of depletion flocculation from LM pectin in skim milk at neutral pH should be determined using analytical centrifugation. The temperature, protein concentration and calcium concentration should be varied to determine the influence of the milk properties.
- To determine if the heat treatment reduces the functional range of pectin, analytical centrifugation should be used to determine the onset of depletion flocculation after varying heat treatment time. To distinguish between milk introduced changes and pectin break down from the heat treatment the heat treatment should also be done without pectin followed by addition of pectin and determination of the onset of depletion flocculation.
- To determine the effect pectin addition during the acidification and gelation process and when pectin is adsorbed to the casein micelles, a combination of oscillating rheology and measurement of electrophoretic mobility should be done. Four experiments should be done combining presence and absence of pectin and heat treatment.
- To test the applicability of dielectric spectroscopy to monitor the yoghurt fermentation process, a fermentation process should be done monitoring pH, oscillating rheology, water holding capacity, and electrophoretic mobility simultaneously with dielectric spectroscopy.

5 Experimental

In this section the preparations, equipment and experimental conditions used in the experimental work of this project will be described. The description of the experiments will be divided in a number of separate experiments, as described above in the Project Definition section. The groups of experiments are listed below. During the experimental work three additional experiments was conducted to examine surprising findings or verify results. These will be described extra carefully in this section. Besides a description of the statistical tools used to analyse the data will be given. To ease the reading the subdivisions of the experiments is maintained in the results and discussion chapters.

Experiments

- Rehydration of skim milk and verification of size distribution
- Intrinsic viscosity of pectin and pectin calcium mixtures
- Phase diagram of pectin calcium mixtures
- Stability of neutral milk-pectin mixtures
- Stability after heat treatment of neutral milk pectin mixtures
- Order of interactions during fermentation
- Fermentation monitored by dielectric spectroscopy
- Statistical analysis

Additional Experiments

- Verification of interaction order results
- Analytical centrifugation of yoghurt
- Comparison of addition of calcium ions and skim milk powder

5.1 Rehydration of Skim Milk and Verification of Size Distribution

In order to be able to vary the protein concentration, maintain milk parameters stable and to mix milk and pectin without heating the mixture the solution chosen was to produce double concentrated rehydrated milk from a single batch of skim milk powder.

The rehydration procedure for 500 mL of double concentrated skim milk (6.8% protein), which has been developed during the project, were performed as described here.
300 mL of demineralised water were heated to 45 °C in a beaker, 94.5 g of skim milk powder (medium heat skim milk powder, 36 % protein, provided by Danisco A/S) were added during vigorous stirring and the suspension was reheated to 45 °C and stirred for 15 minutes while any remaining lumps were crushed. The solution were then transferred to a bluecap bottle, 5.0 mL of 2% NaN3 solution (=0.02% total) was added. The preweighed bluecap was then filled to a total of 500 g solution with heated water. The 45 °C solution was then homogenised for two minutes using a rotor/stator homogeniser (X 1020 international laboratory appliances GmbH, knife diameter 18 mm) with a power regulation at position 3/6. The milk was then cooled to 20 °C on ice and kept at 2 °C until needed. After the bubbles from the homogenisation have separated the size of the particles were determined by DLS (Zetamaster S, Malvern

Instruments, Great Britain) immediately after dilution with deionised water and compared to a sample of commercial skim milk measured at the same time and conditions.
To calculate the size distribution, two fitting methods were applied. One was the monomodal, resulting in the size average, the other was the contin function, resulting in the particle size distribution. It was assumed that particles of less than 30 nm were artefacts, and the results from the contin fit are the intensity distribution above 30 nm.

5.2 Intrinsic Viscosity of Pectin and Pectin Calcium Mixtures

In order to be able to predict the depletion behaviour of protein pectin mixtures, the intrinsic viscosity of the used pectin must be measured at conditions similar to milk at neutral pH (6.7). The conditions determining polymer volume in solution is ionic strength (conductivity) and pH, and besides the contents of calcium is of importance, as LM pectin interact specifically with calcium[Thibault & Ralet, 2003]. To understand the importance of pH and calcium content of milk for the behaviour of pectin in solution, three more points must be determined, namely without calcium at pH 6.7 and both including and excluding calcium at pH 4.5.

Production of pectin stem solution was done by dissolving 10.00 g pectin (LM-pectin, 46 % methyl esterification, Danisco A/S) in 50 °C water by stirring for 50 minutes followed by heating to 60 °C for 3 minutes and cooling on ice to 20 °C to minimise degradation and demethylation of the polymer, followed by addition of 10 mL 2% NaN_3 solution (=0.02%) and water to reach 1000 mL. The stem solution were kept at 2 °C and used for all measurements.

The natural content of soluble calcium in milk at neutral pH is 7.2 mM, equal to 24 % of 30 mM. Intrinsic viscosity is determined from capillary viscometry as described in Appendix 6.
Samples of 100 mL containing 0, 0.3, 0.6, 0.9, 1.2 and 1.5 g/L pectin with and without 7.2 mM Ca^{++} were prepared by mixing different amounts of 10.00 g/L pectin stem solution, 1 M $CaCl_2$ and deionised water to a volume of around 95 mL and adjusting pH and conductivity by careful addition of 1 M NaCl, 1 M HCl and 1M NaOH to match values of pH 4.5±0.05 or 6.7±0.05 and conductivity of 5.5±0.05 mS/cm. As conductivity is very temperature dependent the temperature of the sample is kept at 25.0±0.5 °C during adjustments. Afterwards the sample is topped up to 100.0±0.2 mL with NaCl solution at 5.5 mS/cm. In order to minimise time dependent variation the production order of the samples were randomised. The samples are kept at 25 °C until measured. Measurements were performed in a KPG Cannon-Fenske capillary rheometer(App Nr 49458 K=0.01638, Schott & Gen., Mainz). The Hagenbach correction factors used are a=3732 and b=-2.0666 [Hinge & Yu, unpubl.].
The rheometer was flushed with the sample liquid before the actual 10.0 mL sample was added using a precision pipette. The rheometer was kept in a waterfilled container placed in a water bath with the samples at 25.0 °C. The samples were measured three times using a stop watch and the time was noted at a precision of 0.01 second. Generally the running times were in the range of 60 to 120 seconds and the three measurements had deviations of less than 0.2 seconds. The sample order was re-randomised before viscosity measurements. To check reproducibility one sample was run three times. Additionally the samples were heated to 40 °C and kept for two days before measuring viscosity at this temperature. Similar procedures were used.

5.3 Phase Diagram of Pectin Calcium Mixtures

To determine the phase diagram of pectin versus calcium content, samples were prepared using the samples previously produced for measuring intrinsic viscosity. This was done using the ten non heated, calcium containing samples, five at pH 4.5 and five at pH 6.7. These were calcium enriched adding 1 M $CaCl_2$ to increase the calcium concentration from 7.2 mM(24% of total 30mM) to 15.0, 22.5, 30.0, 37.5, 45.0, 52.5, 60.0 and 67.5 mM. This equals 24, 50, 75, 100, 125, 150, 175, 200 and 225 % of the total natural calcium content of milk. The samples were then shaken and left at first 10 then 25 and 40 °C for at least one hour to obtain temperature equilibrium. The samples was then accessed visually to be either stable or phase separated. The evaluation was done considering if bubbles was trapped in the bulk liquid after shaking, if the water left particles around the edges of the tube after shaking, or even coated the inside of the test tube with an uneven gel like coating after the water withdrew. After two days at room temperature the observations was confirmed by a two phase appearance with a clear water phase on top and a cloudy phase below.

5.4 Stability of Neutral Milk Pectin Mixtures

The analytical centrifuge used was a lumisizer (Lumisizer dispersion analyser, L.U.M. GmbH, Germany) with a radius of 130 mm from centre to sample bottom. The cuvettes used were polycarbonate with a sample thickness of 2 mm and a volume of 500 µL (type 2, prod.nr.110-131xx). When the sample cuvette was loaded, the sample top was at a distance of 105 mm from the centre, experiencing 80 % of the force experienced at the sample bottom, independent of rotational speed. The rotational speeds used were 800 rotations per minute (rpm) resulting in a relative centrifugal force (RCF) of 93, that is 93 times the earth's gravitational acceleration. The determination of RFC is described in Appendix 5. Also used were 1600 rpm (RCF=372) and 4000 rpm (RCF=2325). The analysis programme consisted of running at 800 rpm for one hour based on a test run of 200, 400, 800, 1600, 2400, 3200 and 4000 rpm. At 800 rpm neutral milk remains stable for at least one hour, making it able to detect changes in separation properties.
The software used to control the centrifuge, log and analyse data were Sepview (version 5.1.0.804).

The sample preparation and measurements were done as described in the next section.

Temperature dependence:
Solutions of 0, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900 and 2000 ppm pectin(1000 ppm equals 1 g/L, 0.1% and 1 °/₀₀) in milk at normal milk concentration (3.4 % protein) were produced. Pectin stem solution and water was mixed, shaken and double concentrated milk was added followed by stirring immediately afterwards. The samples were left in 11,5 mL sample tubes in a precision waterbath for at least one hour before analysis. The temperature of the waterbath and the centrifuge was adjusted to 15 °C, a measurement on 500 µL of each sample were preformed as described above followed by re-equilibration and measurement at 30°C, 45°C and 15 °C again to verify that no change occurred because of the time between measurements.

Protein dependence:
Samples at 2.0, 3.5 and 5.0 % protein and the same pectin concentrations as described above were prepaired by mixing varying amounts of pectin stem solution, water and double concentrated milk in the order described above. The samples were kept in a 40 °C water bath for 15 minutes before measurement at 40 °C using the procedure described above.

Calcium dependence:
To test for calcium dependence samples of milk at 3.4 % protein with volume of 10 mL and a pectin content of 1400, 1600 and 1800 ppm were mixed. These were split in four times 2 mL and 0, 45, 75 and 105 µL of 1.0 M CaCl2 were added resulting in samples at 7.2, 30, 45 and 60 mM, equal to 24, 100, 150 and 200% soluble calcium compared to the total amount of calcium in milk.
These were centrifuged in a single run using the same program as the previous samples. Afterwards the samples were poured on a glass plate to observe the properties of the two phases. A photo of this is placed on the data DVD.

The analytical centrifuge and the software used presents data as described in Appendix 5. These are interpreted by looking at the two parameters shown in figure 14.

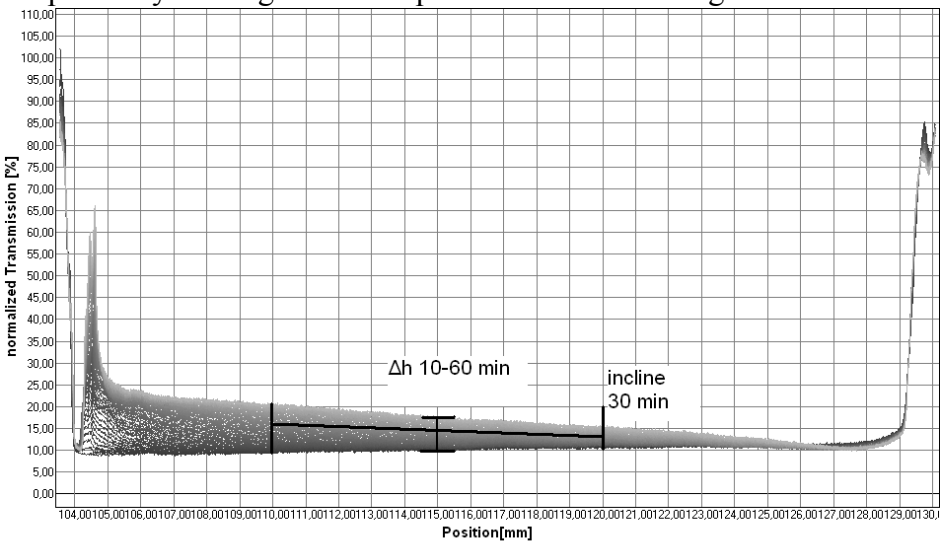


Figure 14: The black marks show the parameters used to determine if a sample is stable or phase separating. The abscissa shows position and the ordinate is light transmission.

The two parameters used to determine if the sample was stable, phase separating or strongly phase separating. The first parameter is the height difference at 115 mm from 10 minutes into the measurement to 60 minutes, when the measurement was ended. The first 10 minutes is left out to let bubbles and other impurities get out of the important area in the middle of the cell. Measurements on pure milk return a difference of 0.2-0.75 when evaluated using this parameter. Unstable samples return a value at least 0.1% higher than the reference and the limit of strong phase separation return more than 2.0% on this parameter. The incline between 110 and 120 mm is used to confirm the trend found from the first parameter as the incline also changes with unstable samples, the normal level is 1.75 to 2.0 and unstable samples return

values of 1.5 or less. Both parameters are subject to variation and hence a combination of the two is used to determine if the sample is unstable.

5.5 Stability after Heat Treatment of Neutral Milk Pectin Mixtures

To observe the effect of the pectin during heat treatment samples were made including pectin to a final concentration of 1600, 1700 and 1800 ppm. The samples were heat treated in a water bath at 85°C for 0, 5, 10 and 15 minutes, samples were extracted and measured in the analytical centrifuge for 60 minutes at 800 rpm and 40 °C followed by further heat treatment to a final time of 20, 40 and 60 minutes, followed by another measurement. Other samples of 1400, 1600 and 1800 ppm final concentration was prepared and heat treated for 0, 5, 15 and 30 minutes, followed by addition of pectin. These were measured in the analytical centrifuge in a way similar to the previous samples.
The analytical centrifuge and stability criteria used are described above.

5.6 Order of Interactions during Fermentation

The purpose of this experiment was to look into the interactions in gelling of acidified milk depending on if the milk was heat treated or pectin was added. This was done combining rheology, and electrophoretic mobility by means of controlling pH.
Sample preparation consisted in bying 3 L pasteurised skim milk (Arla Express) to obtain milk with minimal heat treatment. The milk was mixed and 30 mL 2 % NaN_3 (=0.02%) was added to avoid microbial activity. Then four bottles of 500 g milk was weighed of and to mix pectin into two of them without giving the samples different treatments. All samples were heated to 55 °C for 25 minutes and 350 mg pectin (LM-pectin, 46 % methyl esterification, Danisco A/S) was added to two of the samples. The pectin was dissolved by vigorous stirring and after cooling to 40 °C the samples were homogenised for 60 seconds using the rotor/stator homogenisor described above. The two samples, one with pectin and one without, were cooled on ice while the two others were heat treated by heating in microwave oven to 85 °C and keeping this temperature for 30 minutes before cooling on ice. The samples were kept at 2 °C until analysed.

The pH meter (PHM 290 pH stat controller, Radiometer Copenhagen) using a Blueline 11 pH electrode were calibrated using buffers (Radiometer analytical buffers pH 4.00±0.02 (citrate) pH 7.00±0.02 (phosphate) Hach Lange GmbH) heated to a temperature of 30 °C. The measurements were done in a 400 mL beaker immersed in a precision water bath (cooling: Haake K20, and heating, pumping and temperature control: Haake DC 30, Haake GmbH, Germany). This beaker was stirred by an impeller from above assuring homogeneity and quick dissolution of added solids. The pH electrode was immersed in the liquid and the beaker was covered with plastic film to minimise evaporation. The setup can be seen in figure 15. The pH was logged every 10 seconds using the RS232 port and software written by the author in Delphi specifically for this purpose.
The acidification was done by placing 250 g sample heated to 30°C in the beaker, wait for temperature equilibrium and adding 3.75 g (=1.5%) GdL (D-(+)-gluconic acid δ-lactone

99.0%, Sigma-Aldrich, United States), to the stirred beaker. After dissolution of GdL, 14 mL was transeferred to the rheometer.

Figure 15: The setup used to measure pH during the order of interactions experiment. The water bath also controls the temperature of the rheometer.

Figure 16: The rheometer with a sample injected and the protective cover opened.

Rheological parameters(G' and G'') were measured during the acidification using a dynamic oscillating rheometer (Bohlin CVO 120, Malvern Instruments, Great Britain). The measuring system used was a coaxial cylinder, DIN C25, (24694 Pa/Nm) popularly known as a cup and bob configuration, with a bob diameter of 25 mm. The software used to log and analyse the data was Bohlin software 6.32.1.2. The rheometer can be seen in figure 16.

The temperature of the sample was controlled by the water bath used also for the pH measurement, and hence the sample temperature and thereby pH should be the same. The parameters used for the measurement was single frequency (1 Hz) with controlled stress (0.5 Pa) using continuous oscillation and measuring every 30 seconds for approximately 3.5 hours.

In order to measure the electrophoretic mobility of casein micelles under conditions similar to the ones in the actual yoghurt during fermentation a buffer was prepared. The buffer was constructed to have properties similar to neutral milk, including 7.2 mM Ca^{++} and conductivity of 5.5 mS/cm. The buffer was made from 50 mM acetic acid by addition of 1 M NaOH and 1 M NaCl. Buffer solutions of 100 mL was produced with pH values of 4.50-6.00 with intervals of 0.1 pH unit, additionally pH 6.25 and 6.50 was prepared. Electrophoretic mobility was measured using a zeta master with a pc running (PCS for windows, v. 1.32a, Malvern Instruments) software. Most samples were measured at least eight times, the mean of these have been calculated and the uncertainty given is the standard deviation of these measurements. Results showing zero mobility were sometimes observed in the data sets, these are due to apparatus failure and have been removed before calculations were performed.

The pectin solutions in buffer at concentrations similar to the ones in the samples had a mobility of -0.18±0.04, not significantly different from that of demineralised water (-0.18±0.02). Measurements of these samples took much longer time to do, as the signal was very weak. The samples were extracted from the stirred container at the given pH and diluted

100 times in buffer adjusted to the same pH. The aggregated samples were diluted 200 times to match the concentration window of the apparatus.

Around pH 4.8 the pH stabilised and the gel point had been detected. To obtain the remaining points of electrophoretic mobility down to pH 4.5 the pH was lowered by addition of 0.1M HCl followed by 5 min equilibrium time before a sample was withdrawn and the next pH was reached in a similar manner.

To calibrate the rheology measurements for an observed higher pH, it is assumed that the pH can be expected to follow the same curve, just with different time scales. This means that when a point of pH and time in the rheometer is known, the relative timescale can be calculated by stretching the measured time scale to fit the point, by multiplying with a constant. This is illustrated in figure 17. The line of the original curve is expanded by a linear approximation.

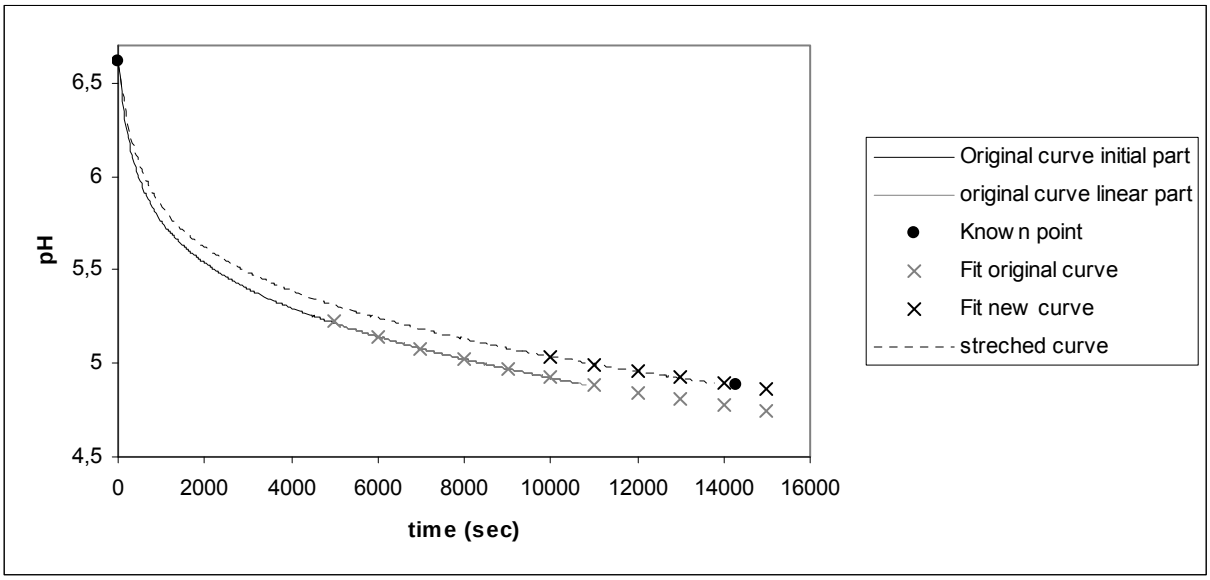


Figure 17: Method of compensation for high pH point measured, the new curve is a copy of the old curve stretched by a constant multiplied to each data point.

5.7 Fermentation Monitored by Dielectric Spectroscopy

The experimental work in applying dielectric spectroscopy to yoghurt fermentation was done in corporation with Ph.D Peter Vittrup Christensen. A sample of yoghurt was supplied and different analyses were conducted to give a basis for comparison with the impedance spectra.
As the fermentation process is a living process with many uncertainties the procedure used was preparation of one sample of yoghurt which was fermented at 37°C in different containers and observed by measurements of dielectric spectroscopy, pH, dynamic oscillating rheology (G' and G''), centrifugation(WHC) and electrophoretic mobility.

In the experiment to apply impedance spectroscopy to yoghurt fermentation, a simple procedure of producing yoghurt was used. It consisted of heating 470 g commercial skim milk(Arla Express) to 85 °C, 500 mg of pectin was added(LM-pectin, 46 % methyl

esterification, Danisco A/S) followed by stirring for 7 minutes, cooling on ice to 40 °C and adding 30 g of commercial skim milk yoghurt with no stabilisers added(Thise Mejeri). This time was considered the start of the experiment. The mixture was then split up and fermented in different closed containers in an oven (model T 5042 E Heraeus GmbH) at 37 °C.

The pH was monitored in a sealed beaker by pH meter using the apparatus described in the previous experiment and calibrated at 37 °C using the buffers described above.

A custom made impedance cell, built by Peter Vittrup Christensen, was preheated and filled with the mixture and put in the oven used for the fermentation. The impedance measurements were made in the range of 1kHz to 10 MHz using a Solartron 1260 impedance analyser. The data for the impedance spectrum was accumulated over three minutes with pauses of ten minutes. Five calibration spectra of KCl solutions in demineralised water was measured at 37 °C according to the varying conductivities of the yoghurt during fermentation and used for calibration of the measured impedance spectra. The solutions were prepared using a 2M KCl stock solution, demineralised water and a conductivity meter (CDM 210 Conductivity meter, Radiometer Copenhagen).

The rheometer used is described above. The temperature was set to 37.0 °C and was controlled with a water based temperature control system (cooling: Haake K20, and heating, pumping and temperature control: Haake DC 30, Haake GmbH, Germany).
The measurement was conducted with the following parameters: Single frequency (1 Hz) with controlled stress (0.5 Pa) using continuous oscillation and measuring every 30 seconds for approximately 3.5 hours.
After this the used parameters were verified by an oscillation amplitude sweep(1 Hz, controlled stress 0.012-5.0 Pa 30 points up and 30 points down on a logarithmic scale, delay between samples 5 seconds, continuous oscillation) and an oscillation frequency sweep(controlled stress 0.5 Pa, 0.1-10 Hz, 20 points on a logarithmic scale, one second delay between samples, continuous oscillation).

The sedimentation behaviour was measured in a centrifuge (model 3-15, Sigma laboratory centrifuges) with a distance of 155 mm from centre to bottom of sample. The rotational speed was 4000 rpm, resulting in a relative centrifugation force of 2740. 10 ml of inoculated milk were placed in each of the eleven 11.5 mL polystyrene sample tube and these were put in the oven at 37°C with the other samples. At varying intervals a tube was removed from the oven, the time and pH was noted, the sample tube was shaken, and a small amount was withdrawn and put in another sample tube for dilution and determination of electrophoretic mobility. The sample tubes were placed on ice for approximately 15 minutes to stop fermentation. Then the sample was shaken and centrifuged at 4000 rpm for 10 minutes and the relative volumes of sediment and clear serum were determined.

The electrophoretic mobility was measured using a Zetamaster S (Malvern Instruments, UK), using data loggong software (PCS v. 1.32a, Malvern instruments Ltd., Great Britain). Each

sample was diluted 20-40 times to reach a suitable concentration for the mobility measurement. The dilution was done using a 5.5 mS/cm KCl solution, at approximately the right pH, directly followed by measurement of pH of the diluted sample and approximately ten measurements of electrophoretic mobility of the diluted sample. The given standard deviation is calculated based on the variation of the measured values of electrophoretic mobility. The measurements sometimes return zero mobility, this is considered an error and these measurements are removed.

5.8 Statistical Analysis

The statistical methods described in Appendix 8 are applied using two different software packages. One is Excel, a part of the Office 2003 package. This software is a spreadsheet used for handling data, making simple calculations and regressions and presenting data in a appropriate way for the report. The other software package is STATGRAPHICS Centurion XV Professional Edition. This software is specifically used for statistical analysis of data and has been used to do the more complicated calculations. Reports of the calculations can be generated from the software and these are available on the data disc.

5.9 Verification of Interaction Order Results

Verification of the interactions found in the experiment "Order of interaction during Fermentation" was attempted. This was done using the heat treated milk without pectin from the experiment. The milk was acidified using slow, dropwise addition of 0.1 M HCl. The milk was placed in a beaker and the pH was reduced from 6.5 to 6.0. Samples were withdrawn and mixed with pectin stem solution-water mixtures resulting in 20% dilution. The final concentrations of pectin were 0, 100, 500, 1000 and 2000 ppm. The pH in the beaker was further reduced to 4.7 and the procedure was repeated. The samples were diluted using the same buffers used in the original experiment and then the electrophoretic mobility was determined following the previous method. The total dilution of the milk from both the pectin-water mixture and hydrochloric acid was around 50%.

5.10 Analytical Centrifugation of Yoghurt

To determine if the water holding capacity of yoghurt produced from the four samples of milk used in the order of interactions experiment exhibited different water holding capacities 50 ml of each milk type were acidified by adding 1.0 g GdL and leaving at 30 °C for 12 hours. pH was measured at the end of acidification after shaking and then approximately 500 µL were extracted with a syringe and placed in cuvettes. These were centrifuged for 90 minutes, 30 minutes at 800 rpm, then 30 minutes at 1600 rpm followed by 30 minutes at 4000 rpm.
The volume of sediment were evaluated as the position (in milimeters) of the phase boundary, liquid top and bottom from the center of the centrifuge at 50 % transmission The calculation of % sediment were done using the following method: (bottom-phase boundary)/(bottom-top)*100% resulting in % sediment in the vial at low, intermediate and hard centrifugation respectively.
Also the samples at pH 4.7 used in the "Verification of Interaction Order Results" with varying amounts of pectin was analysed this way.

5.11 Comparison of Addition of Calcium Ions and Skim Milk Powder

The purpose of this experiment was to compare yoghurts produced with addition of skim milk powder to yoghurts produced using the same amount of calcium contained in the skim milk powder. Two series of substituting skim milk powder were made, one at 150 % and one at 125 % plus a reference. To verify the reproducibility of the methods, the point containing 12.5% extra calcium and 12.5 % extra skim milk powder were made in doublet. This results in the sample scheme seen in figure 18.

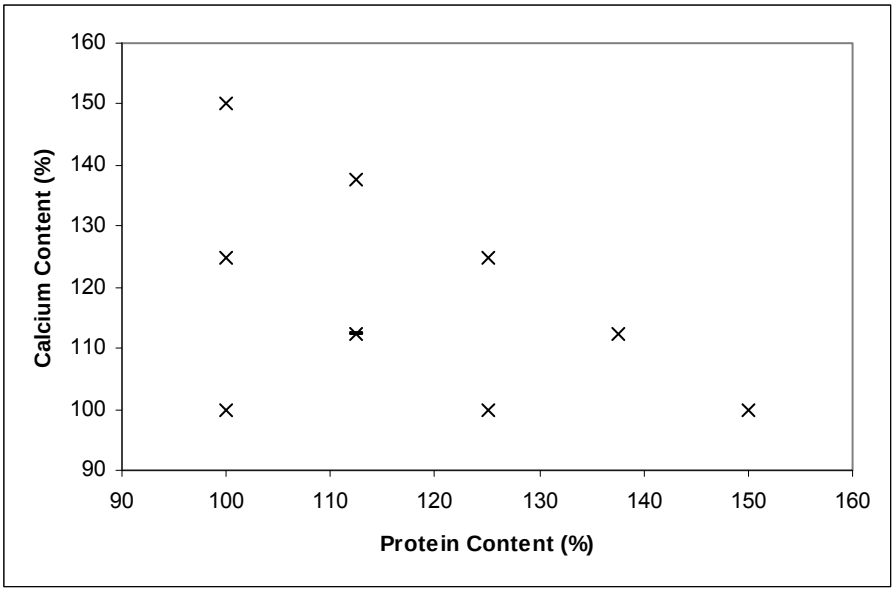


Figure 18: The samples placed on a protein content and calcium content scale, note that the upper row, from upper left to lower right corner, contains the same total calcium content, so does the one below it.

The samples were produced in volumes of 50 mL into greiner tubes and heat treated after mixing, this was done at 85 °C for 30 minutes, followed by cooling on ice. At this point the stability of the samples were analysed, this will be described in detail below. The samples were kept at 30 °C until used and the time from production of the samples to initiation of acidification was noted. The sample order was random. Acidification was done using GdL, by adding 1.50 g to each 50 mL sample (3 %). At addition of GdL the time was noted to make precise comparisons of the samples.

After addition of GdL and shaking for 30 seconds, two samples of 10.0 ml was taken out, put in 11.5 mL sample tubes and left vertically for the next 90 minutes in the 30 °C waterbath. 14 mL was transferred to the rheometer.

The analytical centrifuge used is described above. 500 µL samples of each of the ten preparations were transferred to cuvettes and centrifuged for 30 minutes at 30 °C at 800 rpm followed by 30 minutes at 4000 rpm in the analytical centrifuge. Transmission profiles were measured every 60 seconds After 30 minutes the stability of the samples were evaluated as described above.

After 60 minutes the volume of clear upper phase were evaluated as the position in milimeters of the phase boundary, liquid top and bottom from the center of the centrifuge at 50 % transmission (phase boundary-top)/(bottom-top)*100% resulting in % clear phase in the vial at 60 minutes.

The pH was measured using the pH meter and the experimental configuration described above in the "Order of interaction during Fermentation" section. Before the actual experiments were conducted the pH-time relationship was determined using milk at 3.4 % protein. 250 g milk was first heated to approximately 30 degrees in a microwave oven and left to achieve temperature equilibrium in the beaker for 30 minutes. Then 3 % w/w GdL (7.50 g) was added. The pH was observed and noted at 10-20 points during the next 120 minutes.

Measurements of G' and G'' was preformed using the rheometer, temperature control and configuration described above. The parameters used were a constant shear stress of 0.5 Pa and frequency 1 Hz. The oscillation was kept continuous throughout the measurement and measurements were done every 30 seconds. The temperature was set to 30 °C and to heat up the cup and bob it was filled with water and left to heat for 30 minutes with 100 rpm rotation and then emptied and dried. After mixing with GdL 14.0 mL of milk was added to the cup and bob and the measurement was started. After the 90 minutes the measurement was stopped.

The two samples in the sample tubes were removed from the water bath at 90 minutes, one sample was stirred thoroughly with a spatula and the other handled carefully to not damage the gel. The two samples was centrifuged at 4000 rpm (RCF 2740) in the Sigma centrifuge described above for 10 minutes and the level of liquid and sediment was measured with a ruler, this was later converted to % sediment volume.

The remaining sample in the greiner tube were left at room temperature and later shaken to homogenise the gel, which exhibiting syneresis. These were analysed in the analytical centrifuge using the method described in the "Analytical Centrifugation of Yoghurt" section.

The gel time from addition of acid and the end pH suggests that protein content affects the change in pH in a reducing way. This has been compensated by a different method of finding G' for the gel. This was done first by compensating for later gel time by looking at G' 2000 seconds after gel time. This compensation was not sufficient, and therefore the time after the gel point was multiplied by the protein content. This means that for a sample with 100 % protein G' is determined at the gel point + 2000 seconds, for a sample with 150 % protein the G' is observed at the gel point + 3000 seconds.

6 Results

This chapter contains the results from the experiments done during the project, described in the experimental chapter. To ease the reading, they are presented in the same order as they occur in the experimental chapter.

6.1 Rehydration of Skim Milk and Verification of Size Distribution

Two batches of rehydrated skim milk were produced, A and B. A was used for determining the effect of temperature, calcium content, protein content, and the effect of heat treatment. B was used for comparison of addition of calcium ions and skim milk powder.

The average size of the samples using monomodal fit are shown in table 1.

Table 1: The average size of the particles in rehydrated skim milk and fresh skim milk for batch A and B.

Sample	Size average(nm)
A	
Fresh skim milk	199,1
Rehydrated skim milk homogenised	221,2
B	
Fresh skim milk	200,8
Rehydrated skim milk unhomogenised	221,7
Rehydrated skim milk homogenised	207,9

The size distribution of fresh skim milk measured at the same time, are shown for comparison with the sample size distribution, these are shown in figure 19 and 20. The size distribution before homogenisation is shown for batch B.

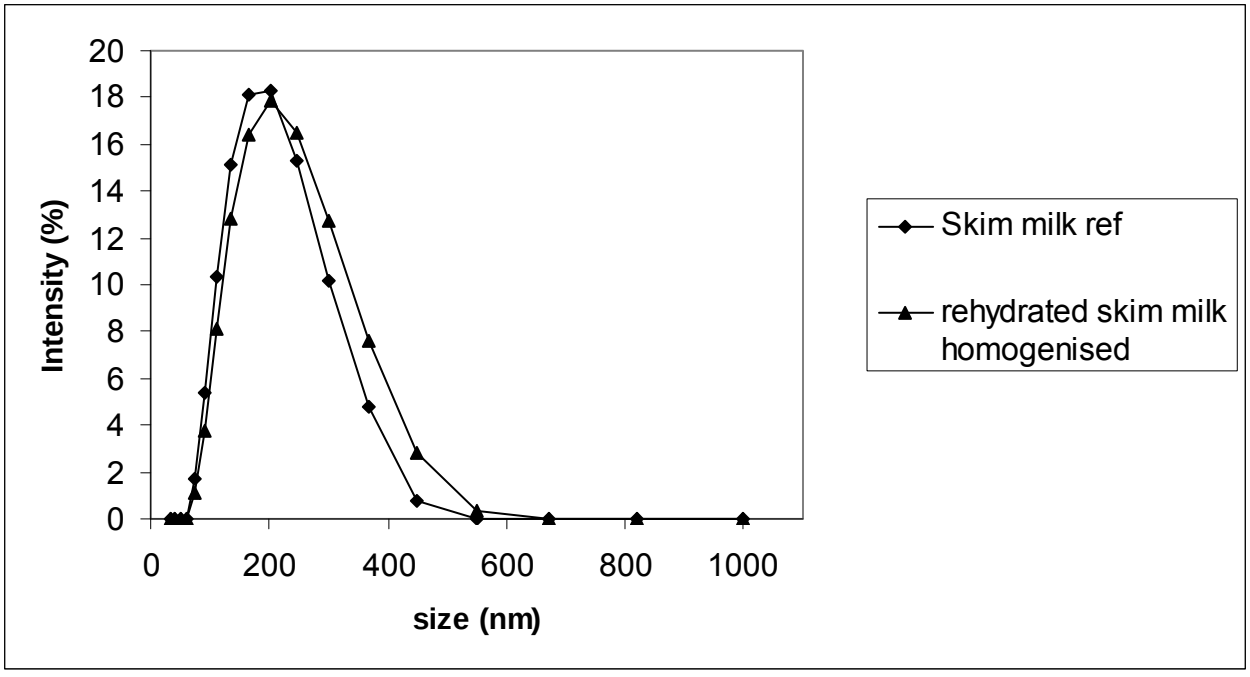


Figure 19: The size distribution of fresh skim milk and rehydrated, homogenised skim milk.

It is seen in figure 19 that batch A has similar size distribution to fresh skim milk.

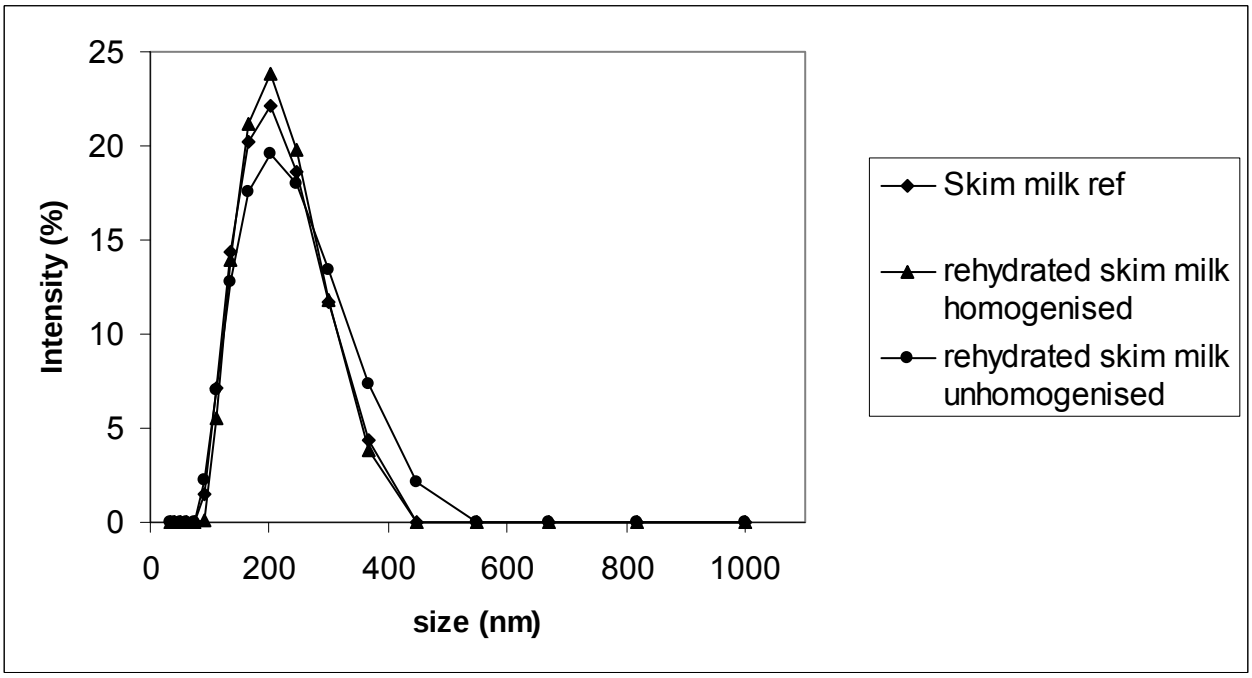


Figure 20: The size distribution of fresh skim milk, rehydrated, homogenised skim milk and rehydrated unhomogenised skim milk.

It is seen in figure 20 that batch B has similar size distribution to fresh skim milk and that the unhomogenised milk is shifted towards larger particle sizes.

6.2 Intrinsic Viscosity of Pectin and Pectin Calcium Mixtures

The relative viscosities have been determined by capillary viscometry, these can be seen in figure 21.

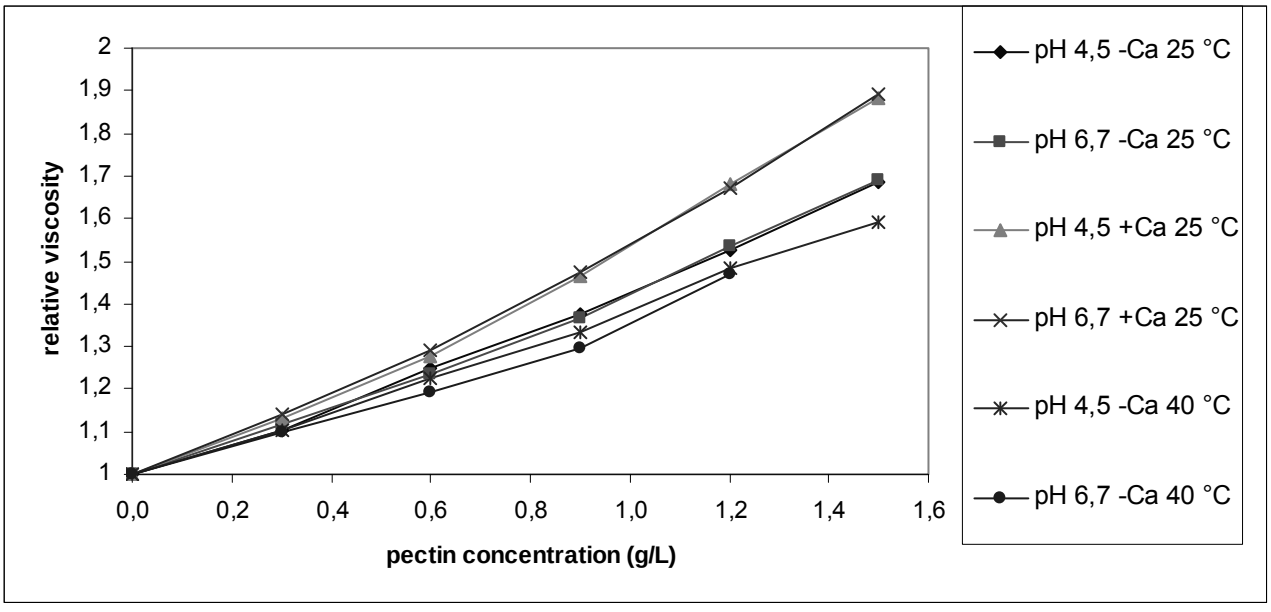


Figure 21: The relative viscosity of pectin solutions at varying pH, temperature and the presence or absence of calcium ions (7.4mM).

In figure 21 the relative viscosities of the samples seems to be grouped in three pairs. The lowest are the pair measured at 40 °C, the middle pair is at 25 °C without calcium and the

upper pair is at 25 °C with calcium. Calcium and pectin concentration is significant when modelling the data, but pH from 6.7 to 4.5 does not result in significant changes in relative viscosity. The data does not show significant changes due to temperature.
The average intrinsic viscosity was calculated using μ_{inh} and μ_{sp} and the average of the two is presented in table 2 with the viscosity average molecular weight $\overline{M}_v$ and DP using the constants and monomer weight values for polygalacturonic acid. The calculations are described in Appendix 6.

Table 2: This table shows the average intrinsic viscosity, the calculated average molecular weight and degree of poymerisation.

pH	Calcium	Temperature (°C)	average [μ] (dL/g)	$\overline{M}_v$ (Da)	DP
4,5	-	25	3,43	$56 \cdot 10^3$	271
6,7	-	25	3,61	$57 \cdot 10^3$	279
4,5	+	25	3,82	$59 \cdot 10^3$	287
6,7	+	25	4,13	$62 \cdot 10^3$	299
4,5	-	40	3,34	$55 \cdot 10^3$	268
6,7	-	40	3,03	$52 \cdot 10^3$	254

6.3 Phase Diagrams of Pectin Calcium Mixtures

This section presents the phase diagrams of pectin calcium mixtures at pH 4.5 and 6.7, with varying temperatures.

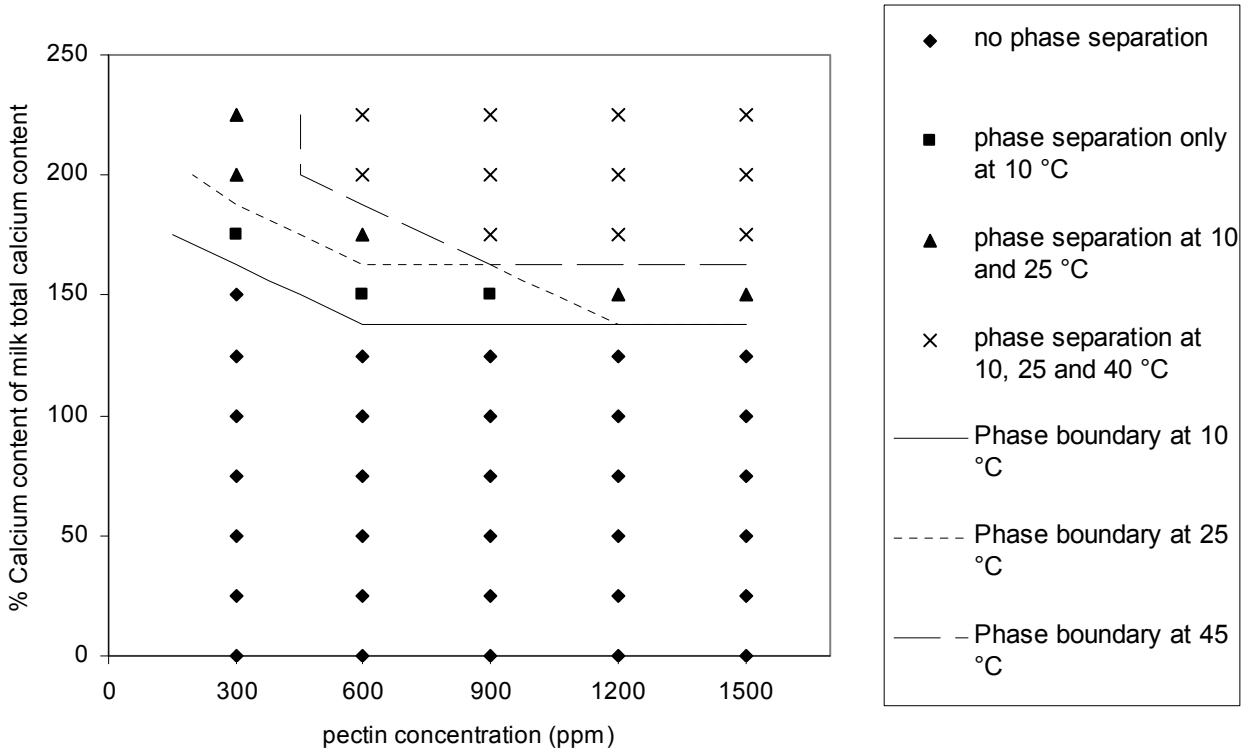


Figure 22: The phase diagram of pectin at pH 4.5 at 10, 25 and 40 °C with pectin concentration on the abscissa and calcium content on the ordinate. The calcium content have been normalised to the total content of milk.

The phase boundary in figure 22 is located at the highest values of pectin and calcium at 40 °C and then the instability boundary is moved to lower concentrations at lower temperature. The same tendency is seen in figure 23. The instability occurs at lower concentrations and is more temperature dependent at pH 4.5 compared to pH 6.7.

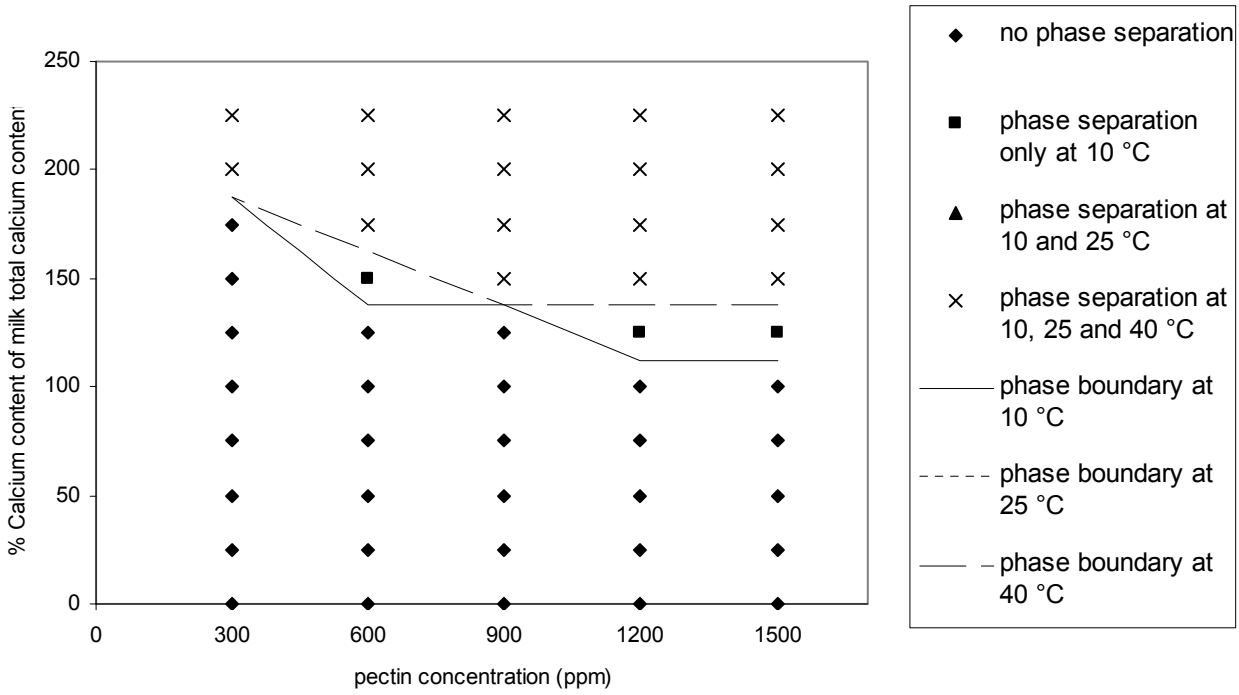


Figure 23: The phase diagram of pectin at pH 6.7 at 10, 25 and 40 °C with pectin concentration on the abscissa and calcium content on the ordinate. The calcium content have been normalised to the total content of milk.

6.4 Stability of Neutral Milk Pectin Mixtures

This section presents the results on stability of milk at neutral pH after mixing with varying amounts of pectin.

The milk system gets modified by temperature, protein content and calcium content and the results are presented as stability diagrams.

The stability is determined as described in the experimental section. The stability diagrams are displayed with pectin on the abscissa and the varied parameter, either temperature, protein content or calcium content, on the ordinate.

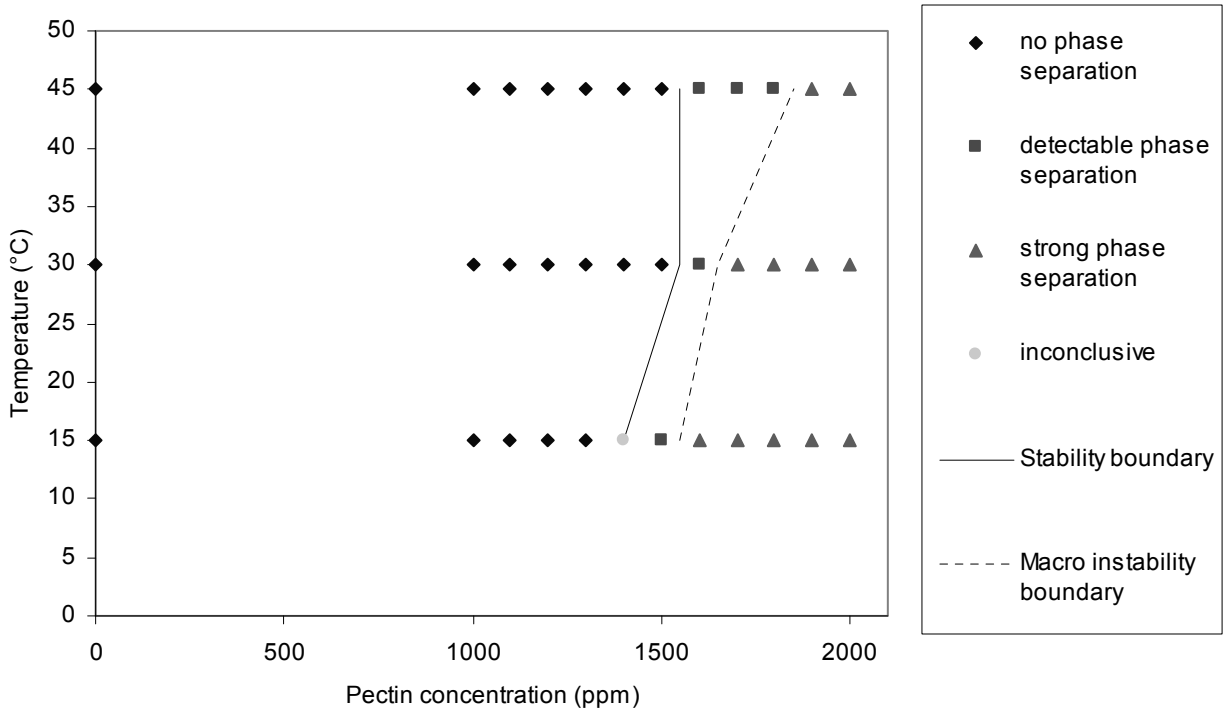


Figure 24: The stability diagram of temperature versus pectin concentration.

It is seen in figure 24 that lower temperature moves the stability boundary towards lower concentration and the degree of instability is lowered with increasing temperature. The limit of phase separation was verified visually as a small amount of dense precipitation at 1500 ppm after three days at 25 °C

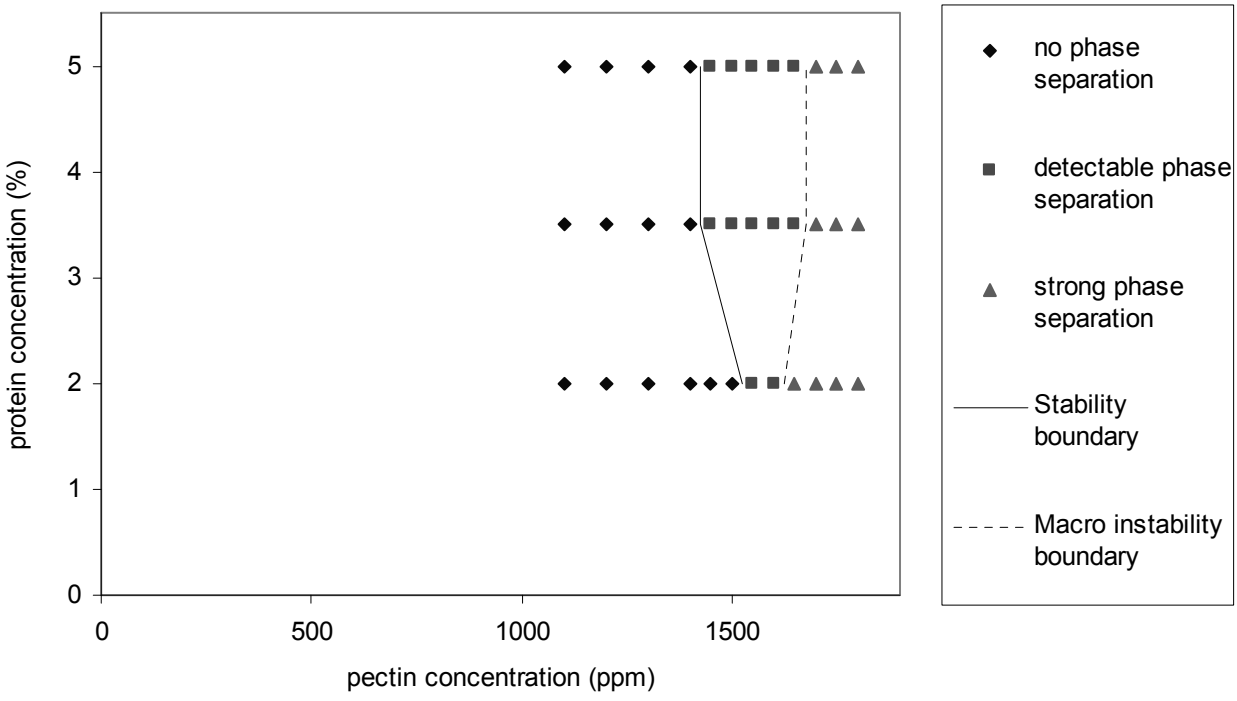


Figure 25: The stability diagram of protein concentration versus pectin concentration.

It is seen in figure 25 that lower protein concentration results in that the stability boundary is moved towards higher concentrations. At low protein concentrations the unstable samples tend to separate more easily, resulting in strong phase separation.

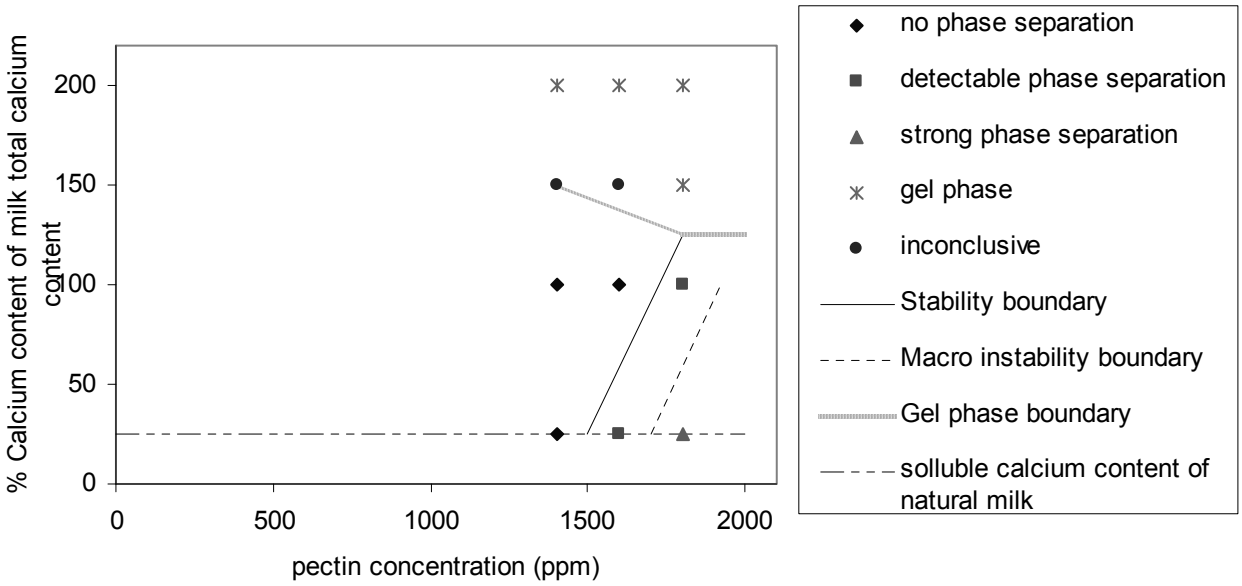


Figure 26: The stability diagram of calcium content versus pectin content. The calcium content is calculated to be % soluble calcium compared to the total calcium content in milk.

The stability diagram in figure 26 shows both instability due to depletion flocculation and gel separation from the serum. At calcium concentration of 100 percent the stability boundary is moved to higher concentration, but at 150 percent gel is beginning to form. This result in a partially transparent gel like phase at the top of the measurement cell.

The gelling of pectin in milk can be compared to the phase diagram of calcium pectin mixtures at the same conductivity, temperature and pH, this is shown in figure 27.

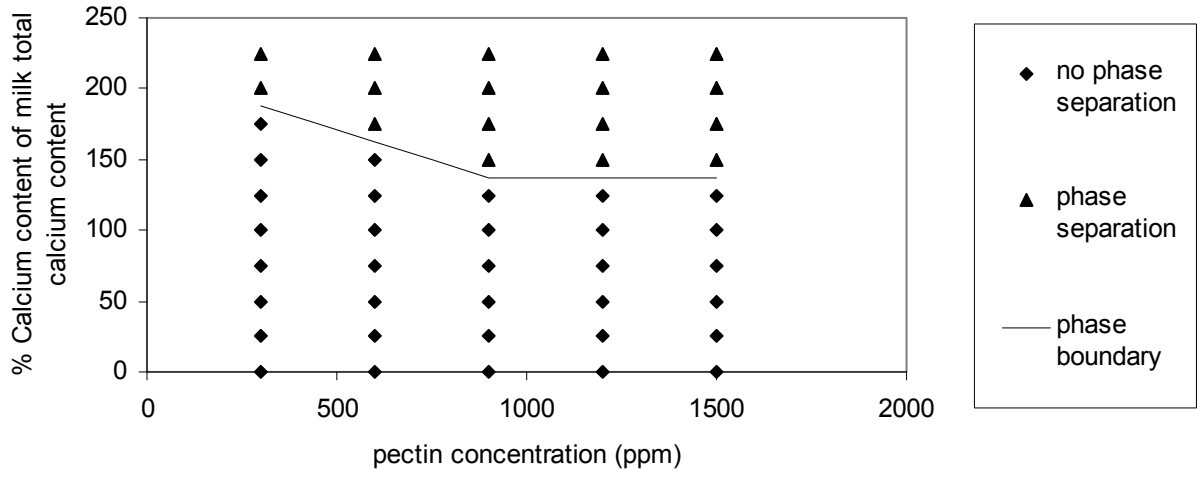


Figure 27: phase diagram of calcium-pectin mixtures at 40 °C with conductivity and pH similar to milk at neutral pH. The pectin concentration is on the abscissa and calcium content on the ordinate. The calcium content have been normalised to the total content of milk.

6.5 Stability after Heat Treatment of Neutral Milk Pectin Mixtures

The effect of including pectin during heat treatment of milk has been examined in the scope of phase stability by analytical centrifugation. Samples were heat treated followed by addition of varying amounts of pectin and determination of stability by analytical centrifugation. This is presented in figure 28. The next experiment included the pectin during the heat treatment followed by analysis of stability by analytical centrifugation and is presented in figure 29.

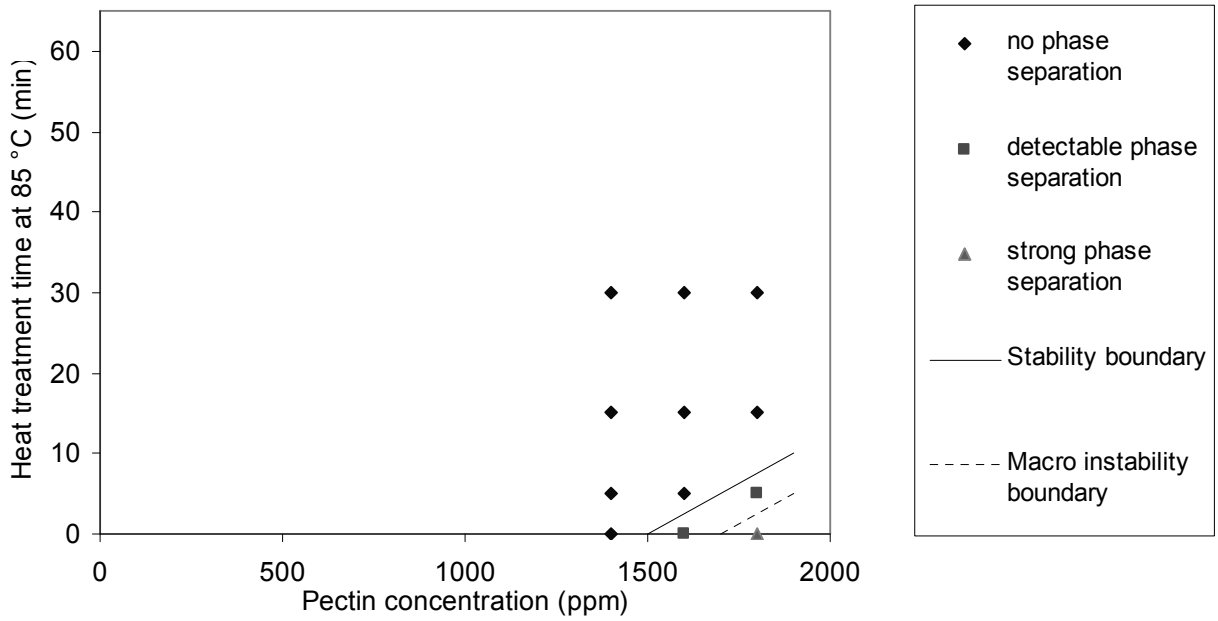


Figure 28: The phase diagram of pectin concentration and heat treatment time without pectin.

Heat treatment without pectin results in a fast increase in stability against phase separation with increase in heat treatment time.

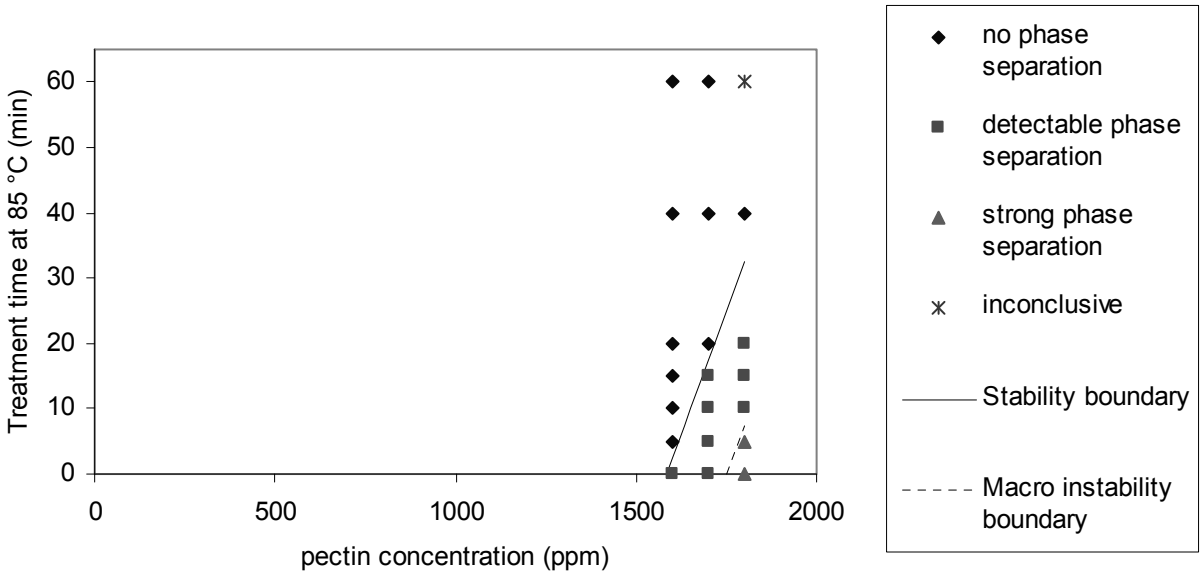


Figure 29: The phase diagram of pectin concentration and heat treatment time with pectin.

Heat treatment with pectin results in a slower increase in stability against phase separation with increase in heat treatment time.

6.6 Order of Interactions during Fermentation

To determine the interaction order of gelation and pectin adsorption and the effect of pectin on the gelation, four measurement series were made. The first was made without pectin or heat treatment (Figure 30), the second was made with pectin without heat treatment (figure 31), the third was without pectin and heat treated (figure 32) and the fourth was made with both pectin and heat treatment(figure 33). The milk used was the same in all cases and the sample preparation procedure was similar. The rheological parameters G' and G'' was measured simultaneously with pH and electrophoretic mobility. The pH was used to connect the results and plot them simultaneously.

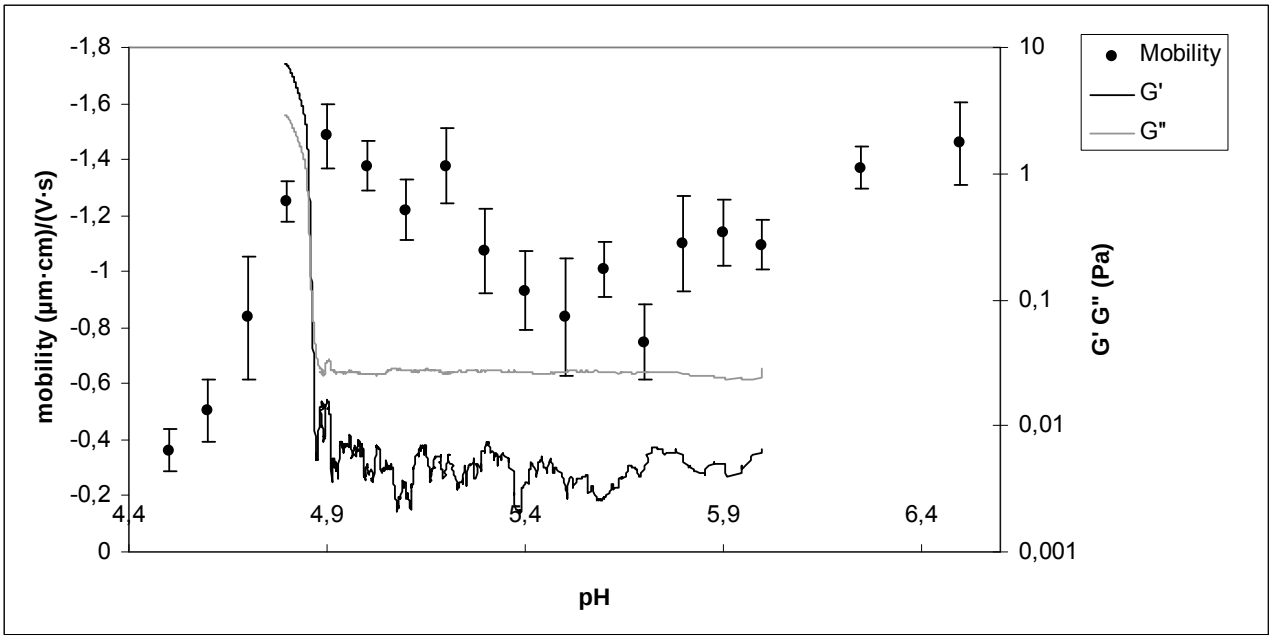


Figure 30: The sample without pectin and without heat treatment. The rheological parameters and mobility plotted as a function of pH. As pH decreases during the acidification the figure should be read from right to left.

The rheological parameters in first part of the acidification in figure 30 is consistent with a liquid with no elastic modulus, as the value of G' is shown on a logarithmic scale it is next to nothing and probably due to limitations of the measuring equipment. This persists until around pH 4.9, where there is a steep increase of G' and G''. The value of G' increases more steeply and passes G''. This is consistent with gelation of the sample and the gel point is pH 4.85. G' levels off around 7 Pa.

The mobility of the casein micelles is decreasing from pH 6.5 to 5.5 followed by an increase to pH 4.9 and then a steep decrease to a level close to zero at pH 4.5.

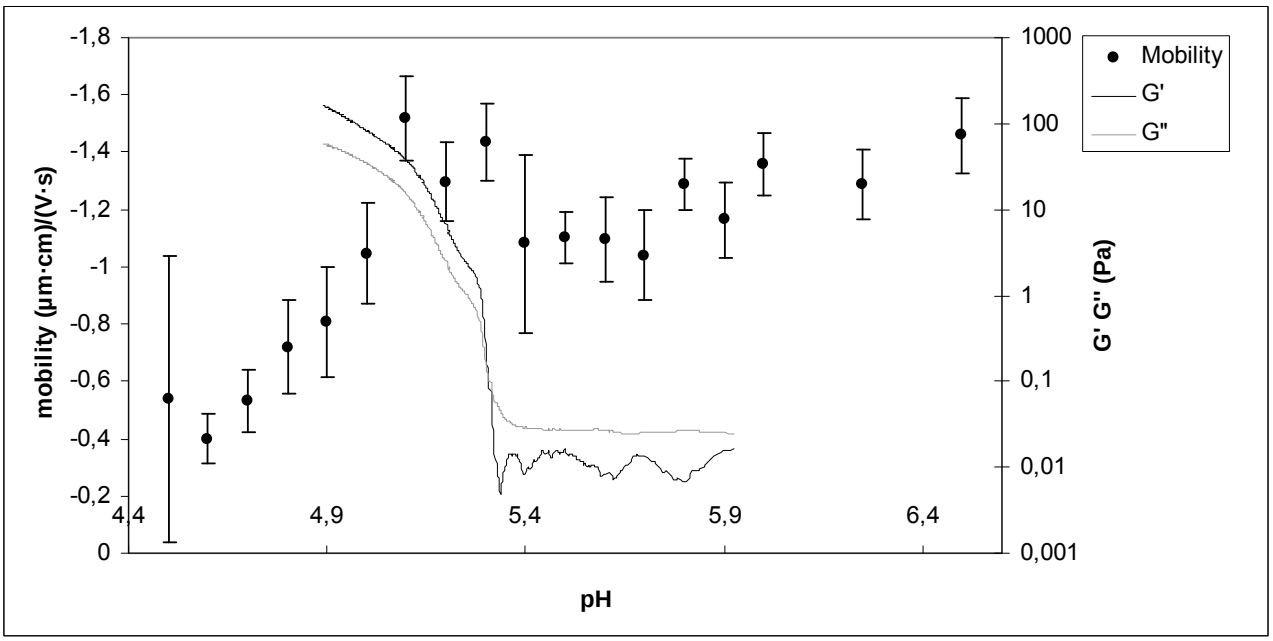


Figure 31: The sample without pectin and with heat treatment. The rheological parameters and mobility plotted as a function of pH. As pH decreases during the acidification the figure should be read from right to left.

The first part of the acidification seen in figure 31 is consistent with liquid, followed by a steep increase around pH 5.3. This point is the gel point and the pH is 5.28. After the initial increase, this is followed by a short flattening of the curve and then another increase to very high values of G'. G' levels off around 160 Pa.

The mobility decreases from 6.5 to 5.5 followed by an increase to 5.1 and then a steep decrease to values close to zero at pH 4.6.

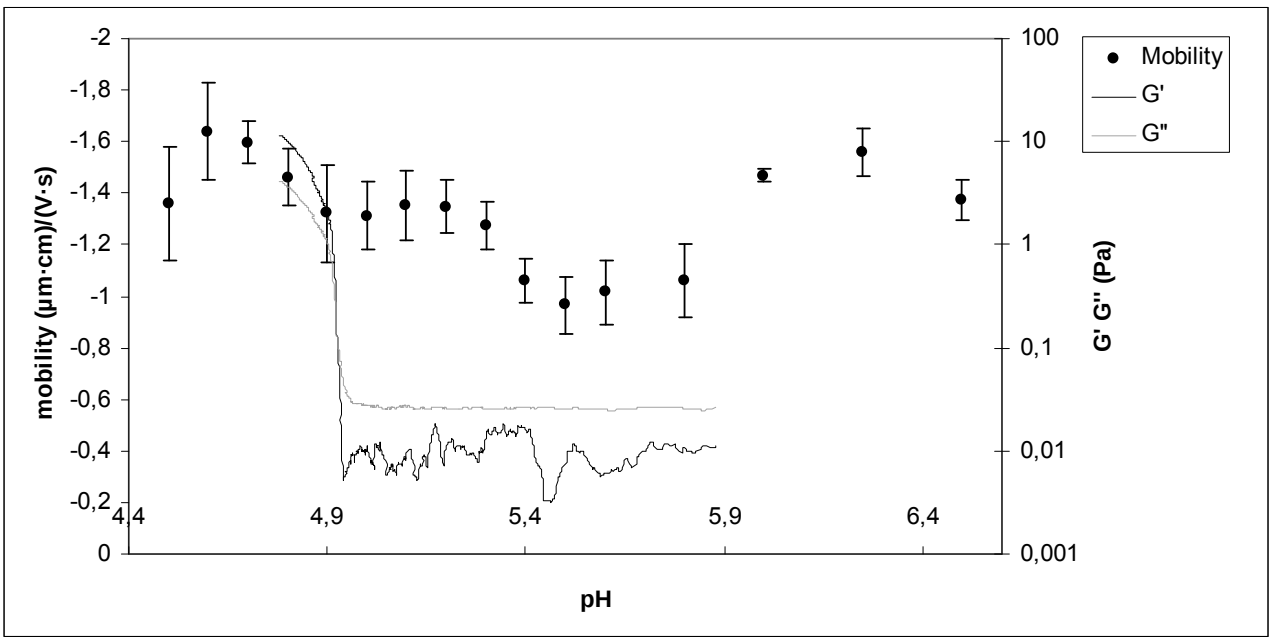


Figure 32: The sample with pectin and without heat treatment. The rheological parameters and mobility plotted as a function of pH. As pH decreases during the acidification the figure should be read from right to left.

The sample seen in figure 32 remains liquid until pH 4.92 where gelation occurs. G' levels off around 11 Pa. The mobility has a minimum at 5.5 followed by an increase to a plateau at pH 5.2 to 5.0 and another increase to pH 4.6.

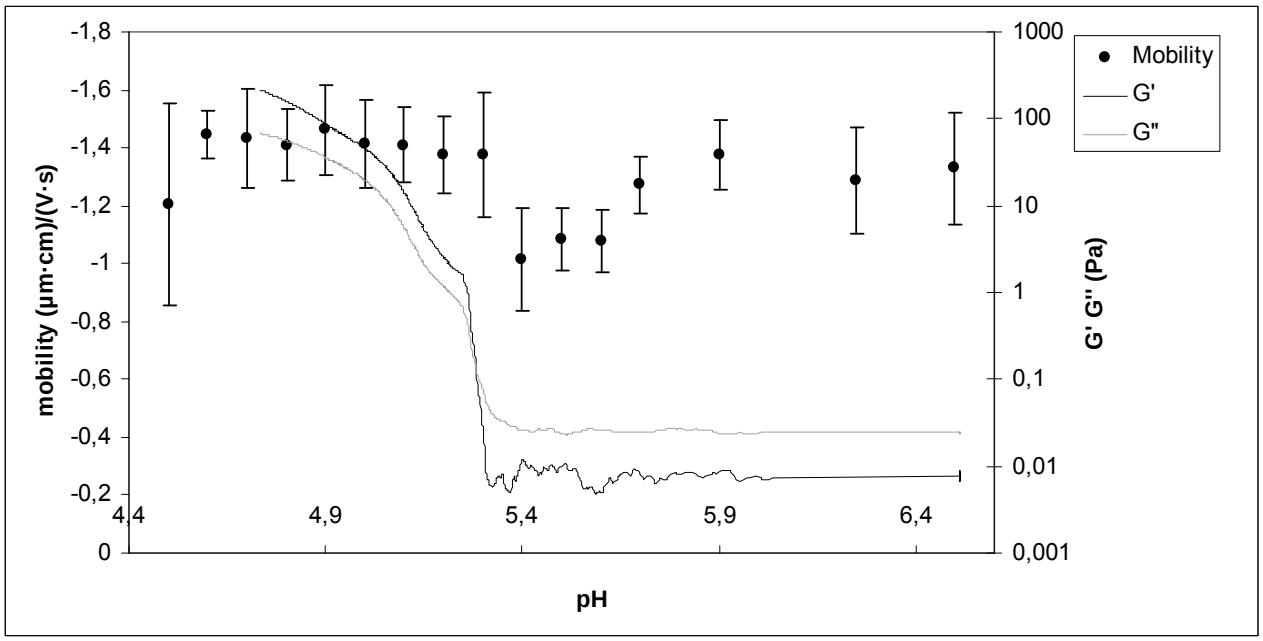


Figure 33: The sample with pectin and with heat treatment. The rheological parameters and mobility plotted as a function of pH. As pH decreases during the acidification the figure should be read from right to left.

In figure 33, the sample remains liquid until the gel point at 5.35. When looking at G'' just before gelation a soft increase occurs before actual elasticity occurs. This can be explained by growth of particle size resulting in increasing viscosity before the particles span the volume and creates a gel. G' levels off around 100 Pa. The mobility decreases to a minimum at pH 5.4 followed by a jump to a stable plateau from pH 5.3 to 4.5.

A comparison of the mobilities is shown in figure 34.

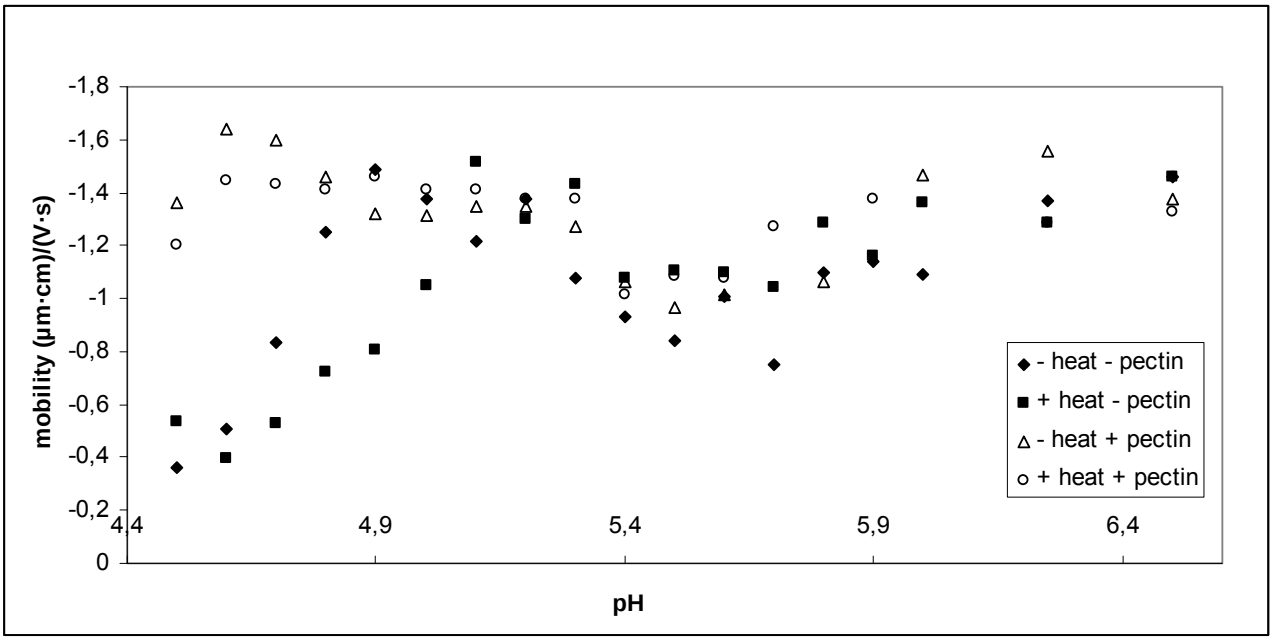


Figure 34: The mobilities plotted as a function of pH.

It is seen that the mobilities have similar values in the interval from 6.5 to 5.2, with a minimum around 5.5. At pH 5.1 the pectin free, heat treated sample starts to decrease, the same happens to the non-heated pectin free sample at 4.8. The two pectin containing samples maintain a stable mobility down to 4.5.

In three of the samples a difference in pH was observed between the stirred beaker and the rheometer. This has been compensated as described in the experimental section, resulting in slightly different gel points. The original and corrected gel points are presented in table 3.

Table 3: The Gel pH and corrected gel pH of the four samples and also the maximum level of G'.

Sample	Gel pH	Corrected	G' level
- heat - pectin	4,85	4,74	7
+ heat - pectin	5,28	5,28	11
- heat + pectin	4,92	4,84	160
+heat + pectin	5,35	5,30	100

The two heat treated samples have similar gel pH values, and so have the two no-heated samples. The values with pectin are slightly higher than the ones without pectin.

The corrections made in the gel pH in table 3 does not change the relative positions of the gel pH when comparing the four samples

G' is an order of magnitude higher for the heat treated samples.

6.7 Verification of Interaction Order Results

This section contains the results an attempt to verify results from the interaction order experiment. This is done by determining the adsorption of pectin at high and low pH. The results of the interaction order suggests that adsorption of pectin only occurs at pH below the gel point. To test thisthe mobility of heat treated milk with increasing pectin concentration was determined at pH 6.0 and 4.7, thi results is shown in figure 35.

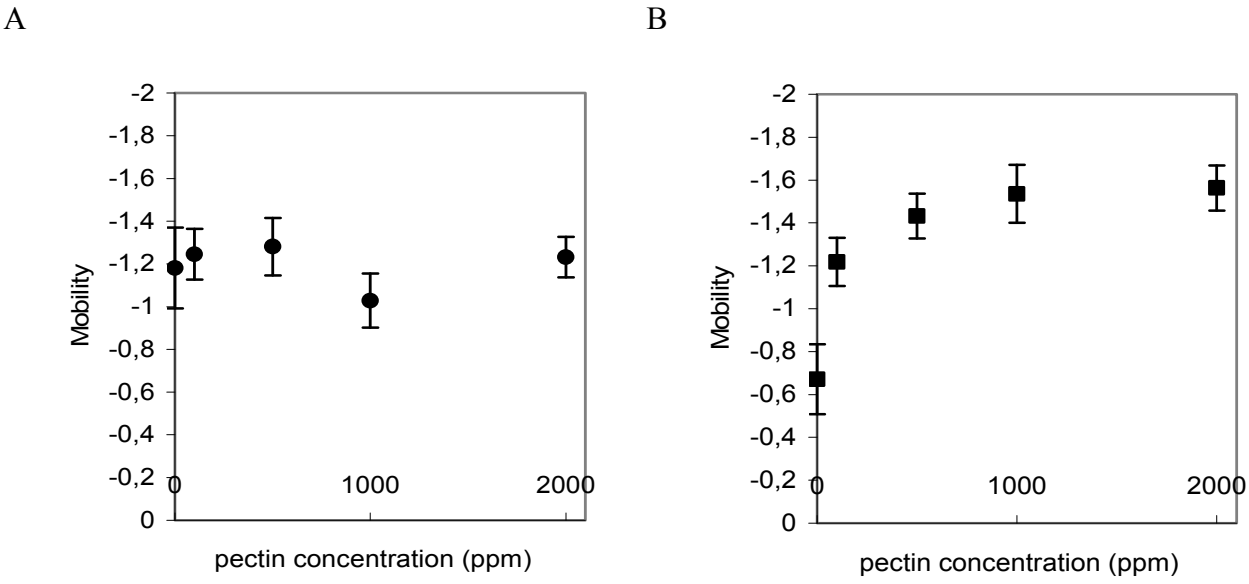


Figure 35: The mobility is plotted as a function of pectin concentration at pH 6.0 (A, left) and pH 4.7 (B, right).

Figure 35 shows that the electrophoretic mobility is stable in the whole pectin interval at pH 6.0 and at pH 4.7 there is an increase towards a steady value from 1000 ppm.

6.8 Analytical Centrifugation of Yoghurt

This experiment tests the applicability of analytical centrifugation to yoghurt at the end of the production process.

The centrifugation of yoghurt made from the samples of the interaction order experiment using 3 % GdL acidification over night to pH 4.1±0.05 is shown in figure 36. The centrifugation of the 5 samples at pH 4.7 from the "Verification of Interaction Order Results" was also run to examine the effect of increasing pectin on the water holding capacity, this is seen in figure 38.

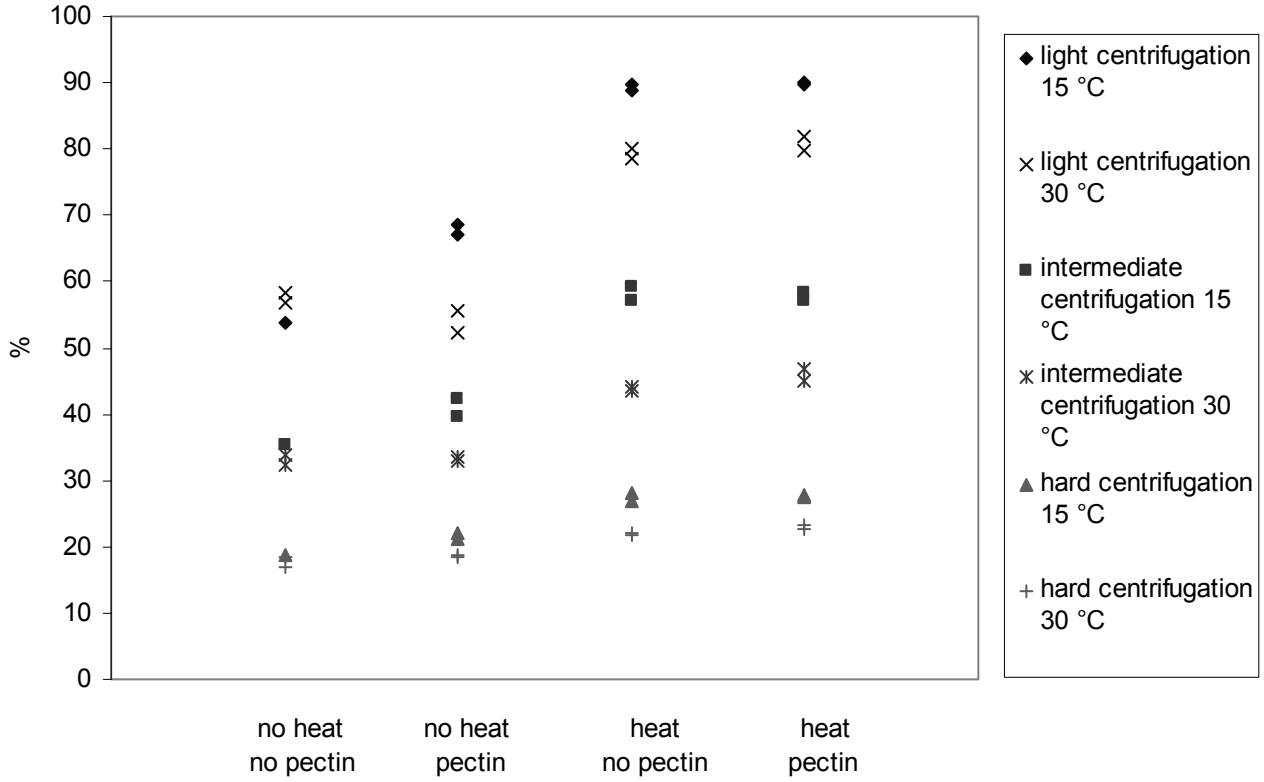


Figure 36: The sediment volume determined as percent of total volume at analytical centrifugation at light (800rpm), intermediate (1600rpm) and hard (4000rpm) centrifugation, each for 30 minutes.

It is seen in figure 36 that with increasing rotation speed of the centrifuge, the sediment volume decreases. When looking at figure 37, which is a plot of the analytical centrifugation results for the sample without heat treatment and including pectin, it is seen that the sediment volume approaches an equilibrium value at all three levels of centrifugation. Statistical analysis have shown that the temperature and heat treatment are the only significant factors in determining the water holding capacity at low, intermediate and hard centrifugation.

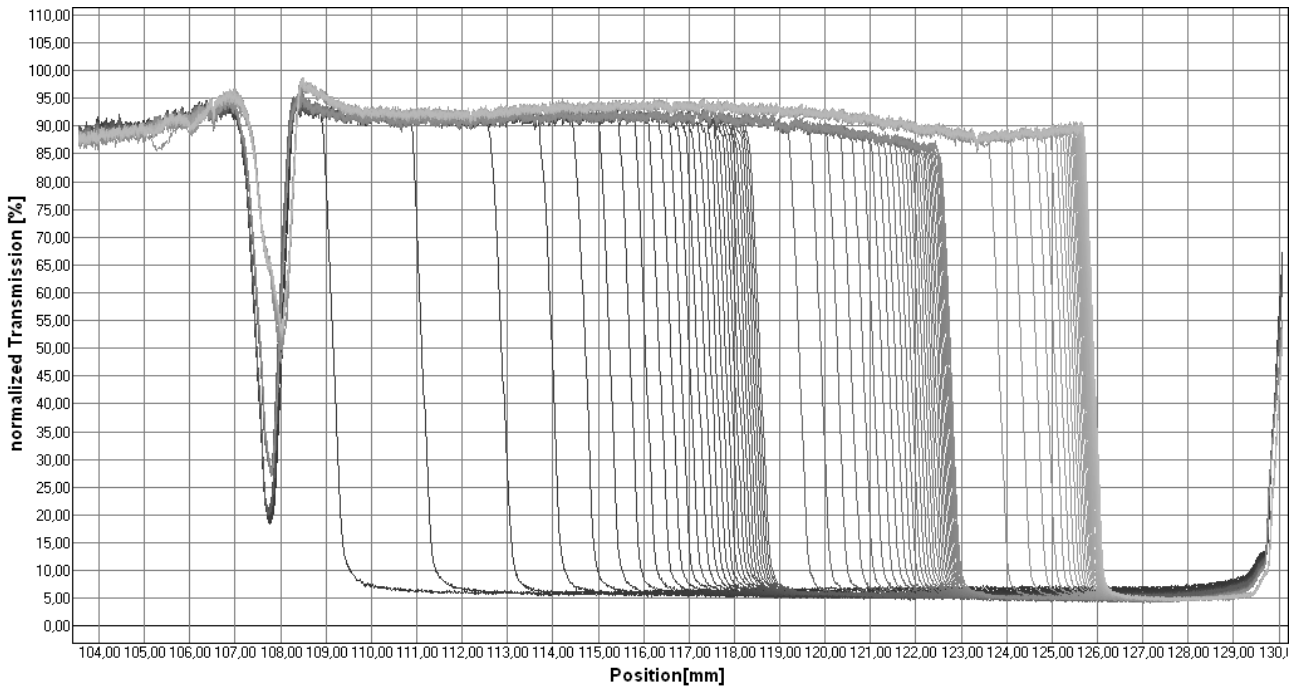


Figure 37: The transmission profiles at increasing centrifugation speed. The sample is no heat with pectin, the milk used was prepared for the interaction order experiment.

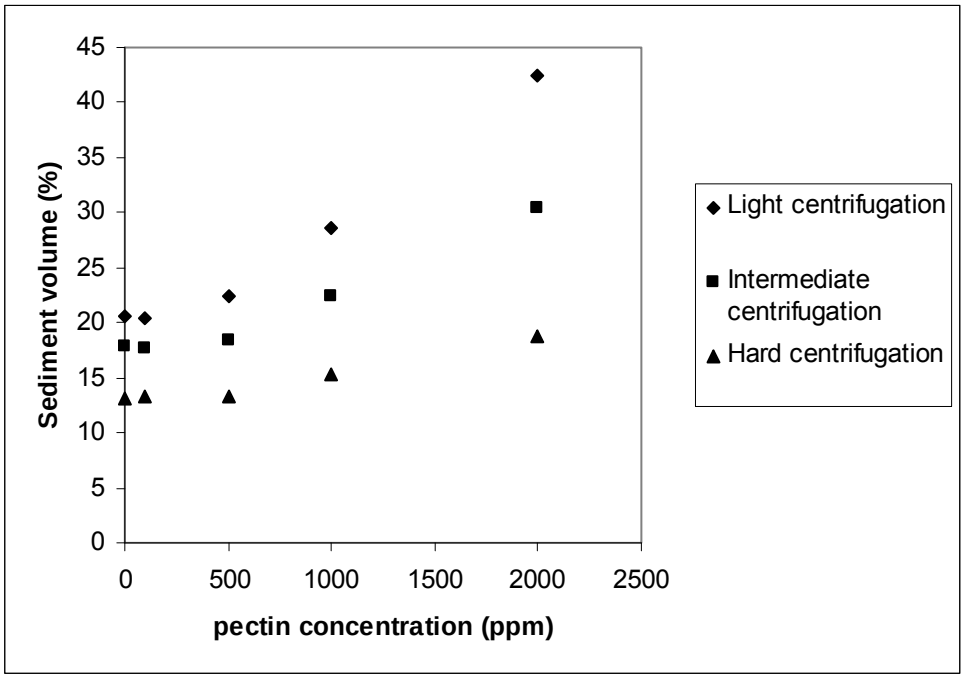


Figure 38: The sediment volume as function of pectin concentration, samples from the experiment "Verification of Interaction Order Results"

It is clearly seen in figure 38 that the sediment volume increases with pectin concentration.

6.9 Fermentation Monitored by Dielectric Spectroscopy

The impedance of the sample was measured every thirteen minutes during the fermentation. This results in a huge amount of data. Relative permittivity at 1 kHz, 10 kHz, 100 kHz and 1 MHz have been extracted and these can be seen on the data DVD. The permittivity is only

shown at 100 kHz. The other frequencies did not supply new information, but 1 kHz is included to aid in the discussion of improving the method.

The results from the comparison analyses will be presented combined with the relative permittivity. First the pH and WHC are displayed as a function of time (figure 39), then the rheological measurements together with the relative permittivity at 100 kHz as a function of time (figure 40). An amplitude sweep made at the end of the measurement is shown in figure 41. Also shown is the electrophoretic mobility and relative permittivity at 100 kHz as a function of pH (figure 42). The pH at each relative permittivity is found from the points of pH value in figure 40. Last shown is the conductivity calculated from the impedance measurements and the relative permittivity at 1 kHz (figure 43).

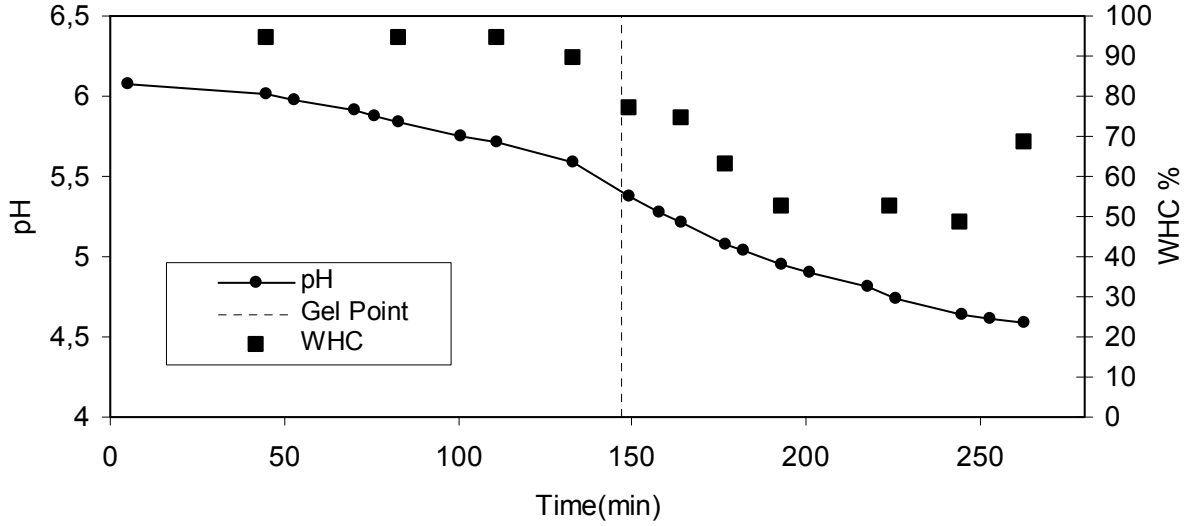


Figure 39: pH and water holding capacity plotted as a function of fermentation time.

The pH of the fermentation, seen in figure 39, progress as expected, with a slow phase followed by a fast fermentation that levels off around 4.5. The WHC starts to decrease around pH 5.6.

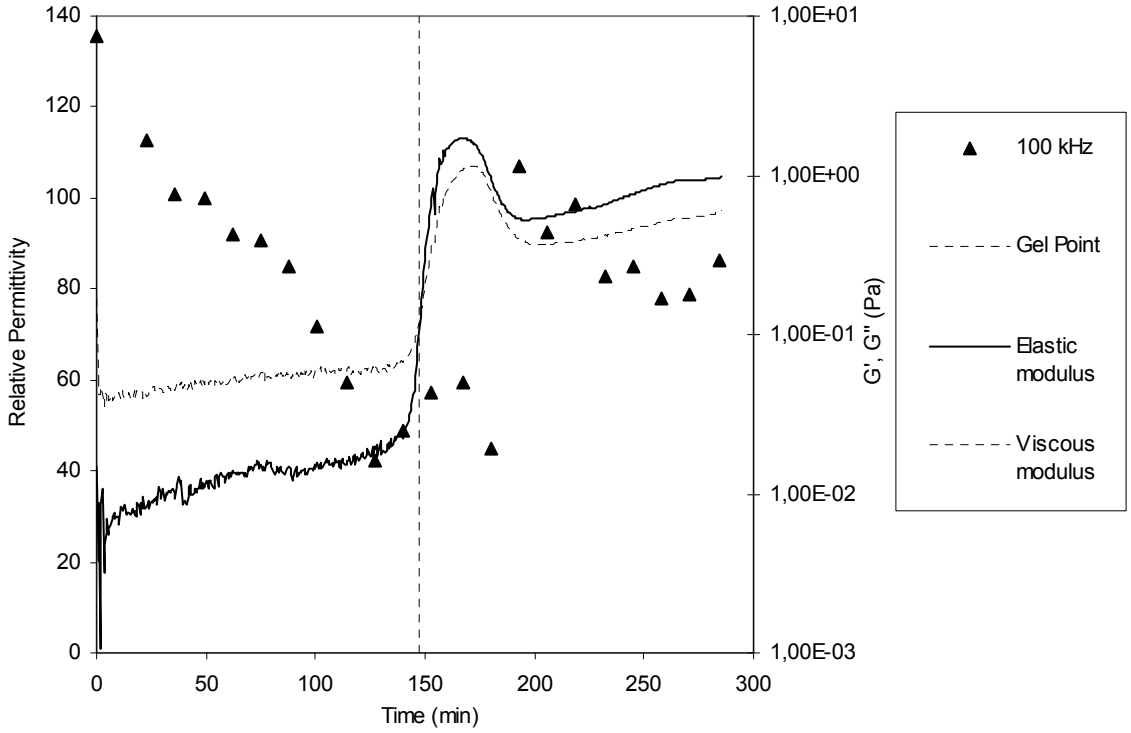


Figure 40: Rheological parameters determined during fermentation together with the relative permittivity at 100 kHz.

The rheological measurement seen in figure 40 is consistent with a liquid followed by gelation at 147 minutes. This is followed by a maxima and then an unexpected drop in both G' and G''. The relative permittivity drops during the first 130 minutes followed by a plateau and a jump to high values around 200 minutes.

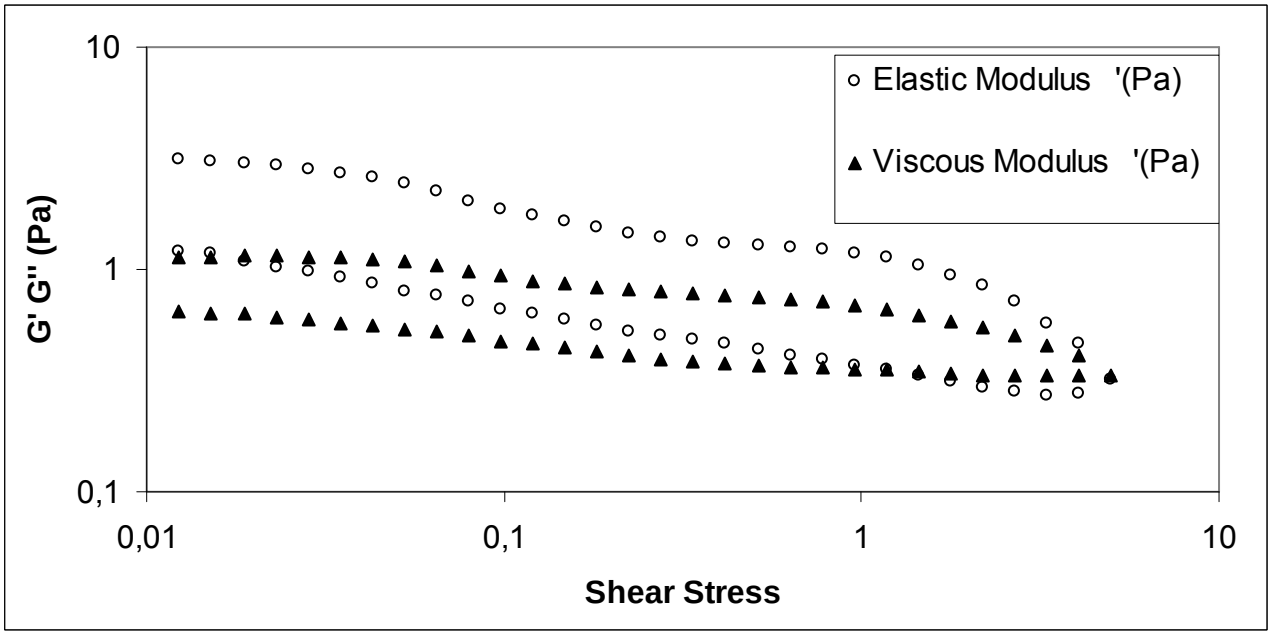


Figure 41: Amplitude sweep after fermentation was finished.

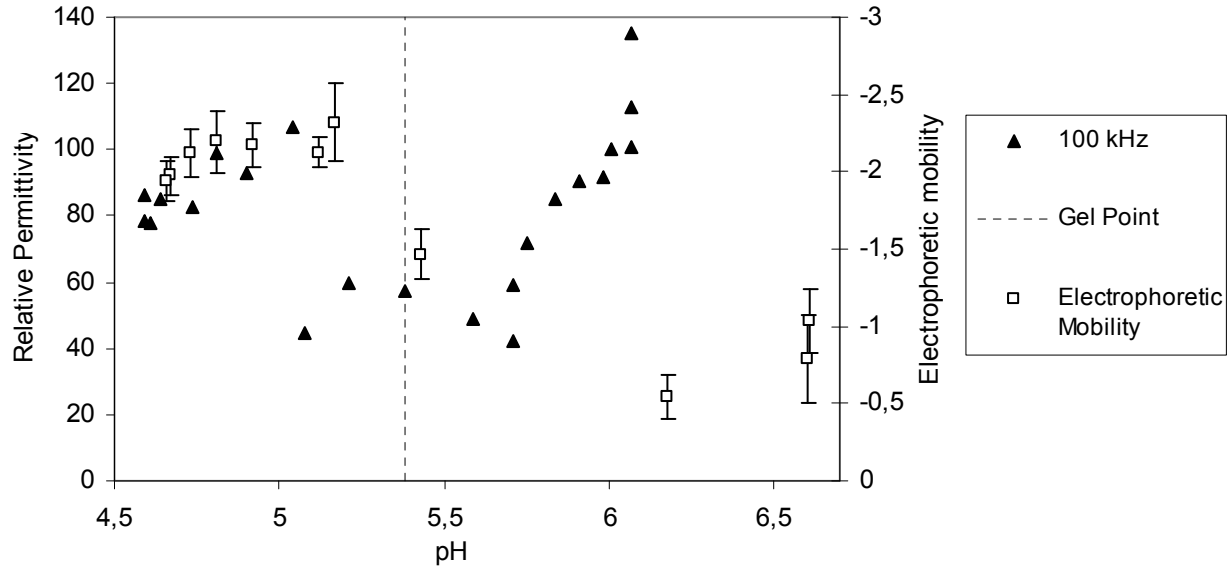


Figure 42: The electrophoretic mobility plotted as a function of pH measured after dilution of the sample, plotted with the relative permittivity at 100 kHz. The fermentation progress from right to left.

The electrophoretic mobility in figure 42 shows low values over pH 6, then an imtermediate point at pH 5.5 and then a plateau with high values from 5.2 to 4.5. The mobility drops with pH until pH 5.7, then a plateau until pH 5.2 and a jump to a high plateau until pH 4.5.

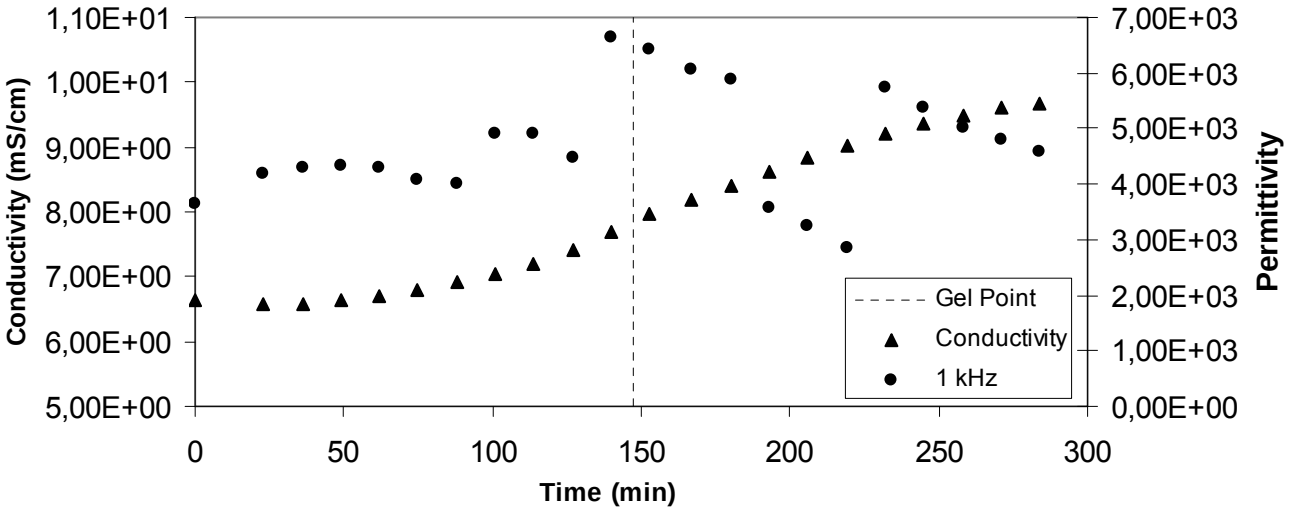


Figure 43: The permittivity at 1 kHz and the conductivity calculated from the impedance.

The conductivity showm in figure 43 shows a plateau until 50 minutes followed by a steady increase for the rest of the fermentation. The relative permittivity at 1 kHz has no clear tendency.

6.10 Comparison of Addition of Calcium Ions and Skim Milk Powder

The purpose of this line of experiments was to examine the effect of the calcium content of the skim milk powder which is added to increase yoghurt quality.

The experiment consists of ten samples with varying calcium and skim milk powder addition.

These samples were analysed before, during and after acidification and the results will be presented in these three sections.

Analyses before acidification:

The samples were analysed by analytical centrifugation at 800 rpm followed by 4000 rpm. The results can be seen in table 4 and figure 44.

Table 4: This table shows stability of samples at light centrifugation.

Protein content (%)	Calcium content (%)	800 rpm
100	100	Stable
112,5	112,5	Stable
125	100	Stable
100	125	Stable
150	100	Stable
137,5	112,5	Stable
125	125	Stable
112,5	137,5	Stable
100	150	Unstable
112,5	112,5	Stable

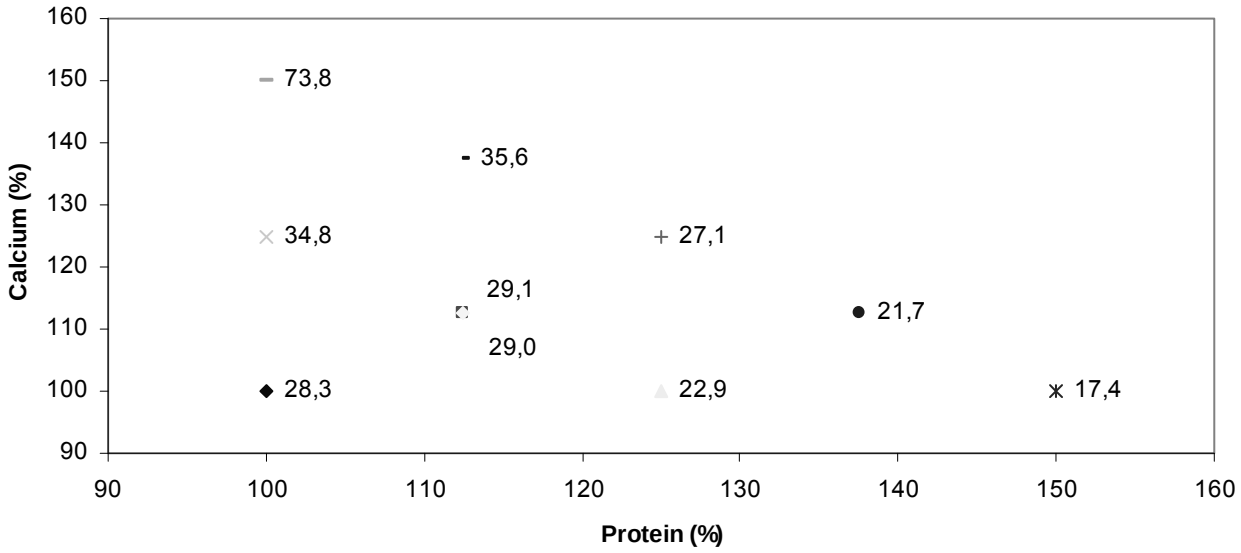


Figure 44: Serum phase of neutral milk-pectin mixtures after 30 minutes of centrifugation at 4000 rpm. The abscissa shows the protein content normalised from natural milk and the ordinate shows the calcium content normalised from the content in natural milk.

At neutral pH the samples are stable, except at 150 % calcium. The sedimentation at 4000 rpm shows that even though the samples appear stable, the calcium and protein affects the properties.

Fitting these sedimentation results using linear models does not show significant interaction with protein, but do so with calcium. The transformation of % sedimentation to ln(% sedimentation) can be modelled with significant interaction with both calcium and protein, and the model R^2 value is increased from 75.8 % to 90.1 %, and thus the model fit is better. Observing the residuals show that the data point of 150% Calcium appears to be much higher

than the others and removing this result in a very good fit(R^2=98.1), especially the one with the logarithmic transformation(R^2=99.0). The model shows that calcium strengthens the sedimentation significantly and protein prevents it.
The sample tube containing 137,5% calcium and 112,5 % protein had a crack and was contaminated with a surfactant, Rodalon, used as an antiseptic in the water bath used for heating the samples. This happened after the analysis by analytical centrifugation at initial pH. Hence, this sample is removed from all following data sets before analysis.

Acidification:

It was controlled if the order of acidification is significant for any of the results, both by blocks of day 1 and day 2 and by time from sample preparation to acidification.
These two variables cannot describe all variation in a linear model, there is no correlation at 95 % confidence. The variables were also included in the modelling process using protein and calcium to model the outputs during acidification and post acidification analysis.

Addition of 3% GdL results in a slow, soft lowering of pH to around pH 4.0, similar to fermentation. Measured values of pH versus time can be seen in figure 45.

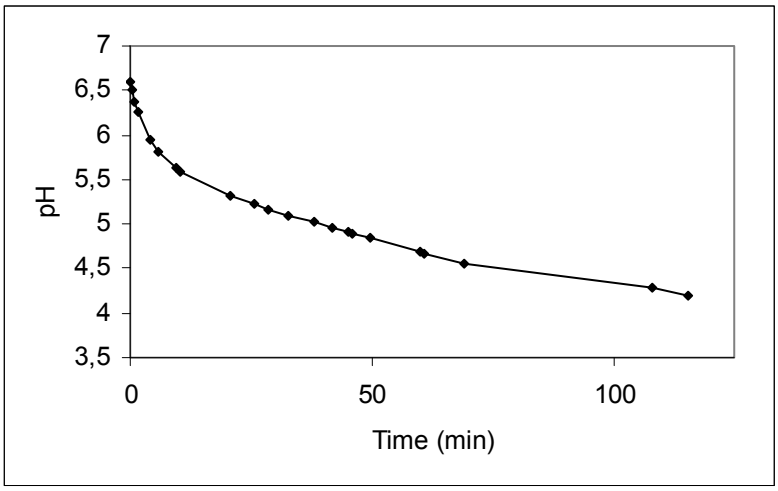


Figure 45: This figure shows the pH in milk after addition of 3% GdL at 30 °C and continuous stirring.

The time from addition of 3 % GdL, to the gel point, determined as a phase angle of less than 45°, can be seen in figure 46.

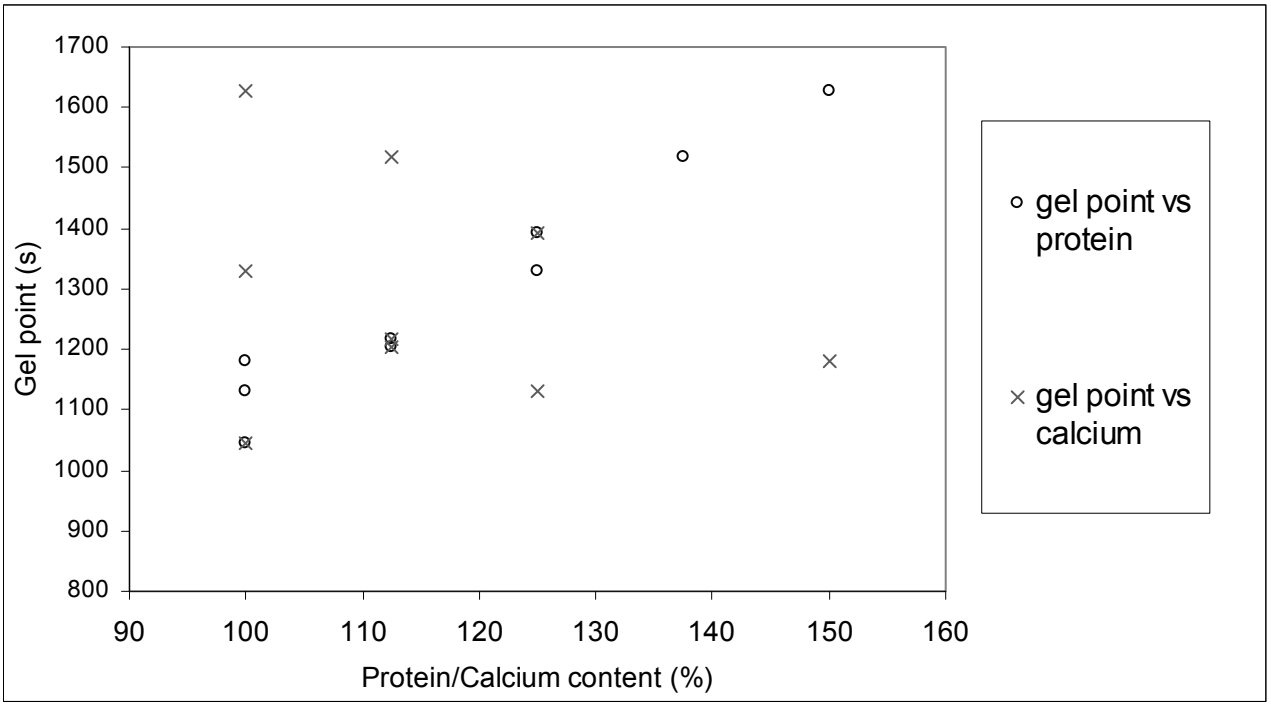


Figure 46: The gel point in seconds after addition of GdL, plotted as a function of calcium content, and protein content

The gel point appears to be strongly correlated with protein content. To counter this trend the gel time has been compensated with protein content, the result is seen in figure 47.

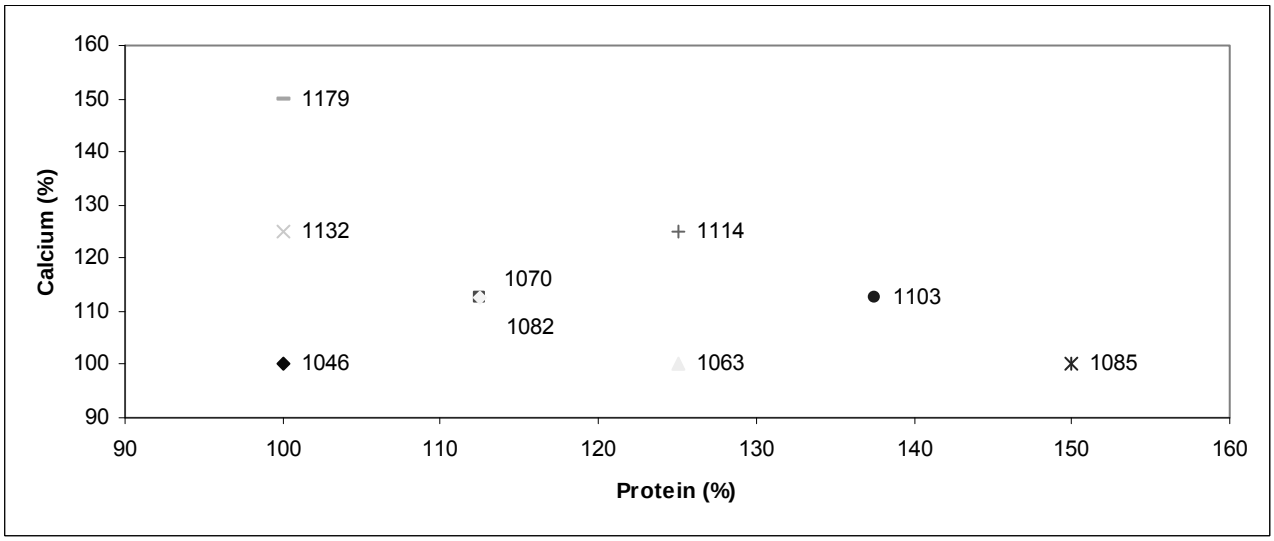


Figure 47: The gel times in seconds after compensation with protein content.

After dividing by protein the trend found with protein is less pronounced and the trend with calcium is easily recognised.

End G', and G' modified for gel time and modified for gel time and protein content, as described in the experimental section, can be seen in figures 48, 49 and 50.

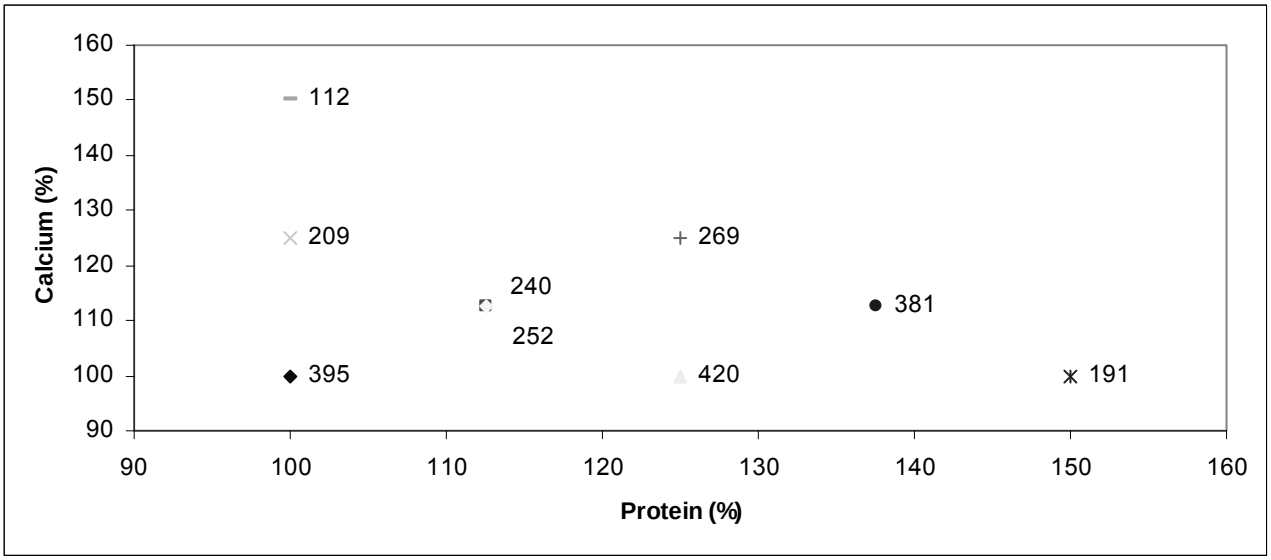


Figure 48: The G' after 1.5 hours of acidification at 30 °C The abscissa shows the protein content normalised from natural milk and the ordinate shows the calcium content normalised from the content in natural milk.

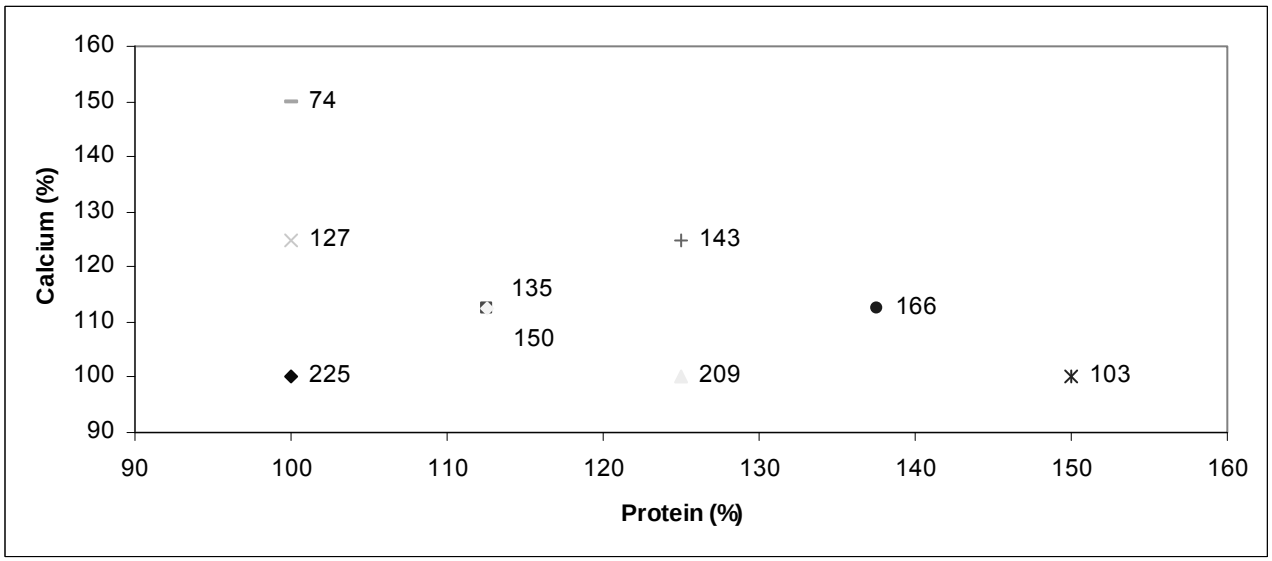


Figure 49: The G' measured 2000 seconds after the gel point to compensate for different gelation times. The abscissa shows the protein content normalised from natural milk and the ordinate shows the calcium content normalised from the content in natural milk.

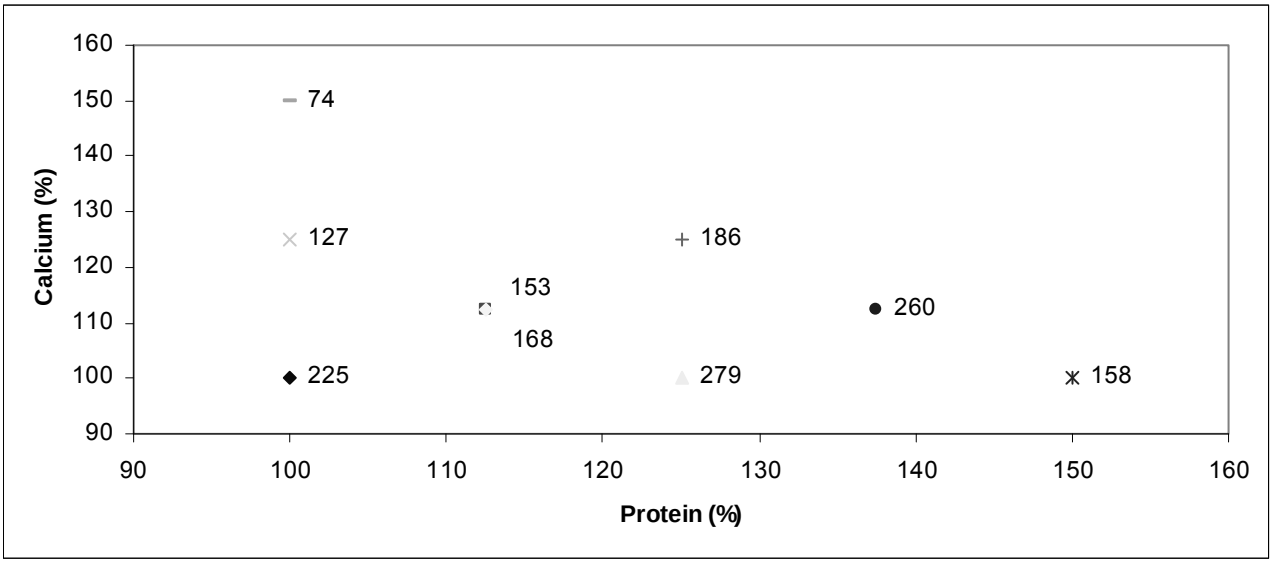


Figure 50: The G' measured 2000-3000 seconds after the gel point. The time of determination is determined as 2000 seconds multiplied by the protein content to compensate for slower acidification. The abscissa shows the protein content normalised from natural milk and the ordinate shows the calcium content normalised from the content in natural milk.

The analysis of the end G' shows that the only significant factor is calcium at a level of confidence of 95%. The addition of calcium decreases the end G'. This is not changed by the modifications described above.

The value of G' at 100 % calcium and 150 % protein appears to be too low, and if this is removed there is significant positive effect of protein and a negative effect of calcium at G' modified for gel time and protein content.

The initial G'' is significantly affected in an increasing direction by addition of calcium and protein.

After Acidification:

Sedimentation of both yoghurt gel and stirred yoghurt, and all three grades of analytical sedimentation can be seen in table 5. The intermediate centrifugation has been blotted in figure 51, and the characteristics of this graph are similar with the other results.

Table 5: this table shows the volume of sediment in percent of sample volume using several methods. These are from left to right: gelled sample centrifugated for 10 min at 4000 rpm, as before, but the sample has been stirred before centrifugation. The last three are results from the analytical centrifuge with increasing force of centrifugation.

% sediment volume						
Protein content (%)	Calcium content (%)	Gelled sample	Stirred gel sample	Light centrifugation	Intermediate centrifugation	Hard centrifugation
100	100	33	28	75	44	25
112,5	112,5	38	37	83	49	27
125	100	35	47	75	48	27
100	125	32	34	75	42	24
150	100	66	51	86	59	33
137,5	112,5	48	49	84	59	33
125	125	53	42	86	55	28
100	150	32	33	78	44	23
112,5	112,5	62	38	83	49	27

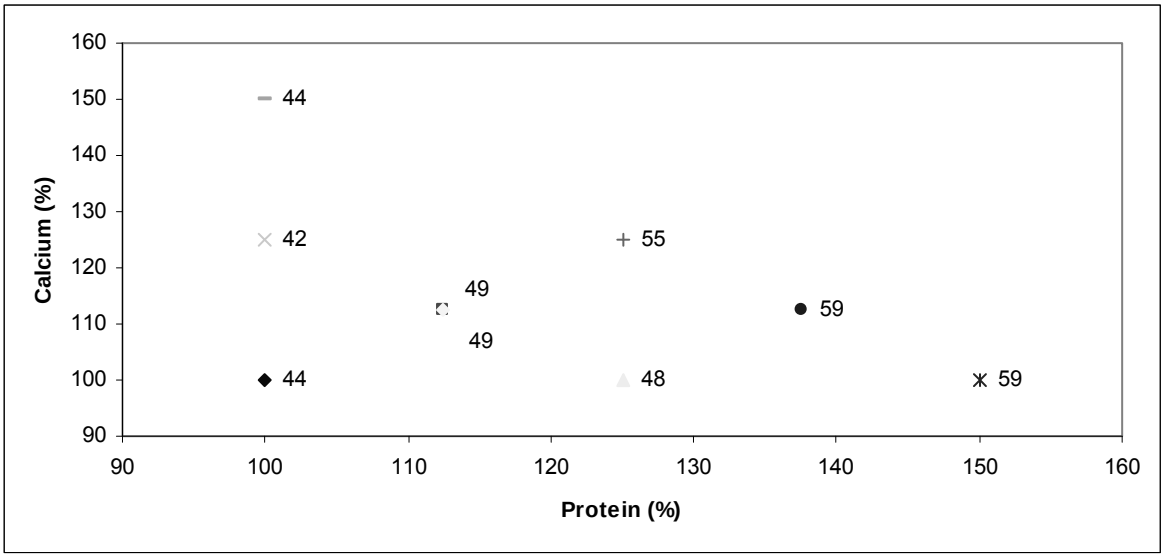


Figure 51: The volume of sediment after intermediate strength centrifugation of stirred yoghurt in the analytical centrifuge. The abscissa shows the protein content normalised from natural milk and the ordinate shows the calcium content normalised from the content in natural milk.

An interaction analysis shows no significant interaction between calcium and protein, even though it may look like that when observing figure 51.

The five variables depend significantly on protein concentration and no other parameters. There are indications that sedimentation of gel and light centrifugation depends on time before acidification but not at the level of 95% confidence.

7 Discussion

This chapter is split up in two sections, interpretation of the results and applicability of methods. The purpose of doing this is to be able to evaluate the three methods, dielectric spectroscopy, analytical centrifugation and electrophoretic mobility independent of the single experiment.

7.1 Interpretation of Results

7.1.1 Rehydration of Skim Milk and Verification of Size Distribution

When observing the rehydrated skim milk particle size distributions in figure 19 and 20 it is seen that the particle sizes are similar to that of fresh skim milk. This means that the experiments are not flawed by effects due to large aggregates of casein micelles. It is also observed that compared to the homogenised sample, the unhomogenised rehydrated skim milk shows a shift in particles size distribution towards higher particle sizes, this may be caused by aggregates of casein micelles.
The size distribution by the contin function contains more information than the monomodal fit, but for verification purposes the monomodal approximation can be sufficient.

7.1.2 Intrinsic Viscosity of Pectin and Pectin Calcium Mixtures

The relative viscosities have been determined with quite high accuracies and statistical analysis has shown that there is no significant difference between the measurements due to pH ot temperature. This is probably due to the number of data points being too low. The results at 40 °C were measured after 48 hours at 40 °C and as described in the theory section, this can result in degradation of the polymer. The relative viscosities at pH 6.7, 40°C appears irregular and lower than at pH 4.5, this could be due to degradation.
When looking at [μ] at 25 °C there is a tendency towards higher values at pH 6.7. This can be understood as the polymer taking up a larger volume at this pH, which is expected due to higher charge of the polymer as it is expected to be partially protonated at pH 4.5.
When comparing the intrinsic viscosities, there is a significant positive change due to calcium. The effect of calcium can both be due to ionic condensation, which would lower the polymer charge and hence the intrinsic viscosity. On the contrary if the calcium makes bridging between pectin chains, these become longer and this results in increasing intrinsic viscosity. This makes it harder to use intrinsic viscosity to predict stability of milk as the precise calcium content and temperature should be known.

When calculating M_w an error is made assuming that the polymer is polygalacturonic acid. Due to lower charge density the polymer is probably longer and has higher M_w than the calculated 55 ~kDa and DP=~275. Besides there is no knowledge of the structure of the molecule, linear or branched.
Improvements of this method is discussed later in this Chapter.

7.1.3 Phase Diagrams of Pectin Calcium Mixtures

The phase diagrams have similarities with the one presented by Axelos and co-workers (1996), shown in figure 8 in the theory chapter, even though no homogeneous, gelled phase have been observed.

The boundary is observed at much higher calcium concentrations and lower pectin concentrations. This is probably due to the lower sodium concentration in milk and it could also be due to more blockiness of the used pectin. The number of protonised acid groups is much higher at pH 4.5, which lowers the number of groups accessible to calcium bridging and this can explain the increased stability at pH 4.5. The lower stability at low temperatures can be due to a shift in the ionic equilibria resulting in more charged groups, but it can also be due to higher stability of the calcium bridges. The effect is well known and used in the production of LM pectin gelled products.

The phase diagrams are determined visually. To improve this, the detection could be done using an objective method. This could be oscillating rheology or absorbance due to refractive indices of the two phases, but it could also be done using the analytical centrifuge to determine the equilibrium sediment volume as this would add the dimension of quantitative results compared to the qualitative ones from absorbance.

7.1.4 Stability of Neutral Milk Pectin Mixtures

The temperature dependent stability diagram shows higher stability at higher temperature. This suggests that the samples will not be exposed to depletion flocculation during heat treatment. It also means that the maximum pectin concentration should be determined at the lowest temperature the pectin-milk mixture is exposed to during the yoghurt production.

The stability diagram of protein concentration is surprising as the addition of higher volumes of casein micelles was expected to increase the depletion interaction and hence lower the boundary from 3.5 to 5.0 % protein in a manner similar to the change observed from 2.0 to 3.5, but instead no change is observed. This may be due to an effect observed in the “Comparison of addition of calcium ions and skim milk powder” experiment. At high rpm the sedimentation of samples with 5.1% protein content is much slower than the ones containing 3.4 % protein. This effect may have delayed the sedimentation due to depletion flocculation, and hence, resulted in false negative results.

When running at high protein concentration more time of centrifugation should be applied.

The calcium pectin shows two types of instability, gel formation at high calcium concentrations and depletion flocculation at high pectin concentrations. The gel particle formation is also observed at similar calcium- and pectin concentrations without the presence of the other milk constituents.

The milk calcium-pectin stability diagram consists of relatively few points. The results of the high calcium content shows a gel phase at the top of the cuvette. In the phase diagram experiment, the tubes showed the pectin phase sedimented. This may be due to high density of the protein rich phase.

The increased stability with low calcium addition is not just shown by one point, it is confirmed by the lower degree of phase separation of the sample at 100 % compared to 25 % calcium at 1800 ppm pectin, and hence it is plausible that addition of small amounts of calcium

(22.5mM) can reduce the depletion flocculation introduced by addition of this particular pectin type.
The points in the phase diagrams are based on a single measurement, and therefore conclusions based on single points should be approached with caution. Conclusions should either be verified by repetition of the measurements or based on tendencies seen in the phase diagram in general.

7.1.5 Stability after Heat Treatment of Neutral Milk Pectin Mixtures

As described in the theory there are two effects of heating milk and pectin at neutral pH. The whey proteins are denatured and adsorbs to the casein micelle surface and the pectin undergoes demethylation and β-elimination. Degradation of the polymer results in shorter chains, and hence a lower [μ] and the critical concentration for depletion flocculation is shifted upwards. The heat treatment of milk results in larger casein micelles, and hence an increased tendency of depletion flocculation can be expected. Besides, at high temperatures, calcium phosphates and calcium citrate precipitate, resulting in lower calcium concentration, resulting in an expected decrease in the critical concentration due to more charged pectin. This effect appears to be countered by less calcium bridges. Pectin has been shown to have a stabilising effect on denaturation of whey protein, and hence may have the ability to slow down the effect of the heat treatment.
The experiment has been done initially without pectin present during the heat treatment, and this, against the theoretical predictions, resulted in higher stability with heat treatment. When adding pectin before heat treatment, the effect of the heat treatment is still increasing stability with heat treatment, but the effect is slower. This can be used to show the importance of the stabilising effect of pectin on whey proteins and on the contrary the breakdown of pectin is of less importance.
To investigate the effect of the heat treatment of pectin it should be heat treated by itself, this could help to disentangle the effects, but it would not change the conclusion that longer heat treatment times are needed when pectin is included in the mixture.

7.1.6 Order of Interactions during Fermentation

The purpose of this experiment was to uncover the interactions occurring between casein micelles and between casein micelles and pectin during acidification. The interactions between pectins have been examined in the previous sections. As the interactions are of electrostatic nature the surface charge of the casein micelles is of interest. The surface charge and gel point is affected by heat treatment, and hence non heat treated samples is also included, these may serve for comparison. By doing this the effect of heat treatment and pectin on gel formation can be determined. It also clarifies when adsorption of pectin occurs and makes it possible to select a method to determine the lower limit of the functional pectin range.
First of all, the results show that gelation occur independent of the presence of pectin.
The results show that the point of gelation is primarily determined from the heat treatment, and this shift the gelation pH from ~4.9 to ~5.3. There appears to be a small effect of pectin, increasing the gelation pH slightly. The correction of the pH values is done as measured pH values in the rheometer were up to 0.1 pH higher than in the stirred container. This can be

caused by a temperature gradient in the rheometer, as the shape of the setup makes it possible for the air to cool the upper part. The equipment is placed close to a window that was sometimes open, and the flow of cold air may have varied from one experiment to another. The compensation method is described in the experimental section. The correction of the pH in the rheometer does not change the conclusions above, but shifts gelation pH of the unheated milk to pH ~4.8. The measurement of G' is a problem as the "knee" and plateau observed right after gelation e.g. in figure 33 may very well be the result of the gel breaking and by careful examination of the curves this is evident in all four experiments. It shows that the gel is very sensitive right after the gelation pH. This may be avoided by using a lower shear stress or using controlled strain measurements. The value of G' can not be used to describe the gel, but it is obvious that heat treatment increases G' by at least a factor 10.

The comparison of the mobilities in figure 34 shows a similar development of the mobility from the beginning of the acidification to the gel point. This consists of a decrease from 6.5 to 5.5, followed by an increase to 5.2.

The minimum observed at pH 5.5 have previously been described by Anema and Klostermeyer (1996) as seen in figure 4 in the theory chapter. The reason for the minimum have been described by Walstra (1990) to be charge neutralisation by calcium from dissolution of CCP combined by loss of structural integrity, which is regained at lower pH.

At pH 5.2 the mobility of the heat treated sample without pectin begin to drop to values close to zero, consistent with the isoelectric point of the whey proteins and caseins. The unheated sample without pectin shows similar behaviour at 4.8, consistent with the isoelectric point of the caseins. These drops in mobility occurs after the gelation of the samples, which suggest that the samples aggregation is connected to the loss of steric stabilisation, as the electrostatic repulsion does not change very much close to gelation.

This can also be the effect of the particle aggregation leaving only the surfaces with the most charges exposed, but even as this might add to the observations, this is not the main effect.

The samples containing pectin shows different behaviour after gelation. The mobility is unchanged with decreasing pH after gelation. The reason for this could be the adsorption of pectin by electrostatic interaction. This hypothesis was tested by a different experiment, the results of this is discussed in the next section.

7.1.7 Verification of Interaction Order Results

The adsorption of pectin at pH 6.0 can be rejected as no tendency to lower mobility occurs when observing the mobility shown in figure 35 A. On the contrary the increasing mobility with increasing pectin concentration in figure 35 B verifies that the pectin adsorption is the reason for the observed difference in mobility between the samples with and without pectin. The mobility reaches a plateau around 500 ppm pectin. The pectin does not appear to adsorb before the point of gelation and this is consistent with that the point of gelation is unchanged by the presence of pectin. The drop in mobility seen in samples without pectin is a combination of negative charges being neutralised and positive charges appearing. The adsorption is due to electrostatic interaction between negatively charged pectin and positive charges on proteins. The results show that a pectin layer adsorbs to the whey proteins.

The point of adsorption means that it is impossible to add the pectin ad the end of fermentation. It is impossible to get pectin into the gel network, and stirring may result in rearrangement of the casein particles before the pectin get to stabilise them.

7.1.8 Analytical Centrifugation of Yoghurt

Analytical centrifugation has been applied on yoghurts produced from the four milk samples used in the interaction order experiment. The water holding capacity of these samples at different degrees of centrifugation can be seen in figure 36. This shows that the heat treatment strongly increases the water holding capacity. At the heat treated samples the results show no significant difference, but with no heat treatment there appears to be an increased stability with pectin at low and intermediate centrifugation at 15°C. To improve this method more rotational speeds can be tested, with different times of analysis and compared with stability during storage. The result with increasing pectin shows that the volume fraction increases with increasing pectin. This verifies that pectin increases the water holding capacity and hence prevents syneresis, but this result can not specify the mechanism alone.

When compared with the electrophoretic mobility it is seen that at 500 ppm the electrophoretic mobility is similar to 1000 and 2000 ppm but the water holding capacity is similar to 0 and 100 ppm. This supports the hypothesis that increasing pectin concentration results in increasing volume fraction, with a quick saturation of the surface, as shown by electrophoretic mobility.

To determine if enough pectin have been added to reach the minimum limit of the functional range using WHC or electrophoretic mobility, a range of pectin-milk mixtures should be allowed to acidify slowly, gel and the pH should reach approximately 4.5. This should be done before disturbing the gel to let the complexation reactions between pectin and the casein network be completed. Then a well defined stirring should be applied at the temperature used in the production. Analysis of the yoghurts should then be done using a method that determines if the surfaces have been fully covered. The obvious choice to do this is measuring the electrophoretic mobility, as this method has proven itself useful to determine the surface coverage, and analytical centrifugation have shows the excess pectin as a higher volume fraction.

7.1.9 Fermentation Monitored by Dielectric Spectroscopy

The procedure used to prepare the yoghurt for dielectric spectroscopy is different from all other experiments done during this project. It uses lactic bacteria instead of GdL for acidification. As the bacteria strains used is unknown, there is produced an unknown amount of EPS, which may adsorb to the casein micelles in a way similar to pectin. Therefore fermentation is unsuitable to quantify casein-pectin interactions. The gelation is observed at 147 minutes, and pH of gelation is determined to be 5.38, this is higher than any other experiment, even the ones including a much more extensive heat treatment. This is probably caused by either too few points on the pH curve, or by a difference in temperature between the oven, where the pH was measured, and the rheometer. The oven was opened repeatedly and was not designed for precision temperature control at temperatures close to room temperature.

The water holding capacity progress as expected. When the gelation occurs, the WHC begins to lower. A considerable increase is seen in the last sample, the reason for this is unknown. The

samples were cooled on ice for approximately fifteen minutes before centrifugation, this means that the actual temperature in the sample varies, both before and during centrifugation, and this further adds uncertainty to the WHC.
The rheology curve shows first a period of liquid behaviour, then an increase in viscous modulus and an stronger increase in elastic modulus resulting in a crossing considered as the gel point after 147 minutes. After around 175 minutes there is a strong decrease in both moduli. This could be the result of syneresis or gel breakage. When considering the amplitude sweep it is clear that no linear, horizontal section appears, suggesting that the gel is broken. The breakage of the gel is clearly visible in the ramp down again, where both moduli is lower than the ramp up. This suggests a brittle gel or that the method of measurement uses too much force. No other measurements show the reduction in G', but they show the same "knee", even though the same setup has been used. This suggests that either this particular gel is very brittle, else the temperature which have been 30 °C in all other measurements makes the gel more stable.
The permittivity of the sample shows a combination of two different effects, one is the flocculation, the other is the particle charge. The changes in permittivity is a small increase for flocculation and a large increase for particle charge density. When considering the relative permittivity compared to rheological measurements it is seen that the decrease is stopped and a small increase initiated around the gel point, followed by a large jump.
The electrophoretic mobility measurements in relation to this experiment have a problem. The dilution is made using KCl solution, and even though this has similar conductivity, it has no buffer properties and this result in large shifts in pH value upon dilution. The means that the measured pH values are either high or low, and the interesting area around the gel point are not examined. The measurements of electrophoretic mobility does show a decrease on particle charge with decreasing pH, from pH 6.7 to 6.2, consistent with the measurements of relative permittivity. It is seen that at low pH values the electrophoretic mobility and the relative permittivity is high, consistent with the adsorption of pectin and possibly EPS to the casein micelles. The permittivity measurements have been put in at the pH values measured at the corresponding times, and this curve contains very few points. This results in a less obvious correlation between the results of the two techniques.

7.1.10 Comparison of Addition of Calcium Ions and Skim Milk Powder

This experiment was to determine if addition of calcium or substitution of protein by calcium addition could be done in stirred yoghurt containing pectin without negative effects on yoghurt quality. The background is the increased stability when adding calcium at neutral pH, and the hypothesis that the effect of adding skim mill powder could be attributed to the calcium content of the powder.

The stability of the samples is unaffected, except at 150 % calcium, where phase separation occurs, setting an upper limit to the amount of calcium that can be added.
The stability at high centrifugation speed described as the serum volume after 30 minutes have been described by calcium content and protein content. The stability is increased by protein

and decreased by calcium. The relation is not linear but logarithmic in nature and at high concentrations of dissolved calcium, the effect is very strong, similar to phase separation.
This effect may be strengthened by calcium-protein ion condensation, resulting in lower stability of the casein micelles.
It appears that the pH –time curve was strongly dependent on protein concentration. This change can be attributed to the increased amount of CCP and protein buffer capacity. The end pH of the samples was highly correlated with protein content, R^2 was 0.99. Hence all measurements is affected by varying end pH and acidification rate. To compensate for this the pH difference a simple compensation has been used. This assumes that the time divided by protein concentration (1.0-1.5) is constant Hence results determined at 1.0 hour for 100 % protein is similar for 1.5 hours for 150 % protein. This has been verified by looking at the gel point. When compensated for protein, the effect of calcium is increasing gel time and a slight increase with protein. The effect remaining with protein is probably a result of the varying end pH, but the effect with calcium is "real".

This shows that adding protein results in a higher end pH, unless it is followed by addition of more lactose to increase the acid production. If ultrafiltration is used then the lactose is removed and the ratio of lactose to CCP is pushed.

The nine gels in this experiment also exhibits the "knee" right after the gel point suggesting a break of the gel. This means that the efforts to measure the original gel strength have failed, but a comparison of the measured values show that after compensation for the gel time and protein content the gel strength is significantly increased with protein content and lowered with calcium.

The water holding capacity is independent of calcium and strongly dependent on protein content with increasing WHC with increasing protein. This means that at this level of pectin addition of calcium does not affect the water holding capacity.

To sum up the results, this experiment shows:
The milk remains stable to a limit between 137 % and 150 % calcium.
The acidification rate and end pH is affected by skim milk powder addition and should be compensated by higher acid production.
The gel was broken due to bad equipment settings, but the value of G' increased with protein and decreased with calcium, both independently and by substitution of protein by calcium.
The water holding capacity was independent of calcium and was increased by protein addition.
Considering the quality parameters of yoghurt it can be determined that addition of protein increases water holding capacity significantly, introducing calcium at high values increases the risk of introducing gel phase separation, and addition of calcium decreases G', reducing gel strength.

7.1.11 Comparison of Phase Stability and Intrinsic Viscosity

Using the analytical centrifuge, the critical concentration of pectin is 1600 ppm at 25 and 40 °C. This is equal to 1.6 g/L or 0.16g/dL

From the measurements of relative viscosity, the intrinsic viscosity at natural milk calcium level at 25 °C is 4.13 dl/g and 3.03 dl/g at 40 °C.

This result in a cop* of 0.66 at 25 °C and 0.48 at 40 °C, with a protein concentration of 34 g/L, at 20 g/L protein the found cop* is 0.45. This suggest that there is an effect of protein concentration that should be considered.

To test the found cop* more pectins should be compared in the same way. To find an actual cop* independent of protein concentration the intrinsic viscosity should be measured in ultrafiltrate of the milk. Besides the importance of the protein content or casein volume fraction should be determined and included in the calculation. Also, the importance of the calcium concentration should be determined. It is assumed that milk pH, protein volume fraction and calcium concentration is the most important parameters in the prediction of depletion interaction. Besides, as the pectins viscosity dependence on calcium concentration, the relation found is only valid for pectins with similar calcium affinity, and hence similar DE and methyl ester distribution.

7.2 Applicability of Methods

7.2.1 Dielectric spectroscopy

The measurements of relative permittivity in yoghurt are made difficult by two properties of yoghurt during fermentation. One is a very high conductivity, the other is that the conductivity nearly doubles during the fermentation, these effects can be seen in figure 43. The high conductivity results in that the phase angle, used to determine the relative permittivity, is small, the precision of the equipment is 0.001° and close to this angle the measurements uncertainty increases. This means that the critical lower frequency (f_{crit}), is higher than usual, and then potentially interesting parts of the spectrum is lost. The change in conductivity means both that even more information is lost, but also that a high number of reference measurements must be measured. An effect of the lack of reference spectra close to each value of conductivity can be seen in figure 43. Which of the five references were used for the calibration can be seen clearly. To improve the measurements of relative permittivity during the fermentation of yoghurt three things should be improved. A high number of reference spectra should be made, e.g. by continuous flow through the cell and addition of KCl. The temperature should be controlled carefully during the measurements. An impedance analyser with higher precision on the phase angle should be used.

The measurements in this project suggests that the flocculation and hence gel point and the charge of the particles can be analysed using dielectric spectroscopy. The separation of these two effects may be done by looking at the relaxation time, related to particle size and the magnitude of the relative permittivity, which is related to charge density. To do this more analyses and higher quality of the data is required.

The perspective is that the pH, acidity, gel hardness, flocculation (gel point) and particle charge and hence pectin adsorption can be determined in one non destructive analysis.

7.2.2 Analytical Centrifugation

The analytical centrifuge has shown to be very useful in determining the critical pectin concentration, which introduces depletion flocculation in the milk at neutral pH. It is a simple method able to do analysis of numerous samples with a minimum of work done. The method should be developed, either by optimisation of rotational speed or time run, to be better at distinguishing between stable and unstable samples.
The analytical centrifuge have also proved useful to determine water holding capacity with high precision, again many samples can be analysed with a minimum of work done.

7.2.3 Electrophoretic Mobility

The electrophoretic mobility has shown useful in determining adsorption of pectin to casein micelles. At the concentrations used the pectin does not have an effect on the measurement of the casein micelle mobility, but when pectin adsorbs to the micelle electrophoretic mobility can be used to detect the adsorption. This makes it a useful tool in the screening of pectin stabilisers. The use of buffers is essential to get the electrophoretic mobility at the pH in the yoghurt. The use of buffers is essential in determining electrophoretic mobility at the specific point during fermentation. The use of acetic acid is advantageous compared to phosphate and citrate that both precipitate as calcium salts. The solubility of calcium acetate is around 400 g/L, and hence no errors due to precipitation occur. The adjustment of the conductivity presents a problem as the environment should be similar to the yoghurt but high conductivity also means that the magnitude of electrophoretic mobility is reduced. The best thing to do must be to select a compromise value and keep it independent of pH.
The electrophoretic mobility in the “verification of interaction order results” experiment show similar values at 1000 and 2000 ppm of pectin, but the water holding capacity increases strongly in this interval. This may be caused by the slipping plane/stern layer moving outwards with addition of more pectin.
The electrophoretic mobility can be used to determine if the minimum amount of pectin have been added to enter the functional range. This is done at the end of fermentation after stirring the milk gel. The mobility will increase at low concentrations reaching a plateau, the first point in this plateau is the minimum concentration in the functional range.

8 Conclusion

The effect of LM pectin in stirred yoghurt as described in the literature has been reviewed with the purpose of identifying the stabilisation mechanism. This has been supplemented by experiments showing that the adsorption of pectin occurs after gelation of the milk. This mechanism has been verified and has been shown to be the same independent of milk heat treatment.

The effect of pectin dosage on stirred yoghurt quality have been described and based on this experiments have been found to determine the functional range of a given pectin. The experiments are simple and this is an advantage as part of the purpose is to screen the efficiency of new pectin stabilisers.

The method of determining the functional range of pectin dose is based on detecting surface charge saturation of the stirred yoghurt gel particles by electrophoretic mobility to find the minimum needed concentration and detecting the depletion flocculation by analytical centrifugation at neutral pH to give the maximum concentration. The concentration limit for depletion flocculation has been shown to depend on temperature, protein concentration and calcium concentration.

The heat treatment of milk is an essential step in the yoghurt production and it has been suggested that pectin have a stabilising effect on the whey proteins, resulting in a demand for longer heat treatments when pectin is present during the heat treatment. It has been shown that heat treatment increases the critical concentration, which means that the upper limit in the functional range should be determined at the lowest temperature before heat treatment.

The measurements of dielectric spectroscopy have shown to be challenging to do in yoghurt systems due to high and highly variable conductivity. On the contrary the technique appears to potentially be able to provide a wealth of information on the progress of the fermentation, the particle interactions and hence the adsorption properties of pectin.

Prediction of the upper limit of functional pectin concentrations has been attempted from the pectin intrinsic viscosity. The intrinsic viscosity depends on calcium concentration and temperature, and therefore the prediction is only valid under specific conditions. To add to this the protein concentration, and thereby casein volume fraction is also of importance. To apply this tool is a challenge, but if the method is established it is an extremely simple way to screen new pectin stabilisers.

Addition of calcium has been shown to reduce pectin introduced depletion flocculation at neutral pH. Experiments have shown that calcium addition reduces gel strength and that WHC is independent of calcium addition. Excessive calcium addition results in phase separation of a pectin gel and this should be avoided. The hypothesis that calcium can partially substitute skim milk addition has been rejected.

The determination of water holding capacity by analytical centrifugation has proven an easy and precise method capable of determining WHC at different centrifugal forces in one run.

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Appendix 1: Dielectric Spectroscopy

A perfect resistor is assumed to follow Ohms law at all current and voltage levels (E=R·I), its resistance is independent of frequency and AC current and voltage signals through a resistor are in phase with each other. Impedance is a measure of the ability of a circuit to resist the flow of electrical current without making the mentioned assumptions [Gamry Instruments, 2007].
Electrochemical impedance is measured by applying an alternating potential to an electrochemical cell and measure the flow of current through the cell.
The impedance (Z) can be found from the applied potential (E) and the resulting current (I) using equation 3.

$$Z(t) = \frac{E(t)}{I(t)} = \frac{E_0 \cos(\omega \cdot t)}{I_0 \cos(\omega \cdot t + \phi)} = Z_0 \frac{\cos(\omega \cdot t)}{\cos(\omega \cdot t + \phi)}$$ Equation 3 [Gamry Instruments, 2007]

where t is time, E0 and I0 are amplitude constants, ω is the radial frequency, equal to 2·π·f, where f is the frequency and ϕ is the phase angle between E and I.
It is also possible to describe the potential and the current response as complex functions, and then the impedance can be described by a complex function using Eulers relationship, this is seen in equation 4.

$$Z = \frac{E}{I} = \frac{E_0 \cdot \exp(i \cdot \omega \cdot t)}{I_0 \exp(i \cdot \omega \cdot t - i \cdot \phi)} = Z_0 \cdot \exp(i\phi) = Z_0 \cdot (\cos(\phi) + i \cdot \sin(\phi))$$ Equation 4 [Gamry Instruments, 2007]

This makes it possible to plot the data in a Nyquist plot as seen in figure 52

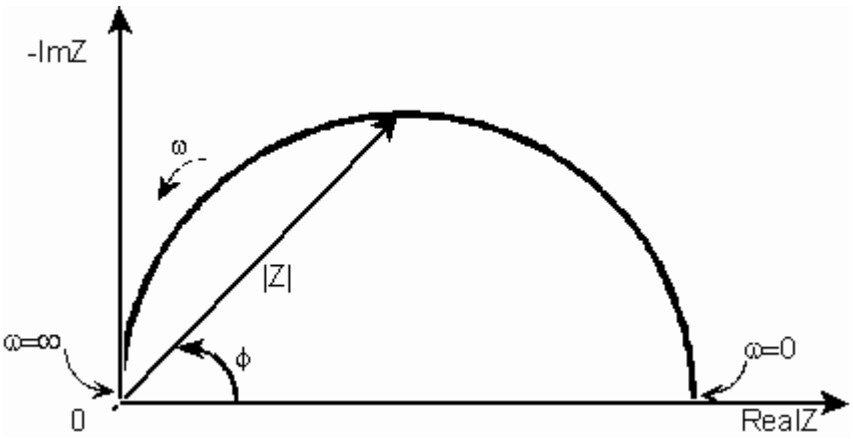

Figure 52: Nyquist plot with the real part of the impedance on the abscissa and negative the imaginary part on the ordinate. The impedance can be seen as a vector at the phase angle [Gamry Instruments, 2007].

In order to evaluate the dielectric properties, of the sample during fermentation, from the measured impedance spectrum, this must be corrected. The correction is done using measurements of the impedance with an empty cell, a short-circuited cell and a cell loaded with KCl solution [Christensen, 2008].
Then the sample measurement can be converted to the equivalent values of conductivity (real part) and relative permittivity (imaginary part) as described in equation 24 in Christensen (2008).

The lower critical frequency(f_{crit}), that is the frequency where the uncertainty becomes too high, is determined by the resolution of the phase angle(ϕ_{res}), the conductivity at a given frequency(K(f)) and the relative permittivity at a given frequency($\varepsilon_r(f)$), as given in equation 5.

$$f_{crit} = \tan(\phi_{res}) \frac{K(f)}{2\pi\varepsilon_0\varepsilon_r(f)}$$ Equation 5 [Christensen, 2008]

where ε_0 is the vacuum permittivity.

This equation shows that when the conductivity increases the critical lower frequency increases, and with high conductivity this results in loss of a considerable part of the spectrum to uncertainty.

Appendix 2: DLVO Theory

The DLVO theory of colloidal stability is a combination of the double layer forces, which are electrostatic in nature and depends on the pH and electrolyte environment and the Van der Waals interactions, which are basically independent of the ionic environment and pH [Israelachvili, 1991]. It is a theory used to predict the interaction forces (V_T) between colloidal particles dependent on the distance between the particles (D) and is a combination of the repulsive (V_R) and attractive (V_A) forces [Israelachvili, 1991].

V_A is determined from the effective Hamaker constant and describes the van der Waals forces, and V_R describes the electrostatic interactions and depends on the particle radius, the ionic composition of the liquid (the Debye length) and the zeta potential.

V_A, V_R and V_T can be plotted as a function of D, this is shown in figure 53.

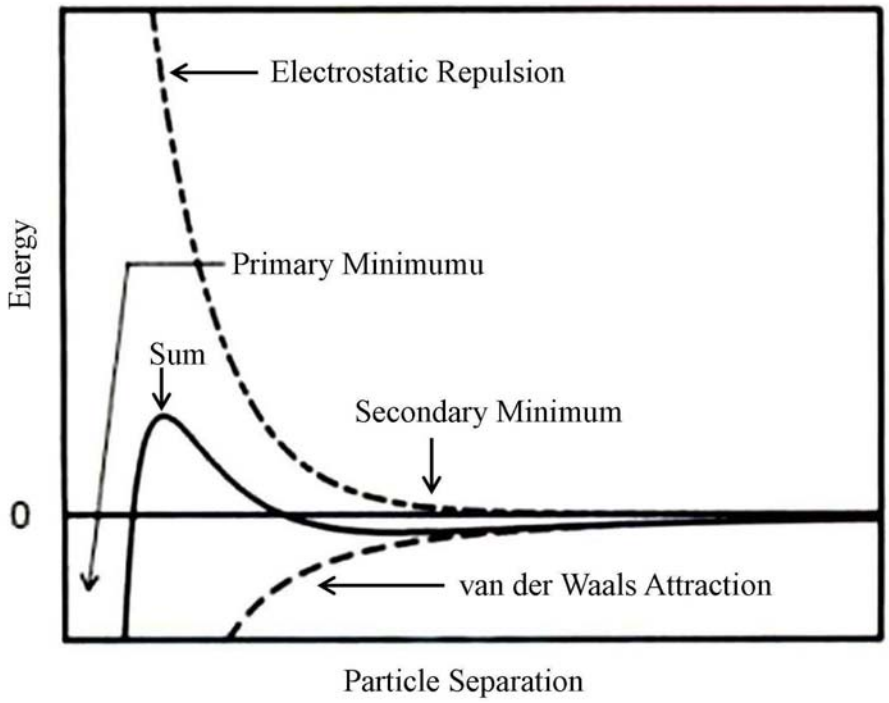

Figure 53: The forces of the DLVO theory plotted as a function of D. note that V_T shows a secondary minimum under some conditions. Adapted from Israelachvili (1991).

If the repulsion of the particles is too high for the particles to reach the primary minimum they remain separated and the suspension is stable. The appearance of a secondary minimum can lead to reversible aggregation of the particles. When the particles are unable to reach the secondary minimum the suspension is said to be kinetically stable [Israelachvili, 1991].

Appendix 3: Dynamic Light Scattering

This technique is used to determine the size distribution of small particles.

It is based on the scattering of laser light by the particles moving around in solution by Brownian motions. This result in fluctuations in intensity of the scattered light, and these depend on the size of the particles through the diffusion coefficient(D) [Atkins & De Paula, 2002].

The diffusion coefficient of a spherical particle can be determined from the Stokes-Einstein equation(equation 6) and Stokes relation (equation 7) [Atkins & De Paula, 2002].

$$D = \frac{k \cdot T}{f}$$ Equation 6[Atkins & De Paula, 2002]

where k is Boltzmann's constant, T is the absolute temperature and f is a frictional coefficient.

$$f = 6 \cdot \pi \cdot r \cdot \eta$$ Equation 7[Atkins & De Paula, 2002]

where r is the particle radius, and η is the solvent viscosity.

The intensity trace from the equipment is fitted using an autocorrelation function, and this is fitted with the equations given above resulting in a size distribution.

Appendix 4: Electrophoretic Mobility

When a charged particle is in solution the charges are compensated by ions of opposite charge. The distribution of these can be explained by the double layer model [Zeta-Meter Inc., no date], this is shown in figure 41.

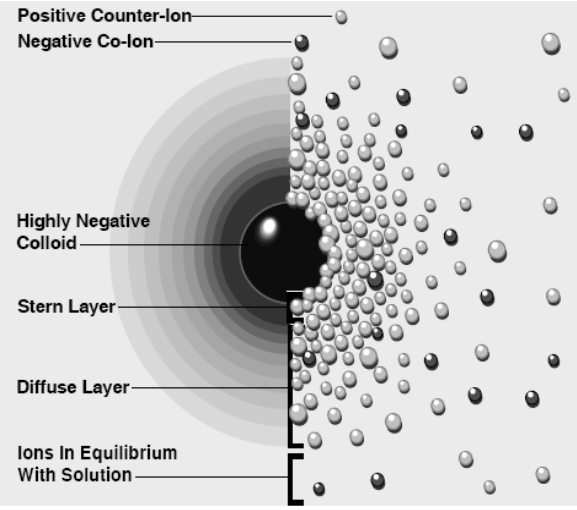

Figure 41: The double layer model [Zeta-Meter Inc., no date].

The double layer is composed of two arbitrary layers, the inner stern layer and the outer diffuse layer. The negative particle attracts positive ions, these are firmly attached to the particle, this is called the stern layer. More ions will be attracted by the negative charges, but these will be repelled by the positive charges and other positive ions approaching the particle, and these constitute an outer layer called the diffuse layer [Zeta-Meter Inc., no date]. This results in an electronic potential between the particle surface and a point in the suspending liquid. The potential drops linearly through the stern layer and exponential through the duffuse layer to zero at the imaginary difference of the diffuse layer[Zeta-Meter Inc., no date].

When an electrical field is applied across the solution, the particle will start to move towards the electrode of opposite charge. This is a result of that some of the associated ions are removed and the particle surface, also called the slipping plane, are charged. Normally the slipping plane is defined as the border between the diffuse and the stern layer[Zeta-Meter Inc., no date]. The movement will be opposed by viscous forces of the liquid, and this results in that the particle will move with a constant speed [Malvern Instruments, no date]. The velocity of a particle in a unit electric field is called the electrophoretic mobility (μ_e), and often have the unit (μm/s)/(V/cm), μ_e is calculated as shown in equation 8.

$$\mu_e = \frac{v}{E}$$ Equation 8 [Lobaskin *et al.*, 2004]

v is the velocity of the particle, E is the electric field per length. The electrophoretic mobility can be used to calculate the zeta potential if spherical symmetry is assumed [Lobaskin *et al.*, 2004].

Appendix 5: Centrifugation

Centrifugation is based on the same mechanisms as sedimentation, and therefore the mechanisms of sedimentation will be described, followed by calculation of the relative centrifugation force, and last the principle of the analytical centrifuge is presented.

Sedimentation

Sedimentation and flotation is basically the same, two phases (gas, liquid or solid) are mixed and one of the phases is continuous. As the dispersed phase often has a different density than the medium, the system tends to separate due to gravity. This assumes of course that the continuous media is liquid or gas. The separation of the dispersed media depends on how concentrated the dispersion is. The separation can be considered in three different phases, with increased volume fraction of dispersed particles, free settling, hindered settling and consolidation [Keiding, unpubl.]. The rate of sedimentation can be considered from as seen in figure 54.

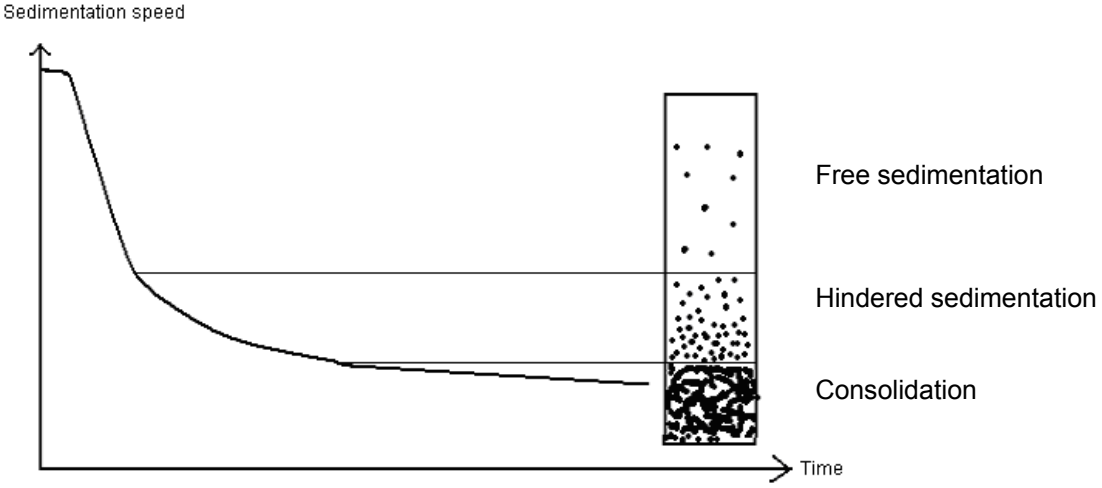

Figure 54: phases of sedimentation and rate of sedimentation

In free sedimentation the rate of sedimentation or flotation(V_s) of a particle can be calculated using Stokes Law, which is given in Equation 9.

$$V_s = \frac{2}{9} \cdot \frac{r_p^2 \cdot \Delta\rho \cdot g}{\mu}$$ Equation 9 [Keiding, unpubl.]

where r_p is the radius of the particle, $\Delta\rho$ is the density difference of the two phases, g is the gravitational force, and μ is the dynamic viscosity.

This equation is only useful when the Reynolds Number is below one, this is the case of compact particles. When the particle is less compact and the Reynolds Number is high, above 400, the sedimentation rate is given by equation 10.

$$V_r = 6{,}8 \cdot \sqrt{\frac{r_p \cdot \Delta\rho \cdot \vec{g}}{\rho_p}}$$ Equation 10

Where ρ_p is the density of the particle. When Reynolds Number is high, the radius is less important.

The Reynolds number(Re) is calculated from equation 11.

$$\mathrm{Re} = \frac{d_p \cdot V_r \cdot \rho_F}{\mu}$$ Equation 11 [Keiding, unpubl.]

Where d_p is the particle diameter, V_r is the velocity of the particle relative to the medium, and ρ_f is the density of the fluid.

This description is not always sufficient, there is another effect that should be considered. This effect is called zone sedimentation. Zone sedimentation occurs when there is a considerable number of adsorbing particles in solution. When a large particle passes a smaller particle, these may aggregate and this results in a zone of particles sweeping down through the media and grabbing all particles on their way, resulting in a clear phase above the sedimentation zone.

When the particle fraction increases at some point the flow of the media past the particles becomes limiting and this is called hindred sedimentation this has a sliding passage to consolidation. At this point a cake is formed, and the limiting factor is the flow of the media through the pores of the cake [Keiding, unpubl.].

These effects are all important when working with fermented milk products. If any fat is present in neutral milk, these particles will float to the surface, a process called creaming. As the density of the casein micelles is higher than the serum these tend to sediment on the bottom, this effect only occurs at strong centrifugation, as brownian motions keeps these homogeneously distributed in the serum under normal circumstances. The case is different when looking at yoghurt. This is described in more detail in the theory section. The focus of this project have not been to derive equations describing the sedimentation properties during centrifugation, instead this have been used as a tool of comparison.

Relative Centrifugation Force

The relative centrifugation force (RCF) is the relation between the force exerted on the sample during centrifugation and the gravitational force experienced on the earths surface, RCF is described in equation 11.

$$RCF = \frac{\vec{F}_{cent}}{\vec{g}} = \frac{\omega^2 \cdot R}{\vec{g}} = \frac{(2 \cdot \pi \cdot N)^2 \cdot R}{\vec{g}}$$ Equation 11

Where $\vec{F}_{cent}$ is the force experienced by the sample in the centrifuge and $\vec{g}$ is the earths gravity(9.82 m/s^2), R is the distance from the centre of the centrifuge to the sample, N is the number of revolutions per second and ω is the angular velocity.
It can be seen from equation 11 that RCF increases linearly with radius.

Description of the Analytical Centrifuge

The analytical centrifuge consists of a temperature controlled centrifuge combined with equipment for measuring the light transmission profile of the sample, as illustrated in figure 55.

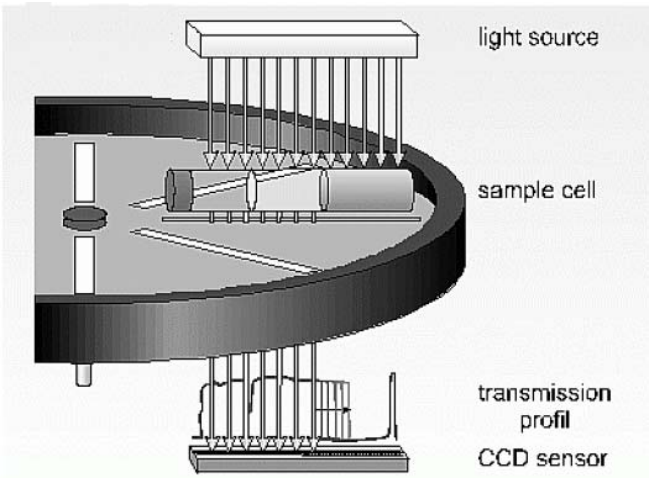

Figure 55: The principle of analytical centrifugation [Lerche *et al.*, 2003]

This centrifuge holds 12 samples and can be programmed to measure the transmission profile of the sample at any interval. Besides it can be programmed to run different rotational speeds. The profiles measured are presented as seen in figure 56.

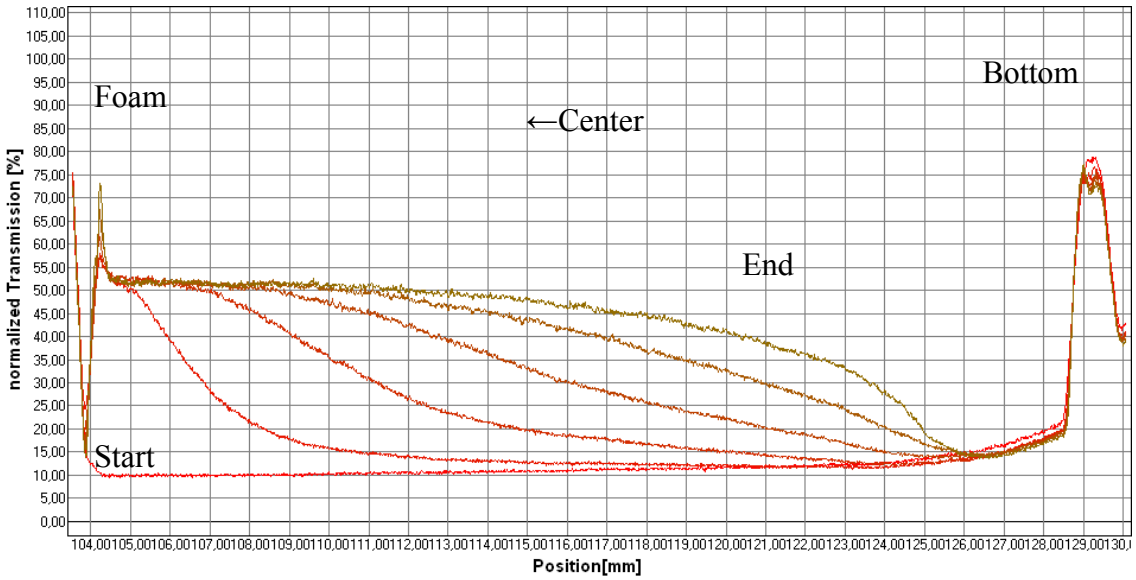

Figure 56: This figure shows a typical analytical centrifugation. The abscissa shows the position in the sample in millimetres from the centre, the ordinate shows the light transmission in percent. The curves are transmission profiles ordered from first to last by colour. The example shows sedimentation with increasing transmission at the top of the sample by time.

Appendix 6: Intrinsic Viscosity

The intrinsic viscosity ([μ]) has units of volume per mass and is the effective volume occupied in solution for a given mass unit of polymer, and it depends on the interactions between the solvent and the polymer. Dissolving polymer in a solvent results in an increase in viscosity of the solution proportional to the concentration of the polymer. As the concentration increases the volume is filled and at some point the polymers start to overlap, this point is known as the critical concentration (c*). This results in a higher increase in viscosity with increasing concentration due to entanglements between the polymers. An example of this is given in figure 57.

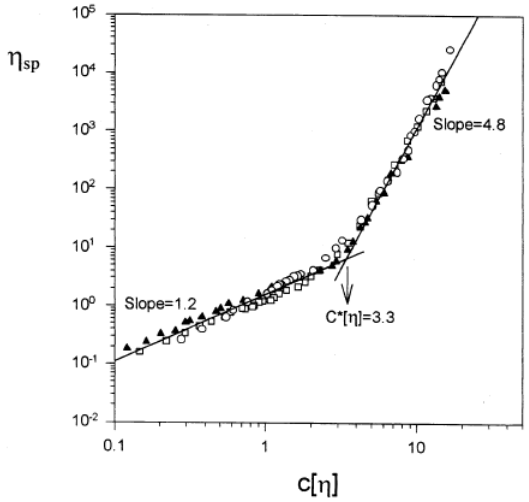

Figure 57: Specific viscosities as a function of [μ]·c for galactomannan solutions at 298 °K [Andrade *et al.* 1999].

When multiplying the intrinsic viscosity with the polymer concentration the resulting parameter, the coil overlap parameter (cop) is polymer length independent (see equation 12).

$[\mu] \cdot c = cop$ Equation 12

This means when plotting cop versus specific viscosity(μ_{sp}) the resulting curve is universal for polymers of a specific type in the given solvent and temperature. At the critical concentration the critical cop (cop*) can be calculated(Equation 13).

$[\mu] \cdot c^* = cop^*$ Equation 13

According to Andrade and co-workers (1999) at the regime under c^*, μ_{sp} is proportional to $cop^{1.2}$ and above c^* μ_{sp} is proportional to $cop^{4.8}$.

The intrinsic viscosity of pectin in aqueous solution can be determined by measuring flow times of an array of diluted solutions of the pectin through a capillary viscometer [Gomes *et al.*, 2000].

These flow times can be corrected for the energy loss due to flow speed by applying the Hagenbach correction factor [Hinge & Yu, unpubl.], as given by equation 14.

$t = t_m - a \cdot t_m^{\ b}$ Equation 14 [Hinge & Yu, unpubl.]

where t_m is the measured run time in the capillary, a and b are parameters depending on the diameter of the capillary and t is the corrected time of running through the capillary.
The corrected run times is then applied in equation 15 and 16:

$$\eta_{inh} = \frac{\ln(t/t_0)}{c}$$ Equation 15 [Gomes *et al.*, 2000]

$$\eta_{sp} = \frac{t - t_0}{t_0}$$ Equation 16 [Gomes *et al.*, 2000]

where t_0 is the corrected run time of the solvent, η_{inh} is the inherent viscosity and η_{sp} is the specific viscosity and c is the concentration.

To determine the intrinsic viscosity two plots are made, the inherent viscosity and the specific viscosity divided by concentration are plotted against concentration. These are related to the intrinsic viscosity by the Huggins and Kraemer equations(equation 17+18) [Gomes *et al.*, 2000].

$$\eta_{inh} = [\eta] + k_{inh}[\eta]^2 \cdot c$$ Equation 17 [Gomes *et al.*, 2000]

$$\frac{\eta_{sp}}{c} = [\eta] + k_{sp}[\eta]^2 \cdot c$$ Equation 18 [Gomes *et al.*, 2000]

where [η] is the intrinsic viscosity and k_{inh} and k_{sp} are constants dependent on the polymer-solvent pair.
According to Gomes and co-workers (2000) fitting the points above by linear approximations results in significantly lower uncertainties than using the second order terms, when the available data consists of few data points. This means that the intersection of a line of the form y=ax+b through the points in the plots described above results in the best approximation of the intrinsic viscosity. This is illustrated in figure 58. The intrinsic viscosity is calculated as an average of the two found values.

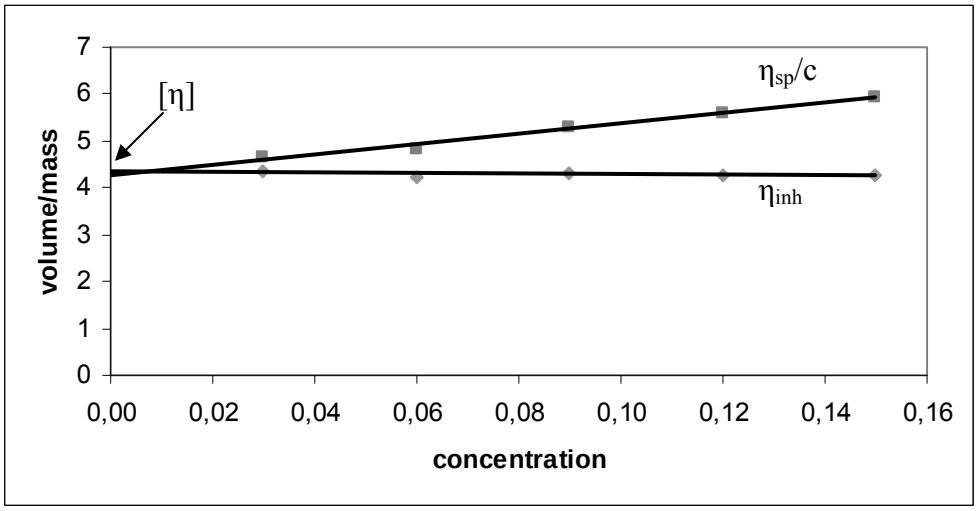

Figure 58: This figure shows an example of the graphical interpretation of determining the intrinsic viscosity from the inherent and specific viscosity of diluted polymer solutions.

The viscosity average molecular weight of a polymer ($\overline{M}_v$) can be calculated using the Mark-Hownik-Sakurade relationship given in equation 19:

$[\eta] = a\overline{M}_v^{\,b}$ Equation 19 [Rudan-Tasic & Klofutar, 1996]

where a and b are constants specific for the polymer-solvent pair. Rudan-Tasic and Klofutar (1996) uses the values $a=4.368*10^{-7}$ and b=1.8737 for polygalacturonic acid. The molecular mass for the monomeric unit of galacturonic acid is 206 g/mol.

Appendix 7: Rheology

The purpose of using rheology in this project is two things, first it is to determine the gel point of milk during fermentation/acidification, and the second is to compare the gel strength of milk gels. For clarity the term "gel strength" refers to the small deformation elastic property, not the fracture property. According to Barnes (2000) oscillatory rheology is the perfect method to monitor and characterise curing and similar processes. To do this it is necessary to know some basics of oscillating rheology, and hence this section aims to give an introduction to the technique.

This section is based on [Barnes, 2000].
If two plates with area A is moved parallel to each other, with a distance (h), at a velocity (V) the shear stress (σ) is the force needed to create the deformation, it is calculated as the force(F) divided by the area(A). The (shear) strain rate ($\dot{\gamma}$) is the velocity divided by the height. The strain (γ) is the deformation (δ) divided by height, and hence it is dimensionless, and it is often given in percent.
These relations are shown in figure 59. The relation between the shear stress and shear strain rate is the dynamic viscosity (η)

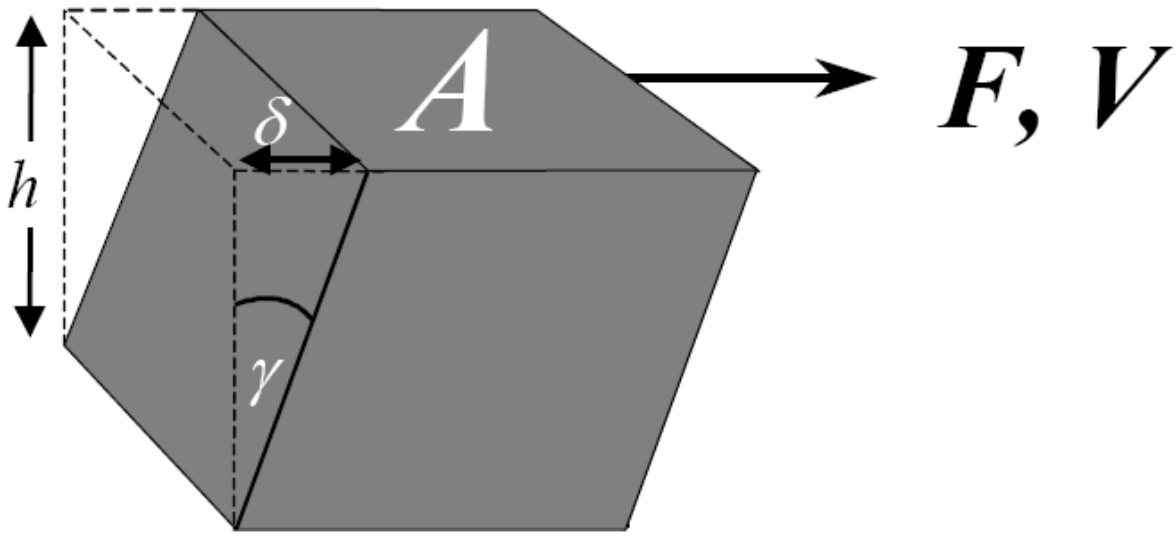

Figure 59: Definition diagram for shear flow.

When analysing viscoelastic materials, a sinusoidal stress or strain should be applied and the other measured. These two methods are called the controlled stress method utilises a constant applied force and determines the rotation rate, and the controlled strain method applies a constant rotation rate and measures the force (stress) necessary to do this. This will result in a response which lags behind the applied sinusoidal wave. The lag may vary from 0°- 90° where 0° is an ideal elastic gel (solid like response) and 90° is an ideal viscous liquid (liquid like response). The angle is called phase angle (φ). An elastic material will, when exposed to stress, stretch (strain) instantaneous proportional to the stress, and when the stress is removed there is instant full recovery. A viscous material will increase V proportional to stress, and dissipate all

the applied energy as heat. The response from a real system, at a given frequency, can be separated in G', the elastic modulus, accounting for the solid like properties and G'', the viscous modulus, accounting for the liquid like properties. The relation between the elastic and viscous moduli and the phase shift (δ_{phase}) may be described by equation 20:

$$\tan(\delta_{phase}) = \frac{G''}{G'} \quad \text{Equation 20}$$

The applied force (stress) during a measurement can be described as torque or moment, torque is independent of geometry of the measuring setup, and hence measurements in different setups can be compared. The ratio of stress to torque is a measuring geometry specific constant with the unit of Pa/Nm.

When measuring with a controlled stress method, it should be determined if the two moduli is within a linear region. This is done by measuring a "frequency sweep" with varying frequency, and an "amplitude sweep" with increasing amplitude followed by decreasing amplitude. If the frequency and amplitudes shows a linear region(constant G' and G'') then a frequency and amplitude is selected within this. Often the amplitude sweep results in a lower value of G' after the maximum have been reached. This is a result of the gel breaking and sometimes the gel breaking point can be observed as a drop in G'.

The fermentation can be considered starting as a stable particle suspension followed by destabilisation of the particles leading to aggregation and growth of particle sizes followed by a volume spanning network, which continue to strengthen, resulting in stronger and stronger particle interactions.

Appendix 8: Statistics

Four questions arise when handling data, are two variables correlated? And when interpreting multiple results from a single experiment: Which variables are significant for a given output variable?
Are two input variables correlated? And are there interactions between two input variables? This section aims to give an overview of the statistical tests and methods used in this project and answer the questions. This section is based on [Ross, 2004]

Analysis of the Correlation of two Variables

To find out if two variables are correlated, e.g. an input variable used in a experiment and the output variable, is done by attempting to use the first variable to model the other. To do this it is a perquisite that the data are arranged in pairs, so that to each data point in the first variable there must be a data point in the second variable((x_1,y_1), (x_2,y_2),… (x_n,y_n)).
It is assumed that the relation between the two variables is linear with an equation of the form

$$y = \alpha x + \beta + e$$

e is the error and it is assumed that it has a normal distribution with average zero and variance σ^2.
y is the variable value calculated from the parameters α, the x value, the constant β, and the error e.
A linear model is then fitted to the data using the least squares method.

$$\hat{y} = ax + b$$

$\hat{y}$ is the estimated value of y based the model with a, x_i and b.
The parameters, a and b, are estimators of α and β, and these are determined by the following equations. They are exposed to the uncertainty of using a limited number of samples.

$$a = \frac{S_{xy}}{S_{xx}}$$

$$S_{xy} = \sum_{i=1}^{n}\left(x_i y_i - \frac{1}{n}\sum_{i=1}^{n} x_i \sum_{i=1}^{n} y_i \right)$$

$$S_{xx} = \sum_{i=1}^{n}\left(x_i^2 - \frac{1}{n}\sum_{i=1}^{n} x_i^2 \right)$$

$$b = \bar{y} - a\bar{x}$$

where $\bar{y}$ is the average value of y_i and $\bar{x}$ is the average value of x_i and n is the number of observations.
The resulting model is a straight line in close proximity to the data points, where the squared distance parallel to the y axis, between the observed data points y_i and the corresponding estimated data points $\hat{y}_i$, is minimised.

To determine how well the model fits the most frequently used value is R^2. This value is a measure of how much of the variability is accounted for by the model. R^2 is calculated from the squared residuals between the model and the data points.

$$R^2 = 1 - \frac{SSE}{SST} = 1 - \frac{\sum_{i=1}^{n}(y_i - \hat{y})^2}{\sum_{i=1}^{n}(y_i - \bar{y})^2}$$

SST is the total sum of squares, the error without applying the model, SSE is the residual sum of squares, the remaining error after applying the model.
The value of R^2 ranges from 0-1 with 1 being the perfect fit.

During the analysis it is assumed that there is a linear relationship between the data and that the variance is constant throughout the data set. When the model is completed the residuals should be observed to verify that the model fits the data and that the variance is approximately constant. This is done by visually examining a plot of the residuals ($y - \hat{y}$) versus x in the case of one input and versus $\hat{y}$ in the case of multiple input variables.
If the residuals appears to not be randomly distributed around zero it is often because the underlying relationship is not linear or the variance is not constant. If there is any knowledge of non linearity of one of the inputs the data should be subjected to linearization. Linearization will not be described in detail in this section, but the approach is basically to apply a mathematical treatment to the non linear data to make them linear and do the fitting procedure again. If the variance is not constant sometimes a weighted least squares procedure is used to fit the line. This will not be described as the sample sizes in this project are too small to justify this approach.

Then it is determined if the found linear relationship is significant. This is done by testing the hypothesis that $\alpha=0$ with $\alpha \neq 0$ being the alternative hypothesis. It must also be decided with how much certainty the result must be, that is choosing a confidence interval. A confidence interval of 95% is most often used and this means that the risk of rejecting a true hypothesis is never larger than 5%.

The regression sum of squares is calculated:

$$SSR = \sum_{i=1}^{n}(\hat{Y}_i - \bar{Y})^2$$

The test statistic F is calculated:

$$F = \frac{SSR/q}{SSE/(n-p)}$$

Where q is the number of independent variables, n is the number of data points and p is the number of parameters in the model. The degrees of freedom is n-p.
If $F > F_{\alpha',q,p}$ the the null hypothesis can be rejected. α' is the level of significance.

The p-value is the probability to make the actual observations when the null hypothesis is true, and is found from the F value. A p-value of less than 0.05 means that the risk of observing a significant correlation between the variables when there is none is less than 5 %. The correlation is significant at the 95 % level.

Determining Which Inputs are Significant to an Output

To statistically prove which input variables are significant for a given output variable a multivariate data analysis is applied. The variables must consist of multiple input variables(x_1, x_2,…,x_k) with one value for each value in the output variable(y) in the form(($x_{1,1}$ $x_{2,1}$ …$x_{k,1}$ y_1)($x_{1,2}$ $x_{2,2}$ … $x_{k,2}$ y_2)…($x_{1,n}$ $x_{2,n}$ … $x_{k,n}$ y_n)). The analysis is based on multiple linear regression and practically the analysis is most often done in a computer program.
A model with multiple inputs of the form $y = \beta + \alpha_1 x_1 + \alpha_2 x_2 + ... + \alpha_k x_k + e$ is constructed and the parameters are estimated, based on the data in a way similar to the method used above. The error is assumed to be normally distributed around zero with constant variance. The resulting model will be in the form:

$$\hat{y} = b + a_1 x_1 + a_2 x_2 + ... + a_k x_k$$

The test of significance of each parameter is performed with a t test as described above, but assuming that all other parameters are significant and the hypothesis that the parameter in question is zero.
This results in a table containing the coefficient value and p values for each input variable. These p-values are the probability of that the parameter is zero. A p value of less than 0.05 means that, at the level of 95% confidence, the parameter is not zero and the correlation is significant. If p values of more than 0.05 exist in the table, the variable is not significant and should be removed from the model. Before this is done one should confirm that the input parameter is not highly correlated with another input parameter, see below. Only the parameter with the least significance, the highest p-value, should be removed from the model and the modelling process should be repeated. If another parameter then appears to be insignificant, the process should be repeated.
When only significant parameters remains the residuals should be analysed as described above. Linearization may be required. If the residuals appear random and evenly distributed around zero the model should be tested.
The model have an R^2 value which shows how much of the variability the model accounts for. If the significance of the model must be confirmed, an F-test is applied in the way described above. The model has an overall p-value, showing the probability that the coefficients of the model all is zero. If this p-value is less than 0.05 it shows that the model parameters affect the output significantly at the 95 % confidence level.

Correlation between Inputs

When considering a model with multiple input variables, it is sometimes experienced that an input which should be very important, e.g. temperature, appears to be insignificant. This may be a result of correlation between two input variables. If two input variables describe the same basic phenomenon, then the modelling process may assign all the importance to one variable, leaving the other insignificant. To avoid this, input variables should be checked for correlation.

This is done as described above in the "Analysis of the correlation of two variables" section. A correlation coefficient may be calculated from the "coefficient of determination", R^2 or directly from the data.

$$r = (sign\ of\ b)\sqrt{R^2} = \frac{\sum_{i=1}^{n}((x_i - \bar{x})(y_i - \bar{y}))}{\sqrt{\sum_{i=1}^{n}(x_i - \bar{x})^2 \sum_{i=1}^{n}(y_i - \bar{y})^2}}$$

r will be within -1 to +1 and describes how correlated the two variables are. If r is positive the variables is positively correlated, if r is negative the data is negatively correlated. The closer the value of r gets to 1 (or -1) the more correlated they are. An interesting property of r is that it is independent of adding or multiplying positive constants to all values of one of the variables, and thus is independent from units and reference values.
When constructing a multivariate model the inputs should be checked for correlation, but whether inputs should be removed from the model based on correlation alone must be addressed in each case.

Interactions between two Variables

Interactions between two input variables mean that the combination of these two input variables affects the output variable more than each of the input variables does alone. This interaction can be both positive and negative, resulting in a cancelling out effect. To analyse for second order or even third order interactions, being of two or three variables, there is extra requirements to the amount of data that must be gathered.
Testing can be done by multiplying the two input variables and including this new variable in the modelling process. If it shows to be significant when both original variables are also included in the model, it can be inferred that the variables interact.