BIOPHYSICAL STUDIES OF MILK PROTEIN INTERACTIONS IN RELATION TO STORAGE DEFECTS IN HIGH PROTEIN BEVERAGES

By Alexandra Grygorczyk
Department of Food Science and Agricultural Chemistry
MacDonald Campus of McGill University

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Master of Food Science

©Alexandra Grygorczyk, 2009
# Table of Contents

Acknowledgements..........................................................................................................................ii

Abstract...........................................................................................................................................iii

Résumé............................................................................................................................................iv

Chapter 1: Introduction....................................................................................................................1

Chapter 2: Literature Review...........................................................................................................2

Chapter 3: Critical Assessment of Sampling Techniques Employed for the Study of Milk Proteins by ATR-FTIR..................................................................................................................33

Chapter 4: Assessment of Storage Stability of Various Protein Ingredients Following UHT Processing......................................................................................................................................59

Chapter 5: The Chaperone Didn’t Show: Interaction Between Native β-lactoglobulin and Caseins Challenges Chaperone Status of Caseins.......................................................................................79

Chapter 6: General Summary.......................................................................................................100

Chapter 7: Future Work...............................................................................................................102

References....................................................................................................................................103

List of Abbreviations...................................................................................................................110

Appendix......................................................................................................................................111
Acknowledgements

I would like to thank my supervisor Dr. Ashraf Ismail for his unrelenting support, guidance and brilliant input throughout my M.Sc. thesis research. As well, I owe a big thanks to Pramod Pandey and Ziad Khoury for their continual encouragement during my studies, both undergraduate and graduate, and for giving me invaluable exposure to the food industry.

I also need to thank Dr. Jacqueline Sedman for editing all the work that I write and Dr. Mark Hancock for his collaboration on the SPR work and for all of his editing help.

I am grateful to the Natural Sciences and Engineering Research Council of Canada (NSERC), for their financial support in the form of a postgraduate scholarship. I would also like to thank Bariatrix Nutrition Inc. for their financial support, also in the form of a scholarship, and for giving me access to their facilities and raw materials.

A big thanks to my good friend Mireille Schnitzer for her statistical consulting and Éric Marseau for editing my résumé.

I’d also like to thank all of my lab mates for their friendship and for making my hours in the lab that much more entertaining.

Finally, I want to extend a special thanks to my mother, father, brothers and my friends for their encouragement, understanding and for providing me with an outlet for my fun side so that I can stay focused and grounded throughout my studies. In particular I’d like to thank Marcin, Ania and Sandra for being there for me and Plamen, Karen, Tanya, Juliana and Anil for making my on-campus life fun.
Abstract

Much like Ultra High Temperature (UHT) processed milk, UHT processed high protein beverages suffer from storage defects including gelation and sedimentation. Although these beverages are made with milk proteins, the food system is very different and much more complex from that of milk. Thus, one cannot assume that the mechanism of development of storage defects is the same as in milk. As such, the goal of this project was to investigate the factors affecting storage stability of high protein beverages.

As a first step, a method was developed to facilitate the study of milk protein solutions by Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) Spectroscopy. Scanning the proteins in solution followed by subtraction of the contribution to the absorbance by water provided the most reliable and repeatable results. Next, the suitability of the data for various forms of spectral enhancement was assessed. Finally the method was applied in a series of experiments in order to determine if the data could provide numerical information. Thermodynamic data for heating of β-lactoglobulin was obtained using three methods: transmission-FTIR spectroscopy in D₂O, ATR-FTIR spectroscopy in D₂O and in H₂O. All techniques provided equivalent results, indicating that analyzing proteins in water by ATR-FTIR spectroscopy can be used to provide quantitative information.

A number of different protein products (α-lactalbumin, β-lactoglobulin, calcium caseinate, WPC, WPI, MPC, MPI) and combinations of these proteins were UHT processed and their storage stability was observed. Unfortunately, the changes were too subtle to be detected by FTIR spectroscopy and we had to rely more heavily on visual observations. From these observations it was determined that altering the protein combinations did not prevent sedimentation of caseinates. However, the presence of β-lactoglobulin changed the consistency of the sediment. Additionally, it was also noted from these trials that proteins alone are most likely not responsible for age gelation of high protein beverages since gelation was only observed when proteins were in the presence of all the other beverage components.
Finally, the interactions between β-lactoglobulin and caseinates were analyzed using FTIR-spectroscopy and Surface Plasmon Resonance (SPR). FTIR spectroscopic analysis indicated that α- and β-caseins exhibited a protective effect on β-lactoglobulin during heating and changed the order of events in β-lactoglobulin’s unfolding. This change in sequence of events suggested that hydrophobic interactions are involved in the casein-β-lactoglobulin interaction. SPR study of casein-β-lactoglobulin interaction demonstrated that there was a specific interaction between these proteins in the order β-casein> α-casein> κ-casein> UHT calcium caseinate. Based on the above results, we propose a model scenario of protein-protein interaction in UHT high protein beverages.

Résumé

Tout comme dans le cas du lait ultra haute température (UHT), les protéines contenues des les breuvages hyper protéinés sont affectées par le traitement à haute température. Ce type de traitement thermique mène à la formation de gel et de sédiments dans ces boissons. Bien que ces breuvages soient à base de protéines laitières, la composition chimique de ces boissons demeure très différente et beaucoup plus complexe que celle du lait. Ainsi, on peut supposer que le mécanisme de formation menant à des défauts de conservation est différent de celui du lait ultra haute température. Le but de ce projet était de se familiariser avec les facteurs affectant la stabilité de conservation des breuvages hyper protéinés.

La première étape du projet consistait à développer une méthode pour étudier les solutions hyper protéinées à base de lait en utilisant la spectroscopie infrarouge à transformée de Fourier en réflectance totale atténuée (RTA-IRTF). Il a été déterminé que le balayage des protéines en solution suivi d’une soustraction du spectre de l’eau était la méthode la plus fiable et la plus reproductible. Une fois l’acquisition des données brutes complétée, celles-ci étaient transformées par amélioration spectrale et évaluées pour déterminer si elles pouvaient être utilisées de manière quantitative. Les données thermodynamiques sur le chauffage de la β-lactoglobuline ont été obtenus en utilisant trois méthodes: IRTF à transmission dans D₂O, RTA-IRTF dans D₂O et dans H₂O. Ces trois méthodes ont produit des résultats équivalents indiquant
que de l’information quantitative pouvait être obtenue lors de l’analyse des protéines en solution aqueuse en utilisant la spectroscopie RTA-IRTF.

Un traitement UHT a été appliqué sur différentes permutations de protéines et nous avons essayé de suivre le vieillissement des protéines avec la méthode développée. Malheureusement, les changements étaient trop subtils pour être détectés par la spectroscopie RTA-IRTF. Les observations visuelles ont donc été utilisées afin de détecter ces changements. Basés sur ces observations, nous avons été en mesure de constater que l’altération des concentrations de différentes protéines ne prévenait pas la sédimentation des caséinates. Toutefois, la présence de β-lactoglobuline a changé la consistance du sédiment. Ces essais de UHT nous ont permis de déterminer que les protéines seules ne sont probablement pas la cause de la gélification des breuvages hyper protéinés, car la formation de gel n’était observée que lorsque les protéines étaient en présence de tous les composants de breuvage.

Finalement, l’accent a été mis sur l’étude de l’interaction entre les caséinates et la β-lactoglobuline en utilisant la spectroscopie IRTF et la Résonance de Plasmon de Surface (RPS). En utilisant la spectroscopie IRTF on a démontré que α-caséin et β-caséin ont un effet protectif sur β-lactoglobuline lors du chauffage et elles changent la séquence d’événements de déroulement du pliage de β-lactoglobuline. Ce changement dans la séquence d’événements suggère la participation de liaisons hydrophobes. L’analyse RPS a démontré une interaction spécifique entre les caséines et la β-lactoglobuline dans l’ordre de puissance β-caséin > α-caséin > κ-caséin > UHT caséinate de calcium. Les résultats de cette étude ont permis de proposer un scénario modèle démontrant de quelle façon les protéines des breuvages hyper protéinées interagissent entre elles suivant un traitement UHT.
Chapter 1
General Introduction

Functional foods are foods which provide health effects that go beyond basic nutritional needs. The functional foods market is growing quickly and expanding at a faster rate than the regular processed foods market (FAO, 2009). High protein foods are classified in this category and there is a current shift towards more protein-rich foods on the global market as opposed to the traditional diet which is heavy in carbohydrate-rich foods (Agriculture and Agri-Foods Canada, 2009). One result of this shift over the last two decades, was the development of specialty high protein foods such as high protein beverages to target dieters, hospital patients recovering from surgery and athletes.

Companies such as Bariatrix Nutrition, SlimFast, Abbott (Ensure) and even Kraft (South Beach Diet) sell high protein drinks and bars. The market is still relatively new and it is extremely competitive. As such, each manufacturer strives to entice customers by improving the taste, appearance and convenience of these products. UHT processed ready-to-drink beverages are the answer to the question of convenience for consumers. However, much like UHT processed milks, high protein drinks which are generally made from milk proteins also suffer from aging defects following UHT treatment.

Although of much interest to the food industry, in-depth scientific studies are lacking on high protein beverages. As such, the objectives of this study were to identify and investigate protein interactions which affect the stability of such beverages. Towards this end, the following thesis is structured in the form of 4 core sections. The first section (chapter 2) reviews the literature concerning the behaviour of UHT processed milk proteins and some of the potential methods to study these proteins, as well as providing background on chaperone proteins. The second section (chapter 3) describes the development of an appropriate sampling technique for the study of high protein beverages by FTIR-ATR spectroscopy. The third and fourth sections (chapters 4 and 5, respectively) describe investigations on protein interactions that can occur during storage of these beverages.
Chapter 2
Literature Review

2.1. Introduction

For many decades whey proteins were seen as a waste by-product of cheese-making. Industry sought the cheapest way to dispose of whey and often opted dispensed it into waterways and onto fields. However as concerns over pollution mounted, these practices became restricted by environmental regulations. As a result, industry was forced to find another solution and began producing whey powders as an inexpensive commodity product. A great deal of research was done to find uses for whey protein powders and soon the great nutritional, biological and functional properties of whey proteins were uncovered. Accordingly, the demand for whey increased dramatically (Smithers et al., 1996). Whey protein powders and other protein fractionates became particularly popular among consumers such as dieters (Bastien, 2004) and athletes (Phillips et al., 2007).

The protein industry started to supply whey and other proteins to consumers in more palatable forms such as in protein fortified meals, and high protein beverages (Bariatrix Nutrition Inc., 2008). High protein beverages have become particularly popular and they are often available either as beverage powder mixes or ready-to-drink beverages. The ingredient list of these beverages often includes more than 15 different items such as various protein blends, flavours, phosphates, colours, gums, salt, sweeteners and carbohydrates. Each of

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oil or cream</td>
<td>3.00%</td>
</tr>
<tr>
<td>MPC</td>
<td>3.00%</td>
</tr>
<tr>
<td>MPI</td>
<td>3.00%</td>
</tr>
<tr>
<td>Calcium Caseinate</td>
<td>1.50%</td>
</tr>
<tr>
<td>WPC</td>
<td>1.00%</td>
</tr>
<tr>
<td>Carrageenan</td>
<td>0.01%</td>
</tr>
<tr>
<td>Carboxymethyl Cellulose, Microcrystalline Cellulose or other gum</td>
<td>0.10%</td>
</tr>
<tr>
<td>Complex carbohydrate (inulin, maltodextrin or starch)</td>
<td>0.30%</td>
</tr>
<tr>
<td>Salt</td>
<td>0.15%</td>
</tr>
<tr>
<td>Colour</td>
<td>0.03%</td>
</tr>
<tr>
<td>Phosphates</td>
<td>0.10%</td>
</tr>
<tr>
<td>Emulsifier</td>
<td>0.10%</td>
</tr>
<tr>
<td>Water</td>
<td>87.71%</td>
</tr>
</tbody>
</table>

Table 2.1: Example of a Typical High Protein Beverage Formulation
these plays an important role in producing a stable and nutritious drink with good sensory attributes. Table 1 is an example of a typical high protein beverage formulation.

High protein beverage manufacturers often combine two or more protein powders in the beverage mix in order to obtain the desired properties in the final product. Properties that are important for the manufacturer include protein content/bulk weight, lactose content, fat content, amino acid profile, solubility, stability, viscosity, colour, flavour and dispersibility. Additionally, protein properties may differ from one source to another and variability may arise from batch-to-batch as a result of the processing methods employed and seasonal difference in milk quality (Opawumi and White, 2004). Below is an outline of some of the most common milk protein ingredients and their properties.

**Caseinate**

Caseinates are formulated as sodium or calcium caseinate. Both are heat stable and easily dispersible. Sodium caseinates have a clean flavour profile, they have high water holding capacity and they act as natural emulsifiers. Calcium caseinates also provide a clean flavour profile and possess a high opacity in water suspensions and good dispersibility. Additionally, the calcium formulate allows manufacturers to lower the sodium content of their product while increasing the calcium content, a decision which is generally considered favourable for nutritional reasons according to consumers. Calcium caseinates however, are more expensive than sodium caseinates (Chandan, 1997).

**Whey Protein Concentrates (WPC)**

WPCs are obtained by separating the whey fraction from the remaining milk proteins then removing some of the minerals and lactose. This formulation contains between 25% and 85% protein content. The fat content increases with protein content and lactose content can be as high as 50%. WPC are highly soluble between pH 2 and pH 8 and provide acid stability and a low viscosity. However, the natural flavour of whey
is usually very strong and difficult to get used to by many consumers. It is often a challenge to mask the taste of WPC high protein products (Chandan, 1997).

Whey Protein Isolates (WPI)

WPIs contain more than 90% protein and have very low lactose and low fat content. Because of costly purification, WPIs are more expensive than WPC and are generally used in more high-end products. They are more suitable than WPC for people with lactose intolerance and for low caloric health products. However, as with WPC, the flavour of whey can be an obstacle (Chandan, 1997).

Milk Protein Concentrate (MPC)

The protein content in MPCs ranges from 56-82%. This protein product can provide foods with opacity, emulsification, heat stability, fortification and a very mild, milky flavour (Chandan, 1997).

Milk Protein Isolate (MPI)

MPI is produced by methods which precipitate whey and casein proteins to achieve 90% protein content with high levels of calcium and low levels of lactose. The casein and whey ratios are very similar to those found in milk thereby providing an amino acid profile comparable to milk. As a food additive MPIs offer dispensability, solubility, emulsification and heat stability (Chandan, 1997).

By identifying these general characteristics and by testing the individual properties of each new protein ingredient received, product developers can prepare protein blends with desired characteristics for use in their beverages. Once the combination is selected, additives such as gums and phosphates are added to further stabilize the proteins in solution and to improve their
texture. The following is a brief description of some of the gums and phosphates commonly used in high protein beverages.

\textit{Carrageenan}

The red seaweed extract, carrageenan, is a complex carbohydrate often used in conjunction with milk-based products. Carrageenan has several fractions called iota, kappa and lambda. The $\kappa$-carrageenan portion requires the presence of calcium ions in order to gel. Since milk products naturally contain calcium, they are a good candidate for gelation by carrageenan. A large concentration of calcium ions is found at the surface of the casein micelle. The net positive charge attracts the negatively charged sulphate groups of carrageenan and forms a network of ionic interactions between carrageenan and casein micelles. This light gel, along with carrageenan’s water-holding capacity, help to keep the milk proteins and other solids in solution over time (FMC BioPolymer, 2008). In addition to its ability to stabilize proteins, carrageenan also has a clean mouth feel and it can be used effectively in beverages at concentrations as low as 200-500 ppm, thereby making it an economical choice (Opawumi and White, 2004).

\textit{Carboxymethyl Cellulose (CMC)}

CMC is a derivative of cellulose formed by reaction of cellulose with alkali and chloroacetic acid. It can be added to beverages to modify the viscosity. It does not gel even in the presence of calcium ions and as such can be used to thicken high protein beverages. It also offers emulsion stability and helps to suspend solids within beverages (Phillips and Williams, 2000).

\textit{Phosphates}

A combination of phosphates is usually added to high protein beverages, particularly in the ready-to-drink variety, to help stabilize proteins. Sodium phosphate is reported to improve the heat stability of proteins during heat processing and polyphosphates and sodium hexametaphosphates act to delay gelation of UHT-processed milk proteins (Datta and Deeth, 2001). It is thought that phosphates interact with milk proteins and
increase their negative charge. Accordingly, phosphates increase protein-protein repulsion and hence make interaction between proteins more difficult and delay gelation (Kocak and Zadow, 1985).

Once the appropriate additives are combined with the proteins to stabilize them and to produce desirable textural characteristics, product developers can then focus on other sensory attributes such as flavour and colour to finalize their product.

The evolution from conception of the product to a finished and ready-to-sell beverage is, in reality, very complex and often very time-consuming. Food systems possess a variety of different ingredients that are subjected to various processing regimes and this can lead to undesirable interactions between components. This often makes stabilization of the final product for long-term storage a great challenge.

The ready-to-drink beverages are popular because of their convenience. They are generally ultra high temperature (UHT) processed for shelf-life extension. Unfortunately UHT-processed high protein beverages made with milk proteins encounter aging problems which significantly limit their shelf-life (Harwalkar, 1982; Datta and Deeth, 2001) and result in large economic losses. Manufacturers generally succeed in delaying problems for a few months by using stabilizing agents described above (Opawumi and White, 2004). However, these additions are based on empirical observations and their mechanisms of action are not totally understood. Moreover, the mechanism and causes of age gelation/sedimentation are also unclear, making it all the more difficult to determine how the additives act to delay the aging process.

To our knowledge no studies to date have been conducted on the storage stability of high protein beverages made with milk proteins. There is however a significant body of literature on the aging of UHT milk and some related to concentrated milk (Harwalkar et al., 1983; Kocak and Zadow, 1985; McMahon, 1995; Datta and Deeth, 2001). For this reason, the ensuing literature review will focus on mechanisms of aging phenomena occurring in UHT-processed milk and concentrated milk, as a starting point for the examination of age gelation of high protein beverages. The review will also describe some of the techniques that have been or could potentially be used to study aging defects in high protein beverages. Finally, we will focus on an interaction between β-lactoglobulin and caseins which is likely occurring in high protein
beverages. This interaction has been recently reported by several authors and appears to protect β-lactoglobulin against aggregation. The work of these authors on this chaperone-like behaviour of caseins will be reviewed.

2.2. Storage Stability of UHT Milk and Concentrated Milk

2.2.1. UHT Milk

The great majority of milk produced in North America is pasteurized to ensure safety and to increase its shelf-life to approximately two weeks. Thus distributors have sufficient time to deliver milk from large processing plants to consumers who live in relatively nearby cities. However, often in industrialized countries which produce milk far in excess of their requirements, milk is processed using harsher treatments to extend the shelf-life to several months for consumer convenience and to allow for milk distribution to more distant or remote locations, particularly to areas where refrigeration may not be available.

Several techniques can be used for heat treating milk to ensure an extended shelf-life. Retorting involves heating milk in sealed containers at 110-120°C for 5-20 min. Because of its harshness, this process results in colour and flavour changes as well as some nutritional losses. High temperature-short time (HTST) and ultra high temperature (UHT) methods have become more common in industrialized countries over the past two decades. These achieve the same degree of sterilization as retorting but result in very little colour and flavour change and little to no nutritional losses. These treatments are performed in continuous flow systems which allow for better surface contact between the heating medium and product, thereby shortening the necessary holding times and generating very fast heat transfer. HTST treatments normally involve heating products to 105-120°C for 30 s-2 min., while UHT treatment involves heating product to 130-150°C for 1-20 s (Muir, 1984). It has been reported that milk proteins that are subjected to HTST or UHT are destabilized and result in age-induced gelation. This limitation has been the largest hurdle for HTST/UHT milk manufacturers (Harwalkar, 1982).

Textural Changes during Aging

As UHT sterilized milk ages, changes occur which lead to the formation of a sediment as well as an increase in viscosity and eventually gel formation throughout the sample. Generally,
sedimentation is minimal and gelation is the main concern which limits the shelf-life of UHT milk. These textural changes should not be confused with increases in viscosity immediately following UHT treatment. As the term, age-gelation implies, these changes are strictly defects which occur with prolonged storage. They generally occur in milk after a period of 3-12 months and depend on the sample composition, processing parameters and storage temperature (Harwalkar, 1982).

There are four stages which lead to the development of a gel during storage of UHT-treated milk. The first stage is quite short and accompanies some product thinning. In the second stage, the change in viscosity remains stable for a longer period of time. In the third stage, there is a sudden change in viscosity which, during the early stages, can still be reversed by agitation. However with time, the viscosity increases until an irreversible custard-like gel forms (Ellertson and Pearce, 1964). Finally, in the fourth stage there is a lowering in viscosity as the gel matrix breaks down resulting in a serum layer and protein curds. It is noteworthy that for reasons unknown, the precise duration of these stages varies widely from batch to batch (Harwalkar, 1982).

Figure 2.1: One mechanism by which gel formation can occur upon aging of UHT milk
**Gel Structure**

The protein gel (the fourth stage of gelation) in UHT-treated milk consists of a three-dimensional (3D) matrix of proteins. There are two pathways through which this gelation is thought to occur. In the first gelation pathway, the events leading to age-induced gelation begins during processing of the milk. The denaturation temperature of $\beta$-lactoglobulin is 55°C. At this temperature, the protein begins to unfold and loses part of its globular structure. As the protein unfolds, the reactive sulfhydryl group of $\beta$-lactoglobulin initiates sulfhydryl-disulfide exchange. A prolonged heat treatment above 80°C leads to the disulfide exchange between all cysteine residues. This occurs extensively during UHT treatment and the reactive sulfhydryl groups of $\beta$-lactoglobulin can react intermolecularly to form $\beta$-lactoglobulin complexes or they may react to form disulfide bonds with $\kappa$-caseins from the casein micelle. Alternatively, the reactive sulfhydryl groups of $\beta$-lactoglobulin may react with other sulfhydryl-containing proteins on the milk fat globule membrane. However, the $\beta$-lactoglobulin-$\kappa$-casein interaction is the most significant event and this is likely due to the fact that $\kappa$-caseins reside on the surface of the casein micelle, making them highly available for disulfide-sulfhydryl interchange with $\beta$-lactoglobulin’s free sulfhydryl group (McMahon, 1995; Datta and Deeth, 2001).

A number of other changes gradually take place during storage of UHT milk. When $\beta$-lactoglobulin forms linkages with $\kappa$-caseins from the casein micelle, as discussed above, it weakens the association of $\kappa$-casein with the other proteins in the casein micelle. This results in the release of the $\beta\kappa$-complex from the surface of the casein micelle. The free $\beta\kappa$-complexes may react to form linkages with other proteins and eventually create a 3D gel matrix. In addition to protein-protein interaction leading to gel formation, enzymes can also play a pivotal role in age-gelation (McMahon, 1995). The enzyme-mediated release of the $\beta\kappa$-complex from the casein micelle involves a two-stage process. In the first stage, proteinases such as plasmin and microbial proteinases, cleave the peptide bond which anchors $\kappa$-casein to the micelle thus releasing the $\beta\kappa$-complex. In the second stage, free $\beta\kappa$-complexes aggregate to form a 3D network of cross-linked proteins. The achievement of a critical concentration of cross-linked $\beta\kappa$-complexes results in the formation of a semi-rigid gel structure (McMahon, 1995; Datta and Deeth, 2001). The relationship between gelation time and proteolytic activity has yet to be quantified; the enzymatic theory has not acquired wide-spread acceptance and a number of theories involving
non-enzymatic mechanisms have been developed (Datta and Deeth, 2001). One theory proposes that storage of UHT milk at higher temperatures mediates the polymerization of whey and casein proteins by means of the Maillard reaction. It has been found however, that UHT milk stored at temperatures above 35°C does not form gels (Andrews and Cheeseman, 1972).

A second pathway for the age-induced gelation may involve casein micelle aggregation. The colloidal stability of casein micelles in milk depends on the amount of surface κ-caseins, the amount of colloidal calcium phosphate, the surface potential and steric stabilization. UHT treatment can cause changes at the surface of the casein micelle. During storage these changes progress and as the casein micelles become destabilized they slowly form aggregates and make a gel matrix. Accordingly, the age-gelation of UHT milk is a result of changes in the free energy of casein micelles. This hypothesis states that during heat processing of milk, the casein micelles are transformed into a meta-stable state with high surface potentials. During storage, spontaneous transformations occur to lower the surface potentials of some casein micelles. Accordingly, not all micelles will have the same potential and the difference in potential among the micelles promotes their aggregation. The extent of aggregation depends on the probability of contact and the number of low-potential micelles lead to a gradual increase in viscosity with increasing storage time (Datta and Deeth, 2001).

**Factors Affecting Age-Gelation**

In an attempt to retard age-gelation of UHT milk, many studies have been conducted to identify the factors that affect the rate and extent of age-gelation. Among the factors, mode and severity of heat treatment, proteolysis, milk production factors, additives and fat content play a predominant role. These factors and their effect on age-gelation will be discussed in this section.

**Heat Treatment Effects**

In a typical UHT process, raw milk is preheated to 80-95°C for approximately 30 s to stabilize β-lactoglobulin. When β-lactoglobulin is denatured at lower temperatures it remains stable in solution and does not separate out. If β-lactoglobulin is denatured at sterilization temperatures it becomes unstable and forms deposits in the high temperature heating section of the UHT system (Datta and Deeth, 2001). The milk then heated to temperatures ranging from 135-150°C for a few seconds to sterilize the milk.
In a study on the effects of pre-heating temperatures on age-gelation of UHT milk, it was found that an increase in severity of the pre-heating conditions resulted in a delay of age-gelation. For example milk preheated at 72°C for 30 s formed a gel sooner than milk which had been preheated at 80°C for 30 min. Additionally, when indirect heating methods were used (such as in a plate heat exchanger or tubular heat exchanger) compared to direct heating methods, age-gelation was delayed. It was observed that for equivalent bactericidal effects, milk that was heated directly experienced gelation within 6-10 weeks at 20°C and milk which was indirectly heated had no gelation or increase in viscosity after 30 weeks at 20°C (Manji et al., 1986; Manji and Kakuda, 1988). Again, the same effect was observed in another study when the sterilization temperature was increased from 142°C to 152°C and the hold time increased from 6 to 12 s (Datta and Deeth, 2001). Payens hypothesized that the delay in gelation caused by increasing the pre-heat temperature is due to β-lactoglobulin precipitating onto the surface of the casein micelle and diminishing the number of sites which are available for interaction (Payens, 1978). Another theory purports that age gelation is initiated by proteinases (either native plasmin or bacterial proteinases) which are not destroyed during heating. A more severe heat treatment inactivates more proteinases and hence slows the age gelation process (Corradini and Pecchini, 1981). A third theory concludes that when milk is treated with severe heat treatment, the extent of chemical cross-linkages within micelles is increased. Thus, the βk-complexes, that were formed by disulfide exchange during heat treatment, are more strongly associated with the micelle and as a result, they are released more slowly from the micelle leading to delayed gelation (Datta and Deeth, 2001).

Proteolysis and Age Gelation

Although it has been clearly demonstrated (Manji et al., 1986) that both plasmin and bacterial proteases catalyse the formation of gels in UHT-treated milk, no study has reported a correlation between the shelf-life of UHT sterilized milk and the extent of proteolysis. Renner reported that although there is more extensive proteolysis in UHT treated milk after storage at 40°C than at 30°C or 20°C (Renner, 1988), gelation is delayed in milk stored at 40°C (Andrews and Cheeseman, 1972). Hence, proteolysis alone cannot be used to explain the differences in gelation times of milk stored at different temperature. Furthermore, gelation is inhibited by the addition of sodium hexametaphosphate but this compound has no effect on proteolysis (Snoeren
et al., 1979). Hence, it has been concluded that although proteolysis contributes to age gelation of milk, other factors must also play an important role.

**Additives**

The effect of various additives on age gelation has been observed (Ellertson and Pearce, 1964; Kocak and Zadow, 1985; Datta and Deeth, 2001). Among the additives investigated were sodium citrate, EDTA, calcium chloride, N-ethylmaleimide and various phosphates.

**Sodium Citrate, EDTA, Calcium Chloride and NEM**

The effects of EDTA and sodium citrate were examined since these compounds are reported to bind metals which are needed as cofactors for proteolytic enzymes. Citrate appears to stabilize the casein micelle and enhances colloidal calcium phosphate linkages, however there is no evidence of EDTA’s involvement in these types of interactions.

The addition of sodium citrate to raw milk prior to processing was found to enhance age-gelation (Snoeren et al., 1979). Milk containing 0.3% sodium citrate gelled faster than the control with no additives but slower than that containing 1%. The addition of 0.1% EDTA appeared to have no effect on the age gelation of UHT processed milk. When the concentration of EDTA was increased to 0.3% however, the onset of gelation was accelerated when compared to control samples (Kocak and Zadow, 1985).

There appears to be conflicting reports concerning the addition of Calcium chloride to UHT-treated milk. According to Kocak and Zadow, the addition of CaCl₂ increased the stability of UHT milk. Milk with added calcium chloride did not gel when stored for over 500 days at 25°C (Kocak and Zadow, 1985). In contrast, Ellertson and Pearce reported that adding calcium chloride to UHT milk accelerated gelation (Ellertson and Pearce, 1964).

N-ethylmaleimide, (NEM) is a covalent chemical modifying agent which is specific for free sulfhydryl groups. As such, it can interact with whey proteins to prevent them from forming intermolecular disulfide bonds with other proteins such as caseins. However, results concerning its effect on age gelation of UHT milk are conflicting. Hong et al. reported that directly heated UHT milk treated with 0.5g/L of NEM gelled later than milk without added NEM (52 weeks with NEM vs. 18 weeks without NEM). However, an opposite effect was observed in indirectly
heated milk; treatment of this milk with 0.5g/L of NEM resulted in gel formation after only 18 weeks whereas without NEM the milk gelled after 40 weeks. This difference in the effects of NEM on directly and indirectly heated milk has not been explained (Hong et al., 1984).

**Phosphates**

The effect of phosphate additives on age gelation of UHT milk has been examined. Sodium phosphate was found to accelerate gelation rate whereas polyphosphates in general were found to delay the rate of gel formation. The extent of phosphate protection appears to increase with increasing chain length and concentration. Polyphosphates with an average of 4.8 phosphorus atoms per chain were found to be the most effective against gelation. Polyphosphates associate with casein micelles and impart a net negative charge, which in turn can increase the association between micelles and βk-complexes through ionic interactions.

The cyclic form of tetrametaphosphate and adenosine triphosphate were found to be more effective than linear compounds. The linear molecules appeared to be converted slowly into orthophosphates which accelerated gelation rate. Additionally, the linear forms are unable to form complexes with Ca$^{2+}$ which can retard gelation by increasing cross-linking between caseins (Kocak and Zadow, 1985).

Snoeren and colleagues reported that disodium hydrogen phosphate and sodium hexametaphosphate (SHMP) act on the second stage of gelation by stabilizing the milk protein system in a manner which is independent of proteolysis (Snoeren et al., 1979). These phosphates may associate with casein micelles and impart a more negative charge. The authors speculated that with the additional negative charges κ-casein would associate more strongly to the micelle and delay the release of the βk-complexes, thereby slowing gelation (Kocak and Zadow, 1985).

**2.2.2. Concentrated Milk**

In the example of sterilized concentrated milk, sedimentation and age gelation or thickening is also observed. Textural changes occurring during aging are very similar to those occurring in UHT-sterilized milk. Sedimentation is less important than viscosity changes and gelation.
The time-dependent progression of gel formation is very similar to that in milk. First, there is an initial decrease in viscosity, followed by a period of stability and then a sudden increase in viscosity and formation of a gel (Ellerton and Pearce, 1964). The on-set of gelation is later than in UHT milk and therefore the product is normally distributed and consumed before gelation occurs. However, it has been observed that concentrated milk from some manufacturing plants gelled sooner and more often, thus generating interest in the factors which affect gelation of concentrated milk (Harwalkar et al., 1983).

**Temperature and Processing Effects**

The production of concentrated milk includes four processing steps: fore warming, concentration, homogenization and sterilization.

The order of these steps can have an effect on aging of the samples; Placing the homogenization step before concentration and sterilization results in a product which gels faster than a product which was homogenized after concentration and sterilization. However, factors which decrease the product’s susceptibility to gelation tend to increase its susceptibility to sedimentation. Thus, homogenization after concentration and sterilization decreases sedimentation but augments gelation. Concentration before sterilization also decreases susceptibility to gelation but increases susceptibility to sedimentation (Harwalkar, 1982).

Fore warming is the process during which raw milk is heated for a specified amount of time to denature whey proteins, yielding a concentrate that is more stable to heat and storage-mediated gelation. Fore warming has been accomplished over a wide range of time-temperature combinations by different manufacturers including 90°C for 10 min, 100°C for 2 min and 140°C for 25 seconds (Muir, 1984). A high temperature-short time combination is desirable because it results in better flavour and colour in the final product. However, Ellerton and Pearce noted that a harsher heat treatment with lower temperature and longer holding times resulted in slightly delayed gelation (Ellerton and Pearce, 1964). Harsher UHT sterilization conditions following fore warming had an even greater impact than fore warming conditions. Figure 2.2 demonstrates the relationship between UHT sterilization holding times and delay of gelation.
Figure 2.2: Average gelation time of 26% total solid evaporated milk as a function of holding time at 270°F (132°C) sterilizing temperature (Ellerton and Pearce, 1964d)

Figure 2.3: Influence of total solids level on gelation rate with sterilization at 260°F (127°C), $F_0= 6.0$ (Ellerton and Pearce, 1964e)
**Concentration Effects**

Ellerton and Pearce investigated the effect of concentration on age gelation of concentrated milk (Figure 2.3). They noted that as the milk was concentrated, it became more susceptible to thickening and gelation. The data shows that by increasing the total solids by just 3%, the shelf-life is decreased by 50%. The authors calculated that a 3% change in total solids represents a 15% change in concentration based on the solids-non-fat to water ratio. Thus, the rate of change in gelation is three times greater than the rate of concentration change. Clearly, concentration plays a very important role in age gelation. However, because concentrated milk is subjected to fore warming and UHT sterilization, it must undergo much harsher heat treatment than milk and therefore gels later than milk even though the protein concentration is higher. In light of these results, it is reasonable to propose that if concentrated milk were processed with the same degree of harshness as UHT milk, the concentrated milk would gel sooner (Ellerton and Pearce, 1964).

**Effect of Composition**

Various researchers have shown that seasonal differences in milk composition have an effect on the rate of gelation of concentrated milk (Ellerton and Pearce, 1964; Harwalkar et al., 1983). Ellerton and Pearce further investigated the effect of milk composition on age gelation of concentrated milk by increasing the protein content and by adding calcium chloride. In the first experiment, the serum protein level was increased by 10% through the addition of acid dialyzed whey protein to the raw milk before processing. The increase in protein level doubled the rate of gelation. The control gelled after 52 weeks while the batch with added whey protein gelled after only 24 weeks. Furthermore, doubling the holding time during sterilization was completely ineffective in delaying the impact of increased protein levels. In the second experiment calcium chloride added to the milk before processing had little or no effect on gelation. During storage at 37°C, calcium addition also had no effect. During storage at room temperature, small amounts (2-6% increase) of calcium had no effect but large amounts (10-14% increase) increased the rate of gelation. Figure 2.5 illustrates this relationship (Ellerton and Pearce, 1964).
Effect of Added Phosphates

Leviton and Pallansch studied the effect of various additives on HTST sterilized evaporated skim milk. The additives studied included polyphosphates, manganese salts, polyhydric compounds and phosphatides. As with UHT milk, when a monophosphate buffer salt was added, the gelation occurred sooner than in its absence. This effect was also observed with exogenous orthophosphate. By contrast, a number of other phosphate compounds delayed gelation. The phosphates tested in order of increasing effectiveness were: pyrophosphate < tripolyphosphate < adenosine triphosphate < polyphosphate (average of 4.8 phosphate molecules per chain) < hexametaphosphate < tetrametaphosphate. The effectiveness of polyphosphates of different chain lengths was also investigated and found that as the chain length increased, the effectiveness in delaying gelation also increased. It appears that phosphate compounds behave the same way in concentrated milk as in regular UHT milk (Leviton and Pallansch, 1962). In a subsequent study, Leviton and colleagues examined the effectiveness of polyphosphates while varying other parameters. The polyphosphates had an average chain length of 4.8 phosphorus units per chain and were added to samples in doses of 0.6 lb per 100 lb milk solids (0.6 w/w). They noted that the addition of polyphosphates enhanced the effect of a decreased storage temperature. Lowering the storage temperature from 37°C (99°F) to 21°C (70°F) nearly doubled the time to reach gelation and this effect was five times greater in samples containing polyphosphates. However, it was also observed that the addition of polyphosphates decreased the amount of dispersible sediment and the sediment was more tightly packed in polyphosphate-containing samples than in the control. Lowering the storage temperature also produced less sediment. When fore warming temperatures were increased, polyphosphates continued to effectively delay gelation but it was observed that with a higher fore warming temperature and shorter holding time, more sediment developed. A similar effect was observed when sterilization temperature was increased from high temperature-short time regimes to ultra high temperature-short time. UHT-sterilized concentrated milk samples possessed more sediment than HTST treated samples and samples with added polyphosphates had less sediment than the controls (Leviton et al., 1963).

Tables 2.2 and 2.3 summarize the factors affecting age-gelation in milk and concentrated milk.
**Temperature** | **Proteolysis** | **Additives**
---|---|---
preheat | harsher delays gelation | Not conclusive. Milk that gelled sooner had higher proteolysis but adding proteinases in controlled fashion did not accelerate gelation. | EDTA | 0.1%↑ gelation 0.3% ↓ gelation
UHT temp | harsher delays gelation | Sodium Citrate | ↑gelation
 |  | CaCl₂ | mixed reports
 |  | NEM | results unclear
 |  | Sodium Phosphate | ↑gelation
 |  | Polyphosphates | ↓ gelation (especially with chain length of 4.8 phosphates and cyclic form)
 |  | Orthophosphates | ↑gelation

*Figure 2.2: Summary of factors affecting rate of age-induced gelation in milk*

*Figure 2.4: Gelation in HTST evaporated milk as influenced by increased calcium content*
2.3. Potential Methods for Studying Aging of High Protein Beverages

In studies on the aging of milk and concentrated milk, many of the findings are reported as visual assessments and sample viscosity as a function of gelation time (Datta and Deeth, 2001). However, in studies where the mechanism of gelation is investigated other techniques become necessary. Some of the most commonly used techniques used to study the mechanism of age-gelation included electron microscopy and SDS-PAGE. In this study however, we report the use of Fourier transform infrared (FTIR) spectroscopy and surface plasmon resonance (SPR) spectroscopy. FTIR spectroscopy has already been successfully applied to study a number of milk products including milk, concentrated milk and cheeses, thus there is great potential for its use in the study of aging of high protein beverages. SPR is a relatively new and powerful technique for studying protein-protein interactions. There are very few investigations on milk products using SPR, one of the foci of this thesis. The methodology and usefulness of these techniques with respect to aging of UHT milk will be discussed below.
2.3.1. ATR - Fourier-Transform Infrared (ATR-FTIR) Spectroscopy

Infrared Spectroscopy is based on the privilege that nearly all materials absorb infrared radiation. Every molecule possessing a dipole moment will absorb specific frequencies of infrared radiation; the frequency at which the molecule absorbs coincides with the frequency at which the bonds within the molecule are vibrating. Every compound has a characteristic IR spectrum and this can be used to identify components in a food sample, to quantitate components (with calibration) or to distinguish between different samples (Wilson and Goodfellow, 1994). In addition, the band position is also sensitive to its environment; this provides added information about the structure and intra- and intermolecular bonding between the moieties within and between the molecules respectively.

Ozen and colleagues employed ATR-FTIR spectroscopy to determine the concentration of plasmin and plasminogen. Even in the presence of a mixture of casein and whey proteins, successful quantification was reported (Ozen et al., 2003). Plasmin and plasminogen are enzymes which cleave milk proteins and could contribute to age gelation of milk, thus ATR-FTIR shows great promise as a technique to investigate factors which accelerate age-gelation.

Kher and co-workers investigated the use of ATR-FTIR spectroscopy for predicting solubility of spray-dried samples of MPC. Although it was not possible to predict solubility of MPC powders after processing and storage, the changes in solubility during storage correlated well with ATR-FTIR spectra. More importantly, it was found that ATR-FTIR was useful in tracking changes in conformation of the milk protein concentrate. Second derivative spectra and PCA (principal component analysis) were the most useful spectral analysis methods in monitoring changes in composition (Kher et al., 2007).

Both ATR-FTIR and transmission FTIR spectroscopy are valuable techniques for the study of the changes in protein secondary structure caused by varying physiochemical conditions. For example, transmission FTIR has been used to study thermal denaturation of whey proteins in milk and changes in milk protein structure during drying and the stabilization of proteins by sugars during freeze-drying or spray-drying. Additionally, spectra of casein and whey hydrolysates have been shown to be useful in predicting physical parameters such as bitterness, solubility, emulsifying and foaming properties (Kher et al., 2007). Accordingly, FTIR
has considerable potential in the study of aging of high protein beverages made with milk proteins.

The main benefits of infrared spectroscopy are that it is very rapid (once calibrated), inexpensive and non-destructive. This is particularly important when a large number of samples must be analyzed. Advantages of ATR-FTIR spectroscopy over transmission FTIR include reduced sample preparation and no need for an instrument mounted sample cell. However, disadvantages include the limited path length which may affect sensitivity, as well as a small sampling area that may not give consistent results for highly non-homogeneous samples (Wilson and Goodfellow, 1994). In addition to this, Oberg and Fink reported interaction of sample components with the ATR ZnSe crystal thereby causing discrete changes in the spectrum (Oberg and Fink, 1997).

2.3.2. Surface Plasmon Resonance (SPR)

Surface plasmon resonance (SPR) is a powerful technique capable of providing real-time, label-free data on the interaction between two binding partners. It can yield valuable information on the specificity of the interaction, association and dissociation kinetics as well as binding affinity (Biacore Systems Corp., 2009). The biosensor group at Pharmacia was the first to produce a commercially successful SPR instrument in 1990, resulting in a steady increase in its popularity over the last two decades (Schasfoort and Tudos, 2008). Today, it is used in pharmaceutical drug discovery, antibody characterization, proteomics, immunogenicity, food analysis and many other life science areas (Biacore Systems Corp., 2009).

The Basic Principle Behind the Technology

SPR technology is based on the phenomenon of excitation of surface plasmons. When polarized light is directed through a prism coated with metal, at most angles of incidence, the light is reflected by the metal film with 100% of its original intensity. However, as the angle of incidence is changed and as it approaches the angle corresponding to the refractive index of the material on the surface of the metal, there is a decrease in intensity of reflected light. This effect is represented in Figure 2.5. in a plot of intensity of reflected light vs. angle of incidence. At these angles, some of the energy of the light beam is absorbed by free electrons in the metal layer to produce an evanescent wave, thus resulting in decreased intensity of the reflected light. The
angle of maximum loss of light intensity is called the SPR angle. Since the angle changes depending on the refractive index of the material on the surface of the metal, protein adsorption on the metal surface will be detected by a shift in the SPR angle, as depicted in Figure 2.6. During the course of an experiment, the SPR apparatus records changes in the SPR angle over time and plots these parameters in a graph referred to as a “sensorgram” (Figure 2.7) (Schasfoort and Tudos, 2008).

Figure 2.5: Loss of light intensity of reflected polarized light beam as angle of incidence approaches angle corresponding to refractive index of material on surface of metal layer. (adapted from: Handbook of Surface Plasmon Resonance 2008).
Figure 2.6: SPR angle shifts following changes in the amount or type of material deposited on metal surface (source: Handbook of Surface Plasmon Resonance 2008)

Figure 2.7: Sensorgram provides real-time data regarding changes in SPR angle over time (source: Handbook of Surface Plasmon Resonance 2008)
**SPR Experimental Set-Up**

In SPR instruments, the prism and metal surface system is associated with a biosensor chip. The chip often consists of a layer of glass coated with gold, and in many cases, the gold layer is carboxymethylated to facilitate the immobilization of molecules onto it. The most common type of immobilization chemistry is amine coupling. It is effective with all proteins and results in random orientation of proteins on the chip’s surface. This is especially desirable if the mechanism of interaction between “ligand” (immobilized protein) and “analyte” (protein interacting with immobilized protein) is unknown. Before commencing the assay, the biosensor chip is inserted into an appropriate holder inside the SPR instrument and the chip surface is conditioned with a buffer. Following conditioning, the carboxymethylated surface is activated and the molecules to be immobilized are injected. The molecules are dissolved in a carefully chosen buffer and at a pH which optimizes binding of the molecule to the sensor chip surface. After each injection there is a washing step to remove non-specifically bound molecules. Injection followed by washing is repeated until the desired amount of substance has been immobilized on the surface. The carboxymethyl groups are then deactivated and the assay is ready to begin. Other immobilization chemistries are also available such as thiol coupling and aldehyde coupling. These alternate immobilization chemistries can be used either to help elucidate the mechanism of binding or to improve binding of “ligand” and “analyte” by ensuring that the binding site of the ligand is always oriented correctly and available for binding (Anton van der Merwe, 2001).

As mentioned previously, SPR technology detects changes in the SPR angle and plots this as a function of time to produce a sensorgram (Figure 2.7). In practice, the changes in SPR angle are usually presented in terms of resonance units (RU) whereby 1000 RU are equivalent to a 0.1 degree shift in the SPR angle. Empirical studies have shown that adsorption of 1ng/mm² of protein onto the metal surface normally results in a response of 1000 RU (Anton van der Merwe, 2001).

Every time molecules are deposited on the sensor chip surface a resonance response in the sensorgram is observed (indicated by the orange arrow in Figure 2.7). The response allows
one to determine how much ligand has been immobilized on the sensor chip surface; the response should remain steady and represents the baseline. Next, analyte is passed over the immobilized ligand and their interactions are analyzed by recording the additional resonance response (Schasfoort and Tudos, 2008).

![Sensorgram](image.png)

**Figure 2.8:** Sensorgram showing the steps of an analysis cycle: 1, buffer is in contact with the sensor (baseline step); 2, continuous injection of sample solution (association step); $\Delta R$ indicates the measured response due to the bound target compound; 4, removal of the bound species from the surface during injection of regeneration solution (regeneration step) followed by a new analysis cycle. A bulk refractive index shift can be observed at $t_1$. (source: Handbook of Surface Plasmon Resonance 2008)

Figure 2.8. shows the typical components of a sensorgram following an assay. Part 1 is the baseline, as described above. Part 2 is the association phase. Its shape reflects the rate of binding; the steeper the curve the faster the association. Part 3 represents the dissociation phase and the steeper the downward curve, the faster the dissociation. The rate of dissociation divided by the rate of association will yield the binding affinity. Part 4 of the sensorgram depicts the regeneration step whereby acids or bases are washed over the surface to remove the analyte. Removal of the analyte, combined with the change in refractive index of the cleaning solutions as compared to the running buffer, result in a depression in the sensorgram. With proper
regeneration, the sensorgram should return to the initial baseline, whereby the ligand is left undamaged and retains the same affinity for the analyte (Anton van der Merwe, 2001).

Calculation of Binding Affinity

The binding affinity or strength of the interaction between an analyte and ligand is expressed as an association constant. The association constant of an interaction \((A + B \leftrightarrow AB)\) is defined as the ratio of product to reactant concentration at equilibrium (Equation 2.1). In SPR, there is a preference to characterize the strength of an interaction by the dissociation constant \((K_D)\), which is the inverse of the association constant and expressed as mol/L. Hence, a strong binding affinity will correlate with a low \(K_D\) value. In order to obtain the dissociation constant for a molecular interaction detected by SPR, a calibration curve must be constructed. The calibration curve is generated by running a series of serial dilutions of analyte over the immobilized surface for a given amount of time at a constant flow rate. The binding response at each concentration is obtained and plotted as a function of analyte concentration.

\[
K_A = \frac{C_{AB}}{C_A \times C_B}
\]

*Equation 2.1.:* Binding affinity is defined as the ratio of product and reactant. In the equation above \(K_A\) is the association constant, \(C_{AB}\) is the concentration of product, \(C_A\) is the concentration of reactant \(A\) and \(C_B\) is the concentration of reactant \(B\). \(K_A\) is expressed as L.mol\(^{-1}\)

In the majority of cases, the data will fit the Langmuir model. This model assumes that the soluble analyte is homogeneous and monovalent, that the immobilized analyte is homogeneous and that all binding events are independent. If all of these conditions are met, the plot of binding response vs. analyte concentration should fit the Langmuir binding isotherm described by equation 2.2. The data is fitted to equation 2.2 by least squares analysis using software such as Origin (MicroCal) or SigmaPlot in order to obtain a value for \(K_D\) and Max (Anton van der Merwe, 2001).
Equation 2.2.: Langmuir binding isotherm. Bound represents the amount of analyte bound at a given concentration expressed in resonance units (RU), \( \text{Max} \) is the maximum amount of analyte able to bind to the surface (expressed in RU), \( C^d \) is the concentration of analyte in mol/L and \( K_D \) is the dissociation constant in mol/L.

**SPR use in Studying Milk Protein Interactions**

A number of studies have been published using SPR to quantify the different milk proteins and to investigate conformations of milk proteins (Venien et al., 2000; Muller-Renaud et al., 2005; Hohensinner et al., 2007; Indyk, 2009). There was, however, only one study which investigated milk protein-milk protein interactions. Marchesseau and co-workers used SPR to quantify casein subunit interactions under conditions of varying pH, ionic strength, temperature and levels of phosphorylation. Each of the casein subunits was immobilized separately on a CM5 sensor chip using amine coupling. The authors noted that at neutral pH, an increase in ionic strength lead to stronger interaction between the caseins thereby demonstrating the importance of electrostatic repulsive forces in preventing casein interaction. Following acidification, increase in ionic strength or dephosphorylation (all of which reduced charge repulsion), casein interactions became stronger. Addition of calcium increased binding response between caseins with the most phosphorylation. Table 2.4 shows the binding affinities of the solubilized casein subunits interacting with immobilized caseins in 1-mM HEPES, pH 7.4, and 150 mM NaCl (Marchesseau et al., 2002).

<table>
<thead>
<tr>
<th>Immobilized casein</th>
<th>Soluble casein</th>
<th>( K_D )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha_s )</td>
<td>( \alpha_s )</td>
<td>5.00±0.14</td>
</tr>
<tr>
<td></td>
<td>( \beta )</td>
<td>6.36±0.22</td>
</tr>
<tr>
<td>( \beta )</td>
<td>( \alpha_s )</td>
<td>2.33±0.09</td>
</tr>
<tr>
<td></td>
<td>( \beta )</td>
<td>5.74±0.16</td>
</tr>
<tr>
<td>( \kappa )</td>
<td>( \alpha_s )</td>
<td>1.12±0.03</td>
</tr>
<tr>
<td></td>
<td>( \beta )</td>
<td>2.12±0.04</td>
</tr>
</tbody>
</table>

*Table 2.4: Kinetic data obtained by the sensorgrams of soluble \( \alpha_s \) - and \( \beta \)-caseins to immobilized \( \alpha_s \), \( \beta \)- and \( \kappa \)-caseins (adapted from: Marchesseau et al. 2002)*
2.4. Molecular Chaperones and Their Relevance to Stability of High Protein Beverages

In 1999, Bhattacharyya and Das were the first to report chaperone-like activity of one of the casein subunits, \( \alpha_s \)-casein (Bhattacharyya and Das, 1999). Recently, Yong and Foegeding (Yong and Foegeding, 2008) have proposed that molecular chaperones could be used as additives in high protein beverages to stabilize whey proteins during processing and storage. The following section will provide some background on molecular chaperones with a focus on milk proteins.

Proteins are often large and very complex molecules whose proper folding is essential to optimal functioning. Because of their complexity, proper folding is unlikely to occur spontaneously and many irregularities exist. In the late 1980’s, protein chemists came to realize that a certain class of proteins existed whose purpose was to ensure proper folding of other proteins. Members of this class of proteins are referred to as molecular chaperones. The discovery of molecular chaperones lead to a significant paradigm shift in the area of protein folding. Previously it was believed that protein folding occurred by spontaneous self-assembly, it is now known that protein folding occurs by means of assisted self-assembly (Berdanier, 2000).

Molecular chaperones are simply defined as a group of proteins that assist in the assembly or disassembly of macromolecules through non-covalent interactions but are not permanent components of these macromolecules during their normal biological functions. The term “non-covalent” in this definition excludes proteins that catalyze co- or post-translational covalent modifications. It is possible however, for a protein to be both a covalent-modification enzyme and a molecular chaperone. An example of this is protein disulphide isomerase whose two activities are carried out at different sites on the enzyme. The term molecular chaperone should be distinguished from the term “chemical chaperone” which refers specifically to small molecules such as glycerol, dimethylsulfoxide and tri-methylamine N-oxide that act as protein stabilising agents (Henderson and Pockley, 2005).

Molecular chaperones mediate protein assembly through processes such as the folding of proteins during their synthesis and after their release from ribosomes, re-folding of proteins that are partially denatured by environmental stresses, the unfolding and re-folding of proteins during their passage across membranes, and the association of polypeptides with each other and with
other macromolecules to form oligomeric complexes. As mentioned previously, some chaperones are also involved in disassembly of proteins such as in partial unfolding and dissociation of subunits and degradation of proteins which are partially denatured. Although it is beyond the scope of this literature review, it is of interest to note that emerging research suggests that some molecular chaperones even work in cooperation with each other in defined reaction sequences to achieve proper folding of a particular protein (Henderson and Pockley, 2005).

There continues to be an increasing number of distinct molecular chaperone families being identified in all types of cells and in most intracellular compartments (Henderson and Pockley, 2005). In 1999, Bhattacharyya and Das were the first to report chaperone-like activity of one of the casein subunits, \( \alpha_s \)-casein (Bhattacharyya and Das, 1999). Today it is known that \( \alpha_s \)-casein, \( \beta \)-casein and \( \kappa \)-casein all exhibit some chaperone-like activities (Zhang et al., 2005; Morgan et al., 2005; Yong and Foegeding, 2008).

Studies by Bhattacharyya et al., Zhang et al. and Morgan et al. as well as others have demonstrated that under certain conditions \( \alpha \)-, \( \beta \)- and \( \kappa \)- caseins can prevent aggregation of various non-dairy proteins (including catalase, lysozyme and insulin) and that \( \alpha \)- and \( \beta \)-casein can re-solubilise partially aggregated proteins. Only a handful of studies examine chaperone-like activity of bovine caseins on other milk proteins. These studies will be outlined below.

Morgan et al. investigated the chaperone-like abilities of \( \alpha_s \)-casein, \( \beta \)-casein and \( \kappa \)-casein by observing light scattering of substrate proteins at 360nm during heat or solvent denaturation in the presence of these casein. During heating of \( \beta \)-lactoglobulin at 70°C for 480 minutes aggregation was reduced by 70% in the presence of \( \alpha \)-casein at a ratio of 1:1. However, as pH increased the effectiveness of \( \alpha \)-casein decreased. When apo and holo \( \alpha \)-lactalbumin were heated at 25 and 35°C for 360 minutes in the presence of \( \alpha \)-casein, aggregation was also successfully reduced and at a ratio of 1:4 of \( \alpha \)-lactalbumin to \( \alpha \)-casein, aggregation was completely inhibited (Morgan et al., 2005).

Bhattacharyya noted that the addition of \( \alpha_s \)-casein to a whey protein isolate (WPI) solution helped to prevent heat-induced aggregation at 70°C as observed by light scattering at 400nm. The authors explained that two structural features of \( \alpha_s \)-casein can be used to explain its’ ability to prevent aggregation in whey proteins: its highly hydrophobic nature and its lack of
sulphur containing amino acids. As whey proteins begin to unfold, they expose hydrophobic groups and undergo thiol-disulfide exchange. The hydrophobic nature of αs-casein quickly attracts it to whey proteins as they begin to unfold and expose hydrophobic groups. As well, the lack of sulphur containing groups on αs-casein does not allow for any thiol-disulfide exchange with whey proteins and instead αs-casein acts as a spacer between the whey proteins thereby preventing whey-whey thiol-disulfide exchange. The hydrophobicity of αs-casein is useful in explaining the fact that αs-casein can not only prevent hydrophobic aggregation of proteins, but it can also re-solubilise proteins which are already hydrophobically aggregated (Bhattacharyya and Das, 1999).

O’Kennedy et al. studied the ability of a mixture of αs1-casein and β-casein to suppress WPI aggregation under varying conditions of pH and ionic strength. Protein denaturation was determined by adding acetic acid/sodium acetate buffer to the sample after the heating/cooling step followed by centrifugation and measuring the absorbance of the supernatant at 280nm. It was noted that the αs1-casein and β-casein mixture was able to suppress heat-induced aggregation of WPI (85°C, 10min, pH 6.0) by >99% when the effective molar ratio of WPI: αs1/β-casein mixture was 1:0.15. As pH was decreased from 6.0 to 5.6, although they retained some chaperone-like ability, the αs1/β-casein proteins became less effective in protecting β-lactoglobulin (O’Kennedy and Mounsey, 2006).

Yong et al. explored whether or not chaperone-like abilities of αs- and β-casein on β-lactoglobulin held up at higher protein concentrations and harsher heat treatments, to mimic the conditions found in high protein beverages in the food industry. Protein aggregation was determined by solution turbidity as the optical density at 400 or 600nm. The authors determined that β-casein is better at preventing aggregation than αs-casein when β-lactoglobulin is used a substrate and it is feasible that β-casein may be used to help stabilize β-lactoglobulin in high protein beverages. Based on their findings, the authors state that chaperone-like ability of caseins can make them useful in high protein beverages by maintaining clarity in the beverages following heat processing and to prevent gelation of β-lactoglobulin when it is used in high concentrations (Yong and Foegeding, 2008).

All of these studies have demonstrated that α- and β-caseins offer β-lactoglobulin some protection against aggregation. However, it has not been clarified whether the reported
interaction begins while β-lactoglobulin is in the native state or whether, as in the case of true chaperones, α- and β-caseins may only interact with β-lactoglobulin once it has been partially damaged. This remains an important question which will be further discussed in this thesis.

2.5. Literature Review Summary and Thesis Objectives

High protein beverages have become especially popular as low-calorie beverages which can provide athletes with the extra protein they need or dieters with the low calories they’re seeking. The on-the-go lifestyle of many consumers has created a demand for products which do not require any preparation and are ready to drink. This demand was met with ultra high temperature processing of high protein beverages. Unfortunately, these milk-based high protein beverages are not immune to the problems encountered when milk proteins are UHT heated and these high protein beverages therefore also suffer from aging defects. Aging of UHT/HTST milk and concentrated milk was discussed as this is the most closely related to the changes which occur in high protein beverages during storage. Over the years, many studies have been carried out to elucidate the mechanism and causes of milk age gelation with the aid of electron microscopy and SDS-PAGE techniques. Although a number of theories exist, to date no mechanism has been developed which can account for the behaviour of these milk protein-based beverages under different conditions. It is, however, generally accepted that the mechanism involves a two-step process during which β-lactoglobulin interacts with κ-casein on casein micelles (through disulfide linkage) followed by dissociation of the βκ-complexes from the casein micelle. Over time, the free βκ-complexes interact with other proteins to form a gel network. A number of factors that affect aging of milk and concentrated milk have been identified. Some of the factors which influence the onset of gelation and sedimentation are milk composition (seasonal factors, total solids, fat content), processing conditions (sequence of processing steps, temperature and time of heat treatments) and additives (polyphosphates, orthophosphates). Some approaches in the delay (but not total inhibition) of age-gelation have also been identified. Most of these findings are now being employed in industry in the production of UHT milk and concentrated milk to extend the shelf-life of these products to 6-12 months.

Many of these same principles have been employed in industry during the production of UHT sterilized high protein beverages made with milk proteins. However, because of the large
differences in composition of milk or concentrated milk as compared to high protein beverages, there is a large potential for high protein beverages to have very different aging behaviour. Since there is no research available on aging of UHT sterilized high protein beverages, this will be investigated during the course of our research.

Because the protein composition of high protein beverages is highly variable it important to first gain a better understanding of the impact of different protein ingredients on storage defects. We have proposed ATR-FTIR spectroscopy as a method for studying high protein beverages and we will attempt to use this technique to relate visible changes in beverages containing different protein ingredients to changes at the molecular level during storage.

Furthermore, we will correlate SPR to FTIR spectroscopic data in order to investigate a specific interaction between milk proteins; the chaperone-like action of caseins on β-lactoglobulin. Using these spectroscopic techniques we will track secondary and tertiary changes in β-lactoglobulin as it interacts with caseins during heating. This will provide insight into the mechanism behind the interaction and hint at its role in storage stability of high protein beverages. Thus, one more piece of the puzzle will be added en route to understanding and controlling storage defects in UHT-processed high protein beverages.
Chapter 3

Critical Assessment of Sampling Techniques Employed for the Study of Milk Proteins by ATR-FTIR

3.1. Introduction

Over the past two decades, transmission-based FTIR spectroscopic techniques\(^1\) have been employed to study dairy products (van de Voort and Ismail, 1991; van de Voort \textit{et al.}, 1992; Sivakesava and Irudayaraj, 2002; Etzion \textit{et al.}, 2004; Upreti and Metzger, 2006; Kaylegian \textit{et al.}, 2007; Koca \textit{et al.}, 2007). The strong absorption of water in the infrared spectral region necessitates the use of very short path lengths (6 – 12 um) in order to examine the amide I band of the proteins. Furthermore, the relatively high viscosity of dairy products can cause the IR beam to be reflected instead of transmitted upon contact with the sample, thus requiring the use of a high pressure homogenizer to deliver the product (van de Voort \textit{et al.}, 1992). Attenuated total reflectance (ATR) spectroscopy (Harrick, 1967) can overcome these limitations and is an increasing attractive technique to study water-based and/or highly viscous products (Nathier-Dufour \textit{et al.}, 1995; Kemsley \textit{et al.}, 1996). Figure 3.1 illustrates the theory behind this technique. Because of the short penetration depth of the IR beam into the sample, when there is water present in a sample, the water absorption band is not nearly as intense as in the case of transmission FTIR spectroscopy thus generating a spectrum with meaningful results. Also unlike with transmission FTIR spectroscopy, high viscosity samples are not a problem in ATR spectroscopy because the IR beam does not need to be transmitted through the sample. Finally, recent advances in ATR spectroscopic instrumentation have greatly improved the repeatability of single bounce (SB)-ATR accessories thus making ATR-FTIR spectroscopy an even more valuable tool in the arsenal of spectroscopic-based analytical techniques (Mossoba \textit{et al.}, 2001; Cocciardi \textit{et al.}, 2004).

\(^1\) Abbreviations used: FTIR, Fourier-transform infrared; ATR, attenuated total reflectance; UHT, ultra-high temperature
Equation 3.1: The depth of penetration is calculated as above where \( \lambda \), is the wavelength of the radiation; \( n_1 \), is the refractive index of the Internal Reflection Element (IRE) material; \( n_2 \), is the refractive index of the medium surrounding the IRE; and \( \theta \), is the angle at which the incident light strikes the interface (Sedman et al., 1997).

![Figure 3.1: Schematic representation of ATR configuration; A focussed IR beam passes into an optically dense crystal. When the beam reaches the interface between the sample and crystal, it is internally reflected once or several times, depending on the mode of ATR spectroscopy used. At every reflection at the interface an evanescent wave is generated which penetrates \(~0.5–5\) microns into the sample. The evanescent waves attenuates the IR beam such that when it reaches the detector, it can be interpreted into an FTIR spectrum specific to that sample (Harrick, 1967a).]
The study of protein structure by ATR-FTIR spectroscopy has been extensively investigated in recent years. However, there are a number of limitations that one must contend with even with the use of the new ATR design. These include:

1. Protein adsorption (surface fouling) on the surface of the ATR crystal which could hamper the quantitative performance
2. Surface-induced protein denaturation
3. Very weak signal necessitating the use of high sensitivity detectors (e.g., liquid-nitrogen cooled detectors).

To overcome the latter, the absorption intensity of the sample must be increased to an extent which makes any disturbances from these limitations negligible. We propose to experiment with three different sampling techniques combined with spectral enhancement in order to overcome these limitations. The experiments will be conducted using milk proteins in aqueous solution to optimise a technique for the study of such samples using ATR spectroscopy. Following optimisation of a technique, thermodynamic experiments will be performed to assess the ability of ATR-FTIR spectroscopy to provide numerical information.

PART I

3.2. Development of a Sampling Technique

As mentioned in the introduction of this chapter, it is essential to have high absorption intensity of the sample in comparison to the spectral contributions of artefacts generated due to the limitations of ATR spectroscopy. Additionally, the water absorption band must be removed from the spectrum to reveal the amide I band, which contains a great deal of information about protein samples. To this end, we have selected three sampling techniques to experiment with: dry powder analysis, thin-film analysis and aqueous analysis followed by water subtraction.
Materials and Methods

ATR Instrumentation

The single-bounce diamond-ATR accessory (DuraScope, SensIR Technologies) was coupled to an FTIR (FTS 3000MX Excalibur, Varian Inc. Boston). The infrared spectrum of the background was recorded of the clean ATR crystal using 128 co-added scans at a resolution of 4 cm⁻¹.

Sample Preparation

Samples were prepared containing a mixture of 5% caseinate and between 0.33-1.5% β-lactoglobulin. The mixtures were pre-heated for 30 s at 90°C followed by homogenization using a Niro-Soavi S.p.A. homogenizer (100 Bar at first stage and 40 Bar at second stage) and ultra-high temperature processing at 140°C for approximately 6.7 seconds in a MicroThermics UHT/HTST Lab Hybrid system. The samples were bottled in 100 mL aliquots under aseptic conditions and stored at 37 °C. An FTIR-ATR spectrum of the protein solutions was recorded on a weekly basis for 6 weeks. When the samples aged and sediment formed, the supernatant was carefully decanted and the sediment and supernatant were analyzed separately.

Dry Powder Analysis

Aliquots of 15 mL were freeze dried. The dry flakes were crushed and ground using a mortar and pestle and a small amount of each sample was pressed with a pressure clamp onto the ATR crystal for analysis.

Thin-Film Analysis

A 5 uL drop of the UHT-treated protein mixture was placed onto the ATR crystal (2 mm diameter) and dried using dry air as shown in figure 3.2. As the sample dried, the spectrum was acquired in real-time in “setup mode”. When the majority of the free water was dried and no more dramatic changes were observed in the spectrum (after approximately 4 minutes of drying), an infrared spectrum of the dried sample was recorded.
To observe the effect of various degrees of hydration on the sample spectrum, a 5 μL drop of sample was placed on the crystal and dried as before. Scans were then performed every 45 s for approximately 20 min under a stream of dry air.

![Diagram of set-up used for drying film of milk protein solution](image)

**Figure 3.2: Set-up used for drying film of milk protein solution**

**Water Subtraction**

A 100 μL aliquot of the protein solution was placed on the crystal. The drop was large enough to flood the crystal and ensure that the sample would not evaporate during scanning. Following data collection, the water spectrum was subtracted from the sample spectrum using a subtraction factor which resulted in an amide I: amide II ratio of 1.4:1.

To verify if there was any sedimentation or protein-crystal interaction during scanning a 100 μL sample of the protein mixture was placed on the crystal and a background spectrum was scanned. Immediately after, scanning of the same sample was done every 45 s for 20 min.
Data Processing

Spectra obtained from all three sampling techniques were normalized to eliminate differences in spectral intensity due to differences in sample thickness. Normalization was achieved by dividing the spectrum by the area between 1800 and 1000 cm\(^{-1}\). Spectral transformations such as second derivative, smoothing and Fourier-self deconvolution were carried out using Omnic 7.3 (Thermo Electron Corp.) Hierarchal cluster analysis was performed using SpectrAnalysis 2.7.6. (Thermal Lube Inc.).

Results and Discussion

Freeze-Dried Sample Analysis

The spectra of the freeze-dried samples of 5% caseinate and 0.75% β-lactoglobulin showed some variation in intensity. Figure 3.3 shows the normalized spectra of six replicates of the freeze-dried protein sample prepared from the same batch of protein. The differences in height of the amide I band between these replicates were within 7%. The variations in peak centre were minimal. The amide I peak centre varied between 1635.7 and 1634.4 cm\(^{-1}\). It is interesting to note however that there is a slight change in the shape of the amide II peak.

As the samples freeze overnight (prior to freeze drying), it is likely that certain protein components may “salt out”, highlighting the importance of proper grinding and mixing of the samples in order to obtain a homogeneous and representative sample before analysis. Unfortunately, freeze-drying of the protein mixture resulted in a fibrous material which was very difficult to grind into small particulates for effective mixing. In addition, because the sample consisted of very light fibers, it became difficult to handle without losing some sample. For the same reason, it was very difficult to cover the ATR crystal evenly. This is the most likely cause of the variations in peak intensity. It is also possible that the sample material did not have equal protein concentrations throughout and due to the texture of the dried sample, it was not possible to mix thoroughly enough to achieve homogeneity.
Figure 3.3: ATR-FTIR analysis of six replicates of freeze-dried samples of 5% casein + 0.75% β-lactoglobulin. Differences in peak height are within 7% of the highest peak.

Figure 3.4: Effect of increasing pressure on spectrum of freeze-dried samples analyzed by ATR-FTIR.
Figure 3.4 shows the effect of tightening the pressure clamp on the intensity of the protein spectrum. Accordingly, care must be exercised when recording IR spectra of powders.

Protein Film Analysis

The thin films of UHT-treated caseinate-β-lactoglobulin mixtures produced spectra which were more reproducible than the spectra of freeze-dried samples. The differences in intensity of normalized spectra were quite small with variations within 5% of the height of the highest peak (see Figure 3.5). Shifts in the peak center of the amide I band remained small between the replicates (within range of +/− 1 cm⁻¹).

Michael Jackson and co-workers reported spectral differences in thin-films of concanavalin when the degree of hydration was varied (Jackson and Mantsch, 1992). In our study, this effect was particularly noticeable in the region between 1520-1510 cm⁻¹, as illustrated in Figure 3.6. In particular as moisture was removed from the sample, the intensity of the peak at 1515 cm⁻¹ increased. This peak was previously assigned to the side chain absorption of tyrosine in a protein spectrum (Kidric et al., 2002). As such one would need to consider the impact of the variability of the extent of hydration on the structure of the protein under investigation.

Alternatively, one could analyze samples which have the same degree of hydration; however this would be very difficult without the use of a humidity chamber (Chan and Kazarian, 2007). Since the use of a humidity chamber is not commonly employed in industry and in most other research studies, this technique would not be representative of the normal procedure used for analyzing protein films.

Another problem with film drying is that there are sometimes variations in the degree of contact between the film and the crystal. During drying, the film sometimes arched very slightly and lost contact with the crystal. There was also the additional problem whereby in rare instances small pieces of the film flaked off and left the crystal partially uncovered. This necessitated close watch over the sample before scanning.
Figure 3.5: Five replicates of film-dried spectra analyzed by ATR-FTIR. Differences in peak height are within 5% of the highest peak.

Figure 3.6: Effect of drying time (degree of hydration) on the spectrum of a thin-film sample of 5% casein + 0.75% β-lactoglobulin
**Water Subtraction Method**

This method was found to be the best of the three sampling techniques tested. The variations in intensity were within 2% of the intensity of the highest peak (Figure 3.7). There were no apparent shifts in the amide I region. In addition, this sampling technique was the least time-consuming as it required no sample preparation at all. The disadvantage of this method is the relatively low signal-to-noise ratio (SNR) which limits processing of data with tools such as derivatives and self-deconvolution, making analysis of the amide I band for elucidation of changes in secondary structure of the protein problematic (Figures 3.13 a and b). It should be noted that increasing the SNR by increasing the number of scans would be problematic since it adds to the risk of fouling the ATR crystal. Some researchers pre-coated the ATR crystal with an antifouling film, but this would not be easily accomplished on a routine basis (Tornkvist et al., 1991).
Schmidt and co-workers used ATR-FTIR spectroscopy to analyze various polymers in aqueous media. The authors noted that some polymers sedimented out of solution and onto the ATR crystal such that the local concentration of the polymers was up to seven times higher on

Figure 3.7: Three replicates of a sample of the 5% casein and 0.75% β-lactoglobulin mixture using the water subtraction method.

Figure 3.8: **bottom set of spectra**: spectra 1.5, 4.5, 7.5 and 10 min after deposition on crystal using sample spectrum as background; **top spectrum**: normal spectrum of 5.75% protein

Schmidt and co-workers used ATR-FTIR spectroscopy to analyze various polymers in aqueous media. The authors noted that some polymers sedimented out of solution and onto the ATR crystal such that the local concentration of the polymers was up to seven times higher on
the crystal surface than in the rest of the solution (Schmidt et al., 2009). This result made it necessary for us to determine if such an event was affecting our spectra. A sample was applied to the clean crystal and a background spectrum was scanned. Next, without removing the sample from the ATR crystal and using the first sample spectrum as a background, the ATR crystal surface was scanned by FTIR spectroscopy approximately every 45 s for 10 min. Figure 3.8 shows a comparison of the intensity obtained when the infrared spectrum of a solution is recorded and compared to a background scan of the solution immediately upon deposition on to the ATR surface. A normal spectral acquisition time of 128 co-added scans requires approximately 1.5 min (at 4 cm\(^{-1}\) resolution). Hence, it is clear from figure 3.8 that the effects of protein-crystal interaction or protein sedimentation during this time frame are negligible. However, 10 min after deposition of the sample, enough protein settles on the surface of the crystal that the effects of protein-crystal interaction contribute almost 9% of the intensity of the normal sample spectrum. Figure 3.9 illustrates the correlation between the time passed since the sample was deposited and the percent contribution of the protein-crystal interaction to peak height.

![Figure 3.9: Percent contribution of protein-crystal interaction over time to intensity of amide I band](image)

**Figure 3.9:** Percent contribution of protein-crystal interaction over time to intensity of amide I band
Summary

Following investigation of three sampling techniques for the study of protein beverages using ATR-FTIR spectroscopy it was established that analysis of proteins in solution followed by water subtraction was the most reliable of the three techniques examined. Although analysis of freeze-dried powders was found to produce minimal peak shifts between replicates, significant variations in intensity were observed and sample handling was quite difficult. Analysis of thin films resulted in rather large variations in intensity and side chain absorption bands were found to be sensitive to the level of film hydration. However, peak shifts were minimal. Milk proteins scanned in solution followed by water subtraction produced the most consistent spectra with virtually no peak shifts or changes in the shape of the bands in the spectrum. There was still a small degree of variation in intensity between replicates but this is most likely attributable to the SNR of the spectra.

PART II

3.3. Data mining of ATR-FTIR spectra of milk proteins

In the above section we have determined that the solution-based spectral acquisition approach provided the most consistent spectral information but at the cost of a substantial reduction in protein peak intensity. Pre-concentrating the sample is more likely to increase the rate of surface fouling. Accordingly, resolution enhancement in combination with accepted spectral analysis techniques were assessed for extracting more information from the spectra.

We have considered three approaches towards this end:

1. Resolution enhancement methods
   a. Derivatization followed by spectral smoothing
   b. Fourier self-deconvolution (FSD) (with varying deconvolution factors)

2. Hierarchical cluster analysis (HCA)

In the following sections, the results of the application of resolution enhancement and data-mining techniques to our samples will be discussed.
Methods

A mixture of 5% caseinate and 1.5% β-lactoglobulin was subjected to UHT processing and storage for a six week period. Samples were analyzed on a weekly basis using ATR-FTIR spectroscopy and the data subjected to water subtraction and normalization (Figure 3.10). The spectral enhancement techniques listed above were applied to the data collected and the usefulness of the various techniques was assessed. As spectral manipulations can result in increased noise and/or reduction in information content, the original data was always analyzed. The band at 1615 cm⁻¹ was employed as a diagnostic indicator of the effect of resolution enhancement techniques. This band represents protein aggregation and should be present whenever β-lactoglobulin is heated (such as in the case of UHT treatment). The amide I band (centered around 1634 cm⁻¹) the replicate spectra also served as an additional indicator of spectral enhancement.

Figure 3.10: Overlaid spectra of a mixture of 5% Casein and 1.5% β-lactoglobulin over a six week period analyzed by ATR-FTIR
Results

Resolution Enhancement Methods

Figure 3.11 shows a comparison of the spectrum before resolution enhancement and two spectra following Fourier self-deconvolution (FSD) using two different bandwidths and one defined smoothing factor (k). When using FSD analysis, an appropriate combination of bandwidth and smoothing factor must be chosen carefully. In figure 3.11 a smoothing factor of 1.9 and bandwidths of 41 and 19 were used as representative examples. In figure 3.11, the spectrum which has been Fourier self-deconvolved using a bandwidth of 41 and a k value of 1.9 is less noisy than the spectrum generated with a bandwidth of 19 and a k value of 1.9. However, using hierarchal cluster analysis (discussed later), we deduced that the former spectrum had in fact been over-processed and important information had been lost.

Figure 3.12, shows the second derivative spectrum of UHT processed 5% caseinate with 1.5% β-lactoglobulin and the second derivative of the same spectrum after smoothing. A smoothing factor of 9 points was selected. This signifies that the smoothed spectrum is composed from one out of every nine points of the original spectrum. Both spectra were multiplied by -1 so that they can be view with the same orientation as an unprocessed spectrum. From this figure, it is apparent that the effect of second derivatives is to enhance the peaks of the
original spectrum. Peaks resulting from noise, however, are also enhanced resulting in a more noisy spectrum. Smoothing can be applied to eliminate some of this noise.

Because the low SNR of the acquired spectra is even further reduced following derivatization, it becomes difficult to ascertain which enhanced peaks are genuine and which are artefacts. Thus, it is wise to compare spectra which have been enhanced using different techniques in order to assign peaks with confidence.

Figure 3.12: 2nd derivative spectrum of UHT processed 5% casein + 1.5% β-lactoglobulin multiplied by -1 and the same spectrum following 9 point smoothing

Comparison of the smoothed second derivative spectrum with the Fourier Self-deconvolved spectrum (Figure 3.13) shows that although the intensity of the peaks is changed by spectral processing, both techniques result in enhancement of almost all of the same peaks (note the similarity in position of the 1634cm\(^{-1}\) and 1618cm\(^{-1}\) peaks/shoulders). This indicates that these peaks are genuine and not artefacts arising from spectral processing. By the same token, those peaks which are present in the second derivative spectrum but not the FSD spectrum may or may not be bonafied. Much like in FSD, choosing the correct smoothing factor after derivatization is essential for balancing noise reduction with peak enhancement. Overall, these
spectral enhancement techniques provided some resolution of the peaks but due to the low signal to noise as compared to transmission FTIR, identification of secondary structures is limited.

*Hierarchal cluster analysis*

Hierarchal cluster analysis (HCA) is a software which compares peak locations and intensities in a set of spectra and classes the spectra into subsets according to how closely related they are (see Figures 3.13, 3.14 and 3.15 for examples). An example of the usefulness of this technique was presented in the previous section, whereby it was used to verify if important data was lost following FSD. This was accomplished by applying FSD to ATR-FTIR spectra (spectral range of 1800-1500cm⁻¹) of a milk protein mixture analysed in triplicate on a weekly basis for six weeks. Two sets of spectra were generated by applying FSD to each spectrum using two different bandwidths. Next, each set was entered into HCA to produce a dendogram. The software was capable of correctly placing the spectra into subsets according to week of storage with one set of spectra (Figure 3.14) but not the other (Figure 3.15). This indicated that the set not clustered into subsets correctly had lost important spectral information following FSD due to an improper selection of FSD factors.

When studying storage stability of proteins, hierarchal cluster analysis may also be used to suggest a trend in structural changes. For example, from the dendograms in figures 3.13 and 3.15, one could speculate that because week 4 is always placed in a completely separate subset from the spectra of other weeks, some pivotal change takes place in the proteins during week 4 of aging. The spectrum of the proteins on week 4 are neither similar to the spectra of the earlier weeks, nor to the spectra of the later weeks.
Figure 3.13: Dendograms of ATR spectra of UHT processed 5% casein + 1.5% β-lactoglobulin following (a) normalization only, (b) normalization and 1st derivative, (c) normalization and 2nd derivative
Figure 3.14: Dendograms of ATR spectra of UHT processed 5% casein + 1.5% β-lactoglobulin following Fourier self-deconvolution using bandwidth 41 and k 1.9 (a) normalization and FSD only, (b) normalization, FSD and 1st derivative, (c) normalization, FSD and 2nd derivative
Figure 3.15: Densograms of ATR spectra of UHT processed 5% casein and 1.5% β-lactoglobulin following Fourier self-deconvolution using bandwidth 19 and k 1.9 (a) normalization and FSD only, (b) normalization, FSD and 1st derivative, (c) normalization, FSD and 2nd derivative
Summary

Various spectral enhancement techniques were presented which could be used to extract data from FTIR spectroscopic spectra, particularly when the signal to noise is low, as is the case in ATR-FTIR spectroscopy.

Fourier self-deconvolution and derivatization combined with spectral smoothing can enhance peak resolution and offer similar final results. In both cases, a balance must be maintained in order to achieve an aesthetic spectrum while retaining all of the data content. To ensure that data has not been eliminated and that genuine peaks are enhanced, the results from both methods can be compared as well as the findings from HCA. The latter technique can also be used to identify a pattern in a series of spectra.

PART III

3.4 Extraction of Quantitative Data: Thermodynamic Calculations

In the previous sections we have shown the potential of ATR spectroscopy in providing qualitative data about milk proteins. Because of the poorer signal to noise of ATR spectroscopy, however, few consider it to be a reliable method for extracting quantitative data. Having developed a repeatable sampling method for the study of aqueous proteins, we will examine the usefulness of the resulting spectra for generating numerical information about the proteins.

Methods

Solutions of 5% β-lactoglobulin in H₂O and 5% β-lactoglobulin in D₂O were heated at 76, 82 and 85°C and the absorbance of the aggregation band at 1615 cm⁻¹ was recorded for the ATR and transmission FTIR spectra. Ln(A) was plotted against the reciprocal of time to obtain the rate constant (k) at 76, 82 and 85°C (see figure 3.16 for a representative plot). Next, an Arrhenius plot was generated by plotting ln(k) against 1/Temp (Figure 3.17) and the slope of the resulting line was then used to determine the energy of activation of β-lactoglobulin aggregation (see Equation 2). The energy of activation could then be used to determine enthalpy (Equation 3). An Eyring plot was generated by plotting ln(k/T) against 1/Temp (Figure 3.18). The intercept of this plot was used to calculate entropy (see Equation 4). Finally, using the calculated change in enthalpy and entropy, the change in Gibbs free energy (ΔG) for the β-lactoglobulin
aggregation reaction was determined (see Equation 5). This procedure was repeated to determine \( \Delta G \) for 5% \( \beta \)-lactoglobulin in D\(_2\)O analyzed by transmission FTIR and ATR-FTIR spectroscopy as well as for 5% \( \beta \)-lactoglobulin in H\(_2\)O analyzed by ATR-FTIR spectroscopy.

\[
k = A e^{-\frac{Ea}{RT}}
\]

*Equation 3.2: Arrhenius equation: \( k \), is the rate constant; \( A \), is the absorbance; \( Ea \), is the energy of activation; \( R \), is the universal gas constant; and \( T \), is the temperature (Blumenfeld, 1976)*

\[
\Delta H = Ea - RT
\]

*Equation 3.3: Entropy is calculated above where \( H \) is the enthalpy; \( Ea \), is the energy of activation; \( R \), is the universal gas constant; and \( T \), is the temperature (Lakshminarayanan et al., 2007a)*

Figure 3.16: 5% \( \beta \)-lactoglobulin in D\(_2\)O heated at 76, 82 and 85°C for 0, 5, 10, 20, 30 and 60 min analyzed in triplicate by transmission FTIR. Error bars indicate standard deviation.
Figure 3.17: Arrhenius plot for 5% β-lactoglobulin in D$_2$O heated at 76, 82 and 85°C for 0, 5, 10, 20, 30 and 60 min analyzed in triplicate by transmission FTIR. Error bars represent standard deviation.

Figure 3.18: Eyring plot for 5% β-lactoglobulin in D$_2$O heated at 76, 82 and 85°C for 0, 5, 10, 20, 30 and 60 min analyzed in triplicate by transmission FTIR. Error bars represent standard deviation.
Results and Discussion

Values of $\Delta G$ for the aggregation reaction of 5% $\beta$-lactoglobulin in D$_2$O analyzed by transmission FTIR and ATR-FTIR spectroscopy as well as for 5% $\beta$-lactoglobulin in H$_2$O analyzed by ATR-FTIR spectroscopy can be found in Table 3.1. Since transmission FTIR analysis is an accepted means of making quantitative inferences about proteins, we assumed that the numbers obtained from this type of analysis were correct numbers. Accordingly, calculations for change in Gibbs free energy from ATR-FTIR spectroscopy resulted in an error of 4.83% if D$_2$O is used as the solvent and an error of 2.62% if H$_2$O is used as the solvent when compared to the transmission FTIR data. The correlation coefficient for $\beta$-lactoglobulin analyzed by transmission FTIR in D$_2$O, by ATR-FTIR in D$_2$O and by ATR-FTIR in H$_2$O was 1, 0.90 and 0.83, respectively. Thus, although the values for change in Gibbs free energy are comparable, there is an inherent larger error associated with analysis of proteins in aqueous solution using ATR-FTIR spectroscopy due to the lower SNR. Galani and co-workers determined $\Delta G$ for heat-induced denaturation of $\beta$-lactoglobulin (4 mg/mL, pH 2) using UV spectrophotometric measurements and they determined that the $\Delta G$ value was 51kJ/mol at 82°C (Galani and Owusu Apenten, 2000). Although our values for $\Delta G$ were closer to 70 kJ, the difference is not so unreasonable considering that our protein is far more concentrated (50 mg/mL) and it is at

\[ \ln \left( \frac{\alpha}{1-\alpha} \right) = \frac{\Delta H}{RT} + \ln \left( \frac{k'}{h} \right) + \frac{\Delta S}{R} \]

\[ \Delta G = \Delta H - T\Delta S \]

Equation 3.4: Entropy is calculated above where $k$ is the rate constant; $T$, is the temperature; $H$, is enthalpy; $R$, is the universal gas constant; $k'$, is the Boltzmann constant; $h$, is Planck’s constant; and $S$, is the entropy. (Housecroft and Sharpe, 2008)

Equation 3.5: The change in Gibbs free energy is calculated above where $G$ is the Gibbs free energy; $H$ is Enthalpy; $T$, is the Temperature; and $S$, is entropy (Lakshminarayanan et al., 2007b)
neutral pH. Based on these experiments, it can be concluded that using ATR-FTIR spectroscopy is capable of providing accurate quantitative data for kinetics experiments regardless of whether the solvent is D₂O or H₂O.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>ΔG of protein in D₂O analyzed by Transmission FTIR</th>
<th>ΔG of protein in D₂O analyzed by ATR-FTIR</th>
<th>ΔG of protein in H₂O analyzed by ATR-FTIR</th>
</tr>
</thead>
<tbody>
<tr>
<td>76°C</td>
<td>69.71 kJ</td>
<td>66.34 kJ</td>
<td>67.88 kJ</td>
</tr>
<tr>
<td>82°C</td>
<td>73.21 kJ</td>
<td>69.48 kJ</td>
<td>70.01 kJ</td>
</tr>
<tr>
<td>85°C</td>
<td>74.96 kJ</td>
<td>71.04 kJ</td>
<td>71.07 kJ</td>
</tr>
</tbody>
</table>

*Table 3.1: Values of ΔG for the aggregation reaction of 5% β-lactoglobulin in D₂O analyzed by transmission FTIR and ATR-FTIR spectroscopy as well as for 5% β-lactoglobulin in H₂O analyzed by ATR-FTIR spectroscopy*

### 3.5 Overall Summary

In order to study changes in proteins during storage of high protein beverages, there was a need to develop an appropriate method that could be used for high volumes of samples and provide reliable information. The capabilities of ATR-FTIR spectroscopic analysis with respect to protein analysis were assessed as a means to this end. It was found that analyzing the samples in solution followed by water subtraction was the most reproducible and quickest sampling method. Unfortunately, it resulted in a low signal to noise ratio necessitating the use of spectral enhancement techniques such as Fourier self-deconvolution and derivatization combined with smoothing. Both techniques, if used incorrectly can lead to artefact development and/or loss of spectral data. The best approach is often to use both techniques and compare the results of both. Thus, if a new peak, which is significantly higher than the noise level, appears following spectral enhancement by both techniques it is most likely a genuine peak. Hierarchal cluster analysis was used as another method of data mining. This technique is useful because separates a set of spectra into subsets according to similarity which can help not only to validate spectral enhancement but also to detect a pattern in a series of spectra. In the final stage of our
assessment of ATR-FTIR spectroscopy, we determined whether the technique can be used to obtain quantitative data about proteins in water and in D₂O. We calculated the change in Gibbs free energy for the aggregation reaction of β-lactoglobulin at 76, 82 and 85°C using spectra acquired from transmission FTIR (β-lactoglobulin in D₂O), and ATR-FTIR (β-lactoglobulin in D₂O as well as in H₂O). It was noted that all three techniques provided comparable results for the change in Gibbs free energy. This showed that ATR-FTIR spectroscopy can be used to make quantitative inferences, albeit with a lower correlation value for some plots (correlation ~0.8 using ATR-FTIR spectroscopy vs. ~1 using transmission FTIR spectroscopy).

We conclude that ATR-spectroscopy has potential as a tool for studying proteins in high protein beverages and it may be used to observe changes in the beverages during storage.
Chapter 4

Assessment of Storage Stability of Various Protein Ingredients Following UHT Processing

4.1. Introduction

High protein beverages are complex systems with multiple ingredients making them very difficult to study. Thus, a “bottom up” approach was undertaken by focussing first on the primary ingredient in these products: the proteins. Before examining the effects of gums or other additives on the proteins, it is important to commence study with a protein system of optimal stability, which eventually can be enhanced by other additives.

As mentioned earlier, to our knowledge, there is no research reported on aging of high protein drinks. Additionally, lot to lot differences in beverage ingredients make it difficult for manufacturers to determine whether reduced gelation in some product batches is a consequence of recipe changes or inconsistencies in ingredients obtained from suppliers. Consequently, it was difficult to develop a theory on how aging defects could be minimized. Thus, the first steps in this project involved conducting UHT trials with varying protein compositions then observing aging of the samples over time. Methodology and results from these experiments will be described below.

4.2. Materials and Methods

Sample Processing and Analysis

The sample permutations for the trials are described in Tables 4.1-4.7. The following is a description of the procedures used for processing of the samples. The dry protein powders were blended together using a whisk then added to 55°C water in an electric mixer. Next, all non-protein ingredients were added to the mixture. Phosphates were pre-solubilised before addition to the bulk mixture by pouring the powders into approximately 100 mL of boiling hot water and blending with a KitchenAid handheld blender until solubilised. Oil and emulsifier were also pre-blended before addition to the bulk mixture by mixing oil, emulsifier and 50 mL water and...
blending with the blender until an emulsion was generated. The batch was mixed in the mixer for approximately 20 min to provide enough time for protein hydration. The mixture was then pre-heated to 80°C in a MicroThermics UHT/HTST Lab Hybrid system and homogenized (100 Bar at first stage and 40 Bar at second stage) in a Niro-Soavi S.p.A. homogenizer. The batches were UHT treated at 142°C for 6.6 s and transferred to sterile 100 mL bottles.

For permutations containing only once UHT-processed β-lactoglobulin, the β-lactoglobulin was mixed as a dry powder with the other protein powders and processed in the manner described above. For permutations containing twice UHT processed β-lactoglobulin, a β-lactoglobulin solution was prepared using the required amount of 55°C water (as stipulated by recipes 4e, 4f and 4g of Table 4.4) and mixed in the electric mixer for approximately 20 min to allow β-lactoglobulin to hydrate. The solution was UHT treated at 142°C for 6.6 s. Next, the β-lactoglobulin solution was combined with the remaining protein ingredients and mixed for 20 min before pre-heating, homogenization and UHT treatment as described above.

Depending on the trial, aging of the samples was followed by visual inspection (and digital photography), measuring the amount of sediment formed using a ruler, pH measurement, viscosity measurement with a Brookfield viscometer, ATR-FTIR spectroscopy, and total solids measurement.

As samples aged a sediment formed so in preparation for analysis by ATR-FTIR spectroscopy, the supernatant was decanted and collected in a fresh tube to be analysed separately from the sediment. During analysis, a 50 μL drop of supernatant or sediment was placed on the ATR crystal and scanned. Each sample was analysed in triplicate and during spectral processing the three replicates were averaged and water was subtracted from the spectrum using a subtraction factor that resulted in a 1.4:1 ratio between the amide I and amide II bands. The resulting spectrum was normalized by dividing the spectral peaks by the area under the curve between 1800 and 1250 cm⁻¹. FSD was accomplished using a bandwidth of 20 and enhancement factor of 1.9. Analysis was performed on a single-bounce diamond-ATR accessory (DuraScope, SensIR Technologies) coupled to an FTIR Spectrometer (FTS 3000MX Excalibur, Varian Inc. Boston). A background spectrum was recorded of the clean ATR crystal (128 scans
were co-added at 4 cm⁻¹ resolution). Each sample spectrum was also composed of 128 co-added scans at 4 cm⁻¹ resolution.

Total solids was determined by pipetting 1 mL of sample into a weighing boat followed by heating to 30°C for several hours until all of the water evaporated. The weight of the weighing boat and the sample were recorded before and after drying. The weight of the dry sample was divided by that of the wet sample and this value was then converted to % (w/w) solids. A statistical program Rv.2.8.1 (The R Foundation for Statistical Computing) was used to determine significant differences between total solids measurements using the linear model function. Samples were analyzed for total solids after approximately 4 weeks of storage using as many samples as were available for each permutation (between 5-10 samples depending on the permutation).

*Enzyme Assay*

Plasminogen (lot#708) was obtained from Innovative Research (Novi, MI) and BIOPHEN Plasminogen (lot#64201-PK:1), a colourimetric plasminogen assay, was obtained from Aniara Corp. (Mason, OH). The colourimetric assay accounted for both plasmin and plasminogen. The assay was performed according to the manufacturer’s instructions which involved addition of urokinase to the sample to activate plasminogen. The activated plasminogen and the plasmin already present in the sample then cleaved a plasmin-specific substrate, SPm41, releasing para-nitroaniline which produced a yellow colour. Because of the opacity of casein-containing solutions, spectrophotometric determination was not possible. Instead, each sample was determined to have either a positive (yellow) or negative (no colour change) reaction. Four concentrations of plasminogen were used as controls: 0.5, 0.75, 1 and 2 mg/L. Two different caseinate products were each dissolved in distilled water at concentrations of 10% (w/v) protein before assaying. The processed samples from UHT trials 4b and c were assayed without any pre-treatment of the samples.
<table>
<thead>
<tr>
<th>Ingredients</th>
<th>1a</th>
<th>1b</th>
<th>1c</th>
<th>1d</th>
<th>1e</th>
<th>1f</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cooking Cream 35%</td>
<td>3.60%</td>
<td>3.60%</td>
<td>3.60%</td>
<td>3.60%</td>
<td>3.60%</td>
<td>3.60%</td>
</tr>
<tr>
<td>MPC</td>
<td>2.23%</td>
<td>2.01%</td>
<td>2.01%</td>
<td>7.15%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPI</td>
<td>3.98%</td>
<td>3.39%</td>
<td>3.39%</td>
<td>6.98%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium Caseinate</td>
<td>1.00%</td>
<td>1.00%</td>
<td>1.00%</td>
<td>1.32%</td>
<td>1.32%</td>
<td>1.00%</td>
</tr>
<tr>
<td>WPC</td>
<td>1.50%</td>
<td></td>
<td>0.26%</td>
<td>0.26%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Lactalbumin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.00%</td>
<td>7.03%</td>
</tr>
<tr>
<td>β-Lactoglobulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.00%</td>
</tr>
<tr>
<td>Carrageenan</td>
<td>0.01%</td>
<td>0.01%</td>
<td>0.01%</td>
<td>0.01%</td>
<td>0.01%</td>
<td>0.01%</td>
</tr>
<tr>
<td>Microcrystalline cellulose + carboxymethyl cellulose</td>
<td>0.12%</td>
<td>0.12%</td>
<td>0.12%</td>
<td>0.12%</td>
<td>0.12%</td>
<td>0.12%</td>
</tr>
<tr>
<td>Inulin</td>
<td>0.30%</td>
<td>0.30%</td>
<td>0.30%</td>
<td>0.30%</td>
<td>0.30%</td>
<td>0.30%</td>
</tr>
<tr>
<td>Salt</td>
<td>0.15%</td>
<td>0.15%</td>
<td>0.15%</td>
<td>0.15%</td>
<td>0.15%</td>
<td>0.15%</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>0.03%</td>
<td>0.03%</td>
<td>0.03%</td>
<td>0.03%</td>
<td>0.03%</td>
<td>0.03%</td>
</tr>
<tr>
<td>Sodium polyphosphate, sodium phosphate mixture</td>
<td>0.11%</td>
<td>0.11%</td>
<td>0.11%</td>
<td>0.11%</td>
<td>0.11%</td>
<td>0.11%</td>
</tr>
<tr>
<td>Sodium polyphosphate, sodium phosphate, sodium citrate mixture</td>
<td>0.11%</td>
<td>0.11%</td>
<td>0.11%</td>
<td>0.11%</td>
<td>0.11%</td>
<td>0.11%</td>
</tr>
<tr>
<td>Diacetyl Tartaric Acid Ester of mono-diglycerides (emulsifier)</td>
<td>0.12%</td>
<td>0.12%</td>
<td>0.12%</td>
<td>0.12%</td>
<td>0.12%</td>
<td>0.12%</td>
</tr>
<tr>
<td>Water</td>
<td>86.74%</td>
<td>87.05%</td>
<td>87.05%</td>
<td>89.19%</td>
<td>89.02%</td>
<td>87.42%</td>
</tr>
<tr>
<td>TOTAL</td>
<td>100.00%</td>
<td>100.00%</td>
<td>100.00%</td>
<td>100.00%</td>
<td>100.00%</td>
<td>100.00%</td>
</tr>
</tbody>
</table>

*Table 4.1: Sample permutations for UHT trial 1*
<table>
<thead>
<tr>
<th></th>
<th>2a</th>
<th>2b</th>
<th>2c</th>
<th>2d</th>
<th>2e</th>
<th>2f</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium caseinate</td>
<td>2%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7%</td>
</tr>
<tr>
<td>α-Lactalbumin</td>
<td></td>
<td>2%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Lactoglobulin</td>
<td></td>
<td></td>
<td></td>
<td>0.20%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPC</td>
<td></td>
<td></td>
<td></td>
<td>2%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WPC</td>
<td></td>
<td></td>
<td></td>
<td>2%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>98.00%</td>
<td>93.00%</td>
<td>98.00%</td>
<td>99.80%</td>
<td>98.00%</td>
<td>98.00%</td>
</tr>
</tbody>
</table>

*Table 4.2: Sample permutations for UHT trial 2*

<table>
<thead>
<tr>
<th></th>
<th>3a</th>
<th>3b</th>
<th>3c</th>
<th>3d</th>
<th>3e</th>
<th>3f</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Lactoglobulin</td>
<td>0.325%</td>
<td>0.75%</td>
<td>1.50%</td>
<td>1.129%</td>
<td>0.75%</td>
<td></td>
</tr>
<tr>
<td>α-Lactalbumin</td>
<td></td>
<td></td>
<td></td>
<td>0.424%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>casein</td>
<td>5.128%</td>
<td>5.128%</td>
<td>5.128%</td>
<td>5.128%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>94.87%</td>
<td>94.55%</td>
<td>94.12%</td>
<td>93.37%</td>
<td>98.45%</td>
<td>99.25%</td>
</tr>
</tbody>
</table>

*Table 4.3: Sample permutations for UHT trial 3*
<table>
<thead>
<tr>
<th></th>
<th>4a</th>
<th>4b</th>
<th>4c</th>
<th>4d</th>
<th>4e</th>
<th>4f</th>
<th>4g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>4.76%</td>
<td>4.76%</td>
<td>4.76%</td>
<td>4.76%</td>
<td>4.76%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Lactoglobulin</td>
<td>0.95%</td>
<td>0.95%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pre-UHT processed β-lac</td>
<td>0.95%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pre-UHT processed and aged β-lac</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.95%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WPI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.30%</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>95.24%</td>
<td>94.29%</td>
<td>99.05%</td>
<td>93.94%</td>
<td>94.29%</td>
<td>94.29%</td>
<td>99.05%</td>
</tr>
</tbody>
</table>

*Table 4.4: Sample permutations for UHT trial 4*

<table>
<thead>
<tr>
<th></th>
<th>5a</th>
<th>5b</th>
<th>5c</th>
<th>5d</th>
<th>5e</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caseinate 1</td>
<td>3.00%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caseinate 2</td>
<td></td>
<td>3.00%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caseinate 3</td>
<td></td>
<td></td>
<td>3.00%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caseinate 4</td>
<td></td>
<td></td>
<td></td>
<td>3.00%</td>
<td></td>
</tr>
<tr>
<td>WPC</td>
<td>1.50%</td>
<td>1.50%</td>
<td>1.50%</td>
<td>1.50%</td>
<td>1.50%</td>
</tr>
<tr>
<td>MPC</td>
<td>5%</td>
<td>5%</td>
<td>5%</td>
<td>5%</td>
<td>5%</td>
</tr>
<tr>
<td>water</td>
<td>90.50%</td>
<td>93.50%</td>
<td>93.50%</td>
<td>93.50%</td>
<td>93.50%</td>
</tr>
</tbody>
</table>

*Table 4.5: Sample permutations for UHT trial 5*
<table>
<thead>
<tr>
<th></th>
<th>6a</th>
<th>6b</th>
<th>6c</th>
<th>6d</th>
<th>6e</th>
<th>6f</th>
<th>6g</th>
<th>6h</th>
<th>6i</th>
<th>6j</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPC</td>
<td>5.58%</td>
<td>5.58%</td>
<td>5.58%</td>
<td>5.58%</td>
<td>5.58%</td>
<td>5.58%</td>
<td>5.58%</td>
<td></td>
<td></td>
<td>5.58%</td>
</tr>
<tr>
<td>β-Lactoglobulin</td>
<td>1.00%</td>
<td>1.00%</td>
<td>1.00%</td>
<td>1.00%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.00%</td>
</tr>
<tr>
<td>β-Lactoglobulin (pre-UHT processed)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.00%</td>
<td>1.00%</td>
<td>1.00%</td>
<td></td>
<td></td>
<td>1.00%</td>
</tr>
<tr>
<td>Calcium caseinate 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.00%</td>
<td></td>
<td></td>
<td></td>
<td>1.00%</td>
</tr>
<tr>
<td>Calcium caseinate 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.00%</td>
<td></td>
<td></td>
<td>1.00%</td>
</tr>
<tr>
<td>Calcium caseinate 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.00%</td>
</tr>
<tr>
<td>Calcium caseinate 4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.00%</td>
<td></td>
<td>1.00%</td>
</tr>
<tr>
<td>Emulsifier</td>
<td>0.14%</td>
<td>0.14%</td>
<td>0.14%</td>
<td>0.14%</td>
<td>0.14%</td>
<td>0.14%</td>
<td>0.14%</td>
<td></td>
<td></td>
<td>0.14%</td>
</tr>
<tr>
<td>Sunflower oil</td>
<td>1.31%</td>
<td>1.31%</td>
<td>1.31%</td>
<td>1.31%</td>
<td>1.31%</td>
<td>1.31%</td>
<td>1.31%</td>
<td></td>
<td></td>
<td>1.31%</td>
</tr>
<tr>
<td>Salt</td>
<td>0.14%</td>
<td>0.14%</td>
<td>0.14%</td>
<td>0.14%</td>
<td>0.14%</td>
<td>0.14%</td>
<td>0.14%</td>
<td></td>
<td></td>
<td>0.14%</td>
</tr>
<tr>
<td>Sucralose</td>
<td>0.01%</td>
<td>0.01%</td>
<td>0.01%</td>
<td>0.01%</td>
<td>0.01%</td>
<td>0.01%</td>
<td>0.01%</td>
<td></td>
<td></td>
<td>0.01%</td>
</tr>
<tr>
<td>Acesulfame potassium</td>
<td>0.01%</td>
<td>0.01%</td>
<td>0.01%</td>
<td>0.01%</td>
<td>0.01%</td>
<td>0.01%</td>
<td>0.01%</td>
<td></td>
<td></td>
<td>0.01%</td>
</tr>
<tr>
<td>Sodium polyphosphate, sodium phosphate mixture</td>
<td>0.11%</td>
<td>0.11%</td>
<td>0.11%</td>
<td>0.11%</td>
<td>0.11%</td>
<td>0.11%</td>
<td>0.11%</td>
<td></td>
<td></td>
<td>0.11%</td>
</tr>
<tr>
<td>Sodium polyphosphate, sodium phosphate, sodium citrate mixture</td>
<td>0.14%</td>
<td>0.14%</td>
<td>0.14%</td>
<td>0.14%</td>
<td>0.14%</td>
<td>0.14%</td>
<td>0.14%</td>
<td></td>
<td></td>
<td>0.14%</td>
</tr>
<tr>
<td>Inulin</td>
<td>0.51%</td>
<td>0.51%</td>
<td>0.51%</td>
<td>0.51%</td>
<td>0.51%</td>
<td>0.51%</td>
<td>0.51%</td>
<td></td>
<td></td>
<td>0.51%</td>
</tr>
<tr>
<td>Microcrystalline cellulose + carboxymethyl cellulose</td>
<td>0.20%</td>
<td>0.20%</td>
<td>0.20%</td>
<td>0.20%</td>
<td>0.20%</td>
<td>0.20%</td>
<td>0.20%</td>
<td></td>
<td></td>
<td>0.20%</td>
</tr>
<tr>
<td>Carrageenan</td>
<td>0.03%</td>
<td>0.03%</td>
<td>0.03%</td>
<td>0.03%</td>
<td>0.03%</td>
<td>0.03%</td>
<td>0.03%</td>
<td></td>
<td></td>
<td>0.03%</td>
</tr>
<tr>
<td>Water</td>
<td>89.81%</td>
<td>89.81%</td>
<td>89.81%</td>
<td>89.81%</td>
<td>89.81%</td>
<td>89.81%</td>
<td>89.81%</td>
<td></td>
<td></td>
<td>89.81%</td>
</tr>
</tbody>
</table>

*Table 4.6: Sample permutations for UHT trial 6*
<table>
<thead>
<tr>
<th></th>
<th>7a</th>
<th>7b</th>
<th>7c</th>
<th>7d</th>
<th>7e</th>
<th>7f</th>
<th>7g</th>
<th>7h</th>
<th>7i</th>
<th>7j</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>β-Lactoglobulin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>β-Lactoglobulin (pre-UHT processed)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>MPC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>WPC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Calcium caseinate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Emulsifier</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Sunflower oil</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Salt</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Sucralose</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Acesulfame potassium</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Sodium polyphosphate, sodium phosphate mixture</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Sodium polyphosphate, sodium phosphate, sodium citrate mixture</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Inulin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Microcrystalline cellulose + carboxymethyl cellulose</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Carrageenan</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Water</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Table 4.7: Sample permutations for UHT trial 7*
4.3. Results and Discussion

Samples from UHT trial 1 were assessed on a weekly basis by visual observation, pH and viscosity measurement and ATR-FTIR spectroscopic analysis. Measurements continued only until week 5 of storage because after approximately three weeks of aging no further changes were observed. Samples were periodically checked by visual observation for eighteen months after processing.

No gelation throughout the sample was observed in any of the samples of this trial. The only storage defect detected was the formation of sediment (see Figure 4.1) which slowly turned into a gel over the course of approximately six weeks. Depending on the sample, the sediment height ranged from 1-6mm and its consistency ranged from a very light gel to pasty and gel-like. In general, when protein ingredients were replaced with ingredients lacking casein, the amount of sediment decreased. It has been reported previously that in UHT milk, the sediment consists primarily of casein (Harwalkar, 1982). The observations from this UHT trial indicate that the same holds true in the case of UHT processed high protein beverages.

From measurements of samples from this UHT trial, it was determined that there were no significant changes in pH (Figure 4.2) or viscosity (Figure 4.3) during aging. Thus, these measurements were omitted during sample analysis in future UHT trials.
Figure 4.2: Representative plot of pH over time. Note that there is no significant change in pH during storage

Figure 4.3: Representative plot of viscosity over time. Note that there is no significant change in viscosity during storage
ATR-FTIR spectroscopic analysis of samples was performed on a weekly basis. Unfortunately, no pattern in the infrared spectra could be detected during storage. When the supernatant and sediment were compared, there were also no significant spectral differences except that the sediment had a higher intensity due to the higher protein concentration.

It was concluded that the system was too complex and changes in the spectra were too subtle to allow study of the system in its entirety. Instead, the ensuing UHT trials contained only protein ingredients to allow examination of the protein ingredients’ individual behaviour during storage.

In UHT trial 2, it was noted that when α-lactalbumin or β-lactoglobulin were UHT processed alone, no sediment was formed (see Figure 4.4), suggesting that caseinates were largely responsible for the sedimentation. Once again, no gelation throughout the sample was observed in any of the permutations in UHT trial 2.
Weekly ATR-FTIR spectroscopic analysis of the samples led to the same conclusions as with the previous UHT trial. Even with a simpler system, no spectral pattern was recognized. The thin-film sampling technique (as described in Chapter 3) was applied in UHT trials 1 and 2. Considering the lack of any detectable pattern in the ATR spectra, the sampling method was refined and a new technique was adopted: analysis of aqueous samples followed by water subtraction.

An important conclusion arising from UHT trial 3 was that ATR-FTIR spectroscopic analysis was not suitable for our samples. Even with an improved sampling technique, no useful information could be extracted from the spectra (see Figure 4.5). Even if a protein ingredient was analyzed alone, most of these contain a mixture of proteins resulting in a system too complex for identification of specific structural changes. Additionally, the low SNR of ATR spectroscopy limited secondary structure analysis even when the samples contained only one or two proteins (β-lactoglobulin, α-lactalbumin) and the changes in protein concentration were too subtle to be detected. Since ATR-FTIR spectroscopy was not useful in quantifying the amount of protein remaining in solution, another method needed to be selected. Various approaches were explored including electron microscopy, fluorescence spectroscopy, UV-Visible spectrophotometry and total solids measurement. In the end, total solids measurement followed by statistical analysis for significance was determined to be most suitable for these samples. However, this total solids technique did not come into use until UHT trial 5. Until then, sediment height (in mm) was used to assess the degree of sedimentation (Figure 4.4).

Another important conclusion from UHT trial 3 was that adding increasing amounts of β-lactoglobulin had a beneficial effect on sedimentation. As β-lactoglobulin concentration was increased, the sediment became less hard. The permutation containing the highest concentration of β-lactoglobulin had sediment which was easily re-dispersed even after five weeks of storage, whereas the permutation without any β-lactoglobulin had a very hard sediment after three weeks of storage, resembling dried up glue. Considering that β-lactoglobulin solutions undergo heat-induced gelation these observations were surprising (Hosseini-nia et al., 1999). This observation gave rise to the idea that the chaperone-like activity of casein (Bhattacharyya and Das, 1999;
Morgan *et al.*, 2005; Zhang *et al.*, 2005) may delay β-lactoglobulin gel formation and in some way limit casein sedimentation.

We postulated that UHT processing slightly damages β-lactoglobulin making it possible for casein to interact with it by chaperone activity. Such interaction could slightly reduce sedimentation by limiting casein-casein interaction. Additionally, the interaction could bring β-lactoglobulin down into the sediment along with casein preventing formation of a casein gel over time.
Figure 4.5: Overlayed spectra of UHT processed 5.13% calcium caseinate and 1.5% β-lactoglobulin weeks 0-6 following replicate averaging, water subtraction, normalization and FSD bw=20, k=1.9.
This theory was investigated in UHT trial 4. Here, β-lactoglobulin which had been pre-treated by UHT processing was incorporated in the protein mix before UHT processing again. Since chaperone proteins have limited interaction with the native form of their substrate we postulated that when β-lactoglobulin was UHT-treated twice, the higher degree of damage would further augment β-lactoglobulin-casein interaction. From this trial, it was observed that samples containing pre-treated β-lactoglobulin had slightly less sediment (again assessed by height in mm) than those with less damaged β-lactoglobulin. However, the difference was quite small (~0.5-1mm) so in an attempt to enhance the effect, all of the other beverage ingredients were added back in UHT trials 6 and 7.

The final question addressed in UHT trail 4 was whether or not there were any milk proteases present in the sample playing a role storage stability. It is known that proteolysis may play some role in age gelation of UHT milk (Datta and Deeth, 2001). However it is not known what amount of proteolytic enzymes is contained in these beverages and hence if they may play a role in their storage stability. To answer this question, an enzyme assay was performed on some samples of UHT trial 4 as well as some calcium caseinate powders used to make the UHT high protein beverages.

Because of the opacity of caseinate-containing solutions it was not possible to analyze the samples using UV-Visible spectroscopic. Instead, the results were presented in terms of visual observation of a positive (yellow colour) or negative (no colour change) reaction. From the results in table 4.8, it is clear that the samples contain less than 0.5 mg of plasminogen/plasmin. Without UV spectrophotometric analysis it is not possible to determine precisely how much plasminogen/plasmin may be present. However, it has been reported by Richardson and Te Whaiti (1978) that any amount of plasmin less than 0.5mg would not have any significant effect on storage defects in UHT milk. Thus, proteolytic milk enzymes do not appear to play any role in the development of storage defects in high protein beverages.
Table 4.8: Results of enzyme assay for plasmin/plasminogen content performed on select samples from UHT trial 4 and caseinate products. A (+) indicates presence of plasmin/plasminogen and a (-) sign indicates absence of plasmin/plasminogen.
The next UHT trial (5), involved investigating the tendency of various caseinate products to sediment. By determining which caseinate products sediment most, it would possible to associate properties of the caseinates with a likelihood to sediment. Thus, manufacturers might be able to screen caseinates based on these properties before using them in products where stability in solution is important. However, from this trial it seemed that all of the caseinates examined produced approximately the same amount of sediment (see Figure 4.6). Figure 4.6
illustrates the total solids measurements obtained from the samples of UHT trials 4, 5 and 6. For each permutation of these trials statistical analysis was performed to test for significance of differences between samples. Table 4.9 (statistical analysis of UHT trial 7) is a representative table of the tables produced for each of the trials.

Because varying the caseinates in trial 5 did not significantly change the amount of sediment formed, the focus was shifted again to the potential application of chaperone-like abilities of casein to improve beverage stability. From UHT trials 6 and 7 it was determined that addition of pre-UHT treated β-lactoglobulin did not improve sedimentation stability of caseinate. Addition of pre-UHT treated β-lactoglobulin not only did not reduce sedimentation but it also

<table>
<thead>
<tr>
<th>Compare</th>
<th>WPC-free product</th>
<th>Product containing β-lac</th>
<th>Product containing UHT-treated β-lac</th>
<th>Original proteins alone</th>
<th>Proteins alone containing β-lac</th>
<th>Proteins alone containing UHT-treated β-lac</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original product</td>
<td>2.92E-01</td>
<td>1.21E-01</td>
<td>4.45E-06</td>
<td>1.40E-03</td>
<td>1.89E-02</td>
<td>7.40E-03</td>
</tr>
<tr>
<td>WPC-free product</td>
<td></td>
<td></td>
<td>8.99E-01</td>
<td>1.47E-01</td>
<td>1.22E-02</td>
<td>2.40E-03</td>
</tr>
<tr>
<td>Product containing β-lac</td>
<td></td>
<td></td>
<td>3.45E-01</td>
<td>1.76E-02</td>
<td>1.85E-02</td>
<td>8.14E-03</td>
</tr>
<tr>
<td>Product containing UHT-treated β-lac</td>
<td></td>
<td></td>
<td></td>
<td>1.21E-03</td>
<td>3.92E-04</td>
<td>7.78E-06</td>
</tr>
<tr>
<td>Original proteins alone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.01E-02</td>
<td>6.76E-01</td>
</tr>
<tr>
<td>Proteins alone containing β-lac</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.43E-02</td>
</tr>
<tr>
<td>Proteins alone containing UHT-treated β-lac</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.9: Statistical analysis of UHT trial 7: Comparison of the probabilities that the total solids measurements for the permutations in question are equal to each other. All values <0.05 are highlighted in violet, indicating that they are significantly different from each other.
produced a more viscous sediment than addition of native β-lactoglobulin before UHT processing. The most surprising result of the last two trials was that gelation throughout the sample was observed in some of the permutations.

In UHT trial 6, each permutation contained all the typical ingredients of high protein beverages (including non-protein ingredients) but the caseinate source was varied. Additionally, half of the permutations had added β-lactoglobulin and the other half had added pre-UHT treated β-lactoglobulin. Interestingly, some samples from every permutation developed a gel throughout the sample after approximately one month of storage.

In UHT trial 7, permutations 7b and 7c developed a gelation throughout the sample after 1 month of storage. In other words, in this UHT trial, only the permutations containing the whole product formulation (including non-protein ingredients) and additional β-lactoglobulin produced a gel. The addition of β-lactoglobulin however, cannot be solely responsible for the development of this gel because the manufacturer of these types of beverages reports that some batches of UHT high protein products have gelation even though none of the products contain pure β-lactoglobulin. The lack of gelation in some lots is often explained by lot to lot differences in the beverage ingredients. Note also that UHT trial 7i, containing all of the beverage ingredients except for the proteins, did not produce a gel. Hence, β-lactoglobulin by itself, cannot be responsible for the gelation. Additionally, this permutation also contained some sediment thus ingredients other than the proteins can add to sediment formation in beverages.

From the very start of the UHT trials, permutations which contained only proteins never had any gelation throughout the sample. Thus, it was stated that gelation of high protein beverages does not appear to be the result of protein-protein interactions alone. We suspect that age gelation of high protein beverages is a result of protein-gum or protein-carbohydrate interaction. Additionally, even without any proteins in the mixture there was some sediment present suggesting that the sediment in high protein beverages is composed of a combination of protein and non-protein ingredients.
4.4. Summary

From this series of UHT trials we have observed that caseinates are likely the primary cause of sedimentation in high protein beverages and that varying the combination of proteins did not prevent sedimentation of caseinates. However, the addition of β-lactoglobulin to calcium caseinate affects consistency of the sediment. In all permutations where proteins were processed without the presence of any other beverage ingredients there was never any gelation throughout the sample. When all the non-protein ingredients were UHT processed in the absence of protein there was some sediment formation and no gel formation. These observations indicated that both protein and non-protein ingredients can cause sediment formation in these beverages. Moreover, they suggest that proteins alone are not responsible for age gelation of high protein beverages. An interaction must occur between proteins and some other beverage ingredient which causes gel development. The culprit interaction is still unknown and provides a future direction for high protein beverage research.
Chapter 5
The Chaperone Didn’t Show: Interaction Between Native β-Lactoglobulin and Caseins Challenges Casein’s Chaperone Status.

5.1. Introduction

In chapter 4 it was noted that an interaction occurs between β-lactoglobulin and caseinates which affects the consistency of the sediment in UHT processed high protein solutions. It was previously reported that disulfide bond formation between β-lactoglobulin and κ-casein is the first step towards age gelation in milk (Datta and Deeth, 2001). From our observations, the interaction occurring between β-lactoglobulin and caseins in high protein beverages is inhibiting gelation (recall a reduction in gelation of the sediment). Thus, we hypothesize that a different interaction must account for this behaviour.

In recent years, several authors (Bhattacharyya and Das, 1999; Morgan et al., 2005; Zhang et al., 2005) have investigated chaperone-like behaviour of bovine casein on various proteins including β-lactoglobulin. Although some have speculated that hydrophobic interactions are critical to these processes, detailed binding mechanisms remain unresolved. Thus, definitive assignments of chaperone behaviour have also not been possible.

In 1989, Ellis and Hemingsen (1989) provided a comprehensive description of molecular chaperones in which they defined them as “proteins whose role is to mediate the folding of certain other polypeptides but which are not components of these final structures”. Pockley (2005) reiterates this definition and goes on to explain that chaperones bind non-covalently to target proteins at sites that are not normally exposed in their native state. Hence, it is not until the target protein is damaged that chaperone interaction may occur. Using this information as a criterion for chaperone status, we set out to determine whether α- and β-caseins’ stabilizing effects on β-lactoglobulin were the result of interactions at sites on β-lactoglobulin which are only exposed in a partially denatured state.
The main objective of this study is to provide a detailed characterization of binding between β-lactoglobulin and casein, a biomolecular interaction that likely occurs in high protein beverages during heating. The secondary objective is to clarify whether or not this interaction exhibits chaperone-like behaviour.

5.2. Materials and Methods

Fourier Transformation Infrared Spectroscopy.

Transmission FTIR Spectroscopic Studies of β-lactoglobulin and casein were carried out on a Nicolet DaIRy Instrument. The background was composed of 256 co-added scans. Variable temperature experiments were performed at temperatures between 25-95°C with 5°C intervals and 10 minute pre-equilibrations at each new temperature before recording the IR spectra. The following protein combinations were examined with 256 co-added scans at each temperature:

<table>
<thead>
<tr>
<th>Protein Combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5% β-lactoglobulin</td>
</tr>
<tr>
<td>3.5% α-casein</td>
</tr>
<tr>
<td>3.5% β-casein</td>
</tr>
<tr>
<td>3.5% κ-casein</td>
</tr>
<tr>
<td>3.5% β-lactoglobulin + 3.5% α-casein</td>
</tr>
<tr>
<td>3.5% β-lactoglobulin + 3.5% β-casein</td>
</tr>
<tr>
<td>3.5% β-lactoglobulin + 3.5% κ-casein</td>
</tr>
</tbody>
</table>

Table 5.1: Protein combinations subjected to an FTIR Spectroscopic variable temperature experiment.

To study the effect of the various casein subunits on denaturation of β-lactoglobulin, the spectrum of a given casein subunit was subtracted from the spectrum of the same casein subunit mixed with β-lactoglobulin, in order to extract the spectrum of just β-lactoglobulin. Next, the absorbance values at 1692cm⁻¹ and 1618cm⁻¹ were recorded at each temperature. The absorbances were normalized according to equation 5.1 and plotted against temperature.
2D correlation analysis of the FTIR spectra was performed as a function of increasing temperature using Grams3D (Thermo Electron Corp) software.

**Surface Plasmon Resonance.**

SPR was performed on a Biacore 3000 system (GE Healthcare Bio-Sciences AB) at 25°C using filtered (0.2 μm) and degassed HBS-EP running buffer (10mM HEPES pH 7.4, 150mM NaCl, 3mM EDTA, 0.05% (v/v) Tween20). α-, β-, and κ-caseins, as well as calcium caseinate ‘ligands’, were immobilized on a carboxymethylated CM4 sensor chip using the Biacore Amine Coupling Kit. β-lactoglobulins, caseins, and control (α-lactoglobulin, BSA) ‘analytes’ were injected over reference and ligand-immobilized surfaces in-tandem, using variable flow rates (10-50 μL/min) and contact times (3-20 min association, 5-20 min dissociation). Between sample injections, sensor chip surfaces were regenerated using 0.05% (v/v) Empigen in Pierce Gentle Elution buffer. To compare relative binding affinities, steady-state binding responses (Req) were plotted as a function of ‘analyte’ concentration; equilibrium dissociation constants (K_D) were predicted by non-linear regression analysis to a steady-state affinity binding model (BIAevaluation v4.1 software).
5.3. Results and Discussion

**FTIR Spectroscopy**

Figure 5.1. shows the overlaid spectra of β-lactoglobulin under the influence of α-casein (i.e. α-casein spectrum was subtracted from the combined spectrum) as it is heated from 25-95°C, following Fourier-self deconvolution. Similar studies were performed for β-lactoglobulin in the absence and presence of β- and κ-caseins. Band assignment was performed according to assignments given by Boye and co-workers (Boye et al., 1996).

![Figure 5.1.: Overlaid Fourier-self-deconvolved spectra of β-lactoglobulin under the influence of α-casein as it is heated from 25-95°C](image)

95°C, following Fourier-self deconvolution. Similar studies were performed for β-lactoglobulin in the absence and presence of β- and κ-caseins. Band assignment was performed according to assignments given by Boye and co-workers (Boye et al., 1996).

Figure 5.2. shows the overlaid plots of FTIR absorbance of the aggregation band at 1618 cm\(^{-1}\) as a function of temperature. Aggregation temperature of β-lactoglobulin (80°C) appeared to be unaltered in the absence and presence of α-, β and κ-caseins.
Figure 5.2: Effect of the various casein subunits on aggregation temperature (1692 cm⁻¹ band) of β-lactoglobulin
Figure 5.3. shows the overlaid plots of FTIR absorbance of the aggregation band at 1692 cm\(^{-1}\) of β-lactoglobulin alone and β-lactoglobulin in the presence of the different casein subunits. This band at 1692 cm\(^{-1}\) likely represents a hydrogen bond between a water molecule trapped in the interior of the β-lactoglobulin calyx and a carbonyl group on a β-sheet which constitutes one wall of the calyx (Boyé et al., 1996). As the molecule is damaged by heat and the tertiary structure is lost, D\(_2\)O enters inside the calyx and H-D exchange occurs, resulting in the loss of the 1692 cm\(^{-1}\) band. As such, this band can be used as an indicator of the degree of tertiary structural damage sustained by β-lactoglobulin exposed to heat (Boyé et al., 1996).

From figure 5.3., it was noted that the tertiary structure of β-lactoglobulin is damaged most easily in the absence of casein. This is apparent because it begins to lose its surface β-sheet structure very quickly after 30°C. In the presence of κ-casein, β-lactoglobulin also begins to lose
its structure at around the same temperature as the control. However, the loss of structure occurs over a broader temperature range than the control. Whereas the 1692 cm\(^{-1}\) band is lost completely by 50°C in the control sample, the band is only totally lost around 60°C in the presence of κ-casein. In the presence of α-casein, β-lactoglobulin only begins to lose tertiary structure after 50°C and in the presence of β-casein this occurs only after 55°C. This indicates that α- and β-casein exert protective effects on β-lactoglobulin and can increase its resistance to heat damage by 20 and 25°C, respectively.

Figure 5.4: Synchronous 2D correlation map of β-lactoglobulin heated from 25-95°C

Next, the spectra of β-lactoglobulin heated from 25-95°C under the influence of α-, β- or κ-casein as well as of β-lactoglobulin alone were analyzed by 2D correlation spectroscopy. For each series of spectra, synchronous and asynchronous 2D maps were generated. Figure 5.4. illustrates the synchronous map for β-lactoglobulin heated in the absence of casein from 25-
95°C. When a peak at a particular intersection of two bands (for example at the intersection of 1692 and 1618 cm\(^{-1}\)) is positive or negative in the synchronous map, this provides the first + or – sign for the correlation of those two bands in tables 5.2 and 5.3.

<table>
<thead>
<tr>
<th></th>
<th>1692 ↓</th>
<th>1683 ↑</th>
<th>1647 ↓</th>
<th>1634 ↓</th>
<th>1622 ↓</th>
<th>1618 ↑</th>
</tr>
</thead>
<tbody>
<tr>
<td>1618</td>
<td>-/-</td>
<td>+/0</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td></td>
</tr>
<tr>
<td>1622</td>
<td>+/-</td>
<td>-/+</td>
<td>+/0</td>
<td>+/0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1634</td>
<td>+/-</td>
<td>-/+</td>
<td>+/0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1647</td>
<td>+/-</td>
<td>-/+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1683</td>
<td>-/-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1692</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Table 5.2.: Table of sequence of events for the unfolding of β-lactoglobulin in the presence of α- and β-caseins*

<table>
<thead>
<tr>
<th></th>
<th>1691 ↓</th>
<th>1683 ↑</th>
<th>1647 ↓</th>
<th>1634 ↓</th>
<th>1622 ↓</th>
<th>1616 ↑</th>
</tr>
</thead>
<tbody>
<tr>
<td>1616</td>
<td>-/-</td>
<td>+/0</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td></td>
</tr>
<tr>
<td>1622</td>
<td>+/-</td>
<td>-/+</td>
<td>+/0</td>
<td>+/0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1634</td>
<td>+/-</td>
<td>-/+</td>
<td>+/0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1647</td>
<td>+/-</td>
<td>-/+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1683</td>
<td>-/-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1691</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Table 5.3.: Table of sequence of events for the unfolding of β-lactoglobulin in the presence of κ-casein and by itself*

This first sign indicates whether the peaks are moving in the same direction (i.e. both rising or both falling) or in opposite directions (i.e. one rising and the other falling).

The second sign obtained in the same manner but from the asynchronous map. Together, the two signs indicate which peak undergoes a change in absorbance first. When the signs are the same, the band at the top of the column is changing first. When the signs are different, the band to the left of the row is changing first. If one of the two signs is missing, this indicates that the data is either not clear enough to assign a positive or negative sign or that the two bands are changing simultaneously. Tables 5.2, and 5.3 depict the sequence of events table for the unfolding of β-lactoglobulin in the presence of α- and β-casein as well as in the presence of κ-casein and in the absence of casein, respectively. Using Tables 5.2 and 5.3, the sequence of events can be represented schematically as follows:
As highlighted in figure 5.5, β-lactoglobulin alone has the same sequence of unfolding events as in the presence of κ-casein. In both cases, unfolding begins with a loss in tertiary structure followed by loss of secondary structures and finally aggregation. In the presence of α- and β-caseins, the sequence of unfolding events in β-lactoglobulin is significantly different. In this sequence, the loss of tertiary structure is delayed and occurs only after the secondary structures are lost. The altered sequence suggests that α- and β-caseins interact with β-lactoglobulin at the opening of its hydrophobic core, thus delaying the exposure of the interior of β-lactoglobulin to solvent. Taken together, these results indicate that both α- and β-caseins can interact with β-lactoglobulin strongly enough to change its behaviour during heating.

**Surface Plasmon Resonance**

One aspect of the chaperone-like action of casein on β-lactoglobulin that has not been clarified by other authors is what degree of heat damage β-lactoglobulin has sustained at the point when casein begins to interact with it. If caseins are genuine chaperones to β-lactoglobulin, they should not interact with it until some damage has been sustained and the sites where
chaperone interaction takes place are exposed. In order to answer this question and to quantitatively characterize these interactions in a label-free, real-time manner, Surface Plasmon Resonance (SPR) was employed. Initially, native and UHT-treated β-lactoglobulin samples, as well as α-lactalbumin control, were screened over immobilized α-, β-, and κ-casein surfaces (~1 ng of each amine-coupled; Figures 5.6, 5.7, and 5.8, respectively).

Using rapid, high-flow screening, reference-subtracted sensorgrams were generated which demonstrated specific binding interactions between the β-lactoglobulin samples and immobilized casein subunits. Moreover, significantly weaker responses with α-lactalbumin (i.e. negative control under identical assay conditions) further validated the observed binding specificity. When comparing all β-lactoglobulin results, the largest binding responses were observed with β-casein, followed by α-casein (moderate response) and finally κ-casein (weakest response).

Next, dose-dependent titration series (Figures 5.9, 5.10, and 5.11) were acquired in order to determine and compare overall binding affinities (equilibrium dissociation constants, $K_D = \frac{k_d}{k_a}$). Biphasic sample heterogeneity, possibly due to the presence of both variant A and variant B of β-lactoglobulin, was observed at the start of the association phase. This excluded fitting of the data to simple 1:1 kinetic model ($k_a = $ association rate constant, $k_d = $ dissociation rate constant). Alternatively, equilibrium binding responses near the end of the association phase (Req, ~1300 sec) were plotted as a function of concentration; non-linear regression analysis to a steady-state binding model (BIAevaluation software) yielded the corresponding $K_D$ values.
Figure 5.6: Rapid screening (50μL/min) of β-lactoglobulin variants (36μM) and α-lactalbumin (control, 36μM) over amine-coupled α-casein surface (~1000 RU).

Figure 5.7: Rapid screening (50μL/min) of β-lactoglobulin variants (36μM) and α-lactalbumin (control, 36μM) over amine-coupled β-casein surface (~1000 RU).
Figure 5.8: Rapid screening (50μL/min) of β-lactoglobulin variants (36μM) and α-lactalbumin (control, 36μM) over amine-coupled κ-casein surface (~1000 RU).
Figure 5.9: Equilibrium analysis (10μL/min) for Sigma β-lactoglobulin AB (0-50μM) binding to amine-coupled α-casein (960 RU); inset, plot of Req versus C predicts $K_D \sim 7\mu M$. 

![Equilibrium analysis graph](Image)
Figure 5.10: Equilibrium analysis (10μL/min) for Sigma β-lactoglobulin AB (0-50μM) binding to amine-coupled β-casein (960 RU); inset, plot of Req versus C predicts $K_D \sim 4 \mu M$.

Figure 5.11: Equilibrium analysis (10μL/min) for Sigma β-lactoglobulin AB (0-50μM) binding to amine-coupled κ-casein (960 RU); inset, plot of Req versus C predicts $K_D \sim 16 \mu M$. 
In the identical manner, equilibrium titrations were acquired for all β-lactoglobulin variants. As a positive control, the casein subunits (and calcium caseinate) were also injected over themselves, as previously reported by Marchesseau et al. (2002). All of the $K_D$ values determined are summarized in Table 5.4.

From a relative comparison of the $K_D$ values, Sigma’s β-lactoglobulin AB exhibited the strongest binding affinity to the caseins. Sigma’s β-lactoglobulin A and β-lactoglobulin B exhibited weaker binding affinities, possibly due to changes incurred during the protein separation. Davisco Foods International Inc.’s β-lactoglobulin had a slightly weaker binding affinity than that of β-lactoglobulin from Sigma, probably due to differences in the preparation method (e.g. Sigma drying technique is lyophilization while Davisco is spray drying). Subsequent UHT treatments of Davisco’s β-lactoglobulin did not significantly alter the observed $K_D$ values, indicating that the binding affinity remains the same even after some heat damage. Of special note, however, the stoichiometry of native β-lactoglobulin binding to casein (8:1) was different compared to UHT-treated β-lactoglobulin (2:1). This stoichiometry appears to correlate well with predicted degrees of β-lactoglobulin self-association. Native β-lactoglobulin has a tendency to form octamers at high concentrations (Verheul et al., 1999). As β-lactoglobulin molecules bind to immobilized casein surfaces, one can imagine that the local concentration of β-lactoglobulin would become significantly higher than what is in the injected solution, hence it would encourage octamer formation and could result in the observed 8:1 stoichiometry whereby every one β-lactoglobulin octamer binds to one casein subunit. Under different conditions, such as upon heating, β-lactoglobulin has a tendency to form dimers (Verheul et al., 1999). When UHT-treated β-lactoglobulin is injected over the immobilized casein surface this generates a 2:1 binding stoichiometry which suggests that heat-treated β-lactoglobulin molecules are binding to caseins as dimers.

For all β-lactoglobulins (from difference sources) binding to all caseins, the general ranking of binding affinities from strongest to weakest is: β-casein > α-casein > κ-casein > calcium caseinate. Each of the casein subunits was injected over itself to verify the method by comparing our results to that of (Marchesseau et al., 2002). We found that our $K_D$ values for κ-casein
binding to the different casein subunits agreed well with their report. However, our affinity constants for $\alpha$- and $\beta$-caseins were stronger than Marchesseau et al. (2002) reported likely due to technical difference in the SPR assays. For example, our study utilized lower surface densities of the immobilized casein subunits to minimize mass transport limitations. Moreover, Marchesseau fit a single concentration of each analyte to a 1:1 kinetic model, whereas our study fit multiple concentrations of each analyte to an overall steady-state affinity model.

Upon examination of the effect of UHT treatment of calcium caseinate on its binding capabilities, it was determined that treatment of calcium caseinate by UHT did not have any effect. Neither UHT-treated nor untreated calcium caseinate consistently ranked as having stronger binding affinities with the $\beta$-lactoglobulins and casein subunits. Differences between binding affinities of UHT-treated and untreated calcium caseinate to any given analyte were quite small and usually within the error margins. Furthermore, binding affinities of untreated and UHT treated calcium caseinates binding to immobilized calcium caseinates agreed well with each other supporting the fact that UHT treatment did not alter binding abilities of these proteins.
<table>
<thead>
<tr>
<th>Equilibrium ( Req vs. C → K_d )</th>
<th>β-Casein ( ~960 RU )</th>
<th>α-Casein ( ~960 RU )</th>
<th>κ-Casein ( ~960 RU )</th>
<th>Calcium Caseinate ( ~980 RU )</th>
<th>UHT Calcium Caseinate ( ~980 RU )</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-lac A (Sigma)</td>
<td>12 +/- 0.9 uM</td>
<td>19 +/- 1.4 uM</td>
<td>108 +/- 37 uM</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>β-lac B (Sigma)</td>
<td>16 +/- 0.6 uM</td>
<td>24 +/- 1.2 uM</td>
<td>57 +/- 6.4 uM</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>β-lac AB (Sigma)</td>
<td>4 +/- 0.8 uM</td>
<td>7 +/- 1.3 uM</td>
<td>16 +/- 2.3 uM</td>
<td>24 +/- 1.3 uM</td>
<td>41 +/- 7 uM</td>
</tr>
<tr>
<td>UHT 1X β-lac AB (Davisco)</td>
<td>6 +/- 0.7 uM</td>
<td>11 +/- 1 uM</td>
<td>8 +/- 1.1 uM</td>
<td>15 +/- 2.1 uM</td>
<td>N.D.</td>
</tr>
<tr>
<td>UHT 2X β-lac AB (Davisco)</td>
<td>5 +/- 0.5 uM</td>
<td>7 +/- 0.6 uM</td>
<td>14 +/- 1.8 uM</td>
<td>26 +/- 3 uM</td>
<td>27 +/- 2 uM</td>
</tr>
<tr>
<td>α-Casein</td>
<td>51 +/- 3.5 nM</td>
<td>55 +/- 3.3 nM</td>
<td>103 +/- 10 nM</td>
<td>41 +/- 2 nM</td>
<td>31 +/- 3 nM</td>
</tr>
<tr>
<td>β-Casein</td>
<td>133 +/- 15 nM</td>
<td>185 +/- 19 nM</td>
<td>219 +/- 12 nM</td>
<td>249 +/- 21 nM</td>
<td>225 +/- 29 nM</td>
</tr>
<tr>
<td>κ-Casein</td>
<td>103 +/- 9 nM</td>
<td>136 +/- 9 nM</td>
<td>168 +/- 14 nM</td>
<td>154 +/- 15 nM</td>
<td>187 +/- 20 nM</td>
</tr>
<tr>
<td>Calcium Caseinate</td>
<td>5.7 +/- 0.6 nM</td>
<td>6.3 +/- 0.6 nM</td>
<td>6.3 +/- 0.6 nM</td>
<td>9.0 +/- 1.0 nM</td>
<td>7.0 +/- 0.9 nM</td>
</tr>
<tr>
<td>UHT Casein</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>18 +/- 1.2 nM</td>
<td>17 +/- 1.0 nM</td>
</tr>
</tbody>
</table>

*Table 5.4: Relative comparison of $K_d$ values determined for binding interactions between β-lactoglobulin and casein variants. Top row represents immobilized “ligands”, left-most column represents “analytes.”*
5.4. Discussion

Table 5.5 summarizes the results of the examination of the interaction between β-lactoglobulin and the casein subunits.

<table>
<thead>
<tr>
<th></th>
<th>FTIR Spectroscopy</th>
<th>SPR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T° of 1692 cm⁻¹</td>
<td>Relative affinity to β-lactoglobulin</td>
</tr>
<tr>
<td>β-casein + β-lac</td>
<td>50°C</td>
<td>Sequence 2</td>
</tr>
<tr>
<td>α-casein + β-lac</td>
<td>45°C</td>
<td>Sequence 2</td>
</tr>
<tr>
<td>κ-casein + β-lac</td>
<td>30°C</td>
<td>Sequence 1</td>
</tr>
<tr>
<td>β-lac</td>
<td>30°C</td>
<td>Sequence 1</td>
</tr>
</tbody>
</table>

Table 5.5: Summary table of the results of our examinations of the interaction between β-lactoglobulin and casein

Using FTIR spectroscopy we successfully confirmed the reports of other authors (Bhattacharyya and Das, 1999a; Morgan et al., 2005a; Zhang et al., 2005b) in that α- and β-caseins alter the heat stability of β-lactoglobulin. β-casein had the strongest effect followed by α-casein, and finally κ-casein had a minor effect. Using 2D correlation analysis we showed that α- and β-caseins, which had the most significant impact on heat stability of β-lactoglobulin, induced a change in its unfolding sequence by delaying loss of tertiary structure. More specifically, the hydrophobic core was protected from exposure to solvent for a longer period of time suggesting that the interaction between these proteins occurs at this opening and may be of hydrophobic nature.

The results of the SPR study correlated well with the FTIR studies by showing that β-casein, which had the largest impact on β-lactoglobulin’s heat stability also had the highest affinity for it. α-casein had an affinity which was slightly lower than that of β-casein and finally, κ-casein’s affinity for β-lactoglobulin was quite weak. Although the results of the 2D correlation study suggest that the interaction is mediated by hydrophobic interaction, surface hydrophobicity cannot be the only factor determining the binding affinity since the order of relative surface hydrophobicities of the casein subunits is as follows β-casein>κ-casein>α-casein (Marchesseau et al., 2002), which does not match the order of binding affinities.
One explanation for this is that the secondary and tertiary structures of α-, β- and κ-caseins alter the availability of the hydrophobic sites for interaction due to steric hindrance. Figure 5.12 displays predicted 3D structures of the casein subunits as reported by Kumosinski and co-workers (Kumosinski et al., 1991;Kumosinski et al., 1993a;Kumosinski et al., 1993b) with the major hydrophobic regions highlighted in pink.

![3D structures of casein subunits](source: Kumosinski 1991, 1993)

Considering the predicted 3D structures of the casein subunits one might imagine that β-casein would be most likely to engage in hydrophobic interactions since it has a strongly hydrophobic region which is easily accessible from many angles. α-casein’s largest hydrophobic region is not quite as hydrophobic as that of β-casein but it also appears to be quite easily accessible from many sides. Finally κ-casein, although it is more strongly hydrophobic than α-casein (~2x more surface hydrophobicity than αs2-casein and ~3x more surface hydrophobicity than αs1 casein as determined by relative retention time in hydrophobic interaction...
chromatography (Lieske and Konrad, 1995), the folded structure presents fewer access points to the hydrophobic portion. Hence, \( \kappa \)-casein would have the weakest binding affinity for \( \beta \)-lactoglobulin during a hydrophobic interaction. This same reasoning holds true to explain the binding affinities of the casein subunits for each other. \( \beta \)-casein, with its well exposed strongly hydrophobic region had the strongest binding affinities for the other caseins, followed by \( \alpha \)-casein and finally \( \kappa \)-casein. Since only a relatively small portion of \( \beta \)-lactoglobulin appears to be involved in this interaction (the opening of the calyx), the lower surface of contact results in the \( \beta \)-lactoglobulin-casein interactions being weaker than casein-casein interactions.

5.5. Conclusions

To achieve the overall objective of this study, we propose a possible scenario for the various interactions occurring in high protein solutions following UHT treatment. Figure 5.13 illustrates a simplified version of this scenario where only small fragments of casein micelles are shown with the calcium phosphate interactions between casein subunits not depicted. Freeman and Mangino explained that following UHT treatment, there is an initial increase in casein micelles size followed by fragmentation and appearance of many smaller diffuse casein particles, particularly with higher processing temperatures such as the ones used in our study (Freeman and Mangino, 1981). One can imagine that these fragmented, diffuse casein particles would no longer retain the well ordered structure in which \( \kappa \)-casein is found all around the surface of the casein micelle and its hydrophilic tails protrude into the aqueous environment and act as an interface between the hydrophilic environment and the hydrophobic core of the casein micelle (Creamer et al., 1998). Such casein fragments would be much more prone to aggregation and could potentially form the type of gelled sediment that we observed in our solutions of UHT processed 5% caseinate. In UHT milk there is a significant fat content and following UHT processing caseins adhere to the fat globule (Hillbrick et al., 1999) and the hydrophobic portions of caseins are more likely to be burrowed in the fat globule and less available for interaction, thus the formation of \( \beta \)-lactoglobulin-\( \kappa \)-casein complexes through disulfide bonds predominates eventually leading to age gelation. In high protein beverages the fat content is very low so the hydrophobic portions of caseins are more available for interaction and these could dominate over the more well-known \( \beta \kappa \)-complex interaction arising from disulfide-sulfhydryl exchange. Since only a small portion of \( \beta \)-lactoglobulin appears to be involved in this
hydrophobic interaction (the opening of the calyx), the rest of the $\beta$-lactoglobulin might act as a spacer between caseins thus preventing casein-casein interactions, and hence, gel formation. We have not proven this theory for the mechanism of interaction through experimentation however this could provide a future direction.

With regard to the secondary objective of our study (i.e. casein’s chaperone status), we show that the interaction may not be the result of chaperone behaviour. If we base ourselves on the original definition of a chaperone and assume that chaperone interaction may not occur until the target protein is partly denatured, we find that caseins do not meet the criteria for a chaperone in this case since they associate with $\beta$-lactoglobulin both in the native and partly denatured form. An alternate explanation could be that “native” $\beta$-lactoglobulin used in this study was not truly native and that its structure was somehow altered during its isolation.

![Figure 5.13: An interpretation of some of the interactions which appear to be occurring in UHT processed high protein solutions. Hydrophobic interactions are highlighted in pink, disulfide bonds are highlighted in purple. Other minor interactions, such as hydrogen bonding, are not shown. $\alpha$-lactalbumin interacts with $\kappa$-casein or $\beta$-lactoglobulin molecules via disulfide bonds. $\beta$-lactoglobulin molecules interact with each other by disulfide bonds and with caseins via some disulfide bonds but mainly via hydrophobic interactions. Caseins interact with each other by hydrophobic interaction.](image-url)
Chapter 6

General Summary

Much like UHT processed milk, UHT high protein beverages produced using milk proteins, suffer from storage defects such as sedimentation and age gelation. High protein beverages may contain as much as 15 different ingredients, thus making them very different and more complex than regular milk. Although in UHT milk, gelation occurs after approximately 12 months of storage in affected batches, gelation of high protein beverages usually occurs after just 6 months of storage. Therefore, these defects significantly limit the shelf-life of UHT high protein beverages and result in large economic losses for their manufacturers. Considering the magnitude of this problem, it is unfortunate that we have not been able to find any literature on the subject.

We approached the task of researching storage stability of high protein beverages with a focus on protein-protein interactions. ATR-FTIR spectroscopy was evaluated as a tool for studying milk proteins. It was found that using this technique it was possible to study milk proteins in aqueous solution and note general changes in the spectra. Additionally, it was possible to successfully obtain thermodynamic data about protein unfolding using ATR-FTIR spectroscopy.

Fifty different permutations of milk protein ingredients (mostly without the presence of the other beverage ingredients) were UHT processed. Storage stability of these solutions was assessed using a variety of techniques. Unfortunately, ATR-FTIR spectroscopy proved not to be useful for following aging of high protein samples. Due to the poorer signal-to-noise as compared to transmission FTIR spectroscopy, even after spectral enhancement using Fourier self-deconvolution or second derivatives, it was not possible to identify specific secondary structures or to detect small changes in protein quantity. Other techniques used included visual observation, pH and viscosity measurements and total solids measurement.
One of the most noteworthy observations we made was that when permutations containing a higher amount of casein were UHT processed there was more sediment, indicating that like in milk, casein is the likely culprit in sedimentation. It was also noted that when the proteins were processed alone, or when all the other beverage ingredients were processed in the absence of protein, there was never any gelation. Sediment was present with any combination of ingredients, whether or not proteins were present indicating that non-protein ingredients also contribute to sediment formation in the beverages. In permutations containing both the proteins and non-protein beverage ingredients gelation was sometimes observed after just 3 weeks of storage. This indicates that gelation in high protein beverages may be an interaction between proteins and some other beverage component, such as complex carbohydrates or gums. Alternatively this may be the result of some involvement of fatty components which block hydrophobic sites when proteins adhere to them, thus encouraging βκ-complex formation and gelation. Finally, one other interesting observation made was that adding increasing amounts of β-lactoglobulin to caseinate prevented the formation of a hard gel in the sediment. This led to another subset of our study.

Without a better understanding of the different interactions occurring in high protein beverages, it would be extremely difficult, if not impossible, to control the storage stability of these beverages. As a first step to understanding the interactions between the beverage ingredients, a study of the interaction between β-lactoglobulin and casein was undertaken. These studies revealed that there was an interaction between native β-lactoglobulin and the casein subunits. The interaction was particularly strong between β-lactoglobulin and β- and α-casein, and it resulted in an enhancement of β-lactoglobulin’s heat stability and a change in its sequence of events during unfolding. Improved protection of β-lactoglobulin’s hydrophobic core suggests that the reaction is mediated by hydrophobic interaction. By combining results of the FTIR spectroscopy study and SPR, a scenario of protein interactions found in high protein beverages following UHT treatment was proposed (Figure 5.9).

Additionally, the observation that binding affinity was unchanged whether β-lactoglobulin was native or slightly heat damaged by UHT processing, suggested that this particular interaction does not meet the criteria for chaperone activity.
Overall, we have acquired a better understanding of the interactions and thermal stability of the various protein components in high protein beverages. We have taken a fundamental approach in beginning to study specific protein interactions occurring in high protein beverages with the hope that some day when more interactions in these beverages are characterized, we can begin to better control the storage stability of high protein beverages.

Chapter 7
Future Work

Having confirmed an interaction between \( \beta \)-lactoglobulin and the casein subunits, it would be interesting to definitively determine the nature of this interaction. Our results to date suggested that hydrophobic interactions play an important role. This could be confirmed in the future using hydrophobic immobilization of casein subunits onto SPR sensor chips. With the major hydrophobic portions of caseins made unavailable, \( \beta \)-lactoglobulin-casein association should be greatly reduced if their interaction is truly hydrophobic in nature.

If our intention is to inhibit gelation of high protein beverages, it is essential to determine what beverage ingredient the proteins are interacting with to form a gel network. The simplest approach would be to execute a series of UHT trials in which the non-protein beverage ingredients are combined one at a time with the protein ingredients. When gelation is observed and the culprit non-protein ingredient is identified, their mode of interaction can be examined and an approach to preventing gelation might be proposed.
References

Ref Type: Report

Ref Type: Electronic Citation

General Electric Company.

Ref Type: Electronic Citation


Ref Type: Electronic Citation


FMC BioPolymer. Carrageenan-Milk Protein Interactions. 2008. Ref Type: Pamphlet


ref Type: Lecture Notes. Ohio State University.

ref Type: Lecture Notes. Ohio State University.


**List of Abbreviations**

3D: Three Dimensional
ANS: Anilinonaphthalene-8-sulfonic acid
ATR: Attenuated Total Reflectance
BSA: Bovine Serum Albumin
EDTA: Ethylenediaminetetraacetic acid
FSD: Fourier Self-Deconvolution
FTIR: Fourier Transform Infrared
HCA: Hierarchal Cluster Analysis
HTST: High-Temperature-Short-Time
IRE: Internal Reflection Element
lbs: Pounds
mL: Millilitre
min: Minute
MPC: Milk Protein Concentrate
MPI: Milk Protein Isolate
NEM: N-ethylmaleimide
nm: Nanometre
s: seconds
SNR: Signal-to-Noise Ratio
SPR: Surface Plasmon Resonance
Temp: Temperature
UHT: Ultra High Temperature
μL: Microlitres
WPC: Whey Protein Concentrate
WPI: Whey Protein Isolate
Appendix

Properties of Major Milk Proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>α-lactalbumin</th>
<th>β-lactoglobulin</th>
<th>αs1-casein</th>
<th>β-casein</th>
<th>κ-casein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of disulfide bonds</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Number of free sulfhydryl groups</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Number of amino acids in sequence</td>
<td>123</td>
<td>162</td>
<td>199</td>
<td>209</td>
<td>169</td>
</tr>
</tbody>
</table>

Table A.1: Comparison of general properties of milk proteins (Source: Mangino and Harper, 2009)

Figure A.1: Crystal structure of bovine α-lactalbumin with a calcium ion bound in the center of the molecule (Source: Troullier et al., 2000. Prepared using MOLSCRIPT (Kraulis, 1991))
Figure A.2: Crystal structure of bovine β-lactoglobulin (source: Brownlow et al., 1997. Prepared using MOLSCRIPT (Kraulis, 1991))
Figure A.3: Full amino acid sequence of bovine α-lactalbumin (Source: Mangino and Harper, 2009b)
Figure A.4: Full amino acid sequence of bovine β-lactoglobulin A (Source: Mangino and Harper, 2009b)
<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>arg</td>
<td>pro</td>
<td>lys</td>
<td>his</td>
<td>pro</td>
<td>ile</td>
<td>lys</td>
<td>his</td>
<td>gln</td>
<td>gly</td>
<td>leu</td>
<td>pro</td>
<td>gln</td>
<td>(glu</td>
<td>val</td>
</tr>
<tr>
<td>30</td>
<td>31</td>
<td>32</td>
<td>33</td>
<td>34</td>
<td>35</td>
<td>36</td>
<td>37</td>
<td>38</td>
<td>39</td>
<td>40</td>
<td>41</td>
<td>42</td>
<td>43</td>
<td>44</td>
</tr>
<tr>
<td>ser</td>
<td>lys</td>
<td>asp</td>
<td>ile</td>
<td>gly</td>
<td>ser</td>
<td>gln</td>
<td>ser</td>
<td>thr</td>
<td>gln</td>
<td>asp</td>
<td>gln</td>
<td>ala</td>
<td>met</td>
<td>gln</td>
</tr>
<tr>
<td>50</td>
<td>51</td>
<td>52</td>
<td>53</td>
<td>54</td>
<td>55</td>
<td>56</td>
<td>57</td>
<td>58</td>
<td>59</td>
<td>60</td>
<td>61</td>
<td>62</td>
<td>63</td>
<td>64</td>
</tr>
<tr>
<td>glu</td>
<td>ala</td>
<td>glu</td>
<td>ser</td>
<td>ile</td>
<td>ser</td>
<td>ser</td>
<td>ser</td>
<td>ser</td>
<td>gln</td>
<td>ile</td>
<td>val</td>
<td>pro</td>
<td>asn</td>
<td>ser</td>
</tr>
<tr>
<td>70</td>
<td>71</td>
<td>72</td>
<td>73</td>
<td>74</td>
<td>75</td>
<td>76</td>
<td>77</td>
<td>78</td>
<td>79</td>
<td>80</td>
<td>81</td>
<td>82</td>
<td>83</td>
<td>84</td>
</tr>
<tr>
<td>ile</td>
<td>gln</td>
<td>lys</td>
<td>glu</td>
<td>asp</td>
<td>val</td>
<td>pro</td>
<td>ser</td>
<td>gln</td>
<td>arg</td>
<td>tyr</td>
<td>leu</td>
<td>gly</td>
<td>tyr</td>
<td>leu</td>
</tr>
<tr>
<td>90</td>
<td>91</td>
<td>92</td>
<td>93</td>
<td>94</td>
<td>95</td>
<td>96</td>
<td>97</td>
<td>98</td>
<td>99</td>
<td>100</td>
<td>101</td>
<td>102</td>
<td>103</td>
<td>104</td>
</tr>
<tr>
<td>leu</td>
<td>lys</td>
<td>lys</td>
<td>tyr</td>
<td>lys</td>
<td>val</td>
<td>pro</td>
<td>gln</td>
<td>leu</td>
<td>glu</td>
<td>ile</td>
<td>val</td>
<td>pro</td>
<td>asn</td>
<td>ser</td>
</tr>
<tr>
<td>110</td>
<td>111</td>
<td>112</td>
<td>113</td>
<td>114</td>
<td>115</td>
<td>116</td>
<td>117</td>
<td>118</td>
<td>119</td>
<td>120</td>
<td>121</td>
<td>122</td>
<td>123</td>
<td>124</td>
</tr>
<tr>
<td>his</td>
<td>ser</td>
<td>met</td>
<td>lys</td>
<td>gln</td>
<td>gln</td>
<td>ile</td>
<td>his</td>
<td>ala</td>
<td>gln</td>
<td>gln</td>
<td>lys</td>
<td>glu</td>
<td>pro</td>
<td>met</td>
</tr>
<tr>
<td>130</td>
<td>131</td>
<td>132</td>
<td>133</td>
<td>134</td>
<td>135</td>
<td>136</td>
<td>137</td>
<td>138</td>
<td>139</td>
<td>140</td>
<td>141</td>
<td>142</td>
<td>143</td>
<td>144</td>
</tr>
<tr>
<td>glu</td>
<td>leu</td>
<td>ala</td>
<td>tyr</td>
<td>phe</td>
<td>tyr</td>
<td>pro</td>
<td>gln</td>
<td>leu</td>
<td>phe</td>
<td>arg</td>
<td>gln</td>
<td>phe</td>
<td>tyr</td>
<td>glu</td>
</tr>
<tr>
<td>150</td>
<td>151</td>
<td>152</td>
<td>153</td>
<td>154</td>
<td>155</td>
<td>156</td>
<td>157</td>
<td>158</td>
<td>159</td>
<td>160</td>
<td>161</td>
<td>162</td>
<td>163</td>
<td>164</td>
</tr>
<tr>
<td>scr</td>
<td>gly</td>
<td>ala</td>
<td>trp</td>
<td>tyr</td>
<td>tyr</td>
<td>val</td>
<td>pro</td>
<td>leu</td>
<td>gly</td>
<td>thr</td>
<td>gln</td>
<td>tyr</td>
<td>thr</td>
<td>asp</td>
</tr>
<tr>
<td>170</td>
<td>171</td>
<td>172</td>
<td>173</td>
<td>174</td>
<td>175</td>
<td>176</td>
<td>177</td>
<td>178</td>
<td>179</td>
<td>180</td>
<td>181</td>
<td>182</td>
<td>183</td>
<td>184</td>
</tr>
<tr>
<td>asp</td>
<td>ile</td>
<td>pro</td>
<td>asn</td>
<td>pro</td>
<td>ile</td>
<td>gly</td>
<td>ser</td>
<td>gln</td>
<td>asn</td>
<td>ser</td>
<td>gln</td>
<td>lys</td>
<td>thr</td>
<td>thr</td>
</tr>
</tbody>
</table>

Figure A.5: Full amino acid sequence of bovine $\alpha_{s1}$-casein (Source: Mangino and Harper, 2009a)
Figure A.6: Full amino acid sequence of bovine β-casein (Source: Mangino and Harper, 2009a)
<table>
<thead>
<tr>
<th></th>
<th>Glu</th>
<th>Glu</th>
<th>Gln</th>
<th>Asn</th>
<th>Gln</th>
<th>Gln</th>
<th>Gln</th>
<th>Pro</th>
<th>Ile</th>
<th>Arg</th>
<th>Cys</th>
<th>Glu</th>
<th>Lys</th>
<th>Asp</th>
<th>Glu</th>
<th>Arg</th>
<th>Phe</th>
<th>Phe</th>
<th>Ser</th>
<th>Asp</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>Lys</td>
<td>Ile</td>
<td>Ala</td>
<td>Lys</td>
<td>Tyr</td>
<td>Ile</td>
<td>Pro</td>
<td>Ile</td>
<td>Gln</td>
<td>Tyr</td>
<td>Val</td>
<td>Leu</td>
<td>Ser</td>
<td>Arg</td>
<td>Tyr</td>
<td>Pro</td>
<td>Ser</td>
<td>Tyr</td>
<td>Gly</td>
<td>Leu</td>
</tr>
<tr>
<td>41</td>
<td>Asn</td>
<td>Tyr</td>
<td>Tyr</td>
<td>Gln</td>
<td>Gln</td>
<td>Lys</td>
<td>Pro</td>
<td>Val</td>
<td>Ala</td>
<td>Leu</td>
<td>Ile</td>
<td>Asn</td>
<td>Asn</td>
<td>Gln</td>
<td>Phe</td>
<td>Lue</td>
<td>Pro</td>
<td>Tyr</td>
<td>Pro</td>
<td>Tyr</td>
</tr>
<tr>
<td>61</td>
<td>Tyr</td>
<td>Ala</td>
<td>Lys</td>
<td>Pro</td>
<td>Ala</td>
<td>Ala</td>
<td>Val</td>
<td>Arg</td>
<td>Ser</td>
<td>Pro</td>
<td>Ala</td>
<td>Gln</td>
<td>Ile</td>
<td>Leu</td>
<td>Gln</td>
<td>Trp</td>
<td>Gln</td>
<td>Val</td>
<td>Leu</td>
<td>Ser</td>
</tr>
<tr>
<td>81</td>
<td>Asp</td>
<td>Thr</td>
<td>Val</td>
<td>Pro</td>
<td>Ala</td>
<td>Lys</td>
<td>Ser</td>
<td>Cys</td>
<td>Gln</td>
<td>Ala</td>
<td>Gln</td>
<td>Pro</td>
<td>Thr</td>
<td>Thr</td>
<td>Met</td>
<td>Ala</td>
<td>Arg</td>
<td>His</td>
<td>Pro</td>
<td>His</td>
</tr>
<tr>
<td>101</td>
<td>Pro</td>
<td>His</td>
<td>Leu</td>
<td>Ser</td>
<td>Phe</td>
<td>Met</td>
<td>Ala</td>
<td>Ile</td>
<td>Pro</td>
<td>Pro</td>
<td>Lys</td>
<td>Lys</td>
<td>Asn</td>
<td>Gln</td>
<td>Asp</td>
<td>Lys</td>
<td>Thr</td>
<td>Gln</td>
<td>Ile</td>
<td>Pro</td>
</tr>
<tr>
<td>121</td>
<td>Thr</td>
<td>Ile</td>
<td>Asn</td>
<td>Thr</td>
<td>Ile</td>
<td>Ala</td>
<td>Ser</td>
<td>Gly</td>
<td>Glu</td>
<td>Pro</td>
<td>Thr</td>
<td>Ser</td>
<td>Thr</td>
<td>Pro</td>
<td>Thr</td>
<td>Thr</td>
<td>Gln</td>
<td>Ala</td>
<td>Val</td>
<td>Glu</td>
</tr>
<tr>
<td>141</td>
<td>Ser</td>
<td>Thr</td>
<td>Val</td>
<td>Ala</td>
<td>Thr</td>
<td>Leu</td>
<td>Gln</td>
<td>Asp</td>
<td>Ser</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>161</td>
<td>Thr</td>
<td>Val</td>
<td>Gln</td>
<td>Val</td>
<td>Thr</td>
<td>Ser</td>
<td>Thr</td>
<td>Ala</td>
<td>Val</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure A.7: Full amino acid sequence of bovine κ-casein (Source: Mangino and Harper, 2009a)