Heat-induced casein-whey protein interactions: influence of protein and mineral composition

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"Simplicity is the ultimate sophistication" attributed to Leonardo da Vinci

"Il semble que la perfection soit atteinte non quand il n’y a plus rien à ajouter, mais quand il n’y a plus rien à retrancher." Antoine de Saint Exupéry
Acknowledgments

Dedicated to,

the people that made me what I am today.

To my untypical wonderful family, that gave me the values that constitute me and that I apply in everything I do. They are my heart, and they do make me proud. To my grand-mother a special thank you for all the more-than-needed interest and support given at all stages.

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With the biggest wish of success to all...

Honestly, Dank u well.
**Resumo**

A estabilidade térmica de leite e concentrados de leite é um tema atual e relevante. Uma mistura de leite magro em pó (SMP) foi sujeita a um tratamento térmico de 90°C durante 15 minutos com adição de caseinatos, cloretos, citratos e fosfatos a pHs 6,5, 6,7 e 6,9. Verificaram-se serem melhores estabilizadores os caseinatos de íons-monovalentes, cloreto de cálcio e citrato de sódio. Concentrados de leite (MPC60, MPC80 e MPC90) foram sujeitos ao mesmo tratamento térmico e pH prévios. As adições proteicas consistiram na adição de proteínas do soro (de leite) ou de isolados de β-lactoglobulina ou α-lactalbúmina. Concluiu-se que as proteínas do soro do leite destabilizam o SMP mas estabilizam os concentrados, sendo que quanto maior o conteúdo proteico do concentrado, maior a quantidade de proteínas do soro a adicionar (até 2% (m/m)). Verificou-se também que a proteína responsável pela estabilização é a β-lg. O processo foi testado à escala piloto com SMP e MPC80 industriais, a pH 6,5, suplementando-os com 1% (m/m) de proteínas do soro e sujeitando-os a quatro tratamentos térmicos: dois num permutador de calor da instalação-piloto (90°C durante 10 minutos e 140°C durante 5 segundos (UHT), num banho-termostatizado laboratorial (90°C durante 15 minutos) e na autoclave (121°C durante 15 minutos). No tratamento em autoclave apenas o SMP permaneceu estável. Devido a indícios de coagulação, o tratamento UHT não foi aplicado ao MPC80. A possibilidade de *scale-up* confirmou-se, uma vez que se revelaram as tendências laboratoriais previamente observadas, pelo tratamento durante 10 minutos de 90°C.

**Palavras-chave:** Leite magro em pó, Concentrados proteicos de leite, estabilidade térmica, coagulação, proteínas do soro de leite, caseínas
Abstract

Heat-stability of milk and concentrates is an actual and relevant problem. A heat treatment of 90°C during 15 minutes in a water bath was imposed to skim milk powder (SMP) with added caseinates, chlorides or citrates and phosphates at the pH values of 6.5, 6.7 and 6.9. Monovalent-ions caseinates, calcium chloride and sodium citrate proved to be the best stabilizers. Milk concentrates MPC60, MPC80 and MPC90, and SMP were subjected to the same thermal treatment and pH values referred. Protein additions consisted of either whey proteins, β-lactoglobulin or α-lactalbumin isolates. It was concluded that whey protein additions destabilize SMP systems and stabilize MPCs. Also, the higher the MPC protein content, the more whey protein is needed (until 2% (w/w)). The whey protein responsible for stabilization was seen to be specifically β-lg. The scalability of this process was tested with industrial SMP and MPC80, at pH 6.5, supplemented with 1% (w/w) of WPI. Four treatments were applied: two at the pilot plant’s heat exchanger (90°C for 10 minutes and 140°C for 5 seconds (UHT), a laboratory’s water bath (90°C for 15 minutes) and the autoclave (121°C for 15 minutes). Autoclave proved to be excessively harsh (only SMP remaining stable). Due to instability indications, the UHT treatment was not assayed for MPC80. The treatment at 90°C for 10 minutes proved the possible scale-up, once laboratory trends perpetuated.

Keywords: Skim Milk Protein, Milk Protein Concentrate, heat-stability, whey-proteins, caseins
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Nomenclature

$\alpha$-lac $\alpha$-lactalbumin

$\alpha_{s1}$-CN $\alpha_{s1}$-casein

$\alpha_{s2}$-CN $\alpha_{s2}$-casein

$\beta$-CN $\beta$-casein

$\beta$-Ig $\beta$-lactoglobulin

$\kappa$-CN $\kappa$-casein

CCP colloidal calcium phosphate

DLS Dynamic Light Scattering

DTT Dithiothreitol

HCT Heat Coagulation Time

HCTemp Heat Coagulation Temperature

HPLC High-Performance Liquid Chromatography

MPC Milk Protein Concentrate

MPI Milk Protein Isolate

pI isoelectric point

SMP Skim Milk Powder

WP Whey Proteins

WPI Whey Protein Isolate
Chapter 1

Introduction

1.1 Relevance to Society

Milk and its derivatives are largely consumed all around the world and have been for at least 8000 years, when cattle started being domesticated [1].

Due to the clear intrinsic role in the current society, the word “milk” is already a synonym for bovine milk, accounting for 84% out of all animal milk production. Considering a total world population of 7.1 billion people, it is remarkable that more than 6 billion consume dairy products either in the shape of milk and/or milk derivatives. Europe, along with Argentina, Armenia, Australia, Costa Rica, Israel, Kyrgyzstan, North America and Pakistan is classified as a high consumption region, with values that reach in average 150 kg, per capita and per year [2].

Forecasts have predicted that a growth of 2% in production would still take place in 2015, in the image of previous years [2].

The described popularity owns itself to two different motives: not only it is a result of its nutritional role, but also of its physicochemical characteristics. Besides being the single most complete food [3], it is also able to give texture and structure to products like yogurt and cheese, and has the capacity to integrate stable air bubbles, originating foams.

Due to the versatility of milk, it can be incorporated into thousands of different applications. Even with globalization and by looking at different countries and cultures, moderated or abrupt changes will be found within dairy products. Those dairy products include of course, raw milk, but also supplemented milks (without lactose, with added calcium, etc.), condensed, evaporated and concentrated milks, infant formulas, ice cream, yogurt, cheese, sour cream or quark and whipped cream, just to name a few. It is also used in more unexpected applications, like meat substitutes, and in more trendy ones like athletes supplements. The character of each final product depends on a lot of factors, starting on the milk source, the microorganisms that were possibly employed to produce it, the additives and also on the heat treatment applied.

In order to avoid the utilization of fresh milk which has a limited shelf-time, and to assure to give origin to all of the aforementioned products, the raw material often used is either milk powders or milk powder
concentrates (MPC), and considering they show some very different properties in the same imposed circumstances, both must be characterized and tested.

The conditions of the heat treatment applied depend strongly on the final product - there are milder heat treatments and intenser ones. For example, for refrigerated products lower temperatures can be enough, while for room temperature kept products, higher temperatures are needed [4] [5].

The heating process that is acknowledged by the public in general is pasteurization, in which a liquid is heated below the boiling point, and that was developed by Louis Pasteur in 1864 for wine. Its commercial use for milk, on its turn, began in the late 1800s in Europe and in the early 1900s in the United States. The purpose of executing this procedure is to increase the safety of milk by destroying pathogens (disease causing microorganisms) that may be present, since milk provides excellent growth conditions for their survival and multiplication. To increase even more the reliability of milk products, harsher treatments can be done, destroying not only disease causing microorganisms but also spoilage microorganisms and enzymes that would contribute to reduce the quality and shelf life of milk. For example, new pasteurization conditions that are still in use today were adopted in order to inactivate *Coxiella burnetii*, becoming them 62.8°C for 30 minutes in a batch process, and to 71.7°C for 15 seconds in a continuous process [5].

Often, especially for concentrated products, intense sterilization treatments are needed, as a result of which physical instability of the product may occur. This instability may manifest itself in essentially two forms; as flocculation and gelation during heat treatment, adopting an yogurt-like appearance, but also in the form of (protein) aggregates formed during heat treatment, that although not directly visible to the naked eye, appear as sediments during storage [4].

The problems of regulating heat stability - the relative resistance of milk upon sterilization that leads to coagulation - appeared over a century ago in the production of evaporated milk. The first patent made for “producing concentrated milk by evaporation in vacuum without addition of sugars and other preservatives” dates back to 1856 by Gail Borden [6].

The commercial production of concentrated milk increased gradually during the First World War and Second World War, and concentrated milk became one of the major dairy products in the 1920s, because of easy transport and long shelf life. To overcome the recurrent problems faced back then - milk’s gelation and excessive thickening of the product during storage - these issues were controlled by carrying out various heat-stability tests on raw milk, and by running pilot sterilization trials on samples from each batch after the addition of various amounts of sodium bicarbonate [6]. This sodium bicarbonate would serve as a buffer, moderating mineral activity, one of the most destabilizing factors, namely it would reason calcium ion activity by binding to available calcium.

The first paper about this subject was published by Sommer and Hart in 1919 [7], and after that accumulating literature has slowly added information to the subject, being the most important contributors Rose, Fox, O’Connell and Singh, among others [8] [9] [10] [11] [1]. Even though the information has been reviewed periodically, the mechanism and pH-dependence of the heat-induced coagulation of milk still intrigues scientists. It is clear that limited progress has been made on explaining either the variability seen or sequence of this physico-chemical behaviour; and it is also unquestionable that problems
related with instability are still found in the industry nowadays, especially with concentrated products [9].

The mentioned instability in form of aggregation also exists in vivo. Naturally, it occurs in the stomach of the neonate in order to delay and improve digestibility. It was by taking advantage of this inherent process that the first humankind reports of cheese production were seen; they involved the use of calf’s stomach or only its natural enzymes, seeking to accelerate aggregation [1]. It is then clear that the referred destabilization can be undesirable in sterilization cases, or it can be exploited in the production of cheese and fermented milk products, among others.

There are dissimilar ways to address the problem of instability. The ideal one would be to understand step-by-step the mechanism by which it occurs. Nevertheless, due to its impracticability, other foreseees are needed. The first step to address the problem is the requirement to have the best knowledge possible of the structures of milk’s components; then, isolated trials are attempted, trying to re-balance milk’s constituents to recognize the possible guiltiness involved of each one of them; and finally, a more practical way, in which empiric tests are executed to decide how to perform in very specific circumstances.

For control purposes, several tests and indicators of the susceptibility of products were created, some more subjective and some more objective. A subjective method that has proven to be both easy and very efficient to evaluate the stability of systems is the eye-recognition of coagulation, in which the aggregates must have around 0.1 mm to be seen. Inside this intuitive method there are then parameters that allow the correlation between samples, specifically the Heating Coagulation Time (HCT) and the Heating Coagulation Temperature (HCTemp), where glass tubes samples are placed in a hot water bath, measuring respectively either the time it takes or the temperature required for coagulation to occur. Even though HCTemp is a more accurate indicator of susceptibility, it is very time-consuming, reason why curves have been mainly drawn explaining time variation, HCT, with pH.

Resulting from these tests, for unconcentrated milks the existence of a maximum of stability at milk’s normal pH was discovered, or in other words, a higher coagulation time (HCT) at a pH of 6.7, followed by decreases in stability (and in the coagulation time) for both higher and lower pHs. For concentrated milks, however, a slightly distinct behaviour appears, where stability will be maintained after the maximum (at a pH of 6.6) and decrease only for lower pHs [11].

The importance of this matter is to try to establish a correlation between pH and instability, since it is known that through the process of heating pH naturally drops, and that this drop of pH will be decisive for other reactions to take place. Adding to this, it is essential to refer that in a general way no signs of instability are verified if milk’s natural pH is maintained during the whole process of heating, even for treatments of more than 15 minutes at 140°C - conditions far extreme than those commercially applied. This backs-up the strong pH-dependence of stability, where even small changes of 0.1 pH units can have big impacts on stability and behaviour [4]. Previous old reports, firstly by Pyne in 1958, and then by Fox in 1981 would also reinforce that milk could take a heating process for at least 3 hours at 140°C without signs of coagulation, just by constantly assuring it to be readjusted to its natural pH [10].

To be able to perfectly control the process of heating without compromising the product, it is important to understand what it includes and what is happening at a molecular level. For that, it is fundamental to be aware of milk’s content and how it reacts upon a certain heat load applied.
1.2 Theoretical Background

According to the 2005 book, Dairy Science and Technology [12], milk is largely constituted by water (87.1%), but also by fat (4%), sugars/lactose (4.6%), proteins (3.3%) out of which 2.6% are caseins and salts (0.85%).

The distinctive carbohydrate/sugar of milk is lactose, a disaccharide composed of glucose and galactose. Lactose is practically the only sugar existent in milk. Regarding the existing fat, it is predominantly made of triglycerides, varying greatly on size and saturation.

Inside protein’s category, it is possible to find a residual wide range of enzymes, besides two very distinct classes, namely caseins and whey-proteins. These two families have very contrasting characteristics. For the caseins, that represent roughly 80% of the total existing proteins, we can find $\alpha_{s1}$-casein, $\alpha_{s2}$-casein, $\beta$-casein, and $\kappa$-casein, also represented respectively by $\alpha_{s1}$-CN, $\alpha_{s2}$-CN, $\beta$-CN, and $\kappa$-CN. Whey-proteins, on the other hand, include $\alpha$-lactalbumin and $\beta$-lactoglobulin, respectively $\alpha$-lac and $\beta$-lg [12].

The main advantage and trick for controlling these proteins is indeed their very dissimilar answer to imposed physical conditions. Caseins coagulate at an acidic pH of approximately 5, and are stable at high temperatures. Whey-proteins, in opposition, coagulate at temperatures higher than 65°C and are stable on acidification. These characteristics are essential for the separation and purification of these components, and also to manufacture some products like cheese. A summary of the characteristics is presented in Table 1.1.

The thermal stability revealed by caseins is due to a loose, ill-defined three-dimensional structure formed by the association of its main proteins, instead of whey proteins’ quite heat labile compact globular structures with unique native conformations. The shape in which they are presented in solution at normal pH and temperature is also very different. Like referred, caseins appear together organized in loosen spherical structures, where thousands of phosphoproteins are linked through calcium phosphate nanoclusters. These colloidal spheres are denominated casein micelles, and have hydrophilic and hydrophobic sections. They are essential in the calcium phosphate transport, and are slowly digested inside organisms, due to long-lasting protein breakdown [12]. A more detailed and enlarged description of these structures and interactions will follow below.

Whey proteins, on their turn, appear generally as individual proteins with little secondary or tertiary structure. Due to their branched-chain aminoacids, they are fast and easily digested, reason why they are consumed by athletes, providing quick available energy. At room temperature, in milk, these two families of proteins do not interact with each-other.

Another category of milk components that have a crucial role are the mineral substances that it contains in solution — primarily K, Na, Ca, Mg, Cl, phosphate and numerous other elements in trace quantities. The organic acids that are present occur largely as ions or as salts - citrate being the principal one. In the end, it can be stated that milk is a set of particles, implying that reactivity can be changed by breaking their natural barriers and compartments or by rearranging components interaction. For this motive, it is fundamental to know milk’s structural composition, as it allows to interpret its physical
Table 1.1: Milk proteins (caseins and whey proteins) main properties.

<table>
<thead>
<tr>
<th>Property</th>
<th>Caseins</th>
<th>Whey proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sedimentability</td>
<td>pH&lt;5</td>
<td>denaturation T&gt;65°C</td>
</tr>
<tr>
<td>Stable</td>
<td>at high Temperatures</td>
<td>at acidic pHs</td>
</tr>
<tr>
<td>Structure</td>
<td>part of calcium phosphate micelles</td>
<td>individual; secondary or tertiary structures</td>
</tr>
<tr>
<td>Digested</td>
<td>slowly</td>
<td>fast</td>
</tr>
</tbody>
</table>

The caseins that occur in the micelles are in its ionic form, caseinate, meaning that they bind with cations, primarily calcium and magnesium but also calcium phosphate and residual citrate [12]. The calcium phosphate existent in the casein micelles is often referred to as colloidal calcium phosphate (CCP).

There is a lot of debate regarding the structure of casein micelles, but four points are consensual between researchers: the existence of both water and calcium phosphate nanoclusters in the core; a structural integrity that is kept by the protein-calcium phosphate interaction; and, finally, that there is $\kappa$-casein located on the outer spherical surface [13].

This $\kappa$-casein that is located at the outside spherical surface has a C-terminal placed in the serum and a N-terminal attaching it to the rest of the micelle. The C-terminal is very important once it is responsible for steric-stabilization - the mechanism that explains the ability to inhibit the coagulation of suspensions [14].

According to the nanocluster model, the casein micelle is a homogeneous matrix of caseins in which the CCP nanoclusters are dispersed as very small “cherry stones”. Attached to the surface of these nanoclusters are the centers of phosphorylation in the caseins (3-5 nearby phosphorylated amino acid residues). The tails of the caseins, much larger than the CCP nanoclusters, then associate to form a protein matrix. This protein matrix is driven by a collection of weak interactions like hydrophobic interactions, hydrogen bonding, ion bonding, weak electrostatic Van der Waals attraction, among other factors (but not the strong calcium phosphate interactions) leading to self association. The association is highly cooperative and leads to a stable casein micelle. Invariably, $\kappa$-casein is thought to limit the process of self association leading to stabilization of the native casein micelle [15]. A representation is displayed in Figure 1.1.

The destabilization that can then take place is strongly related to $\kappa$-casein localization and integrity. Either by enzymatic hydrolysis (chymosin treatment) or by a collapse caused by acidification of the medium or solvent addition (solvent quality being reduced with e.g. ethanol), if the $\kappa$-casein detaches from the micelle, this one loses integrity and disassembles. Enzymatic hydrolysis is not a natural occurring problem, but acidification caused by heating is, and will cause the collapse by protonating the carboxylate groups of the protein residues, namely either the glutamine or the asparagine [16] [6].

Milk equilibrium is a set of interconnected sensible factors, and changes in temperature will strongly affect the existent balance by influencing the behaviour of two relevant actors: proteins and salts. Ratios
of the amount of either solubilized or ionized salts, and ratios of the amount of proteins interacting with other proteins, and lastly ratios of salt-proteins interactions, will determine the state and also appearance of milk.

The main culpable of the problematic addressed is the incapacity of measuring changes in real time. It is known that some changes take place while heating and that they cause coagulation. What is not known is until which extent or in which order they succeed. The exact changes that occur in mineral balance, the absolute amount of denatured whey proteins at real time, or, the amount of denatured whey proteins that associates with caseins, and also ultimately the extent of caseins disruption, is unknown.

The best solution is to measure the physico-chemical properties before and after the heating process, but it may inclusively pass unaware if stronger reactions take place and are then reasoned. This means that there is a need to rely on the evaluation made of the initial raw conditions and on the evaluation made of the measurable changes presented after the cooling down, at temperatures where pH measurements can be taken and tests performed. There is a possibility that some changes are reversible or inverted with cooling.

The unquestioned effect of the heating process is the pH drop. Therefore, another question that is brought up in this problematic is until what extent this pH drop will influence and push other reactions to take place, and until what extent it is the consequence of alternative reactions taking place. On one way, it is possible to point out some thermal acidification contributors: first, lactose degrading into organic acids (including formic acid); then, the fact that temperature increase generates a decrease in calcium phosphate solubility, causing it to precipitate and release $\text{H}^+$; and, lastly, a dephosphorilation of the caseins takes place, precipitating the released phosphate in the molecular form of tertiary calcium phosphate. All these factors will increase deeply and perhaps maybe even cyclically, the acidification of the medium [4].

Even though all these changes may happen, there is a need to evaluate the work conditions imposed and see if all factors would accordingly be influenced to change. For example, lactose degradation to organic acids will only take place with considerable heat load, i.e. by high temperatures (passing 100° C).

Therefore, in a general way, it can be stated that the heat-induced coagulation of milk is the result of
a large number of changes that occur, and that inside those changes only some can be considered as utterly important, being the remaining ones considered consequences. The utterly important changes are the ones whose results will correctly interpret modifications in pH-HCT curves, either by themselves or by some combination of the four, being them: the heat-induced acidification of milk; the heat-induced precipitation of calcium phosphate; the heat-induced dissociation of $\kappa$-casein; and the heat-induced denaturation of whey proteins and interaction with $\kappa$-casein.

According to Fox [10] in 1981, and to cite the conclusions of his revision work and the directions he advised to follow, "the most important single factor is heat-induced acidity, since periodic neutralization delays coagulation more or less indefinitely even though all other heat-induced changes occur normally. Furthermore, zeta potential and hydration decrease with decreasing pH. Maillard browning appears to be relatively unimportant, and whereas whey proteins and soluble calcium phosphate modify HCT and the shape of the HCT-pH curve, coagulation of caseinate occurs readily in the presence or absence of these factors. Hydrolysis of $\kappa$-casein and dephosphorylation and hydrolysis of casein all appear to be important contributors to coagulation in normal heated milk systems, but their significance can be overridden if pH is maintained close to the original". By which citation one can conclude that even though a lot of factors influence the speed and shape of instability, only one is determining on deciding if it finally occurs or not: the pH drop. Also it gives some insights on factors that may be very influencing but not decisive.

Whey proteins and their interaction with caseins when denatured is believed to affect significantly the heat stability of milk, since different behaviours take place while testing regular milk or whey protein-free milk [17]. During heating, whey proteins suffer some changes like the dissociation of non-covalently bounded oligomers and the unfolding of their native structure and accordingly exposition of reactive amino acids. This may result in possible aggregations with either caseins or other whey proteins, via non-covalent interactions or through thiol or disulphide interchange reactions [17]. Since $\beta$-lg is the primary whey protein which contains a free sulfhydryl group, it will be the one involved in these aggregation interactions with caseins.

It has then be claimed that surface properties of casein micelles rather than interior ones are likely to be of greater importance, and that denatured whey proteins may have a stabilizing effect by connecting there to $\kappa$-casein surface extremities [6]. In Figure 1.2 it is possible to observe an schematic representation of this.

![Figure 1.2: Casein micelles, homogeneous matrix of caseins in which colloidal calcium phosphate nanoclusters are dispersed, are represented. Its $\kappa$-casein (blue curvy lines) are in the outer surface and connect to denatured whey proteins (represented by green circles). Possible ways of interactions between this components are showed.(image from NIZO)](image from NIZO)
The electrostatic stabilization of micelles is caused by an existing considerably negative surface charge (being the zeta potential -13 mV at 20°C) due to dissociated carboxyls and some ester phosphate groups. Around this negative surface, on its turn, there is a layer that provides steric stabilization, by means of flexible, hydrophilic-polypeptide chains that consist mostly on C-terminal segments of $\kappa$-casein [18]. It is this hairy layer of $\kappa$-casein that provides a barrier against aggregation unless it is removed.

Inside the micelle, the individual casein molecules are associated by hydrophobic and electrostatic bonds in which CCP plays an important role [6]. The important factors influencing the colloidal stability of milk are calcium ions and pH, both of which diminish electrostatic repulsions and possibly alter the conformation of $\kappa$-casein at the micelle surface (indirectly reducing steric repulsions). Heat treatment will markedly change the serum phase environment around the casein micelles, changing pH and soluble minerals, in particular, calcium ions, breakdown of lactose and urea, as well as changes in the casein micelles themselves (association of whey proteins, changes in CCP, dephosphorylation and casein dissociation) [6]. Heat stability will increase every time calcium ion activity is reduced, since it has effects on $\kappa$-caseins dissociation and on its association with whey proteins [4].

Supporting this is experimental data that verified that at low pH values, most of the denatured whey proteins is found connected with caseins, while at higher pHs the denatured whey proteins are found in the serum, denoting here a distribution pH tendency. Prolonged heating time or temperature has an effect on the extent of denatured whey proteins connection with casein micelles and according to Law and Leaver [19] heat-induced denaturation of whey proteins is enhanced with increasing pH.

Another argument for whey proteins’ importance in stability is the effect that pre-heating may have in this problematic. By pre-heating milk to only assure whey proteins denaturation, by the time the concentration step is performed, milk will withstand a lot better the treatment, increasing the heat stability noted and perhaps even avoiding coagulation.

It is not known exactly which particular changes are directly responsible for coagulation, predispose milk to coagulation or are a consequence of the coagulation process. The initial stages of the heat coagulation process must involve a change in colloidal interactions that allows micelles to approach each other and stay together long enough for chemical reactions to take place. [6]

Essentially, a lot of research in this field has been made. Different conditions have been imposed in a way that most of milk components have been added or taken, to try to explain milk’s stability. It is certain, now, that this instability does not lay on only one factor, but an assembly of factors. These factors, are, for sure, related with the decrease on pH that is verified during heating. The problem in this situation is to define the exact sequence of actions. According to Fox [10] the HCT-pH curve would be influenced by chiefly factors like whey proteins, other heat-denaturable proteins, $\kappa$-casein, colloidal calcium phosphate (CCP), soluble calcium and phosphate, detergents, assay conditions (temperature and agitation), urea, heat treatment, aldehydes, and its concentration or dilution.

At the present work not all factors were evaluated or even considered, but conditions were selected in order to avoid its variability. One example of this is urea, that was not evaluated, and that it is known to have a stabilizing effect once it has a buffer effect that increases stability by diminishing calcium ion
The aim of the performed work is to find how to control casein-whey proteins interactions in both skim milk powder, SMP (spray dried milk) and milk protein concentrates, MPC (milk that has been ultrafiltered) during thermal treatment recurring to mineral or protein additions. By controlling this, coagulation can be avoided or texture building created. Evaluation of how to achieve this is made for three different pHs: 6.5, 6.7 and 6.9.

The heat treatments used were low enough to assure that, for example, lactose and urea do not undergo strong reactions and are kept constants.

Milk Protein Concentrates (MPCs) are powders with high protein content. They are obtained by ultrafiltration sometimes followed by diafiltration. The six existent MPCs are named according to their dry native protein content, a range that goes from approximately 42% to 85%. Instead of considering six MPCs we can, in addition, consider eight. These may include the MPC35, with 35.4% protein, and the MPC90, with 85.8%. The MPC35 is also known as Skim Milk Powder (SMP), and the MPC90 as Milk Protein Isolate (MPI). [20]

Both the names and exact compositions of dry matter, protein, lactose and ash of the MPCs that were used at the developed work are displayed in Table 1.2. Extremely large differences in composition and properties can be found between them, due to the absence or presence of certain components.

Table 1.2: Levels of dry matter, ash, protein and lactose in each of the five Milk Protein Concentrates that were used in the developed work.

<table>
<thead>
<tr>
<th></th>
<th>Dry matter</th>
<th>Protein</th>
<th>Lactose</th>
<th>Ash</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPC35</td>
<td>96.6</td>
<td>35.4</td>
<td>49.6</td>
<td>8.1</td>
</tr>
<tr>
<td>MPC60</td>
<td>96.0</td>
<td>60.8</td>
<td>24.5</td>
<td>7.7</td>
</tr>
<tr>
<td>MPC80</td>
<td>95.4</td>
<td>79.1</td>
<td>6.4</td>
<td>7.7</td>
</tr>
<tr>
<td>MPC85</td>
<td>95.2</td>
<td>84.0</td>
<td>1.8</td>
<td>7.5</td>
</tr>
<tr>
<td>MPC90</td>
<td>95.8</td>
<td>85.8</td>
<td>0.4</td>
<td>7.6</td>
</tr>
</tbody>
</table>

Milk protein concentrates are often used in food industry for the same general applications than milk, which means, for its nutritional or functional properties, like mentioned in the previous chapter 1.1. However, the MPCs can display tailored functionalities, regarding their lack of specific components that are available in milk. The main advantages of using these pass through the fact that their protein is native and through the fact that their natural whey proteins to casein ratio is kept. Also, their low content on carbohydrates and high availability of calcium can be convenient advantage work factors.
At ultrafiltration, milk proteins are increasingly concentrated in the retentate while lactose and some soluble salts are eliminated along with water in the permeate. The level of this concentration will determine which MPC is produced as final product.

The downside associated with the use of MPCs is its low solubility at room temperature - it takes either a long time stirring, a big shear or a higher temperature to fully complete the process of solubilization and to have proper rehydratation. The nature of these interactions and the factors responsible for the loss of solubility are not fully understood yet, but it is known that solubility decreases with protein content increase.

A partial explanation for this loss in solubility must be related with the mineral composition changes - shown in Figure 1.3. With an increase in protein content - and in MPCs' number subsequently - decrease in sodium and potassium (Na, K) relative content takes place, as well as an increase in calcium and phosphorus (Ca, P) content. Inorganic Phosphate, by other side, along with Magnesium is preserved stable.

Figure 1.3: Mineral content variation in the different MPCs is observed with increasing amount of protein, in (w/w)%. (data from NIZO)

In the paper of Crowley et al. [21], MPCs' heat stability is evaluated. It concludes that for a pH inferior to 6.8, the heat stability of MPC suspensions decreases with the increase in protein content of the MPC powders, due to high calcium ion activity. Also that for a pH superior to 6.8, with an increase in protein content of MPC powders, the destabilising influence of calcium ion activity is countered partially by reduced heat-induced κ-casein dissociation. Also that MPC80s’ heat stability could be restored by re-establishing serum composition of skim milk and that fortification with lactose or urea would only affect the heat stability outside the pH region where rapid calcium induced coagulation occurs.
Chapter 2

Methods

The work developed was performed in the laboratory and in the pilot plant. The intent of this transition was to scale up some of the results obtained in the laboratory to discern if they would hold at a bigger scale. Evidently some differences in procedure are seen, due to both the different availability of equipments, but also due to small differences in the materials themselves. In the next sections, laboratory methodology and pilot plant methodology have been separated, explaining methodically the equipments used, the procedure that preceded its utilization and the materials employed.

2.1 Laboratory

It is possible to divide the work performed into different divisional categories according to the main tasks developed, being: the creation of samples by mixing components, a thermal treatment imposed to the samples, the adaptation of the samples to run in a certain equipment - (dilution or reagents/buffer addition, etc.), the methodology of running the equipment and finally, data analysis. An experiment, in the terms defined in this document is defined as the integral of all the aforementioned procedures, starting from the preparation of solutions to the analysis of the data obtained from all equipments. In total, eleven experiments were performed, two in the pilot plant and nine in the laboratory, with one repetition of an experiment to verify the correctness of results.

After the preparation of samples and heat treatment, trials of each sample would be made in four equipments. As described in the following sections, the equipments used were the viscometer Lovis, the particle size meter Zetasizer, the High-Performance Liquid Chromatography and the ultracentrifuge.

Coagulated samples were not tested.

2.1.1 Materials

At laboratory scale the minerals that were used included caseinates from FrieslandCampina DMV, namely sodium caseinate, calcium caseinate, potassium caseinate and magnesium caseinate. Also calcium chloride and magnesium chloride, sodium phosphate and trisodium citrate were used, from Sigma-Aldrich.
Regarding protein additives, whey protein isolate (WPI), and both α-lac and β-lg isolates were supplied from Davisco.

All the chemicals used for preparation of secondary helping solutions that facilitated running the specific procedures for each equipment, were from Sigma-Aldrich. Milli-Q water was also used frequently, from Millipore Corporation.

Concerning milk sources used, the SMP and MPCs for labscale were produced at pilot-plant scale at NIZO food research (Ede, The Netherlands).

### 2.1.2 Preparation of samples

The first step of each experiment was roughly the same for every experiment; to mix at the rightful concentrations all the components involved. Usually dissolving either SMP or MPC in water, and adding a third component in different increasing concentrations. The blank version did not contain the third component. After this, pH was adjusted with either HCl or NaOH and the samples were subjected to thermal treatment. This thermal treatment consisted of 90°C for 15 minutes in almost all experiments.

The heat treatment was performed in two water baths, the first one placed at a temperature of 98°C, only to increase the temperature of the solutions faster, and the second one at 90°C, in order to provide a constant heat load for 15 minutes. To control when to change the samples between the water baths, the temperature inside the flasks was controlled with a digital thermometer.

After this procedure the samples were cooled as quickly as possible at room temperature running water bath.

Initially, in the first experiments, the samples were heated within 100 mL flasks, changing them later to smaller 10 mL tubes to avoid preferential heat patterns and the burning of the liquid contacting with the flask walls.

Storage of samples was made at 4°C in a refrigerated room, at all steps, until next usage.

At Table 2.1 the main experiments performed are described in a schematic resume. The kind of milk source tested, the kind of additives and concentration in which they were added, the pH values that were tested and finally the heat treatment that was performed to the samples, are displayed below.
Table 2.1: Details of all the performed experiments. Second column signalizes if the experiment was executed in the laboratory (L) or in the pilot plant (PP). Also given is the kind of milk source tested, the additives and concentration in which they were added, the imposed pH values and finally the heat treatment that was conducted to the samples. The kind of milk source used was either skim milk powder (SMP) or a milk proteins concentrate (MPC), the mineral additives were either sodium, calcium, potassium or magnesium caseinate (respectively NaCas, CaCas, KCas, MgCas); calcium or magnesium chloride (respectively CaCl\(_2\), MgCl\(_2\)), or sodium phosphate or sodium citrate (respectively Na\(_2\)HPO\(_4\) or Na\(_2\)P and Na\(_3\)C\(_6\)H\(_5\)O\(_7\) or Na\(_3\)C). Protein additives consisted of whey protein isolate, or β-lg and α-lac isolates. The concentrations used were either in % of weight per weight or in mM. The heat treatment recurrently used in the laboratory was a water bath (WB) at 90°C for 15 minutes, but other heat treatments were performed, on the pilot plant in the heat exchanger at 140°C for 5 seconds or at 90°C for 10 minutes. Adding to the pilot plant heat treatments, for the same samples, autoclave was also used (121°C for 15 minutes).

<table>
<thead>
<tr>
<th>#</th>
<th>Milk Source</th>
<th>Additives</th>
<th>Concentrations</th>
<th>pH Values</th>
<th>Heat Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>L SMP</td>
<td>NaCas, CaCas, KCas, MgCas</td>
<td>1% (w/w)</td>
<td>6.5; 6.7; 6.9</td>
<td>90°C for 15min (WB)</td>
</tr>
<tr>
<td>#2</td>
<td>L SMP</td>
<td>CaCl(_2), MgCl(_2)</td>
<td>1; 2.5; 5; 10 mM</td>
<td>6.5; 6.7; 6.9</td>
<td>90°C for 15min (WB)</td>
</tr>
<tr>
<td>#3</td>
<td>L SMP</td>
<td>Na(_2)P, Na(_3)C</td>
<td>1% (w/w)</td>
<td>6.5; 6.7; 6.9 mM</td>
<td>90°C for 15min (WB)</td>
</tr>
<tr>
<td>#4</td>
<td>L SMP</td>
<td>WPI</td>
<td>0.5; 1; 2% (w/w)</td>
<td>6.5; 6.7; 6.9</td>
<td>90°C for 15min (WB)</td>
</tr>
<tr>
<td>#5</td>
<td>L MPC90</td>
<td>WPI</td>
<td>0.5; 1; 2% (w/w)</td>
<td>6.5; 6.7; 6.9</td>
<td>90°C for 15min (WB)</td>
</tr>
<tr>
<td>#6</td>
<td>L MPC60,80</td>
<td>WPI</td>
<td>0.5; 1; 2% (w/w)</td>
<td>6.5; 6.7; 6.9</td>
<td>90°C for 15min (WB)</td>
</tr>
<tr>
<td>#8</td>
<td>L MPC60,80</td>
<td>WPI, β-lg, α-lac, β-lg+α-lac</td>
<td>0.5; 1; 2% (w/w)</td>
<td>6.5</td>
<td>90°C for 15min (WB)</td>
</tr>
<tr>
<td>#9</td>
<td>L SMP, MPC60,80,90</td>
<td>β-lg</td>
<td>0; 0.2; 0.4; 0.6; 0.8; 1; 1.2; 1.4; 1.8; 2% (w/w)</td>
<td>6.5</td>
<td>90°C for 15min (WB)</td>
</tr>
<tr>
<td>#10</td>
<td>PP+L SMP, MPC80</td>
<td>WPI</td>
<td>0; 1% (w/w)</td>
<td>6.5; 6.7; 6.9</td>
<td>140°C 5seconds; 90°C for 10min; autoclave (121°C for 15min)</td>
</tr>
<tr>
<td>#11</td>
<td>PP+L SMP</td>
<td>WPI</td>
<td></td>
<td>6.5; 6.7; 6.9</td>
<td>140°C 5seconds; 90°C for 10min; 90°C for 15min (WB); autoclave (121°C for 15min)</td>
</tr>
</tbody>
</table>
2.1.3 Fractionation by Ultracentrifuge

In this step the main goal was to evaluate the amount, in percentage, of existing sedimentable species existing in each sample, in order to attempt to characterize them qualitatively by assembling the conclusions taken out of this method with the results from the High Performance Liquid Chromatography.

The ultracentrifuge used to perform the fractionation of samples was the Optima L-90K from Beckman Coulter, using SW 41-TI as a rotor.

In this equipment all the samples are centrifuged for 60 minutes, at 100 000 x g and 20°C. After weighting the pellet and supernatant, the supernatant was kept for further analysis in the HPLC.

For this procedure, the weight of each tube placed inside the rotor is taken before filling it with sample and also after the run, for the total weight and distribution between serum and pellet.

The results are then exhibited in the shape of bar charts, reflecting the weighted fractions of pellet and supernatant for each case.

In order to attribute some qualitative meaning to the quantitative results from fractionation, through analysis of particle size and HPLC altogether, it is known that generally native whey proteins, non-micellar-caseins and small aggregates of κ-casein with either denatured whey proteins or non-micellar-caseins will remain in solution, i.e. in the supernatant. In the pellet it is possible to find casein micelles and casein micelles with denatured whey proteins attached.

Having this in consideration, it will be a matter of comparing between different samples’ results of fractionation to try to understand why a higher amount of one of these species has formed instead of the other.

2.1.4 Dynamic viscosity by Lovis

To evaluate the dynamic viscosity of samples at 20°C, a rolling ball viscometer from Anton Paar was used, namely the Lovis 2000 ME equipped with a DMA4100 M density meter. Often alternated with milliQ water (from Millipore Corporation), the samples were placed in the attached autosampler Xsample 122.

The autosampler allows for samples to be placed inside plastic tubes that will automatically be analysed. The instrument itself functions by taking a part of the sample from the tube and passing it through a liquid-filled capillary that is inclined at a defined angle, influencing the displacement velocity of a small metallic ball. The resistance encountered by this ball in the liquid will be measured by three inductive sensors that capt the ball’s passage between defined marks, through transparent or opaque liquids. The liquid’s viscosity is directly proportional to the rolling time.

For the kind of solutions prepared in these experiences, the equipment exhibited some problems and did not always function properly, reason why there is not always accurate or even exhibited data for the viscosity in each experiment.

2.1.5 Particle Size by Zetasizer

The Zetasizer Nano ZS, from Malvern Instruments Ltd. UK, is an instrument that uses Dynamic Light Scattering (DLS) as technique to measure the size of particles, typically in the sub micron region.
The DLS technique measures the brownian motion of particles that are suspended within a liquid, i.e., the random movement that particles make due to the bombardment by solvent molecules. The larger the particle is, the slower the movement will be when affected.

The size of particles is the easiest and quickest parameter to evaluate the destabilization that may be present in the tested samples. The bigger the particles, the closest to coagulation the samples are.

For the samples to be run in this equipment, they were subjected to a 50 times dilution in milk serum. Milk serum is the permeate obtained by a 10 kDa ultrafiltration of skim milk.

Out of this procedure, two kinds of data results are obtained: the average size of the particles that are presented in each sample - results displayed in the shape of chart bars, making easy to compare between different samples - and the size distribution graphics, representing for each size seen, the existing relative amount of particles inside the sample.

2.1.6 Solubility and Denaturation by HPLC analysis

The High Performance Liquid Chromatography (HPLC) is the most accurate equipment for quantification. All the samples in the work developed were analysed in order to quantify its amount of αS1-CN, β-CN, κ-CN, α-lac and β-lg. With the values obtained, it is possible to determine the solubility and denaturation existent in each sample, using the total sample as comparison. The αS2-CN is not quantified in this procedure once it has affinity to the column, binding to it and having “memory-effect”, generating unreliable results.

Considering each mother sample, three different variations of it are tested: the total sample, evidently, but also the 4.6 fraction and the supernatant (resulting from ultracentrifuge’s fractionation). The 4.6 fraction preparation gives an index of the denaturation extent and the ultracentrifuge’s supernatant gives an index of the sedimentability in samples, for each protein.

The 4.6 fraction is prepared by adding to 1 mL of sample, 100 µL of a 10% (w/w) solution of acetic acid, and after a 15 minutes wait, 100 µL of sodium acetate 1 M. This mixture is then vortexed and centrifuged in eppendorfs at 15000xg for 30 minutes, at 20°C. The supernatant is then finally filtered through a 0.22 µm syringe filter, to become ready to be used as a sample for the HPLC run, suffering a second procedure.

The 4.6 soluble fraction procedure has into account that the combination of acetic acid and sodium acetate is a very strong buffer at the pH of 4.6, overruling whatever the natural pH of the sample might have been. Also, that at the pH of 4.6 both α-lac and β-lg are apart from their isoelectric point (pl of 5.2), carrying out some charge. The small amount of charge created will not be enough to take native proteins out of solution, but for already denatured ones it will be enough to cause aggregation, making them sediment.

The supernatant resulting from the ultracentrifuges’ steps is also filtered, considering the importance of avoiding suspended particles in the HPLC instrument. After being filtered it is also ready for a second procedure before running in the HPLC.

After these two pre-preparations, another procedure is followed (also for the total samples). The
exact composition and concentration of the mentioned buffers is available on the article by Hinz et al. [22]. To the 100 µL of sample placed in the tube, 300 µL of E⁺ (a solution constituted by buffer E supplemented with DTT at a concentration of 20 mg/mL) are added. The mixture is then vortexed and waits for 1 hour before 1500 µL of D are joined.

The E⁺ buffer serves to adjust the pH to 7.5 and it consists on a combination of bis-tris, trisodium citrate, urea and Dithiothreitol (DTT); being bis-tris an effective buffer, trisodium citrate to bind to the calcium that is present in micelles, urea to disrupt the hydrophobic interaction and the hydrogen bonds within and between proteins and the DTT to destroy the disulphide bonds. At this point, the proteins are not only unfolded but also in a monomeric state. Lastly, the D buffer serves only to reduce the pH value to the same existent in solvent A, a solvent used during the HPLC analysis itself.

Followed once more by a vortex, the final samples are again filtered into special vials that will later be placed inside the HPLC instruments’ autosampler. Similarly to what is made for the 4.6 procedure, also in this second procedure, the weight after every reagent addition is taken for dilution adjustments in the final peak areas.

After these three variants run in the HPLC, and the results are integrated with the data analyser Chromeleon 7.1 (from ThermoFischer Scientific, USA), some calculations are effectuated to obtain the percentage values for the denaturation and sedimentability of each sample. Firstly, it is necessary to have into account the true dilution to which the samples were subjected to; this is made by weighting the eppendorfs every time some reactant is added. After this, the calculations are made according to the equations (2.1) and (2.2).

\[
\text{Denaturation} \% = \frac{A_{\text{total}} - A_{4.6}}{A_{\text{total}}} \times 100 \quad (2.1)
\]

\[
\text{Sedimentability} \% = (1 - \frac{A_S}{A_{\text{total}}}) \times 100 \quad (2.2)
\]

Where \(A_{\text{total}}\) is the peak area of the total sample, \(A_{4.6}\) is the peak area of the 4.6 soluble fraction, and \(A_S\) is the peak area of the supernatant sample.

The equipment is composed by an Ultimate 3000 pump, a WPS autosampler, a column compartment and a diode array detector, all from ThermoFisher Scientific, USA.

Proteins’ denaturation is an important parameter to perceive, once their natural dimensional shape is needed to have them functioning properly, what means that if they loose their structure they will also lose their initial biological activity. Therefore, to predict the role that each protein interprets after heating it is necessary to know its final conformational state.

Sedimentability, on its turn, gives a notion of the distribution and localization of the protein after heating, what may also be an indication to possible associations and therefore the action that they are executing.
2.2 Pilot Plant

Pilot plant procedure relies on the same general goals and principles applied in the laboratory, but using different equipments and materials.

For pilot-plant performed experiments, MPC85 was supplied by Cremo, WPI by ArlaFoods Ingredients and SMP by FrieslandCampina.

2.2.1 Preparation of Samples

The solutions were prepared by joining to the SMP or MPC, water and WPI in the correct weight proportions and mixing them with a high shear mixer. After pH adjustment and continuous stirring until complete dissolution, samples entered an homogeneizer to ensure full dispersion of the powdered ingredients.

2.2.2 Heat Exchanger: UHT and Pasteurization

UHT treatment was carried out using the heat exchanger from Combitherm available at NIZO food research. The solution passed inside the tubes and the water outside the tubes. Indirect heating with a holding time of 5 seconds at 140°C or 10 minutes at 90°C was carried out.

2.2.3 Autoclave & Water Bath

From the solutions prepared at the pilot plant, unheated mixed samples were taken out in order to stand two different treatments. The autoclave from Zirbus Technologies, was used as an alternative thermal heating procedure to the heat exchanger, executing its heating task at 121°C during 15 minutes. The second procedure was the laboratory’s water bath at 90°C for 15 minutes - the standard procedure used previously.

2.2.4 Pilot Plant Assays

After pilot plant heat treatment, the samples were analysed with the same methodologies than previous experiments at the laboratory scale were, being the fractionation performed by ultracentrifugation, the particle size determined with the Zetasizer and the denaturation and sedimentability of samples calculated trough HPLC analysis.
Chapter 3

Results and Discussion

In general outlines, the work performed had as a goal to control the stabilization of either SMP or MPCs with different mineral compositions or different protein content amounts. In almost all cases, for comparison, three different pH values were tried.

The MPCs were dissolved to a final concentration of 3.5% (w/w) protein content, the same total composition available in milk in its raw state, maintaining the natural ratio between whey proteins and caseins.

Figure 3.1: The average size of the 50 times diluted samples at 20°C, of heat treated (for 15 minutes at 90°C) SMP, MPC60, MPC80 and MPC90 at a pH of 6.5.

In order to understand the experimental results in this chapter and the next ones, it is important to be mindful that by simply heating reconstituted SMP, even if at a pH of 6.5, it will remain stable; and also that by heating reconstituted MPCs without any supplements added, and at a pH of 6.5, its stability will diminish with the increase in protein content level of MPCs. These results are shown in Figure 3.1, where the absence of the MPC90 sample is due to coagulation. It can also be seen that even though MPC80 is present it shows a really high average particle size and that SMP is quite stable (implied by
its low average size). Remaining is MPC60, showing as well to be quite stable, with a really moderate average particles’ size.

Knowing as the samples behave in their unaltered state, comparisons can later be made to its behaviour after having add supplements. The following subsections will be dedicated firstly to the mineral additions tested and then to the protein additions made. Even though pilot plant trials involved only protein additions, laboratory and pilot plant scales are divided in two distinct sections.

3.1 Laboratory

3.1.1 Mineral Additions

In the first set of experiments mineral compounds were added, namely caseinates, phosphates and chlorides.

3.1.1.1 Caseinates

With respect to caseinates, SMP was tested with the addition of 1% weight for weight (w/w) of potassium, sodium, magnesium or calcium caseinate at three different pH values (6.5, 6.7 and 6.9).

Caseinates (ionized caseins) are isolated by adding acid to warm milk, causing a pH drop and therefore a casein precipitation. After the removal, and due to its insolubility in water, a reaction with a basic, ionic salt (or even a metal-base soluble in water) is needed, to give origin to a neutral protein product. The type of alkali that is used to neutralize the acid casein curd will determine what type of caseinate is produced.

As mentioned in the Background, chapter 1.2, with regard to casein micelles, casein is mostly present in the caseinate form and is either connected with calcium or with magnesium.

Adding extra caseinates to milk sources is to simply create a bigger concentration of this ionic components on solution, along with an equal increase in the available minerals.

After the standard heating process, at $90^\circ\text{C}$ for 15 minutes, the first performed test is usually fractionation, resulting of the implemented ultracentrifugation run for all the effectuated experiments, at 100 000 x g for 60 minutes and $20^\circ\text{C}$. This procedure will allow to detect and compare the relative amount of supernatant or of pellet existent in each distinct sample, with the remaining ones.

The observed ratio of the supernatant-pellet division is important considering the resulting ability to compare the sedimentability of components between samples that had different compositions and suffered the same thermal treatment and procedures. The lack or increase of sedimentability are indicators of the type of aggregates that are being formed.

Generally it can be stated that native whey proteins, non-micellar caseins and small aggregates constituted by either $\kappa$-casein, denatured whey proteins or non-micellar-caseins will remain in solution, i.e., in the supernatant. In the pellet it will be possible to find casein micelles and casein micelles with denaturated whey proteins attached.
Observing Figure 3.2 it is possible to notice that the SMP sample at a pH of 6.5 is absent from the data, since it coagulated during heat treatment, being in this case assumed as the maximum example of instability.

Figure 3.2: Fraction of pellet seen after 60 minutes of ultracentrifugation at 100 000 x g and 20°C, of heat treated (15 minutes and 90°C) SMP with either 1% (w/w) of potassium, sodium, magnesium or calcium caseinate, at three different pH values (6.5, 6.7 and 6.9).

Also observable in the Figure 3.2 is that by sticking with one pH and changing caseinate types, or by analysing the whole results as one, there is a trend to increase the amount of the pellet percentage in the order potassium, sodium, magnesium and calcium (K-Cas < Na-Cas < Mg-Cas < Ca-Cas).

What may then be suggested is that according to these results, monovalent cations like potassium and sodium show less pellet and sedimented particles, having therefore bigger supernatants, while divalent cations, like magnesium and calcium have bigger sedimentable pellets and therefore relative smaller supernatants. This fact may be explained by the predisposition to make connections and hence the final size of the particles formed: monovalent cations only form one connection, interacting less and constituting particles that may not be dense enough to sediment on centrifugation, remaining in solution. Magnesium and calcium, on the other side, while divalent cations, can constitute bigger aggregates with higher density and that sediment more easily.

Additionally, inside each caseinate set of results, the verifiable trend is that along with a pH reduction, a pellet increase occurs. In other words, the lower the pH, the higher the amount of sedimented particles will be found as pellet.

Here, and to justify the results, there are two opposite parameters that should be taken into consideration when analysing these trends, once they pull into distinct directions. The first one of them being the dissociation of k-casein from casein micelles surfaces and the second one of them being the calcium ion activity. Both of this effects are pH dependent and conduct the system into different directions in the pH range tested.

The importance of k-casein in the casein micelles is indubitable to maintain its surface structure and stability, like it was thoroughly explained in the Background chapter 1.2; its presence and association on the surface protects and limits each casein micelle from disassembling. By having k-casein dissociation, a bare surface will be left that is prone to coagulation by its willingness to interact and ultimately to
aggregate.

Also, as verified by Crowley et al. [21] and mentioned in the Practical Background, chapter 1.4, it is known that through heating and at high pH values, κ-casein dissociates in a greater extent from casein micelles. Similarly Singh and Fox [23] had published in 1985, stating that the complex β-lg - κ-casein would dissociate from the molecule at pH values greater than 6.7, meaning that presumably with the pH increase, basicity would be responsible for increasing the charge of the constituting molecules of the sub-K-casein layer - near the micelle’s surface - increasing the electrostatic repulsion between the complex and the sub-surface layer, and finally producing the dissociation [23].

There are two possibilities for the dissociation of κ-casein from the micelle, either κ-casein frees itself first with the increase in pH to connect later with the available denatured whey proteins, or otherwise, it establishes first a connection with the available denatured whey protein and then the complex frees itself from the micelle. This can form small particles that remain in the serum and do not sediment, once particles are not heavy enough.

At first sight, for this reason, it could be expected to have more destabilization at a higher pH with micelles disassembling and interacting, forming therefore more sedimentable particles and pellet.

However, the results show bigger pellets at lower pH, reason why the dissociation explained cannot justify it. Even at the maximum work pH (6.9) there is still relatively little heat-induced dissociation of κ-casein, being the micelles therefore still enough stabilized with associated denatured whey proteins.

The other factor for this matter, that explains the pellet results for a low pH is the increase on the calcium ion activity. This other pH-dependent effect being so strong that overcomes the previous tendencies: with the decrease in pH, calcium ion activity increases. This boost in activity is prejudicial and more relevant in disassembling casein micelles (even though they are still connected with κ-casein), causing proteins to form bigger aggregates that include small particles that were in solution before.

In conclusion, the pH drop effect on the calcium ion activity has a bigger effect on destabilizing micelles than the climbing pH has on the increasing disconnection of κ-casein from the micelles (at least, in these pH ranges). Also worth noticing may be that whey proteins are expected to denature more at higher pH values [19], effect that may also contrabalance the possible destabilization existent at high pH; meaning, slightly more available whey proteins to stabilize the slightly less κ-casein attached.

This calcium ion dependency is known to affect mostly αS2-CN, followed by αS1-CN and β-lg, by order of sensibility to precipitation. [18] For κ-casein there is no binding to calcium at any extent [24].

In a way it could be then predicted to have more destabilized micelles due to calcium ion activity at lower pH, resulting in more disassembling, more connections and concluding, in bigger particles, and at higher pH some denatured whey proteins connected with κ-casein in the serum, but still a lot of micelles that are intact enough to stay in solution.

Regarding the Zetasizer’s average particle size results displayed in Figure 3.3, one can observe that once again calcium and magnesium appear as a group, signaling altogether the existence of bigger particles than potassium and sodium, specially looking at low pH (blue bars represent a pH of 6.5). The existence of bigger particles suggests a less stabler state, more predisposed to coagulation. Also, in a general way it is possible to see that bigger particles are related (except for the potassium case) with
the pH of 6.5. This supports the idea that systems are more prone to instability at lower pH values.

Figure 3.3: Average particle size (nm) of the 50 times diluted samples at 20°C, of heat treated (15 minutes and 90°C) SMP with either 1% (w/w) of potassium, sodium, magnesium or calcium caseinate, in three different pH values (6.5, 6.7 and 6.9).

Considering that the SMP sample at a pH of 6.5 completely coagulated (addition-free), it is safe to affirm that the different caseinates stabilize even if remotely the SMP system, at least at low pH. The fact that magnesium and calcium caseinate samples reveal here a higher average particle size, correlates with the fractionation results previously seen, in which they showed bigger pellet percentage results. It was assumed that they had more pellet because they contained bigger aggregates, and in here it is revealed that the existent particles in those samples are indeed bigger. At the higher pH of 6.9, and by regarding the behaviour of the heated addition-free SMP sample, it can perhaps be stated that the system did not need stabilization and therefore the particle size of the caseinates is quite constant and even slightly less stable.

In Figure 3.4, the distribution of the Zetasizer’s particle size is given for each sample, instead of just considering the global average size like in Figure 3.3. Even though the results in this figure allow the same conclusions of results that in Figure 3.3, it is interesting to see more clearly what was already stated for the average results: for a pH of 6.5, for example, there is a shift in the curves indicating that calcium and magnesium contain bigger particles. Likewise for the other two pH values - even though if less marked for calcium - magnesium is clearly continuously deviated. Interesting to notice that theoretically, in solution, the same amounts of interacting species exist, being only the rearrangement of compounds causing the final structural differences seen. For this motive samples will have either towering pikes - higher quantities - of small particles or fewer bigger particles.

Finally, considering the HPLC results two different parameters are evaluated: the denaturation of whey proteins and the sedimentability of both whey proteins and caseins.

The denaturation of proteins, in general, involves the disruption of the secondary and tertiary structures. However, it is not strong enough to break the peptide bonds, remaining the primary structure unaltered.

The heat load applied will increase the kinetic energy, causing the molecules to vibrate until the bonds are disrupted. Whey proteins are sensible to this load, reason why by comparison between the total samples and the 4.6 fraction samples (variation procedure described in chapter 2), their denaturation is evaluated.
Figure 3.4: Distribution particle size of the 50 times diluted samples at 20°C, of heat treated (15 minutes and 90°C) SMP with either 1% (w/w) of potassium, sodium, magnesium or calcium caseinate at three different pH values (6.5, 6.7 and 6.9).

In Figure 3.5 it is possible to observe that as expected, almost complete denaturation of whey proteins, β-lg and α-lac, occurred, concluding then that the heat load was enough to denature them and the 4.6 fraction treatment procedure enough to separate the denatured proteins.

The denaturation seen on β-lg is higher than the one seen for α-lac, being full denaturation reached for β-lg, in most cases. Also interesting to notice is that in α-lac’s case (left chart bar on Figure 3.5), denaturation of added caseinates samples is slightly higher than for pure SMP, and that amongst all, calcium caseinate is in particular somewhat higher. This result proposes that added caseinates may facilitate α-lac’s denaturation; adding caseinates may perhaps increase the connections made with whey proteins during heat treatment, making them more accessible and exposed to denaturation. Alternatively, without the caseinates additions, they would assemble with other whey proteins aggregates making it harder to denaturate. The calcium caseinate would then still increase more this effect, once it frees more caseinates (from the micelles) to be predisposed to connect with denatured whey proteins.

Regarding sedimentability, also for whey proteins, the results are presented in Figure 3.6. Here it is explicit that for both proteins, higher values of sedimentability are seen for lower pH values.

For α-lac and inside each caseinate, a clear decrease in the values of sedimentability is verified with the pH increase. As for the β-lg protein, at higher pH values, sedimentability is either nonexistent or barely visible, meaning that the totality of the proteins must be in the serum phase.

At first sight it is logical to find a smaller quantity of sedimentable whey proteins at higher pH since that at higher pH micelles are still intact in solution and do not sediment as much. Also, κ-casein is more dissociated from casein micelles and therefore in solution, not being pulled (together with whey-proteins)
Figure 3.5: HPLC analysis shows the denaturation of α-lac and β-lg, respectively, in the heat treated SMP samples (15 minutes and 90°C) with 1% (w/w) of potassium, sodium, magnesium or calcium caseinate (KCas, NaCas, MgCas, CaCas, respectively) at three different pH values (6.5, 6.7 and 6.9).

Figure 3.6: HPLC analysis show the sedimentability of β-lg (blue bars) and α-lac (orange bars) in heat treated SMP samples (15 minutes and 90°C) with 1% (w/w) of potassium, sodium, magnesium or calcium caseinate (KCas, NaCas, MgCas, CaCas, respectively), at three different pH values (6.5, 6.7 and 6.9).

by sedimentation time.

As it was observed in fractionation, there is indeed more pellet at lower pH values due to the higher calcium ion activity effect, for which during sedimentation some whey proteins must be pulled to sediment with the remainder particles, in the middle of disruption. Additionally, as it is possible to observe also in Figure 3.6 for a 6.5 pH, again the divalent ion caseinates are the ones presenting a higher level of whey proteins sedimentability. It is assumable then that these divalent ions (calcium caseinate and magnesium caseinate), make indeed more connections, carrying more sedimentable total casein and κ-casein, and that the whey protein associates with these aggregates and thus is sedimentable as well, for what larger quantities of pellet are formed. As the mineral additions are caseinates and caseinates have more affinity with whey proteins than some other mineral additions, along with the lower pH effect that highly influences calcium ion activity - the dismantling of micelles happens, being more caseinates released creating more pellet. Interesting to notice that pure SMP shows more sedimentable whey proteins than samples with caseinates added.

For the surplus proteins, regarding caseins sedimentability now, αs1-casein, β-casein and κ-casein have their results presented in Figure 3.7.

For αs1-casein sedimentability, in general, it is possible to observe that for a lower pH more proteins have sedimented, decreasing the values until a pH value of 6.9, where a minimum is reached. However,
Figure 3.7: HPLC analysis shows the sedimentability of α\textsubscript{S1}-casein, β-casein and κ-casein, respectively, in the heat treated SMP samples (15 minutes and 90°C) with either 1% (w/w) of potassium, sodium, magnesium or calcium caseinate (KC\textsubscript{as}, Na\textsubscript{Cas}, Mg\textsubscript{Cas}, Ca\textsubscript{Cas}, respectively) at three different pH values (6.5, 6.7 and 6.9).

Sedimentability values are quite high, for what it can be probably reasoned that α\textsubscript{S1}-casein is a regular constituent of the pellet.

For β-casein the same tendency is spotted, decreasing the sedimentability with the pH increase. However, the values seen are all around 50%, for which it is concluded that its sedimentability does not change so much with either the pH or the kind of caseinate, as it does for α\textsubscript{S1}-casein or κ-casein. Nevertheless, β-casein must also be considerably present in the core of the pellet.

For κ-casein sedimentability, the differences between pH values are more abrupt; accordingly to the data, for a higher pH there is considerably less sedimentable particles - something that is in conformity with the previously seen results for whey proteins denaturation.

It is expected to have similar distributions of κ-casein and β-lg, since we expect them to be interacting; this means that at low pH, accompanying more sedimented β-lg also more κ-casein will be found, and by pellet’s absence of β-lg at high pH, few κ-casein will also be found sedimented. This means that the previously put hypothesis is present, but that at high pH values it is still possible to find some κ-casein in the pellet that is not connected with whey proteins; all the β-lg must be in the serum interacting either with more β-lg or with disconnected κ-casein.

Like mentioned previously, if these caseinates interact with denaturated whey proteins during heating and then disassemble, or if they connect to each other only afterwards is yet unknown.

By analysing the results it is possible to observe that the effects of adding calcium caseinates are harsher at all tests, when comparing with the other mineral caseinates additions (e.g. HPLC’s sedimentability results of all the proteins), once calcium ion activity must be boosted by the addition of more...
calcium, creating more destabilization.

Once again, it is important to re-refer that two behavioral groups can be distinguished in these results. There is clearly a difference in data, on the different tests, splitting behaviours amongst monovalent ions and divalent ions. In terms of conclusion, it can then be stated that the addition of monovalent ions can have a stabilizing effect at a low pH, since the SMP sample without additions coagulated while the others did not. Also, having into consideration both the average particle size seen and the pellet levels (and additionally even some clues regarding β-lg localization) the data does suggest that adding potassium caseinate may have a really stabilizing effect, while adding divalent ions - especially calcium - can really destabilize SMP.
3.1.1.2 Chlorides: Magnesium & Calcium

A second experiment consisted in the addition of MgCl$_2$ or CaCl$_2$, magnesium chloride and calcium chloride, respectively, to SMP, at four different concentrations, 1 mM, 2.5 mM, 5 mM and 10 mM and three pH values 6.5, 6.7 and 6.9. Samples without additions were also prepared as a control (0 mM). Heat treatment consisted of 15 minutes at 90°C, and some samples were left unheated.

Similarly to what was made for the samples with caseinates additions, fractionation of the samples with chlorides additions was performed, for which the results are presented in Figure 3.8. In this case it is possible to make four types of comparisons between samples; firstly inside the same pH but between the different salts added (magnesium or calcium chlorides), then between samples that have suffered or not thermal treatment, then for the same salt at the same pH but with different concentrations, and lastly a comparison with the control samples (samples without the addition of any chlorides).

What immediately becomes apparent when analysing these data is that there is a general trend for a pellet volume decrease with chlorides concentration increase - specially for calcium chloride.

On the other hand, whilst analysing unheated magnesium chloride samples at the all three pH values, they show similar constant value results at all concentrations except for a very slight decrease at 10 mM.

Figure 3.8: Fraction of pellet that resulted from ultracentrifugation at 100 000 x g for 1 hour at 20°C, of heated (15 minutes and 90°C) or unheated (U) SMP with either 0, 1, 2.5, 5, 10 mM of magnesium chloride (represented by MgCl$_2$) or calcium chloride (represented by CaCl$_2$) at three different pH values (6.5, 6.7 and 6.9).

The absence of a bar for the magnesium chloride sample at a pH of 6.5 and a concentration of 10 mM, is due to coagulation during the thermal treatment.

The comparison between the unheated and heated samples, at a 0 mM concentration of chlorides shows that heated samples have an inferior amount of pellet level that is therefore attributable on the heating process alone. Since the trend is seen for the three tested pH values, it is not pH-dependent. It can therefore only be explained by a larger extent of heat-induced dissociation of κ-casein comparing with a heat-induced association of whey proteins with the micelles.

Considering now samples with added calcium or magnesium chloride and comparing between heated
and unheated samples for each pH, it can be stated that at a pH of 6.5 heated samples have bigger (or equal) pellets than unheated samples. For a pH of 6.7, a lot more steadiness is revealed, being the pellet level roughly constant between heated and unheated samples. For a pH of 6.9, lastly, heated samples show less pellet than unheated samples.

It can then be stated that the 6.9 pH is the only pH in which the natural - without additions - tendency is followed. At other pH values it can be stated that the addition of chlorides creates more pellet than it would be the case in their absence. The lower the pH the more sedimentability occurs, proving the higher instability at lower pH caused by the higher activity of the existent calcium ion and the reduced extent of heat-induced dissociation of $\kappa$-casein.

Another interesting effect is that inside a certain pH of calcium chloride concentrations, following a smaller level of pellet for a concentration of 1 mM, for intermediate concentrations bigger pellet volumes arise, resulting then in a decrease at 10 mM. This does not occur with the addition of magnesium chloride.

As seen above for the caseinates, magnesium chloride seems to be less stable than calcium chloride, i.e. leading to more sedimented particles. By adding these chlorides, and because the medium is already saturated with calcium and magnesium phosphates, some calcium and magnesium will precipitate with phosphate within the micelles, and the general activity of these components will increase. They will facilitate the connection of denatured whey proteins with micelles and promote the $\kappa$-casein connection stability with the micelles. In resume, a bigger protein aggregation and less $\kappa$-casein dissociation will therefore create a bigger level of pellet sedimentation. Why this happens for magnesium in a bigger extent is not known.

Looking at further data may be useful to explain this decrease at 10 mM of calcium chloride. There is a possibility that the decrease is not due to less sedimentable proteins, but to a lower moisture content. Calcium may interact with Glutamine and Aspartame residues reducing the charge and therefore the water binding; also by possibly reducing the $\kappa$-casein on the surface, water binding will be diminished.

About particle size results, they are shown in Figure 3.9, where it is attainable that samples that are unheated do not change relevantly their particle size in spite of the chlorides concentration added. However, the small propensity for high particle sizes in the intermediate concentration values is still a little bit present, like seen for the fractionation.

For heated samples, outcomes are more interesting once some instability starts to appear, specially for lower pH values and higher concentrations. Calcium chloride shows, for all three pH values, lower particle sizes than magnesium chloride samples. This highlights the previous conclusion taken out of the fractionation results, that by adding magnesium chloride, calcium ion activity increases, destabilizing micelles and forming bigger particles.

Also confirmed is that the values from the pH of 6.9 are quite more moderated, being almost equivalent to the unheated values.

Distribution size results of the particles in each sample are presented on the Appendix B.

About HPLC data, Figures 3.10, 3.12 and 3.13 show the sedimentability of each protein at three pH values and five different concentrations of magnesium and calcium chlorides, and heated and unheated
Figure 3.9: Average size of the 50 times diluted samples at 20° C, of heated (15 minutes and 90° C) or unheated SMP with either 0, 1, 2.5, 5 and 10 mM of magnesium chloride (represented by MgCl₂) or calcium chloride (represented by CaCl₂) at three different pH values (6.5, 6.7 and 6.9).

Globally from Figure 3.10, it can be stated that an increase in β-lg and α-lac sedimentabilities occurs with the increase on the concentration of both chlorides. It is also interesting to see clearly two tendencies that were expected in theory: unheated samples have almost zero sedimentation, meaning whey proteins were indeed not denaturated and therefore non-sedimentable before heating; and also that heated samples without additives start from a certain sedimentation percentage, different from zero and that varies with pH. For a higher pH, there is less sedimentation of β-lg, what is indeed coincident with the expected bigger dissociation of κ-casein from the micelles.

This sedimentation percentage increases then considerably with the increase in chlorides concentration, until quite high values - passing however through a slight decrease, that has no justification so far.

Interesting to observe as well, in Figure 3.10 is that for the curves at a pH of 6.5, without any addition of chlorides (yellow and light blue lines), β-lg is completely sedimentable, after which it gets slightly back into the serum for intermediate concentrations, and it finishes back into 100% sedimentability at the really high concentration of 10 mM added chlorides (calcium or magnesium).

Also, the lower the pH, the more sedimentable the particles globally are.

To confirm the expected whey proteins’ denaturation, HPLC graphics are displayed in Figure 3.11, where the results show complete denaturation of β-lg and α-lac in all samples.

Regarding β-casein sedimentability, it is shown in Figure 3.12 a comparison between heated and unheated samples, where it can be stated that there is almost no variation between samples, pH values or concentrations, and that around 75% of β-casein proteins sediments.

As for αs₁-casein sedimentability results, also in Figure 3.12, the comparison between unheated and heated samples indicates that even though this protein is already very sedimentable before thermal treatment, after it, it fully sediments.

On Figure 3.13 it is possible to see κ-casein sedimentability in unheated and heated samples. In unheated samples the values are constant for all concentrations, pH values and chlorides’ type but do
Figure 3.10: HPLC results for the sedimentability of $\beta$-lg and $\alpha$-lac when the addition of either magnesium chloride (represented by MgCl$_2$) or calcium chloride (represented by CaCl$_2$) at 0, 1, 2.5, 5 or 10 mM has been made to SMP, unheated (U) or heated (for 15 minutes and $90^\circ$ C) at the pH values of 6.5, 6.7 and 6.9.

Figure 3.11: HPLC results for the denaturation of $\beta$-lg and $\alpha$-lac when either magnesium chloride (represented by MgCl$_2$) or calcium chloride (represented by CaCl$_2$) have been added at 0, 1, 2.5, 5 or 10 mM to SMP, and suffered heat treatment (for 15 minutes and $90^\circ$ C) at the pH values of 6.5, 6.7 and 6.9.

increase with concentration for the heated ones. The values of sedimentation are also bigger for lower pH values, but in a general way, for all samples there is an uniform climbing trend. Once again this agrees with what was seen for the caseinates, making sense to find more sedimented $\kappa$-casein at lower pH values, once it is also where more pellet is found, instead of at higher pH values where it disconnects from micelles. Again it is possible to notice the slight decrease, here on sedimentation - seen in all the other tests: fractionation, zetasizer and slightly HPLC - before climbing on concentrations, corresponding to moderate concentration values of added calcium and magnesium chlorides.
Figure 3.12: HPLC results for the sedimentability of $\beta$-casein and $\alpha_S$-casein when the addition of either magnesium chloride (represented by MgCl$_2$) or calcium chloride (represented by CaCl$_2$) at 0, 1, 2.5, 5 or 10 mM has been made to SMP, unheated or heated (for 15 minutes and 90$^\circ$ C) at the pH values of 6.5, 6.7 and 6.9.

Figure 3.13: HPLC results for the sedimentability of $\kappa$-casein when the addition of either magnesium chloride (represented by MgCl$_2$) or calcium chloride (represented by CaCl$_2$) at 0, 1, 2.5, 5 or 10 mM has been made to SMP, unheated (U) or heated (for 15 minutes and 90$^\circ$ C) at the pH values of 6.5, 6.7 and 6.9.
3.1.1.3 Sodium: Phosphate & Citrate

The third experiment consisted in the addition of either sodium phosphate or sodium citrate, respectively Na$_2$HPO$_4$ and Na$_3$C$_6$H$_5$O$_7$, (also designated during the present document for simplification motives as Na2P and Na3C) at 1, 2.5, 5 and 10 mM to SMP, at the pH values of 6.5, 6.7 and 6.9. The same samples were heated at 90°C for 15 minutes, or remained unheated.

Phosphate is an inorganic chemical and a salt of phosphoric acid, whose molecular equation is PO$_4^{3-}$. Citrate is a derivative of citric acid, whose molecular form is C$_6$H$_8$O$_7^{3-}$.

The Figure 3.14 represents fractionation results, the division that has resulted from ultracentrifugation and that therefore represents the amount of sedimentable particles for each sample tested in the current experiment. In this case it is possible to make three types of comparisons between samples; firstly inside the same pH but between the different salts added (phosphate and citrate), then between the samples that have suffered or not thermal treatment, and lastly between different concentrations of the same salt at the same pH.

![Figure 3.14: Fraction of pellet that has resulted from ultracentrifugation at 100 000 x g for 1 hour at 20°C, of heated (15 minutes and 90°C) or unheated (U) SMP with either 1, 2.5, 5 and 10 mM of sodium phosphate (represented by Na2P) or sodium citrate (represented by Na3C) at three different pH values (6.5, 6.7 and 6.9).](image)

Inside each pH but comparing different salts it is noteworthy that the samples with sodium citrate show lower levels of pellet than the samples with sodium phosphate, in almost every case. As previously mentioned, this implicates that the citrate containing samples contain less quantity of sedimented particles.

For this reason it seems that on heating added citrate is more stable than added phosphate.

For the phosphate addition it is observable in the results that neither the concentration added or the pH imposed or the thermal treatment have an effect on sedimentability, remaining it constant through out the trial.

For the citrate addition, however, some variation can be detected. Firstly, only taking a look at the heated samples at the three different pH values, one can state that a decrease seems to take place in sedimentation by rising on pH. Moreover, taking a look at the unheated samples, one can state that there exists an increasing sedimentation level with pH increase, specially for the higher concentrations of added citrate. Heat and unheated samples have then opposite behaviours, what will reinforce the
stabilizing effect of added citrate on heating.

This means that by comparing inside each pH for the same heated and unheated samples of citrate, it will be denoted that an increase in pellet quantity with heating takes place at a pH of 6.5, while a decrease (in pellet quantity with heating) seems to take place at a pH of 6.9. For the pH of 6.7, things seem more unaltered, perhaps with a slight increase, specially for samples containing high concentrations of added salts.

Comparing added citrate for the unheated samples between different concentrations, inside each of the three pH values, it is clear that the pellet amount diminishes with the increase in added concentration. For the heated samples, it could be stated that the same lean is present, but in a more modest way. The reason for the decrease with increasing citrate is that it will cause some casein to dissociate from the micelle due to binding of micellar calcium by citrate. For heated samples, this trend will be less pronounced since heating partially reverses the dissociation of caseins and promotes the association of denatured whey proteins with micelles.

The motive for the discrepancy in behaviour of the two salts is that once milk serum is already saturated with calcium phosphate, by adding sodium phosphate, the saturation degree of phosphate will only increase. This will cause calcium ion activity to increase and calcium phosphate to become insoluble, precipitate and associate with the micelles, creating bigger particles. Oppositely, calcium citrate will form a soluble negatively charged complex that captures some of the serum calcium, making calcium phosphate get out of the micelles due to the gradient. With calcium phosphate getting out of the micelles, due to the increase in calcium ion activity, small soluble complexes will stay in solution, diminishing the level of formed pellet.

Particle size was evaluated using DLS analysis (Zetasizer equipment), being the results displayed in the Figure 3.15. In general terms it is correct to state that only small differences are visible. For a pH of 6.5 it is possible to see, for both added citrate and phosphate, that unheated samples are smaller - this is expected once heated samples are predicted to be more unstable, i.e. have bigger particles, at an acidic pH. It is also coincident with fractionation results, where more pellet could be found at a 6.5 pH in heated samples. Contrarily, at the other pH values no relevant differences are observed between heated and unheated samples, conjecturing even a slight tendency to the opposite behaviour - unheated samples having slightly bigger particles. For the pH of 6.9 this is also coincident with the fractionation data obtained in 3.14. It is worth denoting that this behaviour at the higher pH values was already seen for the non addition of supplements in the subchapter of Caseinates.

The distribution results of the DLS analysis are in all respect accordant with the average particle size, reason why they are only presented in further detail in Appendix C.

The dynamic viscosity of samples was also evaluated with the Lovis equipment, as shown in Figure 3.16. Some samples were not included in the results since they did not run satisfactorily. For the points shown, it can be stated that for unheated samples there is an increase in dynamic viscosity with pH increase. As for added phosphate heated samples, no variation is seen in viscosity - constancy matching the previously results seen in fractionation (Figure 3.14) and also partially in particle size analysis (Figure 3.15). Heated samples with added citrate, on the other hand, show some variation
Figure 3.15: Average particle size of the 50 times diluted samples at 20° C, of heated (15 minutes and 90° C) or unheated (U) SMP with either 1, 2.5, 5 and 10 mM of sodium phosphate (represented by Na2P) or sodium citrate (represented by Na3C) at three different pH values (6.5, 6.7 and 6.9).

without a recognizable trend.

Figure 3.16: Dynamic Viscosity for heated (15 minutes and 90° C) or unheated (U) SMP with either 1, 2.5, 5 and 10 mM of sodium phosphate or sodium citrate (represented by Na2P or Na3C, respectively) at three different pH values (6.5, 6.7 and 6.9).

The results of sedimentability of proteins from the HPLC analysis are shown in the Figures 3.17, 3.18 and 3.19, and the results from whey proteins’ denaturation are presented in Figure 3.20.

Regarding the proteins β-lg and α-lac and their sedimentability, Figure 3.17 shows that in this range of concentrations and at the same pH, the curves for added sodium citrate mostly overrule the curves for phosphate. This implies that added sodium citrate generally causes bigger sedimentability than added sodium phosphate, for the same pH. Moreover, in other words, it is possible to find more whey proteins in the pellet of added citrate samples than in their equivalent in concentration and pH with sodium phosphate. Since that for fractionation the amount of pellet was higher for added phosphate samples (Figure 3.14), one can conclude that this was not due to a higher amount of whey proteins attached, what is not a surprise considering whey proteins are quite small in comparison to casein micelles. From these results it can be concluded that citrate contributes for a higher whey protein constituted pellet, even if smaller. Regarding unheated samples, it is interesting to note that only few sedimentability exists, and it is specially for α-lac, low pH values and for samples without phosphates or citrates additives. For concentrations different than zero the sedimentation was barely existent. All of this is coherent since it
is expected for whey proteins to be connected with micelles only if denatured, and unheated samples have not suffered a heat treatment to reach denaturation. Still interesting to keep in mind is that there exists a small denaturation percentage for $\alpha$-lac even without the thermal treatment.

**Figure 3.17:** HPLC results for $\beta$-lg and $\alpha$-lac with the addition of either sodium phosphate or sodium citrate (represented by Na2P or Na3C, respectively) at 0, 1, 2.5, 5 or 10 mM to SMP, unheated (U) or heated (for 15 minutes and 90° C) at the pH values of 6.5, 6.7 and 6.9.

For $\alpha$s1-casein and $\beta$-casein sedimentability, the graphics are displayed in Figure 3.18, and the trend seen here matches the one seen in the fractionation results (Figure 3.14): phosphate samples show a slightly higher sedimentability, specially at higher concentrations. This probably implicates that pellet levels are mostly constituted by these two caseins. It is important to notice, however, that for unheated samples the same trend is present and even more markedly, reaching extremer values - what is also coincident with fractionation results, having unheated phosphates considerable pellets and bigger than the ones seen for citrates.

Analysing the results for the sedimentability of $\kappa$-casein in Figure 3.19, it can be seen that for a pH of 6.5, citrate shows a higher sedimentability until a concentration of 2.5 mM, where the tendency changes and phosphate becomes the highest. For a pH of 6.7, their sedimentabilities are closer and phosphates only overcome citrates’ sedimentability for the highest concentration of 10 mM. For pH 6.9, citrate either dominates or it equalizes phosphate for the concentrations of 2.5 mM and 5 mM. This situation where citrates overstep phosphates’ sedimentability correspond to most fractionation trends.

However, in general the sedimentability of $\kappa$-casein decreased with the increase on concentration of both citrates and phosphates, what corresponds to what happened with the pellet levels in fractionation results. This probably means that both citrates and phosphates have a somewhat stabilizing effect, being smaller particles formed in the medium if they are added. Also again, $\beta$-lg showed the same trend in its sedimentability, reinforcing the existent interaction between denatured whey proteins and these
caseins; its sedimentability also diminished with increase in concentration of citrates and phosphates.

As for the unheated samples, phosphate samples do not change their level of $\kappa$-casein sedimented, while citrate samples decrease it with salt concentration increase. This conclusion is in agreement with the one taken out of fractionation results, where phosphate samples also do not vary their pellet levels, but citrates do, decreasing it. Phosphate addition increases calcium ion activity, precipitating, creating more instability and therefore leading to bigger particles formation and higher amounts of pellet.

Full denaturation of whey proteins was evaluated by HPLC, and results are presented in Figure 3.20. Like previously seen in the former subchapters (Chlorides and sodium phosphate and citrate), this response was expected, since both the heat treatment applied and also the milk sources are the
same in all experiments. For unheated samples some kind of mistake was made in the sample with a concentration of 5 mM of sodium phosphate, since that point is not justified and completely unexpected.

Figure 3.20: HPLC results for the denaturation of β-lg and α-lac with the addition of either sodium phosphate or sodium citrate (represented by Na2P or Na3C, respectively) at 0, 1, 2.5, 5 or 10mM to SMP, unheated (U) or heated (for 15min and 90° C), at the pH values of 6.5, 6.7 and 6.9.
3.1.2 Protein Additions

The approach followed after the mineral additions was the addition of whey proteins as a supplement to both MPC and SMP, in order to evaluate the effects on heat stability.

Usually, by just heating SMP and MPC60, MPC80 or MPC90 without any supplements added, it is verifiable that SMP remains stable while the concentrates turn quite unstable -like seen previously in the Chapter 2 - specially the ones with higher protein content - MPC80 and MPC90. This was seen in the average particle size results presented in Figure 3.1, where MPC90 coagulated and MPC80 showed a really high average particle size when comparing with MPC60 and specially SMP.

However, what was discovered in this set of experiments was that the tendencies of stability or lack of it that were seen during heating could be completely reverted by adding extra whey protein to the dissolved powders. In the past, it had already been verified that in the presence of whey proteins, SMP would lose its stability during thermal treatment; this behaviour was not completely expected, having in consideration the theory behind the connection of whey proteins and $\kappa$-casein. Verifying this event, research was never pursued further on to MPCs.

The relevant discovery made during the present work was that adding whey proteins to MPC90 stabilized it. MPC90 proved to be more stable during heat treatment with added whey proteins than without them - passing from a point of complete instability to a moderate stability. For this reason other MPCs were evaluated and different concentrations of whey proteins were tried.

The following experiments were performed around this discovery.

A trend that is displayed in Figure 3.21 is the increase of the average particle size in SMP results with the increment of added whey proteins, noticing that, indeed, SMP turns out to be less stable with more added whey proteins, showing bigger particles; however, the exact same amounts of added whey protein have the opposite effect if added to MPCs, stabilizing them. The SMP sample at a pH of 6.5 supplemented with 1% (w/w) whey protein is not present in the data because it coagulated; it would have made more sense for this coagulation to have happened also or instead for the 2% (w/w) concentration of added whey protein. It can be seen that at higher pH values SMP continues to be destabilized by the addition of whey proteins, but more moderately. Results for fractionation, Zetasizer's distribution particle size and HPLC are presented in Appendix D.

It is also worth to notice the huge difference in particle size showed by MPC90s at a pH of 6.5, in Figure 3.22, suggesting a promising result, once it turns something completely unstable (for 0% of added whey proteins) - with considerable big particles - into something quite stable (for concentrations above 1% of added whey proteins).

Analysing pH values 6.7 and 6.9, the differences are blurred. The increasing attenuation of instability observed can be explained by the already stable values without added whey proteins. If the system is not unstable, it does not need stabilization, and apparently added whey protein will also not disturb it. The fractionation results for this MPC90 experiment are presented in the Appendix E, and comparing with the fractionation SMP results displayed in Appendix D may be interesting.

Besides the extreme behavioural cases, namely SMP and MPC90, intermediate MPCs were also inspected for their behaviour with whey protein additions, as it is shown in Figure 3.23. The results
Figure 3.21: Average particle size of the 50 times diluted samples at 20°C, of heated (15 minutes and 90°C) SMP with either 0%, 0.5%, 1% or 2%(w/w) of added whey proteins, in three different pH values (6.5, 6.7 and 6.9).

Figure 3.22: The average particle size of the 50 times diluted samples at 20°C, of heated (15 minutes and 90°C) MPC90 with either 0%, 0.5%, 1% or 2%(w/w) of added WPI, in three different pH values (6.5, 6.7 and 6.9).

turned out to be fairly logical, with MPC80 having a behaviour closer to MPC90’s and MPC60 behaving more like SMP.

Coagulated samples are not present in the particle size data (Figure 3.23), viz. the MPC80 without supplemented whey protein at a pH of 6.5. As it has been observed in all experiments, at a pH of 6.9 the differences are a lot less milder, turning out to be quite more interesting to look at lower pH values to spot influences. Here, a first look should be directed at the behaviour of samples when whey protein is absent and their progression with the increasing built on whey proteins’ concentration. In both MPC80 and MPC90, just by adding an amount as small as 0.5% (w/w) of extra whey protein, a huge change is already seen, passing the sample from coagulation to a very reasonable stable state. In MPC60, however, the difference was not so prominent, diminishing a little the average particle size by whey proteins’ addition, although MPC60 showed already reasonably stable without any whey proteins additions.

It can then be stated that MPC80 has a really similar behaviour to MPC90’s and that MPC60 is an intermediate that does not act either as SMP or as MPC90. Thus, so far it is possible to conclude that the higher the protein content of the milk source the more stable a system will turn out to be by the addition
Two questions then remain: the extent to which whey protein should be added to have its stabilizing effect in MPC’s and what exactly, in whey proteins is responsible for the stabilization. To answer the first question different amounts of whey protein were added, evaluating the quantity that should be added for each MPC to reach stability, and to answer the second question the two whey proteins, $\beta$-lg and $\alpha$-lac, were added in separate.

Considering that tendencies and changes are more visible at a pH of 6.5, the experiments were performed at this pH.

Besides adding $\beta$-lg and $\alpha$-lac in separate, a combination of $\beta$-lg and $\alpha$-lac in the correct natural proportions was also tried, specifically adding 2/3 and 1/3, respectively.

From the previous experiments it was already concluded that the concentrations of whey protein required to be added to the MPC60 were smaller than the whey protein concentrations required to be added to the MPC80. For this reason, in Figure 3.24, the concentrations tested in MPC60 of $\beta$-lg and $\alpha$-lac are smaller, varying from 0% to 1% (w/w), while the ones tested in MPC80 start at 1% (w/w). No sample has coagulated in this procedure, but only more sensible concentrations were tested since the goal was to refine conclusions.

Regarding in Figure 3.24 the results for the supplemented MPC60s is then possible to observe that increasing the added concentration of $\beta$-lg stabilizes the system, leading to smaller particles’ sizes. Instead, adding increasing concentrations of only $\alpha$-lac has a destabilizing effect, being these bars higher than any other additions: whey proteins, $\beta$-lg, or $\beta$-lg and $\alpha$-lac added together.

For MPC80, it is also possible to observe that increasing the addition of $\alpha$-lac has no stabilizing effect, while on the other hand, the addition of more $\beta$-lg highly decreases the average particle size, indicating stabilization.
Figure 3.24: The average size of the 50 times diluted samples at 20°C, of heated (15 min and 90°C) MPC60 and MPC80 with either 0.1%, 0.2%, 0.5%, 1% or 2% (w/w) of added WPI, β-λg and α-lac at pH 6.5.

With this data it is clear to conclude that β-λg is the responsible protein for the stabilization observed, since its absence causes results to get unstable. Also that it is not an interaction between the two proteins, but β-λg alone. For this reason experiments continued to be performed with only the addition of this protein, like shown in Figure 3.25, to SMP, MPC60, MPC80 and MPC90 at a pH of 6.5, starting from a concentration of 0%, and increasing cumulatively 0.2% until 2% (w/w) of β-λg. Absent samples coagulated.

Figure 3.25: Average size of the 50 times diluted samples at 20°C, of heated (15 minutes and 90°C) SMP, MPC60, MPC80 and MPC90 with either 0%, 0.2%, 0.4%, 0.6%, 0.8%, %1, 1.2%, 1.4%, 1.6%, 1.8% or 2% (w/w) of added β-λg at pH 6.5.

Similarly to what was seen for the addition of whey proteins, identical tendencies are also found in Figure 3.25 for the addition of only β-λg. Starting by looking at the red bars, that represent SMP, it is possible to see a proportional increase in particle size, and therefore destabilization, with the increase of added β-λg; following the green bars, that represent MPC60, a very slight decrease can be spotted even if in an overall view the samples just remain constantly stable; for the blue bars, that on their turn
represent MPC80 samples, the exact opposite effect seen for SMP is observed, decreasing the particle size proportionally with the increase of $\beta$-lg concentration, stabilizing the system; for the purple bars, that represent MPC90, a very high instability was observed without $\beta$-lg added, leading to coagulation and therefore absent data for the first two lower concentrations - simultaneously, for higher concentrations of added $\beta$-lg, stability starts to be gained.

In Figure 3.26, instead of the average particle size results, size distribution results are shown, to give a clearest representation of how stability changes between samples, and how a big amount of small particles can be transformed into fewer bigger particles, by simply adding whey proteins - or the opposite - in SMP and MPC90, respectively.

![Distribution size of the 50x diluted samples at 20°C, of heated (15min and 90°C) SMP, MPC60, MPC80 and MPC90 with either 0%, 0.2%, 0.4%, 0.6%, 0.8%, %1, 1.2%, 1.4%, 1.6%, 1.8%, or 2% (w/w) of added $\beta$-lg at pH 6.5](image)

Figure 3.26: Distribution size of the 50x diluted samples at 20°C, of heated (15min and 90°C) SMP, MPC60, MPC80 and MPC90 with either 0%, 0.2%, 0.4%, 0.6%, 0.8%, %1, 1.2%, 1.4%, 1.6%, 1.8%, or 2% (w/w) of added $\beta$-lg at pH 6.5

Finally, more in terms or curiosity, another experiment has been performed, which consisted on letting the samples stay in the water bath for some more time, still at 90°C but for 21 minutes. The results can be seen in Figure 3.27, and by comparison with 3.26, it can be stated that the particles size has decreased, in general, except for higher concentrations of added $\beta$-lg in MPC60 and MPC80. This implies that a bigger time imposed on the heat load, diminishes the particle average sizes, what can be explained by a promotion of more $\kappa$-casein dissociation when not enough whey protein is added, and therefore less denatured whey proteins connecting and forming instead smaller particles. This agrees with Law and Leaver [19], when said that prolonged heating time or temperature had an effect on the
extent of denatured whey proteins connection with casein micelles.

![Figure 3.27: Distribution size of the 50x diluted samples at 20°C, of heated (21min and 90°C) SMP, MPC60, MPC80 and MPC90 with either 0%, 0.2%, 0.4%, 0.6%, 0.8%, %1, 1.2%, 1.4%, 1.6%, 1.8%, or 2% (w/w) of added β-lg at pH 6.5](image)

To interpret all these results together there is the need to understand what is physically different between SMP and MPC80, since it must be the responsible for the totally dissimilar behaviours seen. Like mentioned in the Background chapter, how these two powders are produced is quite important and it justifies the differences observed. By ultracentrifugating milk, lactose and soluble minerals will be grossly eliminated (Cl, Na, K, Mg) and the extent to which this is made results in the creation of the different MPCs. This loss of minerals will implicate that the general ionic strength becomes lower, what will increase the calcium ion activity. Since this calcium ion activity is so important to keep micelles integrity, anything that interacts with it may be essential to give further instability or stability.

It is known that κ-casein has an essential role in delimiting micelles, and in that sense, if denatured whey proteins connect to it, the protectorate layer increases. The higher dissociation of κ-casein also has important consequences for size and stability.
3.2 Pilot Plant

3.2.1 Protein Additions

3.2.1.1 Scalability

The scalability of the process is utterly relevant, reason why thermal treatments applied to both industrial SMP and MPC80 with added Whey Protein Isolate (WPI) needed to be attempted at the pilot plant. At the resemblance of the others experiments, the reconstitution of the milk powders was made to a final native protein concentration of 3.5% (w/w) before WPI addition. The addition of WPI was made in two concentrations, 0% or 1% (w/w), and the pH was kept at 6.5. Both SMP and MPC80 were tested with and without the addition of WPI and besides the two thermal treatments performed at pilot plant scale, with the same samples heat treatments like autoclave or water bath were additionally performed at laboratory scale. Also unheated samples were kept. At the pilot plant the two heat treatments performed were the UHT, 5 seconds at 140° C, or the heat exchanger, 10 minutes at 90° C. After the thermal conditions were applied, testing was made in the form of the previously executed experiments (fractionation, Zetasizer’s DLS analysis and HPLC).

The absence of the sample "SMP+1% (w/w) WPI AUTOCLAVE" in Figure 3.28 is due to its coagulation during the thermal process. With reference to the remaining samples, it can be stated that the scale up was quite successful, being the tendencies previously observed in the laboratory maintained. Here it is possible to compare the pure samples with their whey protein supplemented ones, as well as to compare between SMP behaviour and MPC80’s, as well as between the different thermal treatments.

![Figure 3.28: The average size of the 50 times diluted samples at 20° C of SMP or MPC80 supplemented or not with 1% (w/w) WPI at pH 6.5. Different heat treatments were applied to this samples: either no treatment at all (U), the standard water bath for 15 minutes and 90° C in the laboratory, the autoclave (121° C for 15 minutes), the heat exchanger in the pilot plant that heated at 90° C for 10 minutes, and the UHT treatment that consisted in 5 seconds at 140° C.](image)

Firstly it is possible to observe that MPC samples without whey protein present really bigger particles, effect that is not seen for supplemented samples. In these supplemented samples, even the one
that passed through the heat exchanger is quite stable. However, it is not advisable to push the heat exchanger to the limit, once that coagulation inside the tubes can cause some problems, motif why the UHT was not applied at the MPC80, with or without whey proteins.

It may also be interesting to notice that for MPC80 samples, the values of the average size were higher in the hot bath treatment than in the heat exchanger. This may even indicate that effects are less marked in the pilot plant, what is convenient.

None of the samples tested, except for pure non-supplemented SMP, survived non-coagulated to autoclave due to the severity of the heat treatment.

Regarding SMP, the conclusion that added whey protein causes instability in the samples during heating is also reinforced once it is possible to see for all supplemented samples higher zeta-average sizes than for non-supplemented ones.

The problem encountered in performing the present experiment in the pilot plant is also the motive why it is interesting and useful to do so: besides the possible unexpected behaviour of samples that sometimes occurs with scale-up, the acceptable level of coagulation that samples are allowed to reach is quite different. Inside small tubes in a water bath, even if samples coagulate completely, the worst case scenario will be that they cannot proceed to further analysis on the testing equipments. At a pilot plant scale more attention needs to be payed; if it is predictable that the sample may coagulate completely, then the test should not be run due to clogging risk. The absence of UHT of the MPC80 with and without WPI is due to this risk prevention: once the exit flow rate had already shown some coagulation indication signs for the MPC80 in the heat exchanger treatment, and since the UHT is a harsher treatment, it was advisable to do not perform it. Supporting this decision is the non-supplemented SMP's difference in particle size between the UHT and heat exchanger treatments; disregarding the addition or not of whey protein, harsher treatments cause more instability, having the particle size increased its size in the UHT. Also supporting the decision is the fact that whey protein supplemented samples of SMP already shown instability.

Looking at MPC80 unheated samples, with or without the whey protein supplement, it can be seen that the average particle size is smaller in the WPI supplemented sample, what may give some insight to whey proteins' behaviour in solution when not denaturated. Perhaps native whey proteins stay in the serum and connect to other components, forming smaller particles and decreasing then the average size of particles. Even if whey proteins aggregate with other whey proteins, the final particle size created would still be small when comparing with aggregates formed with casein micelles.

In Figure 3.29, samples' fractionation is displayed by showing pellet's percentage in each case. As it is possible to spot, between unheated samples differences are not very discernible, but in heated samples some variation is seen. In here, regarding each treatment at a time, it is possible to witness firstly a higher amount of sedimentable particles in all the samples to which WPI was added, and secondly a more evident discrepancy of values between added or absent WPI in the heat exchanger treatment. The most significant difference was observed for the MPC80.

This strongly indicates that casein micelles covered with whey protein are getting more out of solution and sedimenting.
Once the Zetasizer results already have proven that the expected tendencies for added whey protein to SMP and MPC80 were also seen in this case, the justification in this increase of pellet for the supplemented samples can only be that smaller particles are also sedimenting, enlarging the total quantity of pellet.

Figure 3.29: Fractionation resulting from ultracentrifugation at 100000 x g for 1h at 20°C, of SMP or MPC80 supplemented or not with 1% (w/w) WPI at pH 6.5. Different heat treatments were applied to this samples: either no treatment at all, the standard hot water bath at 15min and 90°C in the laboratory, the autoclave (121°C for 15min), the pilot plant heat exchanger treatment (H.E.) at 90°C for 10 minutes, and the UHT treatment that consisted in five seconds at 140°C. Also unheated samples are showed (U).

The results of the distribution of particle size and fractionation are presented in Appendix F.

3.2.1.2 ratio or absolute amount of whey protein-caseins?

From the previous results it is clear that whey proteins in excess, i.e. a higher amount of whey proteins in solution have a stabilizing effect on MPCs behaviour during thermal treatment.

Since milk’s (natural) total protein content is around 3.5% (w/w), and adding extra 1% or 2% (w/w) of whey proteins is a considering amount in proportion, it was suggested to preserve the total protein content but alter the ratio caseins-whey proteins. Instead of having around 80% of caseins and 20% of whey proteins out of the total 3.5% protein content, (meaning 2.8% of caseins and 0.7% of whey proteins), whey proteins would represent 1.7% and caseins 1.8%, and effects would be evaluated.

A final experiment was therefore performed around this principle to evaluate if the whey protein stabilizing effect was due to the absolute amount of more denatured whey proteins connecting to micelles, or if indeed the ratio whey proteins-caseins was more important.

By the delivery time of the present document not all data had been collected, reason why there are no conclusions for the subject. However, it remains a question that deserves further investigation.
Chapter 4

Conclusions

Regarding SMPs and how to stabilize them, three types of additions were made inside mineral additions: caseinates, chlorides, phosphates and citrates.

For caseinates addition it can be stated that monovalent ions caseinates (potassium and sodium) create more stability than divalent ions caseinates (calcium and magnesium). Also that probably harsher effects are seen for calcium caseinates, and more stabilizing effects for potassium caseinates.

Concerning chlorides addition, supplementing SMP with calcium chloride creates more stability during heating than adding magnesium chloride. The reason that explains this matter, why magnesium forms bigger particles, is yet unknown.

Finally, the addition of either sodium citrate or sodium phosphate, verifies that sodium citrate has a more stabilizing effect to the system.

With respect to protein additions, whey proteins and both $\beta$-lg and $\alpha$-lac isolated additions were assayed to SMP and different MPCs.

As a conclusion, it was seen that whey proteins’ addition destabilizes SMP systems, during thermal treatment, disregarding the pH; and also that whey proteins’ addition stabilizes the different MPCs - at least for the heat load applied of 90°C for 15 minutes. More specifically, the responsible for this stabilization are not whey proteins in general, but only $\beta$-lactoglobulin, as $\alpha$-lactalbumin, when added alone, has no stabilizing effect.

It can be concluded as well that the higher the MPC protein content, the more added whey proteins needed to stabilize it, and therefore a higher stabilizing effect will be obtained (since in this case samples are more unstable without any added protein).

With these new knowledge obtained on what can serve as a destabilizer or stabilizer source, additions and similar approaches can be executed into specific cases where these goals are present.

A more theoretical conclusion can also be taken by having verified with the data results showed that indeed it exists a higher level of $\kappa$-casein dissociation at higher pHs, contributing to form smaller particles that do not sediment; another important conclusion was to verify calcium-ions’ activity as very important in determining the stability of the system, contributing to higher levels of sedimented particles, where whey proteins and $\kappa$-casein could be found in a bigger extent, connected with the remaining
casein-micelles.

The scalability of this achievement was also tested, and SMP and MPC80 at a pH of 6.5 were supplemented with 1% (w/w) of WPI and gone through four different treatments: two treatments on the pilot plant at the heat exchanger - at 90° C for 10 minutes and 140° C for 5 seconds (UHT), the usual water bath at 90° C for 15 minutes in the laboratory and the autoclave for 121° C for 15 minutes. Autoclave proved to be the harsher treatment, for which only SMP remained stable. The UHT treatment was not even rehearsed for MPC80, since indications were present that it would not withstand such a treatment - perhaps a higher concentration of added whey proteins would avoid coagulation in the equipment. The treatment at 90° C for 10 minutes proved scale-up to be possible, once the previous trends seen at the laboratory were maintained.

The heat load applied is also important for the conclusions taken, mainly because it not as harsher as it could be, not influencing some remaining factors that could modify the observed effects. This means that it is important to have present that the conclusions taken are valid inside the conditions applied.

4.1 Achievements

The major achievement of the present work was to discover that MPCs can be stabilized during heat treatment by simply adding β-lg. Considering that ingesting more proteins is a current concern of developed and non-developed societies, and that alternatives to animal direct sources are actively searched, to add this extra isolate that could not only facilitate the processing of the products but also increase its nutritional content, will for sure bring increased value.

Some other components bring stability to the system, but they are either not advisable to consume (being the result of only laboratory research) or are not as efficient as whey proteins in stabilizing. Also, since milk protein concentrates may be simpler to use than skim milk powder in some applications, it is advantageous to manage and control its properties correctly.

4.2 Future Work

Ideally, further development of techniques to monitor changes in milk in situ during heating would significantly advance the understanding of the heat-induced coagulation of milk.

It would also be advisable to conduct more tests to get sharper conclusions. Regarding mineral additions, it could be suggested to try them in MPCs, for example.

Different thermal treatments should be tried to verify the validity of results, different pHs should be tested to define ranges of behaviours and scale-up should be refined once it is the main final goal. Some behaviours were slightly unequal to the ones seen at the laboratory, and by regarding the importance of predicting correct behaviours, more information should be collected.
Bibliography


Appendix A

#1: Caseinates

Figure A.1: Lovis Dynamic Viscosity for heated (15 minutes and 90°C) SMP with either 1% (w/w) of potassium, sodium, magnesium or calcium caseinate, at three different pHs (6.5, 6.7 and 6.9).
Figure B.1: Distribution particle size of the 50 times diluted samples at 20°C, of heated (15 minutes and 90°C) or unheated SMP with either 1 or 2.5 mM of magnesium chloride (represented by MgCl₂) at three different pHs (6.5, 6.7 and 6.9).
Figure B.2: Particle Size Distribution of SMP with magnesium chloride)Distribution particle size of the 50 times diluted samples at 20°C, of heated (15 minutes and 90°C) or unheated SMP with either 5 or 10 mM of magnesium chloride (represented by MgCl$_2$) at three different pHs (6.5, 6.7 and 6.9).

Figure B.3: Distribution particle size of the 50 times diluted samples at 20°C, of heated (15 minutes and 90°C) or unheated SMP with either 1 or 2.5 mM of calcium chloride (represented by CaCl$_2$) at three different pHs (6.5, 6.7 and 6.9).
Figure B.4: Distribution particle size of the 50 times diluted samples at 20°C, of heated (15 minutes and 90°C) or unheated SMP with either 5 or 10 mM of calcium chloride (represented by CaCl$_2$) at three different pHs (6.5, 6.7 and 6.9).

Figure B.5: Distribution particle size of the 50 times diluted samples at 20°C, of heated (15 minutes and 90°C) or unheated SMP at three different pHs (6.5, 6.7 and 6.9).
Appendix C

#3: Phosphates & Citrates

Figure C.1: Distribution particle size of the 50 times diluted samples at 20°C, of heated (15 minutes and 90°C) or unheated (U) SMP with either 1 or 2.5 mM of sodium citrate (represented by Na3C) at three different pHs (6.5, 6.7 and 6.9).
Figure C.2: Distribution particle size of the 50 times diluted samples at 20°C, of heated (15 minutes and 90°C) or unheated (U) SMP with either 5 or 10 mM of sodium citrate (represented by Na3C) at three different pHs (6.5, 6.7 and 6.9).

Figure C.3: Distribution particle size of the 50 times diluted samples at 20°C, of heated (15 minutes and 90°C) or unheated (U) SMP with either 1 or 2.5 mM of sodium phosphate (represented by Na2P) at three different pHs (6.5, 6.7 and 6.9).
Figure C.4: Distribution particle size of the 50 times diluted samples at 20° C, of heated (15 minutes and 90° C) or unheated (U) SMP with either 5 or 10 mM of sodium phosphate (represented by Na2P) at three different pHs (6.5, 6.7 and 6.9).
Appendix D

#4: SMP + Whey proteins

Figure D.1: Fraction of pellet that has resulted from ultracentrifugation at 100 000 x g for 1 hour at 20° C, of heated (15 minutes and 90° C) SMP with either 0.5%, 1%, 2% (w/w) of added whey proteins at three different pHs (6.5, 6.7 and 6.9).
Figure D.2: HPLC results for the sedimentability in SMP with either 0.5%, 1%, 2% (w/w) of added whey at three different pHs (6.5, 6.7 and 6.9).
Figure D.3: Distribution particle size of the 50 times diluted samples at 20°C, of heated (15 minutes and 90°C) or unheated (U) SMP with either 0.5%, 1%, 2% (w/w) of added whey at three different pHs (6.5, 6.7 and 6.9).
Appendix E

#5: MPC90 + Whey proteins

Figure E.1: Fraction of pellet that has resulted from ultracentrifugation at 100 000 x g for 1 hour at 20°C, of heated (15 minutes and 90°C) MPC90 with either 0.5%, 1%, 2% (w/w) of added whey proteins at three different pHs (6.5, 6.7 and 6.9).
Appendix F

#10: SMP, MPC80 + WPI

Figure F.1: Distribution particle size of the 50 times diluted samples at 20°C, of SMP or MPC80 supplemented or not with 1% (w/w) WPI at pH 6.5. Different heat treatments were applied to this samples: either no treatment at all, the standard hot water bath at 15min and 90°C in the laboratory, the autoclave (121°C for 15 minutes), the pilot plant heat exchanger treatment (H.E.) at 90°C for 10 minutes, and the UHT treatment that consisted in five seconds at 140°C. Also unheated samples are showed (U).
Figure F.2: Fraction of pellet that has resulted from ultracentrifugation at 100,000 x g for 1 hour at 20°C, of SMP or MPC80 supplemented or not with 1% (w/w) WPI at pH 6.5. Different heat treatments were applied to this samples: either no treatment at all, the standard hot water bath at 15 minutes and 90°C in the laboratory, the autoclave (121°C for 15 minutes), the pilot plant heat exchanger treatment (H.E.) at 90°C for 10 minutes, and the UHT treatment that consisted in five seconds at 140°C. Also unheated samples are showed (U).