Effect of Ohmic Heating on Colloidal Stability of Cheese Powder Dispersions

Carolina Pombo Andrade

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Biotechnology

Supervisors: Prof. Mohammad Amin Mohammadifar
Prof. Marília Clemente Velez Mateus

Examination Committee

Chairperson: Prof. Nuno Gonçalo Pereira Mira
Supervisor: Prof. Mohammad Amin Mohammadifar
Members of the Committee: Prof. José António Leonardo dos Santos

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Preface and Declaration

Preface

The work presented in this thesis was performed at DTU National food institute, in the reesarch group for Food Production Engineering, during the period February - October 2022, under the supervision of Assoc. Prof. Mohammad Amin Mohammadifar, and within the frame of the Erasmus programme. The thesis was co-supervised at Instituto Superior Técnico by Prof. Marília Clemente Velez Mateus.

Declaration

I declare that this document is an original work of my own authorship and that it fulfills all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

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Abstract

Cheese powder is manufactured by mixing melted cheeses with water and emulsifying salts (ES) to form a homogeneous and stable emulsion, denoted cheese feed, until and during spray drying. The presence of emulsifying salts influences several critical events, including pH adjustment, calcium chelation from the casein micelle by exchanging sodium ions, consequent solubilization of proteins, and subsequent fat emulsification. However, demands for reduction of sodium in foods makes production of cheese powder without emulsifying salts desirable, hence new strategies for stabilization of cheese feed are needed. The advantages of ohmic heating (OH) as heat treatment are considered an attractive option for the dairy industry and so, the present study aimed at understanding the effect of this technology over the use of conventional heating (CH) methods on the stability of cheese emulsions. Cheese powder dispersions with no ES were prepared and processed with different conditions: electricity (OH or CH), temperature (85ºC or 60ºC), and number of homogenizations. OH had a great impact on calcium diffusivity from the casein micelle, which can be related to disturbances on charge organization within protein structure. Overall, ohmic heating is an exciting opportunity to manipulate calcium partitioning between colloidal and serum phase in cheese powder dispersions, and so it could be used to modulate protein functionality by affecting casein micelle structural integrity. However, results indicated that ohmic heating alone is not sufficient to reach the desired quality characteristics. Therefore, charge distribution of the proteins should also be adjusted to an optimum value by other treatments.

Keywords: Ohmic heating, cheese emulsions, stability, calcium solubilization

Abstract in Portuguese

O queijo em pó produz-se ao misturar intimamente queijos derretidos e sais emulsificantes (SE), formando uma emulsão estável e homogénea, denominada "massa-de-queijo", mantida durante a secagem por pulverização. Os SE influenciam diversos eventos críticos, incluindo ajuste do pH, quelação de iões cálcio da micela de caseína e troca pelos de sódio, solubilização de proteínas, e emulsificação de gordura. Contudo, para reduzir o teor de sódio na comida, é desejável produzir queijo em pó sem SE. Por isso, são necessárias novas estratégias para estabilização da massa-de-queijo. O aquecimento óhmico (AO) é uma opção de tratamento térmico atrativa nas lacto-indústrias. Este estudo pretende comparar os efeitos desta tecnologia e de métodos de aquecimento convencional (AC) na estabilidade das emulsões. Prepararam-se dispersões de queijo em pó sem SE e processaram-se com diferentes condições: eletricidade (AO ou AC), temperatura (85ºC ou 60ºC), e diferente número de homogeneizações. AO demonstrou ter um grande impacto na difusão de cálcio da micela de caseína, o que poderá relacionar-se com perturbações na organização de cargas na estrutura das proteínas. No geral, AO é uma oportunidade entusiasmante para manipular a partição de cálcio entre as fases coloidal e solúvel nas dispersões de queijo em pó, e pode ser usado para modular a funcionalidade das proteínas por afetar a integridade estrutural da micela. Individualmente, AO parece ser insuficiente para alcançar as características desejadas, pelo que a distribuição de cargas das proteínas deverá ainda ajustar-se ao valor ótimo através de outros tratamentos.

Palavras-chave: Aquecimento óhmico, emulsões de queijo, estabilidade, solubilização de cálcio

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List of Symbols and Acronyms

Symbols

σ	Eletrical conductivity
E	Electric field strength
V	Voltage
L	Distance between electrodes
$V_{\max}$	Maximum voltage

Acronyms

LAB	Lactic acid bacteria
O/W	Oil-in-water
β - Lg	β - Lactoglobulin
BSA	Bovine serum albumin
CCP	Colloidal calcium phosphate
MFGM	Milk fat globule membrane
ES	Emulsifying salts
IC	Intact casein
SC	Sodium caseinate
BMP	Butter milk powder
OH	Ohmic heating
CH	Conventional Heating
FAST	Fluorescence of advanced Maillard products and Soluble Tryptophan
H	Homogenization
NFC	Nitrogen conversion factor

NMR	Nuclear magnetic resonance
ICP-MS	Inductively coupled plasma – Mass spectrometry
PSD	Particle size distribution
S-MLS	Static multiple light scattering
T	Transmission
BS	Backscattering
CLSM	Confocal laser scanning microscopy
DM	Dry Matter
$D_{[4,3]}$	Volume-weighted mean diameter
PSD	Particle size distribution
$d_{(0.1)}$	10% of the total amount of sample is smaller than this size
$d_{(0.5)}$	50% of the total amount of sample is smaller than this size
$d_{(0.9)}$	90% of the total amount of sample is smaller than this size

Chapter 1

Introduction

Cheese powder is a convenient flavouring agent that can be applied in a wide variety of food products [1], and its processing include cutting different kinds of cheese, adding water and emulsifying salts, mixing, heat treatment, and spray drying. To obtain a stable, without protein precipitation or creaming, homogeneous and pumpable emulsion, named cheese feed, before atomisation, emulsifying salts (ES) are introduced due to their pH adjustment and calcium sequestering abilities, which removes a large portion of calcium attached to the casein micelle and replaces it by sodium. This event breaks intra- and inter-casein molecular cross-links and increases negative charges. Consequently, a more open reactive casein conformation is favored, with superior water-binding capacity than natural cheese, leading to fat emulsification [2, 3]. Nonetheless, dairy producers are under increasing pressure from health conscious consumers to reduce or remove salt in their products, and there is also an increasing interest in the production and application of natural food ingredients, which can lead to "clean labelling" of the final food products. Hence, new strategies for stabilisation of cheese feed without ES are needed [4].

Optimization of processing parameters may improve emulsion stability during production of processed cheese, and so, similar effects could be expected on the stability of cheese feed [5]. The heat treatment step present in cheese powder production, responsible for melting, is one of the processing conditions that has a major effect on the calcium equilibrium, which can interfere with the casein conformation and aggregation state. Usually, high temperatures lead to a decrease in diffusible calcium from the micelle, due to its precipitation [6], which has a negative effect in protein solubilization and fat emulsification. Therefore, new heating systems should be tested to evaluate their effect on emulsion stability.

Ohmic heating (OH) is considered an emerging processing technology that is able to overcome the problems that arise from the use of conventional heating. These last methods, despite being the most used to ensure microbial safety, can lead to overheating of foods, which consequently causes loss of nutritional compounds and sensory changes [7]. Since OH is a process wherein electric current passes through materials, the increase in temperature is due to internal energy transformation (from electrical to thermal), leading to a rapid and uniform heating of the food, when compared to the traditional methods

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[8, 9]. Besides ensuring nutrient retention capacity and sensory attributes, this technology is easier to control, presents a great variety of designs, and is more energy efficient and environmental friendly. Hence, the use of this new technology as a heat treatment is an attractive option for the dairy industry [10].

1.1   Thesis aim and hypothesis

The overall aim of the present study was to evaluate the effect of ohmic heating on colloidal stability of cheese powder dispersions. The use of electricity and process parameters, such as frequency and electric field, may influence casein micelles and protein conformational structures [11], as well as the equilibrium between the soluble and insoluble calcium. Hence, as OH influences ionized materials, we hypothesize that its usage may increase the diffusibility of calcium, even at high temperatures, present in the micelle, solubilizing the proteins, which can then emulsify the fat droplets, allowing the manufacture of a stable emulsion without addition of emulsifying salts. Due to being more convenient and ready-to-use, the experiments were done on cheese powder dispersions to mimic the cheese feed formed during production. The samples were then processed using OH and conventional heating and the stability was characterized through different parameters, such as particle size distribution, rheology, and physical stability, and the microstructure was evaluated by confocal laser scanning microscopy. The calcium, protein and fat content of the sediment and serum phases after processing were analysed. No other study has been reported regarding the use of this emerging technology on cheese feed stability, making this investigation a first step to obtain a knowledge platform that provides understanding on the possible effects of OH on cheese feed, and in the future, in powder properties and functionality.

1.2   Thesis outline

This thesis is organized according to Figure 1.1. Chapter 2 gives a brief overview about the history of cheese, and cheese manufacture, as well as the components that make up this product. It allows the reader to gain knowledge regarding the topic so that it is easier to understand the experience and to interpret the results obtained. Chapter 3 concerns the experimental procedure of this project, where the materials and the experimental and analytical methods used are explained. In Chapter 4, the results obtained are presented and explained, and one can evaluate the effect that ohmic heating has on the stability of cheese powder dispersions and on the diffusibility of calcium, protein solubility, and fat emulsification.

Chapter 2
State of the art

2.1. Cheese history, manufacture, and components
2.2 – 2.3. Cheese powder production and cheese feed stability
2.4. Ohmic heating

Chapter 3
Experimental procedure

3.1. Experimental design
3.2. Materials used during this project
3.3. Procedures for sample preparation
3.4. Procedures to characterize the emulsion stability and protein, fat and calcium distribution

Chapter 4
Results and discussion

Overview of the experiments and their outcomes and discussion of the results obtained

Chapter 5
Conclusions

5.1. Review of the project and main conclusions taken
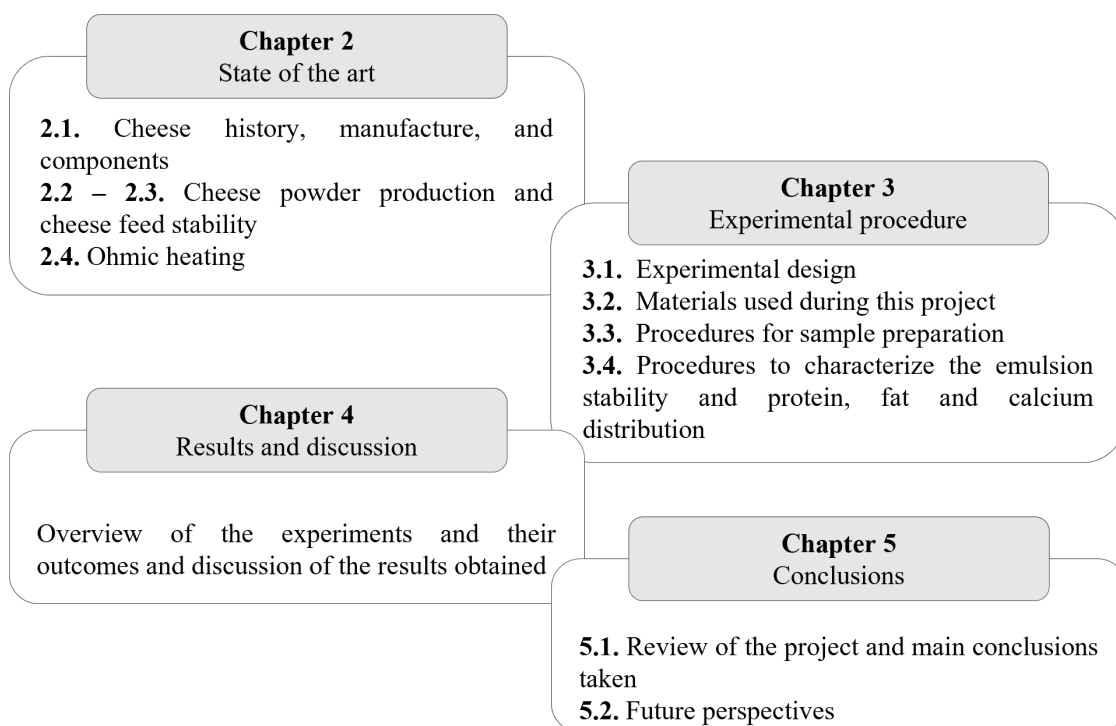**5.2.** Future perspectives

Figure 1.1: Schematic representation of this thesis

Chapter 2

State of the art

"Cheese. The adult form of milk. Hard and soft; new, mature, and overripe. Graced with a spectrum of smells from wispiness to shocking assault upon the nostrils."

Richard Condon, *A Talent for Loving*, 1961

2.1 Cheese

2.1.1 History

For us, that live in the twenty-first century, it is quite hard to imagine how such an important and popular product as Cheese was not part of the day-to-day life. The actual time and place of the origin of cheese and cheesemaking is unknown, however, new advances in a diversity of fields of research have led to new insights into humanity´s earliest experiences with cheese [12]. It is commonly believed that it started around 8,000 years ago, in a region known as the "Fertile Crescent" between the Tigris and Euphrates rivers, in Iraq, during the "Agricultural Revolution", a period characterized by the cultivation of plants and domestication of animals. Despite being the dominant dairy species in most part of the world nowadays, cattle were not the first dairy animals being domesticated. Goats and sheep were small, gregarious, and easily herded, used mainly to supply meat, hides and wool [13]. Humans soon realized the nutritive value of milk produced by these animals, and dairy products became important components of the human diet [14].

It is thought that the first fermented dairy foods may have been produced accidentally. Milk is a rich source of nutrients for contaminating bacteria, being some of them known as lactic acid bacteria (LAB) as they use the sugar, lactose, as a source of energy, producing lactic acid as a by-product. Bacterial growth and acid production most likely occurred in milk during storage or to dry milk due to the dry climate, to produce a more stable product. When a certain amount of acid is produced, caseins, the principal proteins in milk, coagulate at ambient temperature, around their isoelectric point (pH 4.6), forming a gel in which the fat and aqueous phases of milk are entrapped. This acid-induced milk gel is quite stable

when left undisturbed but, if broken, due to the movement of storage vessels, for instances, it separates into curds and whey. The first ones could be consumed fresh or stored for future use, while acid whey is a refreshing drink to be consumed immediately [14].

Lactic acid produced *in situ* is believed to have been the original milk coagulant, but proteolytic enzymes were also recognized as an alternative mechanism. It started with the practice of storing milk in stomachs of animals, before the development of pottery, as these provided a ready-made, easily sealed containers. As the stomach contains enzymes, such as chymosin and some pepsin, known as rennet, milk would extract them from the tissue, leading to its coagulation during storage [14].

The conversion of the principal constituents of milk to cheese brought some advantages in terms of storage stability, easiness of transport, and diversification of the human diet, which led to the parallel spread of cheese manufacture and civilization throughout the Middle East, Egypt, Greece, and Rome. This is proven by the several references in the Old Testament to cheese, e.g., Job (1520 BC, where Job remarks to God " did Thou not pour me out like milk and curdle me like cheese"; Job 10.10). Milk and dairy products formed an important part of the diet of people of the Near East during Biblical times. Furthermore, cheese is represented in the tomb art of Ancient Egypt and in Greek literature [14], e.g. Homer (1184 BC) and Aristotle (384 – 322 BC) [13]. In this last country, it was used in the basic daily Greek meal, but also enjoyed in the form of cheesecake by the aristocracy during the symposium, or drinking party [15]. In Classical Rome, cheese manufacture was well established, and this ingredient was included in the rations of Roman soldiers. The movements of Roman armies and administrators promoted the spread to cheese throughout the world, and the migrations of people throughout Europe after the fall of the Empire led to the expansion of cheese manufacture [13].

Nonetheless, the major contributors to the development of cheese "technology" and to the evolution of cheese varieties during the Middle Ages (5th to the 15th century) were the monasteries. Along with the spread of Christianity and expansion of knowledge, the monasteries highly contributed to the advancement of agriculture in Europe and to the development and improvement of food commodities, such as wine, beer, and cheese [14].

The 17th century arguably marked a turning point in the history of cheese, giving rise to the last step in the diffusion of cheese worldwide. This resulted from the explosive growth of urban populations in rapidly expanding cities, the establishment of global trade networks by major European powers as they competed to colonise North and South America, Oceania, and Africa, and from the scientific advances that stimulated the scientific and industrial revolutions [12, 14]. Afterwards, cheese became an item of considerable economic importance in a number of these countries, notably the USA, Canada, Australia, New Zealand, and Argentina [14].

Nowadays, there is a highly efficient, large-scale, technology-intense industrial manufacture in most of

the world´s cheesemaking regions, but the production of hand-crafted artisanal cheeses on small scales using traditional practices still persists in specific geographic regions [12].

2.1.2 Manufacture

With this being said, cheese production has a long history which is reflected in the diversity of cheese varieties and in the wide range of technologies used for their manufacture [16]. Most varieties evolved by accident due to particular local circumstances, such as the breed of the dairy animal, the chemical composition or microflora of the milk supply, or the growth of moulds during storage. These accidents were then incorporated into the manufacturing protocol, which has undergone a series of evolutionary changes and refinements over time [13].

Cheese is considered the most diverse, interesting, and challenging group of dairy products. These last are biologically and biochemically stable if properly manufactured and stored, whereas cheeses are considered dynamic, undergoing changes in flavour, texture, and functionality [17].

Cheesemaking can be subdivided into two phases, manufacture, and ripening, both involving a complex series of consecutive and simultaneous microbiological, biochemical, and chemical events. It may lead to products with the desired flavours, physicochemical characteristics, functional properties, and structure, when balanced, or off-flavours, when unbalanced events happen [17]. The manufacture step corresponds to a dehydration step in which the fat and protein present in milk are concentrated to different degrees depending on the cheese variety. It determines the nature and quality of the finished cheese. In its turn, it is during the ripening phase that the characteristic flavour and texture of the individual cheese varieties develop. In general, a similar raw material, milk, is subjected to a manufacturing protocol, whose general principles are common for the majority of cheese varieties, demonstrated in Figure 2.1, making it fascinating how such a diverse range of products is created [14].

2.1.3 Milk

As cheese is a dairy product, it all starts from milk, which is a fluid secreted by the female of all mammals that provides all the nutritional requirements to the neonate of the species. Therefore, this complex oil-in-water (O/W) emulsion supplies energy through fat and sugar (lactose), amino acids from proteins, vitamins, minerals (soluble and insoluble) and water. It also contains minor constituents that serve physiological functions, including antimicrobial systems (immunoglobulins, lactoperoxidase, lactoferrin), enzymes and inhibitors, cell growth and control factors [18–20]. The milk of the principal dairying species (cow, goat, sheep, and buffalo), and the human milk are very well characterized, but for this thesis the bovine milk was considered.
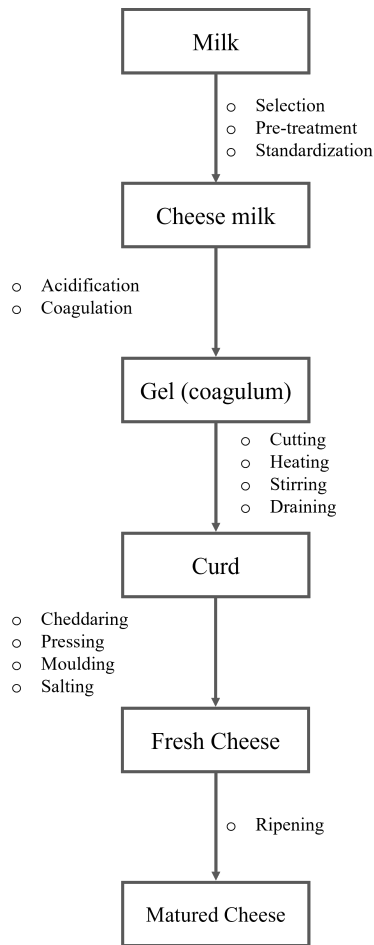
6

Figure 2.1: Flow diagram of the main steps in cheesemaking. Variations are done to produce different types of cheese.

From a cheesemaking point of view, the proteins of milk are its most important constituents and belong to two main categories: the whey proteins and the caseins. Whey proteins consist of four main types – β-lactoglobulin (β - Lg), α-lactalbumin, immunoglobulins, and bovine serum albumin (BSA), and they exist as globular soluble proteins [19, 21]. The fact that whey proteins are denatured by heat and acidity is technologically exploited in the production of whey cheeses, as in Ricotta, and smooth-textured cheeses as Quark [21].

Caseins comprise approximately 80% of the protein content in milk. There are four primary casein molecules α_{s1}, α_{s2}, β and κ-casein, and the mass proportion in milk is 30, 10, 36 and 16%, respectively. These four present different amino acid sequences and exhibit distinct behaviour due to post-translational modifications (phosphorylation for all caseins and glycosylation for κ-casein) [22]. In their native form, the caseins, together with small ions of calcium and phosphate (colloidal calcium phosphate - CCP) form aggregates of several thousand individual protein molecules with an average diameter of 200 nm, known as casein micelles [23, 24], constituting stable and highly hydrated particles [25]. They are synthesized in vesicles within the secretory cells of the mammary gland and then expressed by exocytosis of the vesicles [25].

Regarding the structure of the micelles, the interior is thought to be mainly occupied by α_{s1}, α_{s2} and β-caseins, as these contain a higher amount of serine phosphate residues, which causes them to bind calcium and calcium phosphate strongly. Herewith, these molecules are considered calcium-sensitive caseins and precipitate at relatively low calcium concentrations, including the calcium level present in milk. In turn, κ-casein is located at the surface, with its hydrophobic region inside the micelle, and its glycosylated hydrophilic tail protruding outside, as Figure 2.2 demonstrates. The stability of the micelles is provided by protein-protein hydrophobic interactions, the negatively charged tail of κ-casein, that repel micelles from each other, and the protein-chain cross links mediated by the CCP that held the micelles together [20, 26, 27].
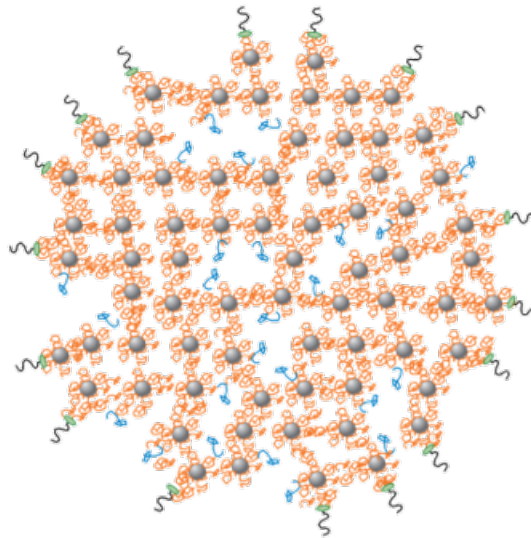

Figure 2.2: Schematic representation of a micelle structure. The α_{s1}, α_{s2}, β-caseins are represented in orange and attached to the calcium phosphate nanoclusters, the grey spheres. Some β-caseins, shown in blue, hydrophobically binds to other caseins. The green spheres together with the black line represents the para-κ-casein and its macropeptide chain on the outermost parts of the surface. The blank spaces represent the water channels within the structure. Reproduced from [25].

Another important milk constituent are lipids, existing in an average of 3.5 g/L in bovine milk. Triglycerides correspond to the predominant type of lipids present in milk, also existing diglycerides, monoglycerides, fatty acids, phospholipids, sterols (mainly cholesterol) and trace amounts of fat-soluble vitamins (A, D, E and K). Fat exists as globules of 0.1 - 20 µm in diameter, surrounded by a structured membrane referred to as the milk fat globule membrane (MFGM), consisting mainly of phospholipids and proteins, which allow the emulsification of lipids in milk [20].

In its turn, salts of milk, despite being quantitatively of minor constituents, they are of major significance to its technological properties. Regarding cheesemaking, calcium and phosphate represent the most important ones. The calcium in milk exists in a dynamic equilibrium between the soluble forms (free calcium ions and soluble undissociated calcium complexes with phosphate and citrate) in the aqueous phase

and the insoluble form, associated, as seen, with the casein micelles, forming the CCP. The precipitation of calcium phosphate to form these nanoclusters is largely influenced by pH and temperature. Decreases in pH usually cause partial solubilization of CCP, while high temperatures leads to a decrease in calcium solubility, leading to the formation of heat-induced CCP, and so, the occurrence of these events throughout cheese manufacture causes an equilibrium between the soluble and insoluble calcium. This equilibrium is commonly termed the "calcium equilibrium" of cheese, and can be disturbed by alteration of manufacturing steps [28].

Herewith, cheese composition is strongly influenced by the milk content of fat, protein, calcium, and pH, which is a critical factor in several aspects of the manufacture and ripening of cheese curd. In turn, these constituents of milk are related with the species, breed, individuality, nutritional status, health, and stage of lactation of the producing animal. When selecting the milk, one should avoid the presence of chemical taints and free fatty acids, as these can cause off-flavours in the cheese, and antibiotics which inhibit bacterial cultures. Furthermore, the milk should be of good microbiological quality, in order to not cause defects or public health issues [29].

2.1.4 Pre-treatment of milk

The majority of the manufactures include a pre-treatment step to improve consistency and for safety reasons. Consistency is one of the most important attributes in the manufacture of quality products. Despite the cheese moisture content and so the amount of protein and fat being determined by the cheesemaking process, the fat:casein ratio results from the fat:casein ratio present in milk [21, 29]. As seen before, the milk components vary in composition, making standardisation crucial, once it maintains compositional uniformity between batches of cheese manufacture, which is important to attain a desired composition of the cheese. This can be achieved through centrifugation of the raw milk, that separates into skim milk and cream. The first is then combined with whole milk or the cream to obtain the desired level of casein and fat [17, 30].

To minimize the health hazards associated with pathogens, pasteurization has been included as a pre-treatment method. The high temperatures at which milk is exposed, as well as the relatively short time (72 ºC; 15s is the standard practice), allows to kill microorganisms and to inactivate some enzymes. The process of pasteurization alters the indigenous microflora of milk which enables the quality of cheese to be more uniform [14, 17, 31, 32]

Afterwards, milk is transferred to vats (or kettles) that can vary in shape, size and be open or closed, and is converted to cheese curd, a process that involves three basic operations: acidification, coagulation, and dehydration [29].

2.1.5 Acidification and coagulation

The coagulation of caseins is an essential step in the manufacture of all cheese varieties, where a gel that entraps the fat is formed. It can be achieved by three possible methods: acidification by a starter culture or acids, and proteolysis using enzymes such as rennet, or by heat/acid coagulation [17]. These ways were used to basic categorise the cheeses into acid-coagulated, rennet-coagulated or heat/acid-coagulated.

Acidification can be achieved as a consequence of two methods. One consists in the *in situ* production of lactic acid through the fermentation of the milk sugar, lactose, by lactic acid bacteria. It previously relied on the indigenous milk microflora to produce acid, but the rate of acidification was unpredictable, which would lead to different qualities of cheese [21]. While this traditional technique is still being used for certain artisanal varieties, selected cultures of LAB, also known as starter cultures, were introduced and have been progressively improved and refined, being globally used in industrial cheesemaking [21, 29].

The alternative method consists in using acid, usually lactic or HCl, or acidogen, such as gluconic acid-δ-lactone. Despite being more controllable than biological acidification, the use of starter bacteria also provides important functions during the ripening process. Therefore, chemical acidification is mainly used for cheese varieties for which texture is more important than flavour [29].

The manufacture of acid-coagulated cheeses is based on the property that caseins are insoluble at their isoelectric point (pH 4.6) forming a gel in which fat is entrapped. Acidification leads to a number of physicochemical changes that promote hydration/dispersion or dehydration/aggregation effects on the casein micelle. The ratio of these effects changes with the reduction of pH, as the onset of coagulation occurs at pH 5.1, and a further reduction to 4.6 leads to the formation of a continuous gel structure [21].

The production of acid at the appropriate rate and time is a key step in the manufacture of good quality cheese, and besides acting as coagulant, as previously referred, it also plays a number of important roles [21, 29], as it:

- Affects the denaturation and retention of coagulant in the curd

- Controls the growth of many non-starter bacteria in cheese

- Influences the activity of enzymes during maturation, affecting both the cheese flavour and quality

- Affects the dissolution of CCP, helping to determine the level of calcium in the cheese curd

- Promotes gel syneresis

The second corresponds to a traditional coagulant used in cheese manufacture. Rennet is primarily made up of the enzyme chymosin, an acid proteinase, also containing other enzymes such as pepsin, but in smaller quantities. The conversion that milk undergoes during rennet coagulation can be classified as a two stage process [17].

The primary phase of the rennet action involves the production of para-κ-casein and glycomacroproteins. The hydrolysis of the κ-casein at the Phe_{105}-Met_{106} site, which is several times more susceptible to acid proteinases than any other peptide bond in the milk system, releases the highly charged, glycosylated hydrophilic region, representing the C-terminal of κ-casein, which protrudes from the micelle surfaces, destroying the principal micelle-stabilizing factors (electrostatic and steric) and their colloidal stability [17, 26].

The second phase is a non-enzymatic stage that involves the aggregation of the casein micelles, which will lead to the formation of a rigid, water insoluble gel like network, usually known as curd. This depends on the concentration of Ca^{2+}, which may act by crosslinking micelles via serine phosphate residues or by charge neutralization. [17, 33, 34]

A small group of cheeses are produced by a combination of heat and acid, being Ricotta the most known and most important member of this group. They are typically fresh soft cheese varieties, just as acid-coagulated cheeses, but the coagulation of milk usually occurs at higher pH (> 5.2) and at temperatures higher than 75ºC [35].

The fat globules, previously dispersed in the milk, become surrounded by the casein as the coagulum forms, being trapped together with serum, which is called whey and contains the water soluble components, including, lactose, minerals, and whey proteins [36].

2.1.6 Post-coagulation processes

Afterwards, the gel is subjected to a variety of treatments that can be generically classified as dehydration with the purpose of removing whey from the gel and to concentrate the casein, fat and micellar calcium phosphate to the desired degree according to the cheese variety [17]. The coagulated gels are quite stable when left undisturbed but when cut or broken or subjected to external pressure, a process called syneresis is enhanced. This consists in the squeezing out the whey, due to the para-casein matrix contraction [17, 37].

Syneresis allows the cheesemaker to control the moisture content of the cheese and, consequently, the activity of microorganisms and enzymes, the biochemistry of ripening and the composition, stability, and quality of the finished cheese [38].

The variety of treatments exploited by the cheesemakers include:

Cutting: after the gel attains sufficient firmness, it is cut with special knives. This process allows to increase the rate of syneresis, once the smaller the curd pieces, the greater the surface area available for loss of whey and the lower the moisture content [38].

Cooking: The curd/whey mixture is stirred, heated and, eventually, separated. The cooking of the mixture plays a role in the control of syneresis by influencing curd shrinkage and acid development [32].

The time and maximum temperature of heating is characteristic of the variety and governs the moisture content and consistency of the curd before it is made into cheese [38, 39]. For most varieties, cooking is performed by circulating steam or hot water through the jacket of the cheese vat. During cooking, the mixture is stirred with the purpose of facilitating the cooking, preventing the curd pieces from matting (strong negative effect on syneresis), and promoting syneresis, which is directly proportional to the intensity of stirring [38].

Draining: process used to separate the curd from the whey [30].

Shaping: After removing the whey, the curds mat to form a continuous mass. The treatment of this mass varies according to the type of cheese intended to be formed and may involve pressing, turning, and pilling blocks of curd, traditionally known as Cheddaring, and inverting the mass of curd in the moulds [38].

Salting: The addition of salt presents several functions in cheese. It preserves it, enhances safety, contributes to flavour (saltiness), and impacts the overall quality of cheese, due to its effects on water activity, microbial growth, protein hydration within the gel matrix, and enzymatic activities. These last, in turn, influence biochemical changes and the development of texture and flavour. The contribution of salts depends on the amount and the method of addition of salt, the variety of cheese, and the ripening conditions [40, 41].

At the end of the manufacture process, one ends up with fresh cheese, which are mostly produced by acid-coagulation. Rennet-coagulated cheese goes through the most crucial technological step in cheese manufacturing, **ripening** (maturation), as the unique characteristics of the individual cheeses develop during this process [40, 42]. Due to several biochemical events, including glycolysis, proteolysis, and lipolysis, there is a change in composition and structure, and the rubbery and tasteless curd is transformed into a desirable cheese with a characteristic appearance, taste, and aroma [21]. Ripening can last for a period of 3 weeks to over two years, and the duration is inversely related to the moisture content [29, 36].

2.1.7 Cheese varieties

"There is a cheese for every taste preference and a taste preference for every cheese" [43] is a sentence that demonstrates the great range of cheese varieties that can be produced from a limited amount of raw materials (milk, starter cultures, coagulant and salt) [44]. Despite the number of cheese varieties produced worldwide not being known yet, it is believed to exist more than 1000, and a number of attempts have been made to classify them, in order to facilitate their study [21, 44].

The classification of cheese varieties exploits certain characteristics of the cheese, as the texture, method of coagulation and ripening indices. The arrangement according to texture is based in the rheological properties, more precisely on the moisture content of cheese, and these are classified as very hard, hard, semi-hard and soft cheeses . Although this is widely used, it suffers from a serious drawback since it groups together cheeses with extensively different characteristics and manufacturing protocols. Regarding

the method of coagulation, being one of the fundamental events in cheese manufacture, it is considered a clear primary criterion for classification, Figure 2.3. Cheeses can be integrated into three "super-families": rennet, acid, and heat/acid cheeses. Rennet-coagulated cheeses are the most major international varieties, representing around 75% of total cheese production and almost all ripened cheeses. In their turn, acid-curd cheeses represent 25% of total cheese production and are usually consumed fresh. Coagulation by a combination of heat and acid is used for a limited number of varieties. Lastly, the possibility of classifying cheese according to the extent of chemical breakdown during ripening was presented, but it is still not possible to do so reliably, due to the dynamic system that is ripening, and so the age at which the cheeses are fingerprinted creates a major problem of definition [16, 44].

Camembert cheese was the one used in this present project. It was originated in Normandy, France, in the 18th century. As Figure 2.3 indicates, it is a surface mould-ripened cheese variety, which are generally soft cheeses characterized by the growth of the white mould *Penicillium camemberti* on the surface of the cheese. During ripening, these cheeses soften from the surface towards the centre, which often leads to a spreadable, almost fluid, consistency [44].
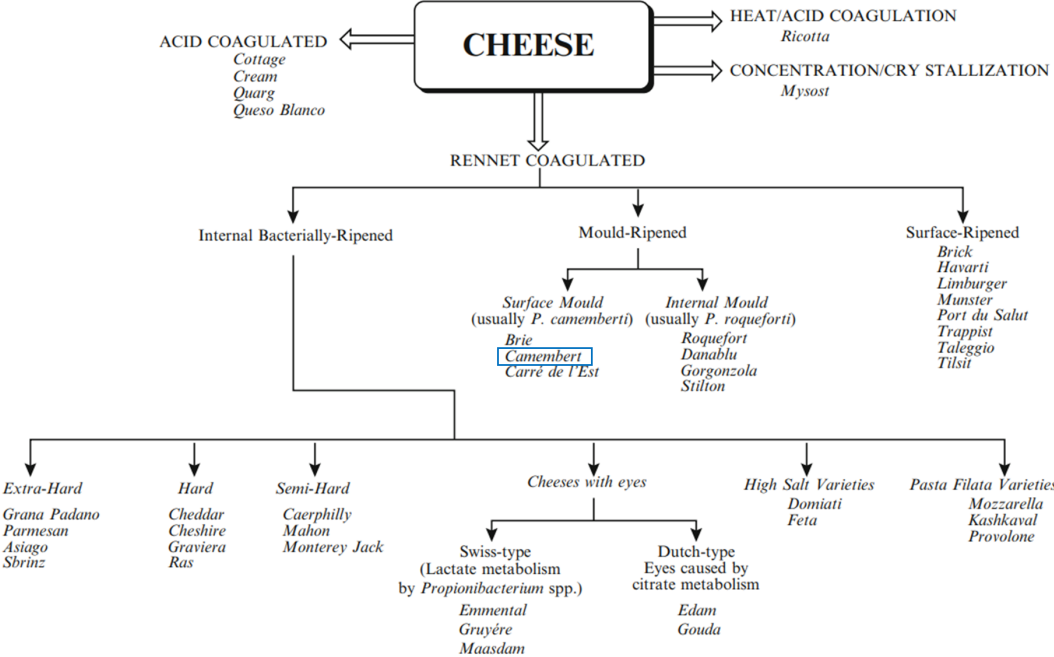
Figure 2.3: Schematic representation of cheese varieties. 13 super-families characterize cheese based on the method of coagulation used. Further sub-categorization is done based on the principal ripening agent and/or characteristic technology. Camembert is a rennet-coagulated surface-mould ripened cheese. Reproduced from [44].

2.1.8 Cheese consumption and benefits

World production of cheese is about 17 Mt per year, being Europe the principal production region, followed by North America [3]. The advancements made in cheese production regarding the isolation and

characterization of the principal constituents of milk, the microbiology of raw milk, the mechanism of coagulation, among other factors, allowed the production of cheese with the desired functionality and nutritional value. There has been a consistent increase in consumption of cheese in most countries, due to its functional properties such as diversity of flavours and textures, appearance, mouthfeel, adhesive properties, and long shelf-life [45–47].

Cheese popularity is also a result of being considered a nutritious and versatile food, that plays an important role in a healthy diet. The precise natural composition varies among types of cheese, but generally, this ingredient contributes with a significant amount of both saturated and total fat to the diet. It also has a protein content ranging from 3 to 40%, depending on the variety, being a source of numerous bioactive peptides with important properties, including anti-microbial, anti-inflammatory and antioxidant. Furthermore, cheese is a good source of vitamin A, riboflavin, and vitamin B12, as well as a good source of bioavailable calcium, phosphorus, and magnesium [48].

Furthermore, cheese presents a great flexibility in use [3]. The natural cheese produced is generally consumed as table cheese, which means it is eaten on its own or as an accompaniment to bread during a meal. However, the volume of cheese used as an ingredient in the preparation of culinary dishes has substantially been increasing over the years, giving rise to the commercial use of the terms, ingredient cheese and cheese ingredient [49].

The production of ingredient cheese involves alterations in the manufacture protocol, so that one can obtain cheese with targeted functionalities designed to optimize its quality as an ingredient in specific applications. The functionalities of the unheated and heated cheese are key quality determinants of ingredient cheese, as these allow the cheese to be included in a vast range of dishes, including desserts, salads, sauces, lasagne, among others [49].

In turn, cheese ingredients derive from natural cheese subjected to secondary processing treatments, which can be minimal (drying and grating) or more extensive (heating, shredding). They are used as flavourings in an extensive array of snack and formulated food products. Cheese powder, which is the main topic of this study, is included in this category [49].

2.2   Cheese powder

Cheese powders are dehydrated cheese products, which are industrially-produced cheese-based ingredients, with major economic importance due to their widespread use as flavouring agents and nutritional supplements in ready meals, sauces, creams, soups, bakery products, pasta dishes, among others [1, 46]. Several factors favour the use of cheese powders over natural cheese. The convenience is one of them. Powders can be easily applied to the surface of snack foods or incorporated into food formulations, whilst natural cheeses require size-reduction. The lower water activity of powders allows them to have a longer

shelf-life, and to be stored for long periods of time without alteration or deterioration of quality. Lastly, cheese powders can provide a greater diversity of flavours, due to the use of different types of cheese [49]. Nowadays, over 100 varieties of cheese powder can be found in the market and can be produced using a wide range of cheeses, including Cheddar, Danbo, Camembert, Gouda, and Emmental. These powders are formulated by either a combination of several of those cheeses, or just by the use of a single one, producing a pure cheese powder [50, 51].

2.2.1   From cheese to powder

The manufacture of cheese powder essentially involves the four main steps below [2, 46, 49, 52]:

i. **Determination of the blending formulation.** This consists mainly of comminuted natural cheese, water, emulsifying salts, and, optionally, different additives, including dairy ingredients, starch, flavours enhancers, antioxidants, colouring agents, and filling agents, including whey, skim milk solids, maltodextrins. The types and levels of ingredients included are determined by the type of cheese powder intended to be produced, natural or extended, the flavour required, and its application.

ii. **Processing of the blend.** This refers to the heat treatment of the blend, usually by direct or indirect steam injection while constantly agitating. The blend is processed until the emulsion formed is homogeneous in colour and consistency and free of lumps and non-hydrated material. Processing constitutes an important step as it kills any potential pathogenic microorganisms, extending the shelf-life, and eases the physico-chemical and microstructural changes that transform the blend into a stable oil-in-water emulsion, named cheese feed.

iii. **Homogenization.** This technique is optional but is commonly practiced ensuring homogeneity and to promote a finer dispersion of fat droplets, leading to a smoother and creamier hot blend.

iv. **Spray drying of the cheese feed.** The design of the drier and operation conditions influences the physical and the flavour characteristics of the cheese powder.

Regarding this last operation, spray drying is a suspended particle processing technique and has become one of the most important methods for drying fluid foods (milk, whey, coffee, and infant formula) [53]. This method involves the atomization using either a rotary (wheel) or a pressure nozzle atomizer of the cheese feed into droplets by spraying, followed by the rapid evaporation of the sprayed droplets into solid powder by hot air at a certain temperature and pressure [47, 54].

There are different spray dryer processes (single stage or two stage, for instance) and equipment configurations (tall-form, filtermat, silo-form) that can be used in food powder production [49], as Figure 2.4 display. However, single- and two-stage spray dryers are more commonly used for dairy products. In the first one, the product is dried to the final moisture content in the spray drying chamber and can be

characterised as single powder particles with high bulk density. The two-stage drying combines initial spray drying with a second stage in which the final moisture content is obtained. The feed enters the drying chamber via a set of high pressure nozzles and the air flow patterns in the drying chamber directs the particles to the second drying, which can be a fluidized bed or a belt dryer [1, 47, 55].
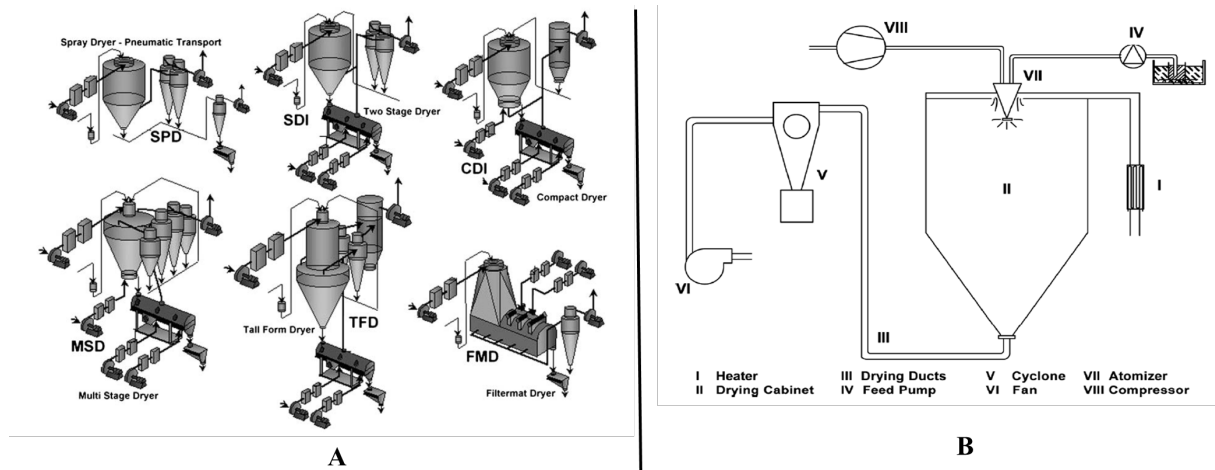

Figure 2.4: A) Various types of spray driers. Reproduced from [56]. **B)** Schematic representation of a spray dryer, specific for co-current mode operation. Reproduced from [57].

Powder is then cooled and recovered from the exhaust air, using a cyclone separator, bag filter, or a wet scrubber, and packaged [13, 47]. The design of the drier, as well as the operating conditions, such as the atomizer type and pressure, direction of air flow, air inlet and outlet temperature and air humidity, must be optimized and determined based on the feed, the product being produced, and the purpose, so that undesirable physical, chemical, and biochemical transformations do not occur [13, 58].

2.2.2 Powder properties

The final quality of cheese powder can be evaluated through different properties with significant inter-relationships between each other, that can be divided into physical, chemical, and functional properties.

The first two are related to the feed composition (physicochemical characteristics, viscosity, thermos-sensitivity, availability of water, and flow behaviour), and also to the spray drying conditions applied (type, nozzle/wheels, pressure, agglomeration, and thermodynamic conditions of the air). A few of the physical and chemical properties correspond to particle size distribution, moisture, composition, water activity, colour, thermos-stability, free fat, microstructure [59].

Powder functional properties are related to its final application. These include instant, foaming, reconstitution, and emulsification, and are directly influenced by the combination of physical and chemical properties [59].

Previous studies [1, 50, 60] determined the effect of various compositional parameters, such as the cheese age, the addition of dairy ingredients, the spray drying conditions, on the final quality of cheese powders. However, the study on the effects on the cheese powder manufacturing properties, i.e. the characteristics of cheese feed, as stability, rheology, microstructure, and homogeneity, is very limited [51].

2.3   Cheese feed

The manufacture of cheese feed deconstructs the casein network of natural cheese, in order to solubilize the protein, enabling it to bind the free water and emulsify the free fat released during heating and shearing, forming a concentrated oil-in-water emulsion [2].

Emulsions are defined as colloidal systems composed of two immiscible liquids, where one forms the continuous phase of the system, and the other is dispersed in droplets. Oil-in-water emulsions are employed in a wide range of areas such as food, pharmaceutical, cosmetic, among other industries, as a result of their ability to transport or solubilize hydrophobic components in a continuous water phase [61–63].

The process that allows the mixing of two immiscible liquids into a single one is called homogenization and is usually achieved by applying intense mechanical agitation, using, for instance, a high shear mixer, high pressure valve homogenizer, an ultrasonic homogenizer, as well as other techniques [64, 65].

To form cheese feed, different types of natural cheeses are added together with water, emulsifying salts (ES), and, optionally, with other additives, including dairy ingredients, starch, maltodextrins, flavours enhancers, antioxidants, and, sometimes, colouring agents [1, 52, 66]. The cheese emulsion that results from the processing is required to be homogeneous, uniform, stable (without protein precipitation and cream separation) and pumpable, until and during spray drying [4, 67].

2.3.1   Emulsion stability

Emulsion stability is the ability to resist changes in its physicochemical properties over time, and it is always important to identify the main physical and/or chemical mechanism responsible for the instability, so that it is possible to determine the most effective strategy to improve it [65]. Food emulsions may become unstable and the water and oil phases may separate due to different physicochemical mechanisms, that are often interrelated with each other, such as gravitational separation, flocculation, coalescence, Ostwald ripening and phase inversion [64], Figure 2.5.

Gravitational separation is the most common instability mechanism and includes both the creaming and the sedimentation processes. The density of the droplets present in an emulsion is different from that of the liquid that surrounds them, and so a net gravitational force act upon them. Therefore, in **creaming**, the droplets move upward, once they present a lower density compared with the surrounded liquid, while in **sedimentation**, the droplets move downward, due to the opposite reason. In oil-in-water emulsions, the

droplets tend to cream, once edible oils (in their liquid state) present lower density than that of water [65].

Flocculation is the process where two or more droplets aggregate with each other, while maintaining their initial integrity. The tendency for droplet flocculation to occur depends on the balance of attractive and repulsive forces acting between the droplets: if the attractive interactions dominate the long-range repulsive interactions, but not the short-range repulsive interactions, droplets remain close together (flocculate), but do not merge into each other [65].

Coalescence is the process where two or more droplets merge together to form a single larger droplet, causing the droplets to cream or sediment faster due to the increase in their size. In oil-in-water emulsions, this process eventually leads to the formation of a layer of oil on top of the material, which is referred as oiling off. Coalescence may occur as direct result of droplet collisions, or as a result of two or more droplets remaining in close contact for long periods of time, for instance in concentrated, creamed, or flocculated emulsions. In practice, this last reason is more likely to happen in food emulsions [68, 69].

Ostwald ripening is the process whereby larger droplets grow at the expense of smaller droplets due to mass transport of dispersed phase material through the continuous phase. It occurs because the solubility of the material in a spherical droplet increases as the size of the droplet decreases [65, 70].

Lastly, **phase inversion**, as its name indicates, is the change of an oil-in-water emulsion to a water-in-oil emulsion, or vice versa. This process is essential in the manufacture of certain food products, including butter and margarine, but undesirable in other foods, as it has an adverse effect on product appearance, texture, stability, and taste [71]. It is usually triggered by some alteration in the composition or environment of an emulsion. The physicochemical basis of this process is immensely complex, as it involves aspects of droplet flocculation, coalescence, and disruption [65, 72].
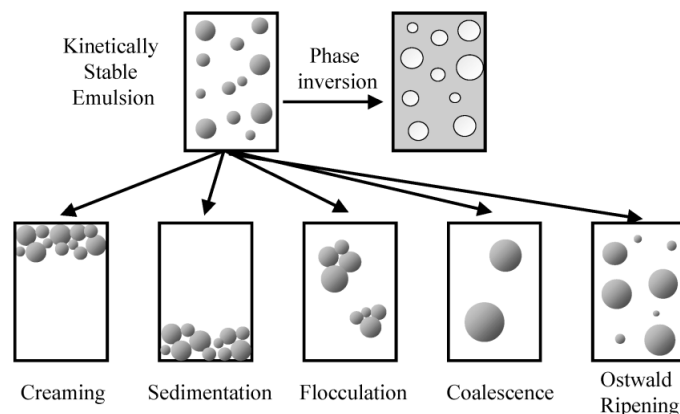

Figure 2.5: Physicochemical mechanisms that lead to unstable emulsions. In order to remain stable, an emulsion has to resist to these changes, otherwise phase separation may occur. Reproduced from [65].

Herewith, one of the main concerns and challenges is to keep the emulsion stable with minimal structural changes during manufacture and storage [64].

2.3.2 The role of emulsifying salts

Heating cheese to a high temperature generally results in some oiling-off and moisture exudation. These effects reflect shrinkage and dehydration of the casein network and coalescence of fat and so, the search to overcome the heat-induced destabilization of cheese began, leading to the important discovery of the addition of "melting salts", known as emulsifying salts. Emulsifying salts are not true emulsifying agents *per se*. They promote, along with the heating and the shearing, physicochemical changes in the blend, turning the insoluble calcium phosphate and para-casein soluble. The hydrated proteins can act as emulsifiers and form a membrane layer around the liquid fat released during heating and shearing, contributing to the formation of a smooth, homogeneous, and stable emulsion [2, 73].

The preparation of stable emulsions requires the incorporation of substances known as emulsifiers. Ideal emulsifying characteristics are possessed by salts which consist of monovalent cations (sodium) and polyvalent anions (phosphate or citrate), where sodium salts of phosphate, polyphosphates (eg: pentasodium tripolyphosphate – $Na_5P_3O_{10}$), citrates (eg: trisodium citrate – $2\ Na_3C_6H_5O_7 \cdot 1\ H_2O$), or a combination of them represent the most commonly used. They are able to influence several critical events [2, 73–77], and a visual representation of these is presented in Figure 2.6.

pH adjustment: the use of ES usually increases the pH of the cheese, which contributes to an enhanced dissociation and calcium-sequestering ability of the ES, and an increased negative charge on the paracaseinate. This allows to create an emulsion that remains stable until and during spray drying.

Calcium Sequestration: this involves the ability of ES to chelate calcium from the casein matrix by exchanging sodium ions, which results in the conversion of insoluble calcium paracaseinate into soluble sodium paracaseinate. The effectiveness of ES in binding calcium depends on the valency, type of ionic species forming the emulsifying salt, pH, ionic strength, temperature, among other effects.

Casein Hydration: The sequestered complexes disrupt the major molecular forces that cross-link the various monomers of casein in the network. This disruption, in conjunction with heating and mixing, leads to hydration and partial dispersion of the proteins, which can help emulsification by coating the surfaces of dispersed free fat globules.

Fat Emulsification: Within the matrix, sodium paracaseinate acts as an emulsifier due to having both hydrophobic and hydrophilic regions in their peptide chain, which they position in the oil phase and aqueous phase, respectively. This property allows them to lower the interfacial tension and the global free energy of the system. The increase in viscosity as a result of water binding by the paracaseinate also enhances emulsion stability by restricting the mobility of the emulsified fat particles.
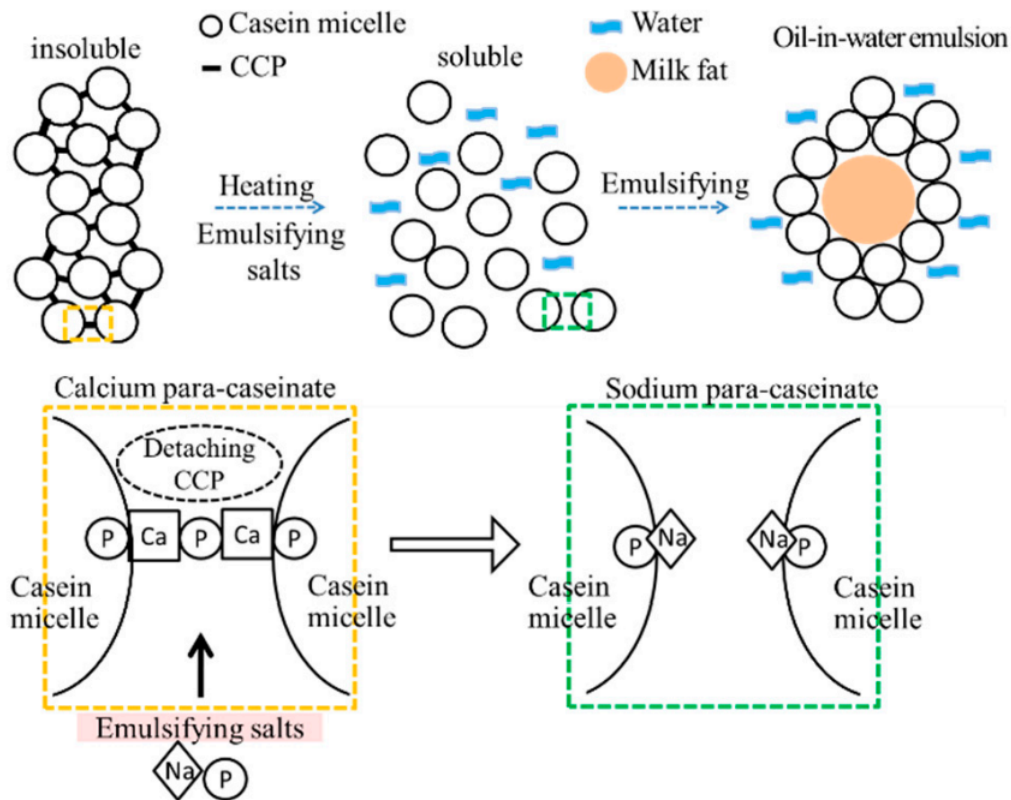
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Figure 2.6: Role of emulsifying salts on the stability of cheese emulsions. Due to its calcium sequestering ability and exchange by sodium ions and pH adjustment, proteins turn soluble and can act as emulsifiers, forming a membrane layer around the liquid fat. Reproduced from [78].

Water is added to the cheese blend to work as a vehicle for the emulsifying agents and to adjust the dry matter content in the final product [75].

Varming et al., 2014 [67], showed the importance of emulsifying salts, as the cheese feed stability generally decreased when the ES concentration was reduced . This can be explained by a periodic shift in the mineral (calcium) balance of the system, promoted by the presence of ES, furnishing a continuous dispersion of casein and subsequent protein-protein interactions and fat emulsification [79].

2.3.3 Clean label

However, the addition of ES leads to an increase in the sodium and phosphate content, and so the healthy conscious consumers and authorities are putting the dairy producers under the pressure to reduce the salt content in their products [4]. Therefore, the development of cheese powder with reduced amount or without ES has been investigated. There are other parameters that may affect the emulsion stability during production of processed cheese, including type and composition of the cheeses used, addition of other dairy ingredients, and processing parameters, and similar effects could be expected on the stability of cheese feed [67].

<u>Cheese</u>

Natural cheese is one of the most important ingredients used in cheese powder production. The selection of the natural cheese used for the manufacture is of extreme importance as it influences the required composition for cheese powder, as well as its flavour, maturity, consistency, and texture [66]. The choice depends on many of the gross composition of the natural cheese (fat, protein, and moisture content), calcium content and intact casein (IC) level [2]. Based on these factors, Varming et al., 2014 [67] demonstrated that feeds produced with a combination of Cheddar and soft white or Camembert cheeses had a generally higher stability when compared to feeds produced only with Cheddar cheese. This may be related to a lower concentration of calcium, a higher level of intact casein, and pH increasement, as compared with the Cheddar cheese alone .

Age is another characteristic that varies from cheese to cheese according to the variety, due to differences in manufacture, in its composition, and ripening conditions. During this last process, the paracaseinate is hydrolysed to peptides and free amino acids, which leads to a decrease in the level of intact casein. This consequence may lead to a decrease in the firmness of the cheese powder produced and owes to the fact that the peptides give a lower degree of fat emulsification than intact casein [2, 66]. In accordance, Ray et al., 2016 [80] studied the effect of Cheddar cheese age on the stability of cheese feed without ES and concluded that feeds made from 3 months old Cheddar were significantly more stable than those made from 5 months old cheese. A similar increase in emulsion stability was also observed for cheeses of 7 months of age compared with 12 months, indicating the relevance of cheese age on feed stabilization .

<u>Addition of dairy ingredients</u>

Another strategy that has the potential to provide cheese feed stability and be an ES replacer is the addition of other dairy ingredients. Herewith, the influence of sodium caseinate (SC), butter milk powder (BMP) and their combinations on emulsion stability were studied. SC consists of a mixture of the four types of casein and is an interesting ingredient in food emulsions due to its excellent emulsifying properties [4]. BMP contains phospholipids, casein, whey protein, minor peptides, and lactose, and has emulsification ability due to its protein and milk fat globule membrane content [81]. The research indicated that emulsions containing equal amounts of both dairy derived ingredients presented better stability when compared to the presence of one of the ingredients solely.

<u>Processing conditions</u>

Cheese feed is also controlled by the processing conditions. Temperature, time and shear provided during manufacture play a major role in the emulsion formation and degree of fat emulsification, as well as, together with the conditions of spray drying, in the functional properties of cheese powder [2, 66].

In cheese powder production, the homogeneous slurry formed is held at 60°C for up to one hour, heat treated, and then spray dried. Hougaard et al., 2015 [79] characterized the stability of model cheese feeds with and without the presence of emulsifying salts during different holding times at 60°C. An increase in

stability was verified for cheese feeds produced with ES as the holding time also increased, but no changes in stability were observed for cheeses without ES. Therefore, approaches, other than holding, are needed to increase stability . A promising suggestion, with a limited number of studies so far, is the effect of heat treatment.

Cheese manufacturers use different types of cookers with various designs and operating conditions. These differ on the type of mixing and agitation systems, and the type and mechanism of heating, which was already stated to be a relevant step in cheese emulsions and cheese powder production. Heating is usually done through direct steam injection and indirect heating [66].

These last correspond to conventional thermal processing techniques, being the most used to ensure microbial safety of processed foods and enhance shelf-life through destruction of enzymes, toxins, etc. These traditional methods require heat energy to be generated externally and then transferred to food though conduction, convection or radiation. For food products containing particulates, these heating transfer methods require time to heat the coldest point of a system, which is usually the center of the largest particle, overheating the remaining particles and the surrounding liquid. This over processing leads to loss of nutritional compounds and sensory changes, such as flavour and aroma [7]. Moreover, the combustion of fossil fuels to generate heat causes economic and energy losses [82]. These drawbacks, along with the increasing consumer demand for nutritious and delicious food has challenged the scientific community to develop innovative and adequate processing techniques to ensure safe products with high nutritional values and a "fresh like taste" [83–85]. Emerging processing technologies are defined as technologies that a) are in the research and development stage, with significant potential to be commercialized or b) are already commercialized but represent only a small percentage of the market for applications in the food industry. They are used as alternatives for food processing to develop safe foods with minimal damage to nutritional and sensory properties [85, 86]. One of these novel technologies is ohmic heating (OH).

2.4 Ohmic heating

2.4.1 Overlook

OH is a thermal technology that is able to overcome the problems that arise from the use of conventional heating (CH). Despite being considered a novel technology, it has been applied to foods since the 19th century, being first used in the pasteurization of milk, but felt in disuse due to the costs of electricity, lack of inert materials to make the electrodes and difficulties in controlling the process parameters [87]. However, the studies regarding this process led to significant improvements, being currently used in different fields, such as blanching, evaporation, dehydration, fermentation, sterilization, pasteurization, among others [83].

It is defined as a process wherein alternating electric current (usually alternating) is passed through materials via two electrodes inserted in the food, demonstrated in Figure 2.7, with the primary purpose of

heating the materials. When electrical current flows through a conductor, the motion of charges within the material results in agitation of molecules and consequently, in increased temperature. The heating occurs in the form of internal energy transformation (from electrical to thermal), which allows this technology to not depend on heat transfer [8]. The most important processing variable corresponds to the electrical conductivity (σ), which is a measure of how well a material accommodates the movement of an electric charge. It is not a constant value as it depends on the material temperature (normally linearly), and this variation is the result of the increased ionic mobility at elevated temperatures [82]. For ohmic heating to occur, it is important that the electrical conductivity of the material is non-zero, which means that completely nonpolar materials, such as oils, cannot be heated as they do not conduct electricity. However, most food materials are aqueous-based, and possess some electrical conductivity, which allows them to be heated. Another important parameter is the electric field strength (**E**) which is a function of the applied voltage or the interlectrode gap. Therefore, even when electric conductivity is low, it is possible to heat the material by using devices with low electrodes gaps and increasing the voltage, which increases the electric field strength to compensate [10].
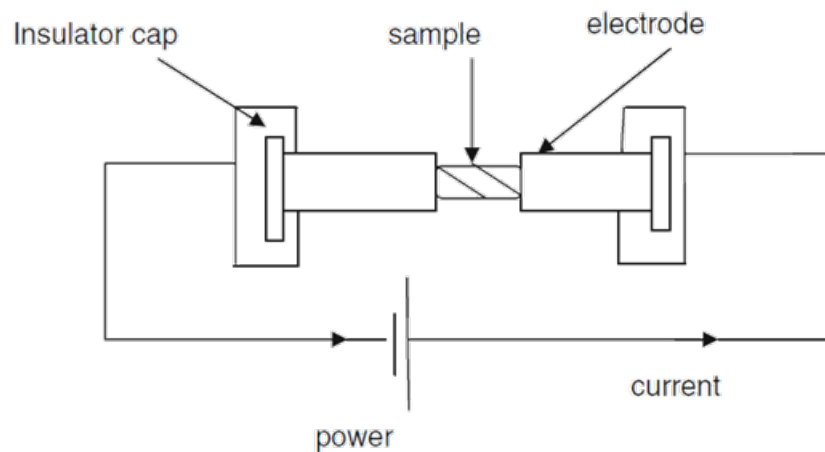

Figure 2.7: Representation of the ohmic heating technology. It is defined as a process wherein alternating electric current is passed through materials via two electrodes inserted in the food. Reproduced from [7]

One of the main advantages of ohmic heating over conventional heating methods is the uniformity and velocity of heating. As seen previously, in conventional heating heat is transferred from an external medium through heat exchange walls and a carrier fluid before reaching the solid phase, while ohmic heating relies on internal energy generation, allowing heating the solid phase at the same rate as the fluid. This ensures greater nutrient retention capacity and sensory attributes [9]. Another advantage is the fact that ohmic heating reaches higher temperatures than conventional heating, where the medium temperature is the highest attainable. It is a process easier to control due to instant switch-on/off, and offers the possibility to handle the heating rate online by changing the voltage applied, which favours the convenience of this technology. It promotes in a less extent fouling, which is a major problem in thermal processes of milk products [83]. Additionally, the cost and the wide variety of designs and heater sizes

also contribute to chose OH over CH technologies. Finally, is a more energy efficient technology, once nearly all the energy delivered to the food is used, and efficiencies above 90% and above are common. Thus, this environmental friendly technology is an attractive option in an industrial setting [10].

2.4.2 Applications in the dairy industry

The use of ohmic heating has been recommended for the processing of a wide range of food products because of abundant quantity of water and polar components, such as minerals and proteins in them. This composition description makes milk an ideal candidate for ohmic heating. The majority of studies conducted so far are related to the effect of this technology on microbial inactivation of dairy products to ensure safety. The main mechanism of microbial inactivation caused by OH is the thermal effect on membrane structure and enzymes of the microorganisms. An additional non-thermal effect produced by OH has also been reported, where a mild electroporation of the cell occurs, leading to pore formation in the cell membrane, which reduces the thermal resistance of the microorganism. Other important studies are concerning the influence of this technique on quality and sensory factors of the dairy product, in order to evaluate the benefits of OH and to define the optimum process parameters. The indicators studied include the free fatty acid content, protein denaturation and aggregation, fluorescence of advanced Maillard products and soluble tryptophan (FAST) index, among others [83].

There is a lack of studies on processed dairy products, such as cheese, butter and fermented milk, and these attain disadvantages when compared to milk, once they contain non-conductive substances in the composition, such as fat globules, which promotes non-uniformity in heating generation. However, these should be seen as an opening to a range of research opportunities, such as the optimal conditions of OH for these products and new applications for this technology that could integrate one of the processing steps of one of these projects. [83].

Chapter 3

Experimental procedure

3.1 Protocol

The experimental outline presented in Figure 3.1 aims to give a visual representation of the conditions and procedures used to analyse the stability of cheese powder dispersions.

3.2 Materials

Cheese powder of medium-maturated Camembert and similar types of cheese without emulsifying salts (38.10% fat, 47.98% protein, 0.67% calcium, 8.15% moisture) was provided by Lactosan A/S, Ringe, Denmark.

3.3 Experimental methods

3.3.1 Cheese powder dispersion preparation

Cheese powder at a concentration of 17.5% (w/w) was added to deionized water and mixed for 2 hours on a stirring plate. The sample was then placed at 4ºC overnight to establish a good hydration. The preparation procedure for the five samples prepared is displayed in Table 3.1. The effect of different parameters on the stability of cheese powder dispersions were studied in this experiment: electricity, through the use of ohmic heating and conventional heating, temperature, some samples were processed at 85ºC and others at 60ºC, and homogenization, a second homogenization was added in the processing of two samples. For each condition, two replicas were produced.
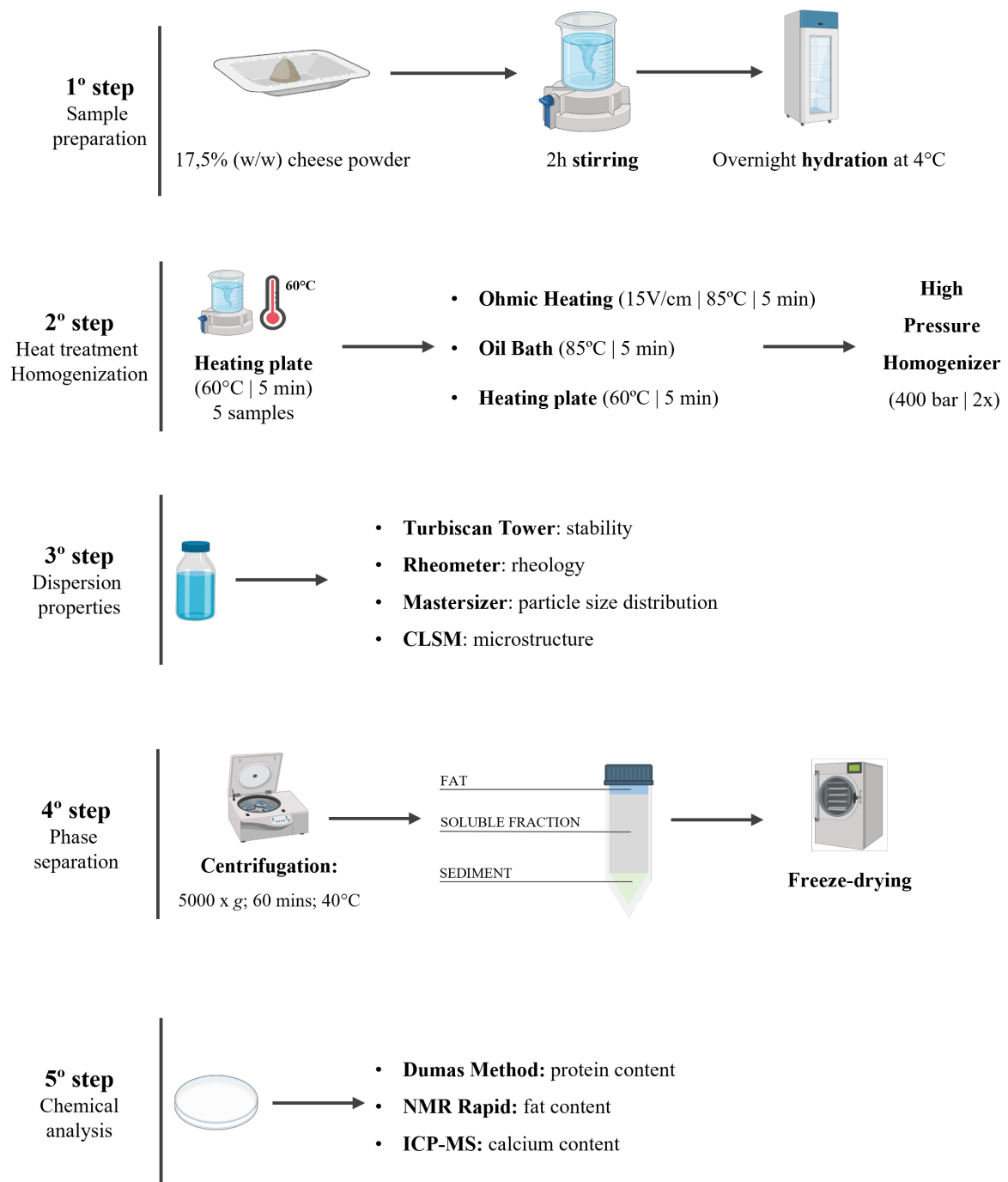
1º step
Sample preparation

17,5% (w/w) cheese powder   2h **stirring**   Overnight **hydration** at 4°C

2º step
Heat treatment
Homogenization

Heating plate
(60°C | 5 min)
5 samples

- **Ohmic Heating** (15V/cm | 85ºC | 5 min)
- **Oil Bath** (85ºC | 5 min)
- **Heating plate** (60ºC | 5 min)

High Pressure Homogenizer
(400 bar | 2x)

3º step
Dispersion properties

- **Turbiscan Tower**: stability
- **Rheometer**: rheology
- **Mastersizer**: particle size distribution
- **CLSM**: microstructure

4º step
Phase separation

Centrifugation:
5000 x g; 60 mins; 40°C

FAT
SOLUBLE FRACTION
SEDIMENT

Freeze-drying

5º step
Chemical analysis

- **Dumas Method:** protein content
- **NMR Rapid:** fat content
- **ICP-MS:** calcium content

Figure 3.1: Experimental design followed throughout this project. The procedures and analytical methods are further described in more detail.

Table 3.1: Procedures used (+) in the preparation of all the five samples. The procedures are showed in order of occurrence from left to right.

Sample ID	Heating plate - 60ºC	Homogenization (H)	Heating plate - 60ºC	Ohmic Heating (OH) - 85ºC	Oil Bath (OB) - 85ºC	Homogenization (H)
OH + H	+			+		+
H + OH + H	+	+		+		+
OB + H	+				+	+
60 + H	+		+			+
H + 60 + H	+	+	+			+

3.3.2 Heat treatment

Ohmic heating (OH)

An ohmic heater (BCH ltd., Lancashire, United Kingdom) with an ohmic unit consisting of a holding cell made of W500 grade polyethylene-polypropylene with variable size adjustment and mountings for temperature loggers (K-type) was used, Figure 3.2. A maximal supply at 230 voltage using alternating current (60 Hz, sinusoidal) was installed with the ohmic heater and a titanium electrode with high corrosion resistance in chloride environments [88] was used. The distance between the electrodes was set at 8 cm apart and the width of the chamber was 9.5 cm. After the system was closed, 250 mL of sample were added to the ohmic heater. The voltage gradient used was 15 V/cm. The voltage gradient (E) is the ratio of applied voltage (V) to the distance between electrodes (L). The applied voltage was the percentage of the total voltage supply (V_{max} = 230 V). The voltage gradient was set by changing the applied voltage (V) at fixed L for all experimental setups. Voltage gradient was calculated as $E = \frac{V}{L} = \frac{\% V_{max}}{L}$ The final temperature to reach was 85ºC. The time required for the samples to come up to the mentioned temperature was recorded. For the samples OH + H it took 18.5 ± 0.5 s and for the samples H + OH + H it required 38.5 ± 1.5 s to reach the temperature (mean ± SD, where n=2). These differences in time are due to the decrease in temperature of the sample after one homogenization, which increases the come up time to reach 85ºC. After a holding time of 5 min, samples were rapidly transferred to the high pressure homogenizer.

Conventional Heating (CH)

Samples were subjected to two types of conventional heating:

i. Oil bath

Samples in an Erlenmeyer flask were immersed in an oil bath at 85ºC, where the temperature was controlled through a t-type thermocouple, for a holding time of 5 min. Oil bath was used instead of a heating plate so that the come up time would be similar to the one reached when using OH. OB + H
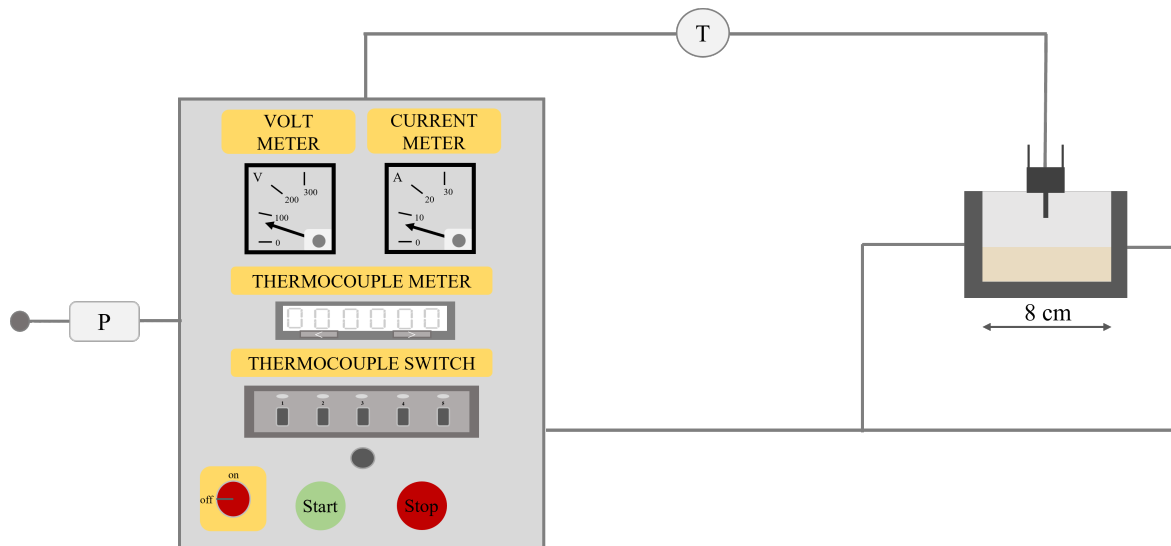
Figure 3.2: Representation of the laboratory scale ohmic heating set. The equipment was constituted by a power supply (0-230 V, 60 Hz) that is connected to the control unit, where the voltage and the temperature measured by the thermocouples can be regulated and the equipment can be switched on-off. The samples were placed in a chamber in between electrodes 8 cm separated.

samples reached 85ºC after 69 $\pm$ 1 s (mean $\pm$ SD, where n=2).

    ii. Heating plate

Samples that were not subjected to either ohmic heating or oil bath, continued being heated up at 60ºC in the heating plate, so that the exposure time to temperature would be the same.

3.3.3 Homogenization

To disrupt the fat globules, the samples were run in a Rannie High Pressure Laboratory Homogenizer Model MINI-LAB (APV, Izmir, Turkey) at 400 bars. To evaluate the effect of homogenization, some samples were subjected to this procedure before and after heat treatment.

3.3.4 Centrifugation

Subsequently to homogenization, the stability, rheological properties, and particle size distribution of the cheese powder dispersions were analysed. The remaining sample was centrifuged in a 4-16Ks centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) equipped with rotor and insertions for 50 mL centrifuge tubes with conical bottom. Centrifugation was performed at 40ºC, with a speed of 5000 x g during 60 mins. The accelerated instability provided by centrifugation was observed as separation into three phases. One can assume that fat was primarily found in the top layer, precipitated protein in the bottom phase, leaving an opaque watery phase in between upon phase separation, where soluble protein can be found.

3.3.5 Freeze-drying

The three phases obtained after centrifugation were then separated and freeze-dried (Thermo Fisher Scientific Inc., Massachusetts, USA). In this process, also known as lyophilization, the water in the form of ice under low pressure is removed from a material by sublimation. Freezing water in the material prior to lyophilization inhibits chemical, biochemical, and microbiological reactions, which allows the retention of its quality [89]. The fat, protein, and calcium content were then analysed.

3.4 Analytical methods

3.4.1 Chemical analysis of cheese powder dispersions

i. Protein content

Total protein content was analysed according to the Dumas method by using rapid MAX N exceed (Elementar Americas, Inc., Ronkonkoma, New York, USA). The basis of the Dumas method is the conversion of all nitrogen forms in the sample to nitrogen oxides through combustion at high temperatures in an oxygen atmosphere, reduction of these forms to nitrogen gas (N_2), and subsequent measurement by use of a thermal conductivity detector [90]. Dry samples were placed in iron tips. The system was calibrated daily before analysis by running the following sequence: three blanks (empty crucibles), five aspartic acid standards and two run-in samples. Protein content (%) was calculated from the nitrogen content of the material, using a nitrogen conversion factor (NFC) of 6.38 [91], which is the most appropriate value for cheese. The value NFC cannot be identical for all sources of food proteins, as it is related to the amino acid composition and presence of side groups covalently bound to some amino acids of the protein chain [92]. Protein content was analyzed in the sediment and soluble phases. For the fat phase, the protein content was calculated by subtracting the values obtained from the total protein content.

ii. Fat content

The measurement was done through the Rapid Nuclear Magnetic Resonance (NMR) Fat Analyzer (CEM Corporation, Matthews, NC, USA) that is provided with a technology that completely isolates the detection of the proton signal in fat molecules from all other compositional proton sources. The method "Dairy Powders", already present in the equipment, was used for the analysis. The fat content was measured in all three phases.

iii. Calcium content

Calcium content was measured using an Inductively Coupled Plasma – Mass Spectrometry (ICP-MS). This technique uses an argon plasma, the ICP, to convert the sample into ions, which are then separated by MS by their mass-to-charge ratio. The detector counts the number of selected ions per second which allows the instrument to determine the concentration of the chosen element [93]. Calcium content was measured only in the sediment phase, and the value obtained obtained was subtracted from the total calcium content to obtain the soluble phase value. It was considered that the amount of calcium in the fat

phase was minimal.

3.4.2 Particle size measurement

The measurement of particle size distribution of oil droplet in cheese feed samples was based on the Static light scattering method and done through Mastersizer 2000 (Malvern Instruments Co. Ltd., Worcestershire, UK). Particles pass through a focused laser beam and scatter light at an angle that is inversely proportional to their size. The angular intensity of the scattered light is then measured by a series of photosensitive detectors. The map of the scattering intensity versus angle is the primary source of information used to calculate the particle size, accurately predicted by the Mie scattering model. Samples were placed at the feed hopper at room temperature (sample temperature $40\pm5°C$). The applied refractive index of the dispersed phase for all samples was set to 1.469 and for the continuous phase (water) was 1.330.

Statistical dimensions of size were expressed through the $d_{(0.1)}$, $d_{(0.5)}$ and $d_{(0.9)}$ values, which means that 10%, 50% and 90% of the total amount of sample is smaller than this size, respectively. The two sizes $d_{(0.1)}$ and $d_{(0.9)}$ enclose the range of particle sizes of the sample. From these values, it is possible to obtain the Span by the following equation:

$$Span = \frac{d_{(0.9)} - d_{(0.1)}}{d_{(0.5)}} \tag{3.4.1}$$

If the Span value is closer to 0, it is possible to infer that the respective sample is more uniform and the size consistency is better. Particle mean diameter was expressed as volume-weighted mean diameter $D_{[4,3]}$.

$$D_{[4,3]} = \frac{\sum_1^n D_i^4 \nu_i}{\sum_1^n D_i^3 \nu_i} \tag{3.4.2}$$

3.4.3 Rheological properties

The flow behaviour of the cheese feed samples was measured using the Discovery Hybrid Rheometer 2 (DHR-2, TA Instruments, New Castle, USA) using a rotor conical/DIN SST SMART-SWAP (diameter 28 mm) and a fixed lower concentric cylinder cup. For each measurement, approximately 25 mL of cheese feed was placed into the interior cup and the temperature was precisely controlled at 40 °C. Flow curves for each sample were measured over 10 min with shear rate continually increasing from 0.1 to 200 s^{-1}.

3.4.4 Physical stability

Turbiscan Tower (Formulaction, Toulouse, France) was used to investigate the stability of the cheese feed. This scanning method is based on Static multiple light scattering principle (S-MLS), where an infrared light source with a wavelength of 880 nm illuminates the sample, and 2 sensors collect the backscattering (BS) and transmission (T) signals. The stability is measured by the amount of light that is backscattered and sent back to the detector. Samples were prepared on a 20 mL glass vial. The BS

and T signals were acquired repeatedly over 2 hours every 2 minutes at the whole sample height and the temperature of the equipment was set to 40ºC.

3.4.5   Confocal laser scanning microscopy

The emulsions were dyed with Nile red [94] (Sigma-Aldrich Denmark A/S, Søborg, Denmark), dyeing the lipid phase. The dye was excited at 561 nm by a filter at 607 nm. FCF fast green (Sigma-Aldrich Denmark A/S, Søborg, Denmark) was used to dye the proteins and was excited at 640 nm by a filter of 700 nm [95]. The sample was imaged using a 40x lens (Nikon Apo LWD water 40x NA 1.15) on a spinning disc confocal microscope constituted by an inverted microscope (Nikon Ti2) equipped with a laser source (405 / 488 / 561 / 640 nm), a confocal spinning disc module (Yokogawa CSU-W1, 50um pinholes), a quad-band emission filter (440 / 521 / 607 / 700 nm) and an sCMOS camera (Photometrics Prime95B).

Chapter 4

Result and Discussion

4.1 Influence of processing conditions on particle size

Various statistical parameters used to understand the particle size of emulsions are expressed in Table 4.1, and a particle size distribution diagram for all 5 samples is presented in Figure 4.1. A monomodal particle size distribution was observed for the sample OH + H, whereas bimodal particle size distributions were noticed for the other samples. Independently of the type of heat treatment used, ohmic heating or oil bath, a high temperature of 85ºC led to to the highest particle sizes, with a $D_{[4,3]}$ of 8.83 µm for the OH + H sample and a value of 6.46 µm for the OB + H sample. The great majority of whey proteins are removed during cheese production, however, there is still some that remain, especially β-lactoglobulin since it is the main whey protein present in milk. At a temperature of 85ºC these proteins denature and become associated with casein micelles, through interaction with κ-casein, via hydrophobic interactions and disulphide bonds, which can justify the increase in particle size with the increase in temperature [96]. However, the homogenization of the sample prior to ohmic heating (sample H + OH + H) decreased the particle size to one similar to the one obtained by the sample treated at 60ºC with only one homogenization (60 + H). At 60ºC, the use of a second homogenization showed a decrease in particle size (3.19 ± 0.29 µm). These two last results indicate that a homogenization step prior to processing is positively correlated with particle size reduction, through disruption of protein aggregates and fat droplets. Besides, as referenced in Subsection 2.4.2, the presence of non-conductive materials, such as fat droplets, may be a disadvantage when it comes to the use of ohmic heating. This additional homogenization may help to promote a more uniform heating generation, by creating a more homogeneous sample, and so treatment can be more successful. In terms of uniformity, which is correlated with the Span value showed in Table 4.1, samples heated at 85ºC demonstrated a better size consistency compared to the ones heated at a lower temperature. The $d_{(0.5)}$ value for the 60 + H and H + 60 + H samples are lower compared to the samples heated at higher temperatures, especially for the last sample, which $d_{(0.5)}$ value is 0.56 ± 0.11 µm which means that the sample presents a high number of small particles, once 50% of them are smaller than 0.56 µm, and then a few bigger particles that occupy the same volume as the small ones. This can be seen through the bimodal particle size distribution diagram where the two peaks have the same height and explains the

heterogeneity of this sample. These samples having a lower particle size also corroborates the effect of higher temperatures on the aggregation of proteins.

Table 4.1: Average particle sizes of cheese dispersions. Samples processed at high temperatures, independently of the heat treatment used, demonstrated particles with a bigger size. In both cases, homogenization was able to reduce the particle size. Values are mean $\pm$ SD (n=2).

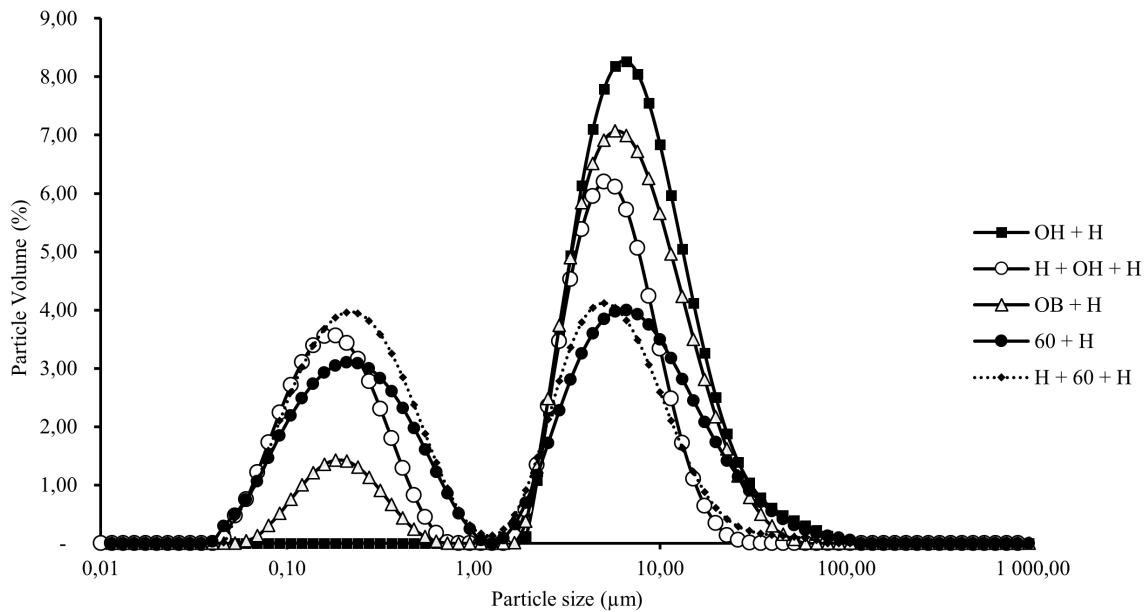| Sample ID	Span	$d_{(0.1)}$ (µm)	$d_{(0.5)}$ (µm)	$d_{(0.9)}$ (µm)	$D_{[4,3]}$ (µm)
OH + H	2.01 $\pm$ 0.10	3.17 $\pm$ 0.03	6.66 $\pm$ 0.01	16.53 $\pm$ 0.63	8.83 $\pm$ 0.31
H + OH +H	3.07 $\pm$ 0.38	0.14 $\pm$ 0.02	4.31 $\pm$ 0.66	13.63 $\pm$ 3.69	5.94 $\pm$ 1.73
OB + H	2.16 $\pm$ 0.02	3.01 $\pm$ 0.04	6.67 $\pm$ 0.22	17.40 $\pm$ 0.38	6.46 $\pm$ 0.65
60 + H	4.96 $\pm$ 0.04	0.12 $\pm$ 0.00	2.84 $\pm$ 0.19	14.22 $\pm$ 0.86	5.49 $\pm$ 0.42
H + 60 + H	15.72 $\pm$ 2.52	0.11 $\pm$ 0.00	0.56 $\pm$ 0.11	8.68 $\pm$ 0.31	3.19 $\pm$ 0.29

Figure 4.1: Diagram of the Particle size distribution obtained. A monomodal particle size distribution was observed for the sample OH + H, whereas bimodal particle size distributions were noticed for the other samples. The plots represented correspond to only one of the replicas produced.

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4.2 Rheological properties of cheese powder dispersions

Viscosity is a measure of the resistance of a fluid towards being deformed when under shear stress and is defined as the ratio of the shear stress to shear rate [97]. The viscosity properties of emulsions before spray drying influences the atomization of the feed and so the characteristics of cheese powder [55]. Data for fluids are often presented tin the form of viscosity – shear rate diagrams, as Figure 4.2 presents for the 5 samples produced. It is possible to observe that the viscosity is independent of time and shear rate for all samples, meaning they all present a Newtonian behaviour [98].
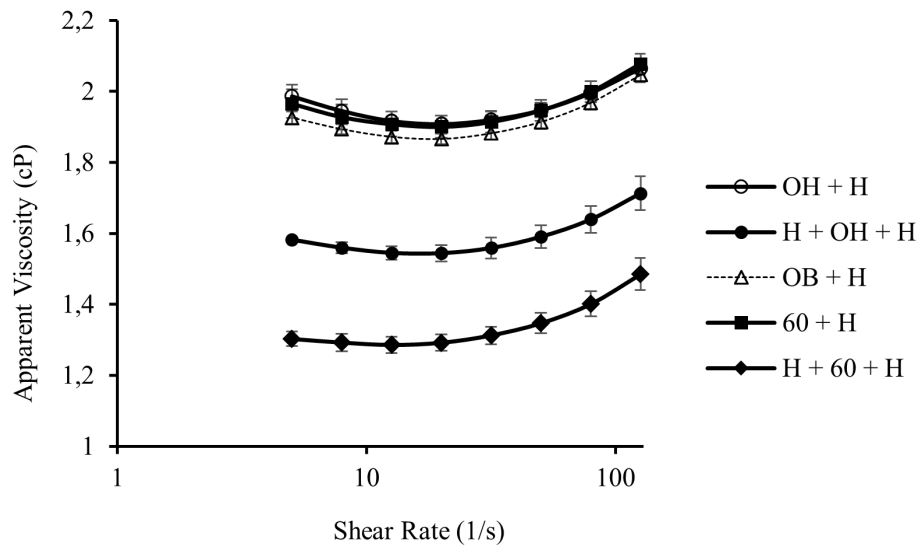

Figure 4.2: Apparent viscosity variation of the different cheese ppowder dispersions as a function of shear rate. All samples demonstrated a Newtonian behaviour once the viscosity did not change as a function of shear rate over time. Samples with a second step of homogenization exhibited a lower viscosity value. The results correspond to the average of the two replicas.

A similar apparent viscosity value was obtained for the 60 + H, OB + H and OH + H samples. The rheogram for the samples with double homogenization shifted downwards, with a maximum shift for the sample treated at 60°C. The viscosity of cheese powder dispersions was thus decreased by an homogenization step prior to heat treatment. Usually, the smaller the particles, the greater the surface area available for interaction, and so the higher the viscosity [99]. However, this was not observed in the results obtained, as the sample with smaller particles presented the lowest viscosity. Lower viscosities are usually related with unstable samples, which is proven by the fact that the presence of ES leads to an increase in apparent viscosity, when compared to samples with lower amount [4, 55]. Differences in the rheological behaviour can be assumed to be due to particle interactions and structural changes [100] and so, the presence of ES has a positive effect on protein hydration and formation of a continuous protein network structure, which causes a degree in viscosity and fat emulsification [55]. The overlapping results obtained for the other samples indicates that neither the high temperature, nor the type of heat treatment had a significant effect on the flow behaviour of the emulsions.

An adequate viscosity is also required for spray drying, as a too viscous sample can cause problems in pumping and droplet formation during atomization. The high viscosity also has high implications on the powder properties, once it results in a solubility decrease, an increase in particle size and less free flowing powder [4]. Urgu et al., 2018 [55] considered an emulsion too viscous to feed to drier when its apparent viscosity showed results from 5000 to 500 centipoise (cP) . Therefore, all samples produced in this experiment retain the appropriate viscosity for spray drying.

4.3 Influence of different processing conditions on the stability of cheese powder dispersions

The stability of the cheese powder dispersions prepared with different heat treatments can be characterized through the backscattering (BS) profiles obtained over a period of 2 hours. An increase in the BS percentage at the bottom of the vial is associated with protein precipitation that sediment, while an increase at the top is usually related to the presence of fat droplets in a process called creaming.

To produce cheese powder, it is a major criterion that the intermediate cheese emulsion does not show any type of instability [55].However, all samples displayed changes in stability over time, through sedimentation and/or creaming. Samples H + OH + H and H + 60 + H demonstrated a higher sedimentation, seen by the intensity of the BS signal captured (around 11%), when compared to the other samples (around 6%). This could be related to an excess level of insoluble protein that ends up precipitating. However, the opposite behaviour for creaming is observed. We hypothesize that the low creaming layer may be related to the presence of smaller fat droplets that take longer to cream, or to the interaction between fat globules and proteins. The width of the sedimentation layer should also be taken into consideration. As seen in Figure 4.3, the OH + H, OB + H, and 60 + H present a higher sedimentation layer, which may indicate differences in particle densities, compared to the other two samples, that present a more compact layer.
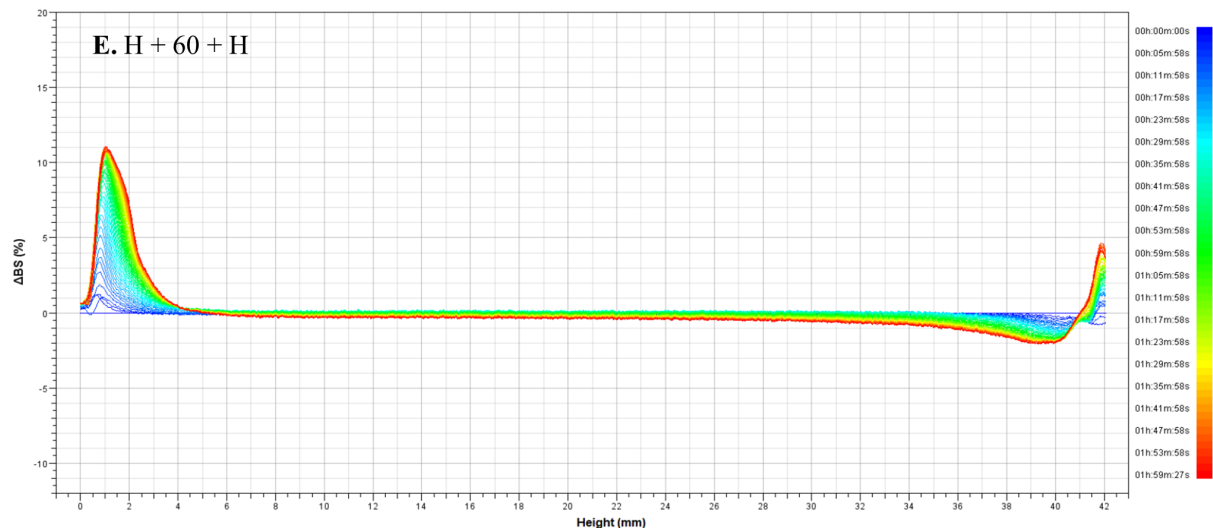
B. OB + H

C. 60 + H

D. H + OH + H

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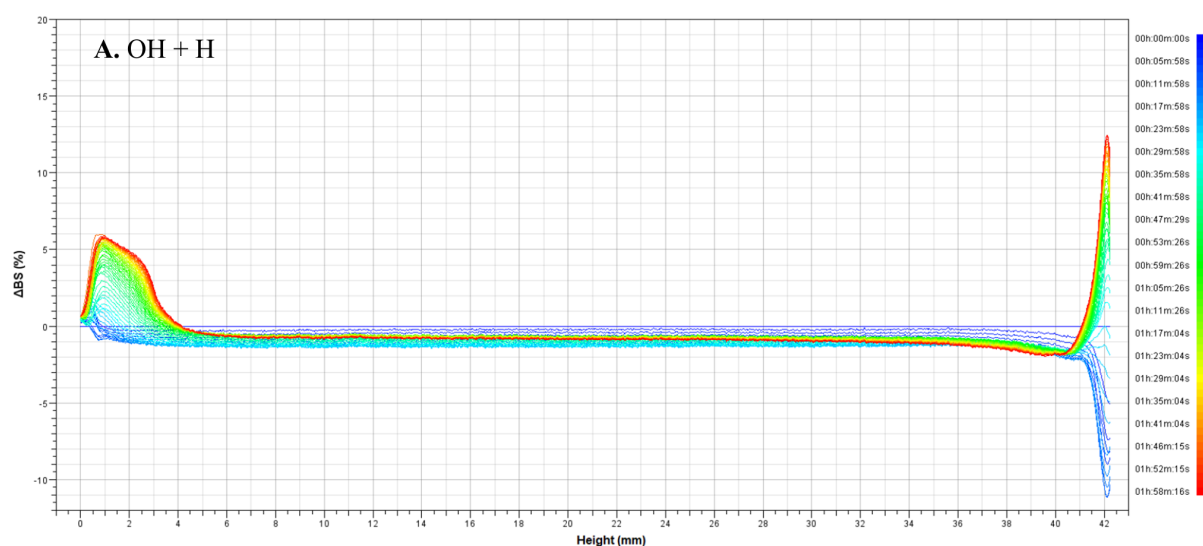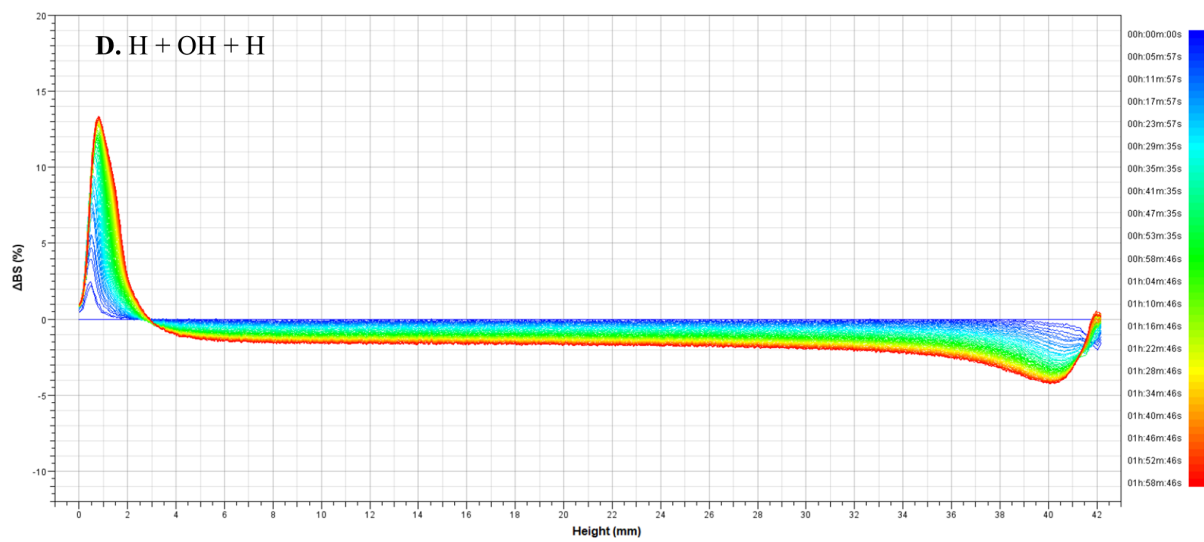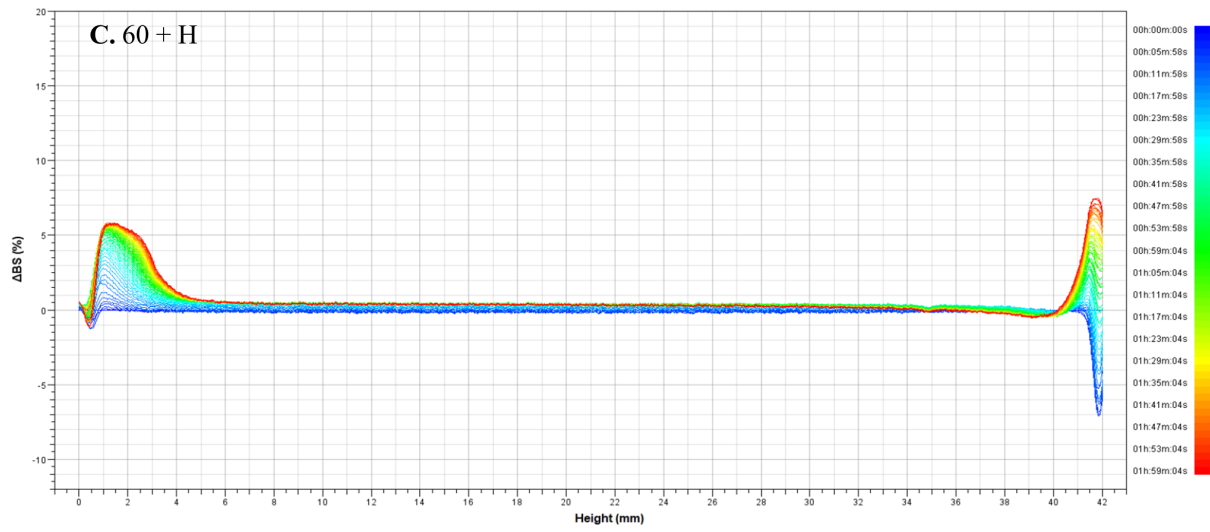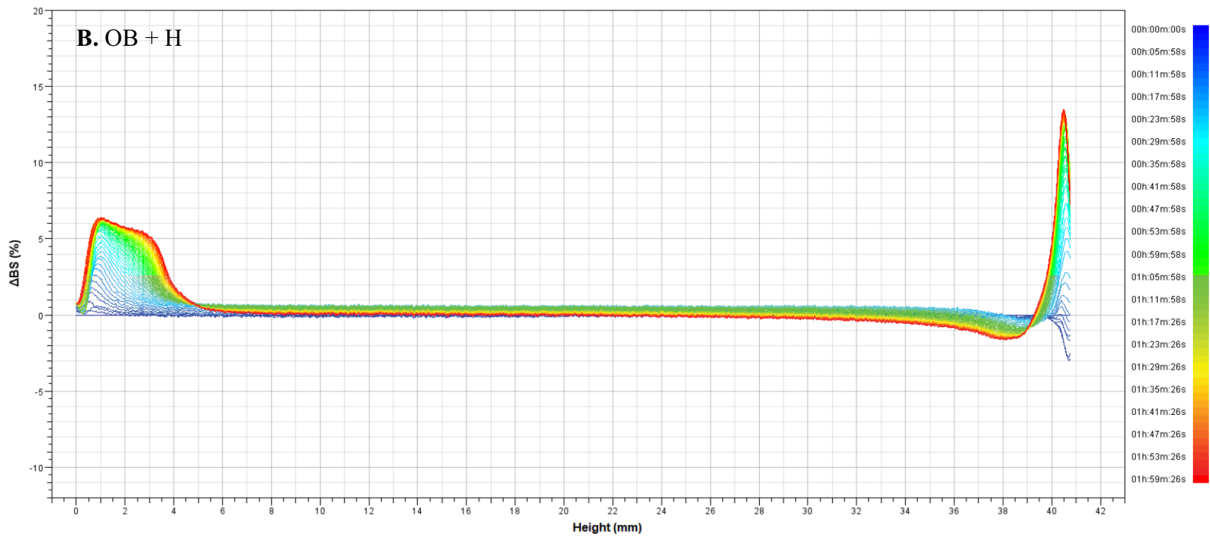
Figure 4.3: Physical stability (percentage of backscattering versus vial height). Sedimentation of proteins is associated with an increase in BS% at the bottom of the vial (left part of the graph), while an increase at the top is usually related to the presence of fat droplets in a process called creaming (right part of the graph). Samples with a second step of homogenization exhibited more sedimentation, whereas the other samples presented more creaming.

4.4 Microstructure of cheese disperisons evaluated by CLSM

Confocal Laser Scanning Microscopy was used to confirm the effects of the different processing conditions used on particle size, rheological properties, and stability behaviour, through the visualization of the microstructure of the samples, Figure 4.4. The CLSM images for the H + OH + H and H + 60 + H samples exhibited evenly distributed protein particles (green), and small fat droplets (red), which confirms the positive effect of homogenization. For the H + OH + H sample it can also be observed that the surface of the protein particles are coated by fat droplets in a thin layer (yellowish), which may indicate the interaction between fat and proteins, hypothesized in the previous Section 4.3 for the stability results obtained. Kelimu et al., 2017 [4] tested the effects of different dairy ingredients on the stability of cheese emulsions. In the absence of ES, the emulsions with sodium caseinate (SC) added demonstrated a similar microstructure image, with fat droplets coating the protein particles, which had a remarkable impact on the stability against creaming.

Both these samples demonstrated a better microstructure when compared to all the others, including the control one. All the other CLSM images revealed irregular protein aggregates, void area (black) which is mainly water and fat clusters (orange), where protein and fat droplets were disconnected from each other with little indication of emulsification.
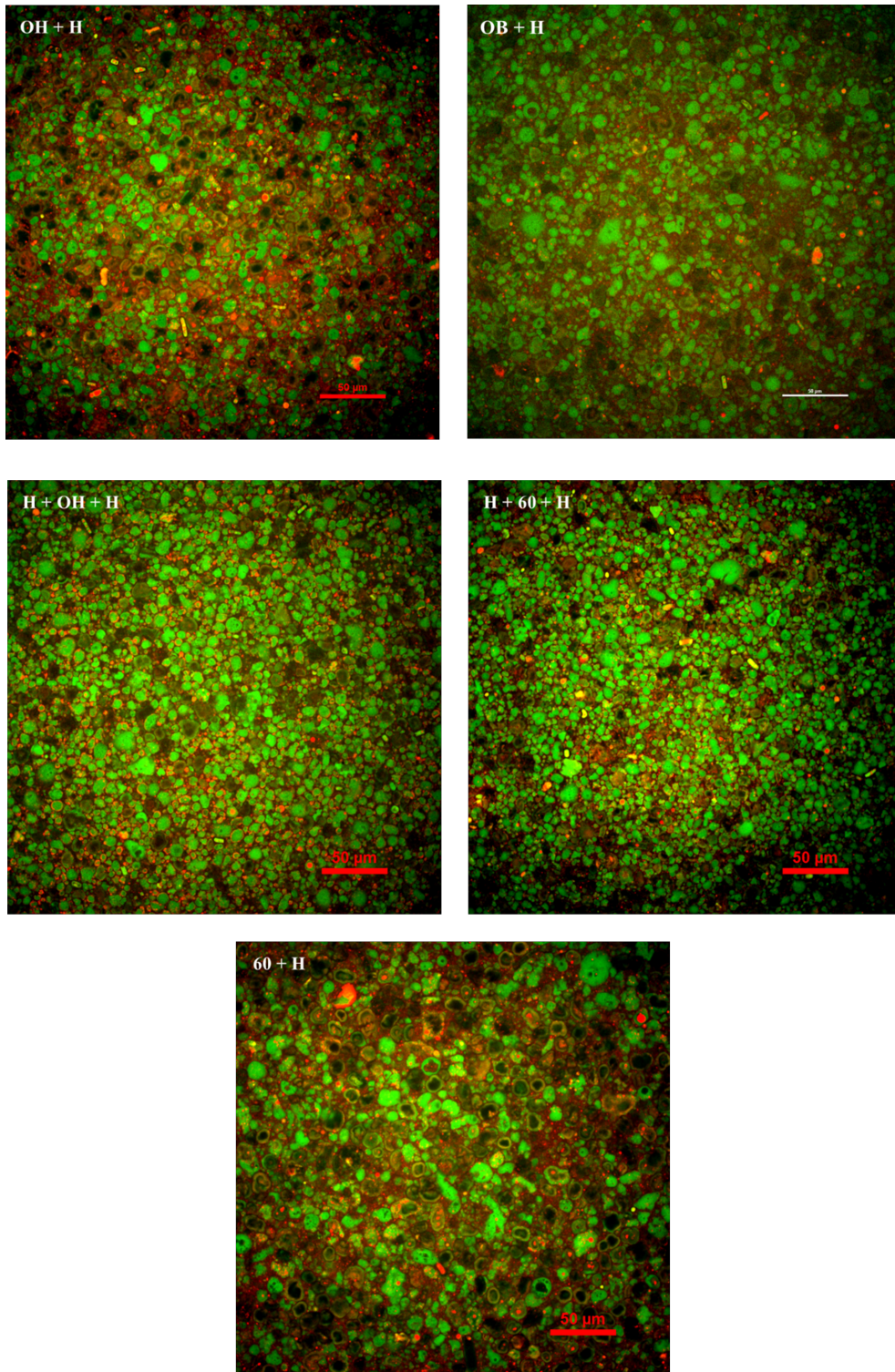
Figure 4.4: Microstructure of cheese dispersions visualized by CLSM. Green coloring represents protein and orange represents fat. Representative images are chosen from a series of images available for each sample composition.

4.5 Protein, fat and calcium balance

Up to now, the particle size, rheology, and stability results provided a general idea on how the different processing conditions affected the behaviour of the 5 cheese powder dispersions. However, fat emulsification and protein stabilization play equally important roles in keeping cheese emulsion stability, and so, the investigation of the shift in the calcium balance of the system, is an important step to comprehend the dispersion of casein, protein-protein interactions and consequent fat emulsification [79].

The cheese powder used for the production of the present cheese powder dispersions does not contain emulsifying salts, which are known to have the ability to sequester calcium from the casein matrix, through the exchange by sodium ions, leading to casein solubilization, and further fat emulsification [2, 73, 75]. It is possible to correlate the emulsification stability of a cheese feed with the powder properties, and so, as seen in Subsection 2.2.2, a good quality cheese powder presents a low amount of free fat and a high protein solubility, which means that the optimal processing condition needs to be able to diffuse the calcium from the casein micelles, solubilizing it, and provide a high amount of fat in the soluble phase, as it would indicate interaction with soluble protein.

For this purpose, the differences in intact and soluble protein, calcium balance and fat content after the different heat treatments were calculated based on the dry matter (DM) content, Table 4.2. After centrifugation, three layers were obtained, and one can assume that fat was primarily found in the top layer, precipitated protein in the bottom phase, leaving an opaque watery phase in between upon phase separation, where soluble protein can be found.

Heating has a great effect on calcium equilibrium and its interaction with caseins, and it is agreed that high temperatures lead to a decrease in diffusible calcium, due to precipitation of calcium phosphate. Therefore, it is expected that samples treated at a temperature of 85ºC demonstrate a lower amount of soluble calcium, once this mineral is forced inside the micelle, as caseins are effective stabilizers of CCP [6]. Under our experimental conditions, the sample heated at 85ºC in an oil bath (conventional heating) corroborated this theory, as it showed a value of 22.95 ± 9.29 % of soluble calcium. However, this does not apply to both samples treated with OH (54.23 ± 5.50 % and 45.27 ± 9.18 % of soluble calcium in H + OH + H and OH + H, respectively), which presented values of soluble calcium closer to the one obtained by the sample treated at a lower temperature (60 + H - 48.05 ± 16.23 % of soluble calcium). Since both OB and OH samples were treated at 85ºC and there is a significant difference in the results obtained, it is possible to infer the positive impact that the ohmic heating technology has in the shift in mineral balance, enabling the calcium to dissolve from the micelle and migrate to the serum/soluble phase. It can be suggested that the moderate electric field may have imposed, even subtly, disturbances on the charge organization within protein structure and its orientation in the direction of the applied electric field [101], which facilitated the diffusion of calcium.

Table 4.2: Chemical analysis of the cheese powder dispersions. Fat, protein, and calcium content (%) based on the dry matter content (%) for each layer obtained after centrifugation. Values are mean ± SD (n=2).

Sample ID	Layers	Dry Matter %	Fat %	Protein %	Calcium %
OH + H	Top	13.69 ± 0.27	33.28 ± 0.74	5.99 ± 1.14	
	Water phase	62.18 ± 0.75	52.15 ± 1.57	58.50 ± 1.47	45.27 ± 9.18
	Sediment	24.13 ± 0.48	7.88 ± 0.83	35.51 ± 0.10	54.73 ± 9.18
H + OH + H	Top	13.76 ± 0.90	29.59 ± 1.84	10.65 ± 0.59	
	Water phase	59.07 ± 2.28	47.18 ± 2.51	53.32 ± 0.68	54.23 ± 5.50
	Sediment	27.17 ± 1.39	17.27 ± 0.46	36.02 ± 0.09	45.77 ± 5.50
OB + H	Top	15.96 ± 0.48	38.76 ± 1.13	6.07 ± 0.74	
	Water phase	55.52 ± 0.27	45.34 ± 0.28	52.06 ± 1.85	22.95 ± 9.29
	Sediment	28.52 ± 1.74	12.44 ± 0.87	41.87 ± 2.59	77.05 ± 9.29
60 + H	Top	16.23 ± 0.39	39.77 ± 0.69	9.71 ± 3.11	
	Water phase	60.88 ± 2.46	50.11 ± 1.27	55.94 ± 1.02	48.05 ± 16.23
	Sediment	22.89 ± 2.08	6.83 ± 0.14	34.35 ± 4.13	51.95 ± 16.23
H + 60 + H	Top	12.59 ± 0.29	30.22 ± 0.62	12.92 ± 1.94	
	Water phase	64.20 ± 3.19	57.69 ± 1.22	54.99 ± 2.42	37.16 ± 1.34
	Sediment	23.21 ± 3.59	9.74 ± 1.20	32.09 ± 4.37	62.84 ± 1.34

The high standard deviation values obtained between replicas concerning the calcium content can be explained by the fairly rapid reverse of the changes provoked by heat treatment. As Andrew J.R. Law [96] demonstrated, 1.5h after heat treatment and storage at ambient temperature, the concentration of colloidal calcium was very similar to the one found in raw milk . Therefore, the centrifugation step of replicas may have occurred at different times after the heat treatment, some of them over 1.5h later, which may have caused a reverse change of the calcium balance, leading to different results.

In this study, soluble protein was defined as the one that did not sediment after centrifugation at 5000 x *g* for 1 hour. All samples demonstrated a similar trend regarding the protein content, with a higher percentage in the soluble phase, followed by the sediment, and then the top layer, which is mainly constituted by fat. Regarding the fat content, all samples displayed a higher amount in the soluble phase compared to the other layers. It is difficult to correlate the results between samples, once many parameters were changed. However, the effect of ohmic heating can be evaluated by comparing the OH + H sample with the OB + H sample, as both were treated at 85ºC for 5 min with one homogenization step, varying only the heat process used. By looking at the results of the protein and fat content and the calcium balance all together, the positive impact of OH in all of these variables is undeniable. As said before, the ideal process would lead to an increase in the diffusible calcium, which was already discussed that the OH technology had a positive impact, and would increase the amount of soluble protein (58.50 ± 1.47 % for OH + H, compared to 52.06

± 1.85 % for OB + H) and soluble fat (52.15 ± 1.57 % for OH + H, compared to 45.34 ± 0.28 % for OB + H), demonstrating a higher protein solubilization and fat emulsification. The H + OH + H sample presents the higher amount of fat in the sediment (17.27 ± 0.46 %) which is in relation with the stability results obtained and with the assumption that fat was interacting with insoluble protein, that ends up precipitating To produce cheese powder, this type of sample could be mixed and spray-dried quickly, and once the fat is surrounded by protein it is protected against oxidation, but for other applications, as for solubilization of the powder, it would not be ideal. Therefore, the solubility of proteins still needs to be improved.

The solubility and aggregation state of proteins depend on a balance between temperature and pH. Therefore, we suggest complementing this present study with the variation of pH, with the aim of controlling the surface electric charge of proteins and aggregation process, so that we can benefit from both the presence of some aggregated particles, responsible for the increase in viscosity, and from non aggregated proteins that will act as fat emulsifiers. Casein micelles are stable at high temperatures, and moderate heating does not cause aggregation between micelles nor disruption of their internal structure. However, changing the pH can easily destabilise the micellar integrity. Proteins are usually insoluble at their isoelectric points, which is pH = 4.6 for caseins. Increasing the pH from the isoelectric point gives rise to more negatively charged micelles, which strengths the repulsive forces of the caseins chain, increasing solubility, and micelle size, due to producing loose and expanded structures in the micelles. Additionally, by acidifying the solution, the solubilisation of colloidal calcium phosphate from the micelles increases. Since this material is largely responsible for maintaining the integrity of the micelle, the loss of CCP may be possibly accompanied by the dissociation of caseins from the micelles [102, 103], that may interact with fat droplets. The mineral balance is very dependent on pH and temperature, and so it would be interesting to look at the set effect of these two physicochemical parameters.

Chapter 5

Conclusions and outlook

5.1 Conclusions

Cheese powder is a dehydrated cheese product with major economic importance due to their widespread use as flavouring agents, nutritional value, and higher convenience and longer shelf-life compared to natural cheese. During cheese powder production, an oil-in-water emulsion, denoted cheese feed is formed. As it is required to remain stable until being spray dried, emulsifying salts are added. These promote physicochemical changes in the blend, which lead to solubilization of calcium, and consequent hydration of caseins that can then act as emulsifiers and form a membrane layer around the liquid fat released during processing, forming a smooth, homogeneous, and stable emulsion. However, there is an increase pressure from conscious consumers and authorities to reduce the salt content in food, and so the dairy industry has been investigating new strategies for stabilisation of cheese feed without the presence of ES.

This master thesis opens a new knowledge platform regarding the effects of ohmic heating on cheese feed stability, hypothesizing that the use of this technology as a heat treatment to process the blend may change the protein, fat, and calcium balance, creating a stable emulsion. The ohmic heating treatment was compared with the use of conventional heating and different parameters were used, such as temperature, 60ºC and 85ºC, and number of homogenizations. For both heating treatments, higher temperatures led to an increase in particle size, which we hypothesize to be due to aggregation of casein micelles and β-Lg. An extra homogenization prior to heat treatment was able to decrease the particle size, through disruption of protein agglomerates and fat droplets. In terms of rheologic behaviour, all conditions produced emulsions with Newtonian behaviour and with values of apparent viscosities appropriate for spray drying. Regarding the stability of the emulsions, all samples displayed instability mechanisms, through sedimentation and creaming. Samples processed with a two step homogenization led to a higher sedimentation intensities, which could be due to the presence of an excess of insoluble protein that ends up precipitating. The creaming layer was lower for these two samples, which we hypothesize to be related to fat droplets interacting with protein forming complexes that remain stable.

The positive impact of ohmic heating compared with conventional heating was seen, however, in the differences between the calcium balance and fat and protein contents, by comparing the results obtained for the two samples heated at 85ºC for 5 min with only one step homogenization (OH + H and OB + H). Despite the high temperature, which is known to hamper the diffusion of calcium, the use of moderate electric field led to a significant higher solubility of calcium, compared with the conventional heating technique. This is though to be related with the disturbances on the charge organization within protein structure and its orientation in the direction of the applied electric field, which facilitated the diffusion of calcium. Fat emulsification and protein stabilization play equally important roles in keeping cheese emulsion stability, and so the higher values of soluble protein and fat when OH was used, also demonstrates the great potential this technology shows on emulsion stability.

Overall, ohmic heating is an exciting opportunity to improve the quality of food, and continued innovation will benefit both food manufacturers and consumers. Results obtained in the present research clearly show that ohmic heating technology is a powerful tool that mainly interferes with calcium solubilization, which consequently affects fat emulsification by proteins. However, there is still room for improvement, as the technology still demonstrated the appearance of instability mechanisms, such as sedimentation and creaming, and appeared to have an adverse effect on protein solubility, as fat is interacting with insoluble protein.

5.2   Future perspectives

This study is just the "Once upon a time" of a story where ohmic heating is the main character. The plot still needs to be written, and other parameters still need to be casted. It would be very interesting to investigate more about the effect of ohmic heating on caseins itself, by understanding the effect of this technology on protein charge, which can be done through the measurement of ζ-potential, and also by investigating which individual casein proteins are affected by OH, through SDS-PAGE. In this study, OH was used at 85ºC to investigate the effects of extreme conditions. Looking at the effect of a broader range of temperatures and voltages would be a way to find the ideal conditions for emulsion stability. In order to improve protein and calcium solubility, it would be appealing to combine the effect of temperature and electricity with pH variations once it is known to alter solubility of proteins and calcium phosphate. Furthermore, the use of OH should also be linked with the use of different cheese types and compositions, different ages, and different addition of ingredients once these seem to also have an effect on emulsion stability.

There are no studies regarding the effect of ohmic heating on cheese feed stability for cheese powder production, which makes this is an innovative investigation that opens doors to a whole new field where this new technology is used as processing condition and has a substantial potential to change and enhance the dairy industry. It still requires a great amount of research, and I would leave the suggestion for the next experiments to be carried out using natural cheese instead of cheese powder, as it would reproduce a more authentic and more studied cheese emulsion. Recreating cheese feed using cheese powder just forms

a more complex system that may influence the results in a certain way the results. Furthermore, more than two replicas should be made for each sample, so that statistical analysis can be performed.

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Colophon

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