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Ecole Doctorale des Sciences de la Matière, du Rayonnement et de l'Environnement – SMRE

Unité Matériaux et Transformation – UMET

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To obtain the PhD degree in Food Science from Université de Lille

Specialization: **Agri-food Biotechnologies, Food Science, Physiology**

Thesis presented and defended on June 26, 2020 in Villeneuve d'Ascq - France.

Márcio Henrique NOGUEIRA

RELATION BETWEEN STRUCTURE AND VISCOSITY IN DEMINERALIZED CASEIN MICELLES

Jury

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Milena CORREDIG, Professor, Aarhus University, Aarhus, Denmark.

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Université de Lille. Faculté des sciences et technologies

Ecole Doctorale des Sciences de la Matière, du Rayonnement et de l'Environnement – SMRE

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THÈSE DE DOCTORAT

Pour obtenir le titre de Docteur en Sciences de l'Alimentation, Université de Lille

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Márcio Henrique NOGUEIRA

RELATION ENTRE STRUCTURE ET VISCOSITÉ DANS LES MICELLES DE CASÉINE DÉMINÉRALISÉES

Jury

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“**…What we do in life… echoes in eternity.”**
General Maximus Decimus Meridius

“I don't want to believe. I want to know.”
Carl Sagan

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Section 3.1 → Nogueira, M. H., Ben-harb, S., Schmutz, M., Doumert, B., Nasser, S., Derensy, A., … Peixoto, P. P. S. (2020). **Multiscale quantitative characterization of demineralized casein micelles : How the partial excision of nano-clusters leads to the aggregation during rehydration**. *Food Hydrocolloids*, *105*. https://doi.org/10.1016/j.foodhyd.2020.105778.

Section 3.2 → It will be submitted to the journal entitled “Powder Technology”

Section 3.3 → It will be submitted to the journal entitled “Food Hydrocolloids”

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ABSTRACT

The ready-to-drink beverages with high protein content are a market trend and bring together a product with nutritional advantages associated with a facility to consumption. In this context, the whey proteins are still the major type of protein in use. However, casein micelle (CM), which is protein assembly with several colloidal calcium phosphate nanoclusters displaying many water cavities, represents a better option for this application because of its natural abundance and its unusual resistance to heat treatments. However, the use of CMs presents show drawbacks; CMs produce more viscous dispersions than whey proteins (at equal protein concentration) and display a slower rehydration time (after spray-drying).

It was presented in some bibliographies that demineralization could be an alternative to changing the apparent viscosity of CMs dispersions and also reduce the time necessary to rehydrate the casein-based powders. However additional experiments required to be provided to strengthen this hypothesis (i) to ascertain that this demineralization step carried out before spray drying is still operating at molecular level after modified casein-based powders have been re-dispersed at high protein content and subjected to UHT treatment, as these two unit operations are usual in ready-to-drink beverages; (ii) to precise the underlying mechanisms involved explaining such possible gains in techno-functional properties (rehydration and apparent viscosity). To measure the impact of the calcium-demineralization in CMs structure and the consequences in term of techno functionalities of the casein-rich dispersions, different calcium-demineralized casein-based powders were employed: (i) commercial ones provided by Ingredia and produced at lab-scale; (ii) specific demineralized casein based-produced at pilot-scale from Ingredia.

Concerning commercial casein-based powders, it has been confirmed that calcium-demineralization affects the CMs organization at different molecular levels, resulting in the release of some reactive chemical groups (hydrophilic and/or charged ones) that favors the formation of hard-to-dissolve particles. It was shown that the effect could be stronger (the particles formed are harder to dissolve) whether rehydration temperature is elevated. Analysis of rehydration behavior of native CM under different temperature conditions has allowed us to propose a fast rehydration protocol (50°C/1H +homogenization step) that mimics aggregates size distribution and apparent viscosity of native CMs, obtained at ambient temperature without homogenization.

The study of the pilot-scale casein-based powders has allowed us to evaluate the structural and functional changes associated with two recombined processes applied to produce dense dispersions (like ready-to-drink high-protein beverages) which are: (i) the powder rehydration; (ii) the commercial sterilization by ultra-high-temperature. From structural investigations, it has been shown that CMs display two types of nanoclusters, one strongly associated with the CMs proteins and another one weakly attached-to-CMs, allowing of improving present knowledge of micellar casein models. It is shown that the removal of the first type of cluster has little impact on the CMs structure, and the removal of the last type induces a greater disorder in the CMs nanostructure.

On the other hand, the main impact of UHT on the CM structure is the internalization of calcium phosphate ion by the CM. After UHT, the concentration of calcium and phosphate ions decreases in the soluble phase, and it increases within CMs. This effect is significantly greater in weak-to-moderate demineralized samples than in the native one. In contrast, for the most demineralized sample, UHT induces a greater structural change and a loss of nanoclusters.

As far as technological properties are concerned, for samples displaying weak-to-moderate degrees of demineralization, UHT is responsible for producing the less viscous dispersions.

Based on this study the following mechanism is proposed: Calcium internalization during UHT likely neutralizes the reactive chemical groups (hydrophilic and charged sites), on the surface of the CMs, which are likely to favor a sticky interaction between. In contrast, for the most demineralized sample, UHT induces a significant increase in viscosity. In this case, the further increase in disorder induced by UHT likely favors a further exposition of reactive sticky chemical groups. Thus, the UHT dependent liberation of these groups favors the sticky interaction between CMs increasing the viscosity of the dispersion. This study suggests that demineralization degree have to be finely tuned.

To conclude, these studies bring an improvement in the understanding of the CM structure and important information to the development of ready-to-drink high-protein beverages using CMs as a major protein source.

RÉSUMÉ

Les boissons prêtes à boire à haute teneur en protéines sont une tendance du marché et réunissent un produit aux avantages nutritionnels associés à une facilité de consommation. Dans ce contexte, les protéines de laits du lactosérum restent le principal type de protéine utilisé. Cependant, la micelle de caséine (CM), qui est un assemblage de différentes caséines comportant plusieurs nano clusters de phosphate de calcium colloïdal et contenant de nombreuses cavités d'eau, représente une protéine intéressante pour cette application en raison de son abondance naturelle et de sa résistance inhabituelle aux traitements thermiques. Cependant, l'utilisation de CM présente des inconvénients; les CM produisent des dispersions plus visqueuses que les protéines de lactosérum (à concentration de protéines égale) et affichent un temps de réhydratation plus lent (après séchage par pulvérisation).

Il a été supposé que la déminéralisation pourrait être une alternative pour réduire la viscosité des dispersions de CM et réduire la durée de réhydratation des poudres à base de caséine. Cependant, des expériences supplémentaires devaient être accomplies pour renforcer cette hypothèse (i) afin de s'assurer que cette étape de déminéralisation effectuée avant le séchage par pulvérisation fonctionne toujours au niveau moléculaire, une fois que les poudres à base de caséine modifiées ont été redispersées à une teneur élevée en protéines et soumises au traitement UHT (car ces deux opérations unitaires sont incontournables dans les boissons prêtes à boire); (ii) préciser les mécanismes sous-jacents expliquant ces gains possibles de propriétés techno-fonctionnelles (réhydratation et viscosité apparente).

Pour mesurer l'impact de la déminéralisation du calcium dans la structure des CM et les conséquences en termes de fonctionnalités des dispersions riches en caséine, différentes poudres à base de caséine déminéralisée en calcium ont été utilisées: (i) certaines sont des poudres commerciales livrées par Ingredia; (ii) d'autres sont des poudres déminéralisées spécifiques produites à l'échelle pilote par Ingredia

Concernant les poudres commerciales à base de caséine, il a été confirmé que la déminéralisation du calcium affecte l'organisation des CM à différentes échelles moléculaires (enveloppe des caséines, sous micellaire), entraînant la libération de certains groupes chimiques réactifs (hydrophiles et / ou chargés) qui favorisent la formation de particules difficiles à dissoudre. Il a été démontré que l'effet était plus fort si la température de réhydratation est élevée. L'analyse du comportement de réhydratation des CM natifs dans différentes conditions de température nous a permis de proposer un protocole de réhydratation rapide (50 ° C / 1H + étape d'homogénéisation) qui imite la distribution granulométrique et la viscosité apparente des CM natifs, obtenues à température ambiante sans étape d'homogénéisation.

L'étude des poudres à base de caséine à l'échelle pilote nous a permis d'évaluer les changements structurels et fonctionnels associés aux deux opérations unitaires de reombinaison systématiquement appliquées pour produire des dispersions denses

que sont les boissons riches en protéines : (i) les réhydratation en poudre; (ii) la stérilisation commerciale par ultra-haute température.

Des études structurales, il a été démontré que les CM présentent deux types de nanoclusters, l'un fortement associé aux protéines des CM et l'autre faiblement attaché aux CM, ce qui a permis d'améliorer la connaissance sur l'organisation micellaire. Il est démontré que la suppression du premier type de cluster a peu d'impact sur la structure des CM alors que la suppression du second type induit un effet plus important dans la nanostructure des CM.

D'autre part, le principal impact de l'UHT sur la structure du CM est l'internalisation de l'ion phosphate de calcium par le CM. Après l'UHT, les concentrations d'ions calcium et phosphate diminuent dans la phase soluble et augmentent au sein des CM. Cet effet est significativement plus important dans les échantillons déminéralisés dit - faibles à modérés que dans les échantillons natifs. En revanche, pour l'échantillon le plus déminéralisé, l'UHT induit un changement structurel plus important et une perte de nanoclusters.

En ce qui concerne les propriétés technologiques, pour les échantillons présentant des degrés de déminéralisation faibles à modérés, l'UHT est responsable de la production de dispersions moins visqueuses.

Sur la base de cette étude, le mécanisme suivant est proposé: l'internalisation du calcium pendant l'UHT neutralise probablement les groupes chimiques réactifs (sites hydrophiles et chargés), à la surface des CM, qui sont susceptibles de favoriser une interaction collante entre eux. En revanche, pour l'échantillon le plus déminéralisé, l'UHT induit une augmentation significative de la viscosité. Dans ce cas, l'augmentation supplémentaire des troubles observés par l'UHT pour ces déminéralisations élevées favorise probablement une exposition supplémentaire des groupes chimiques collants réactifs. Ainsi, la libération dépendante UHT de ces groupes favorise l'interaction collante entre les CM augmentant la viscosité de la dispersion. Cette étude suggère que le degré de déminéralisation doit être finement controllé pour obtenir les propriétés escomptées.

Pour conclure, ce travail de thèse contribue à la fois à apporter une amélioration de la connaissance de la structure des CM (déminéralisées ou non) et des informations importantes pour le développement de boissons riches en protéines prêtes à boire souhaitant utilisés les CM comme source majeure de protéines.

CHAPTER 1 - GENERAL INTRODUCTION

General Introduction

The concerns about the correct nutrition have increased in the last decade (Demaio & Branca, 2018). In this context, as discussed by different international health organizations and researchers groups, correct nutrition is associated with a decrease in the risks of some chronic diseases (WHO and FAO, 2003).

The correct nutrition is associated with a well-balanced diet, which should contain a variety of different foods, capable of providing a wide range of nutrients necessary to promote the good development of all of our physical and psychological capacities that our bodies need (Burgess & Glasauer, 2004).

In a well-balanced diet, the proteins represent an important food component that is responsible for promoting different processes in the human body, such as regulation of the body's cell functions, tissues, and organs (Lonnie et al., 2018). The proteins are formed by a chain of amino acids which provide essential components for living organisms, such as nitrogen, hydrocarbon skeletons, and sulfur and cannot be replaced by any other nutrients, is an essential precursor for the synthesis of proteins, peptides, different regulator metabolites, such as urea and creatine and in the hormonal secretion (e.g., dopamine and serotonin) (Wu, 2009).

Regarding the increase in health concerns and the demand for a higher amount of proteins, the market of food focused on hyper protein products become a trend in the last years (Henchion et al., 2017). As projected (Henchion et al., 2017; Lagrange, Whitsett, & Burris, 2015), it is expected an increase from a total of about 650 millions of dollars in 2016 to 1.05 million of dollar predicted in 2021 in this market of food with a high amount of proteins until 2050, associated with the increase of the population to 9.1 billion and an increase in the food production to about 70%.

The milk proteins represent one of the most important sources of proteins which can be used in a wide range of food products, such as cheese (Fox et al., 2016), yogourt (Tamime & Robinson, 2000), desserts (Rybak, 2014) and food supplement (Wilborn et al., 2013).

In cow's milk, the most abundant protein are the caseins, present as a sponge-like form (Rebouillat & Ortega-requena, 2015), denominated as casein micelles (CMs). The CMs represent about 80% of the total protein (Dalgleish & Corredig, 2012; De Kruif et al., 2012; Fox & Brodkorb, 2008; Phadungath, 2005a).

The natural structure of the CMs (pH 6.7) is formed by the association of four different casein fractions (α_{s1}, α_{s2}, β and κ) in different proportions, being distributed in different regions of the CMs. This association is stabilized by the colloidal calcium phosphate (CCP) nanoclusters and the polyelectrolyte brush formed by the κ-casein on the surface of the CMs (De Kruif & Zhulina, 1996; De Kruif et al., 2012; Phadungath, 2005a).

As announced previously, the CCP is one of the most important agents responsible for stabilizing the micellar structure of CMs. The changes in the amount of calcium or their interaction affect the structure of CMs, as observed after some technological treatments, such as addition of chelating salts (Pitkowski, Nicolai, & Durand, 2008; Ye & Harte, 2013), demineralization (Meletharayil et al., 2018) and pH changes (Silva et al., 2013).

These changes in the CMs structure are responsible for some usual technological problems, such as instability to heat treatments (Lucey et al., 2001; Mohammed & Fox, 2014; Perveen et al., 2015; Trejo & Harte, 2010). However, some interesting properties of the demineralized CMs have been observed, such as better rehydration properties of casein powders (McCarthy et al., 2017) and better viscosity of the dispersion made with caseins (Bienvenue et al., 2003; Meletharayil et al., 2018; Pandalaneni et al., 2018; Schkoda, Hechler, & Kessler, 1999).

These two properties changes can be important to the application of the CMs as a food ingredient to manufacture high protein beverages, as demonstrated by the bibliography (Corredig et al., 2019; Pandalaneni et al., 2018). However, to produce this kind of products (high protein beverages) with CMs, it will be necessary to trespass some problems associated with the use of the CMs powder as an ingredient, such as their poor wettability and long time required for their rehydration (Ji et al., 2016; Richard et al., 2013) and its difficulty to produce low viscosity dispersions, desirable for

beverages, with a high amount of proteins, over 10 % (w/w) (Amelia & Barbano, 2013; Bouchoux et al., 2009).

This thesis document has as objective to fill this gap; the present manuscript has mainly elaborated with two objectives: i) the evaluate the changes in the CMs structure with different degree of demineralization ii) to quantify its impact on the functional properties (apparent viscosity, and heat stability) in high concentrated dispersions made of CMs demineralized powders.

The demineralized powders were produced with the help of INGREDIA, which support partially this Ph.D. thesis (March 2017-June 2020) work with the help of ANRT (which refers in French to Association Nationale Recherche and Technologie). The experimental part of this work (structure and functional characterizations) was mainly performed at INRAE (which stands for French National Institute for Research on Agriculture, Food and Environment) and namely at the joint UMET laboratory (which refers to Unit Material and Transformation) within the PIHM‘s team (which stands in French to Processus aux Interface and Hygiène des Matériaux). After the foundation (March 2018) of the joint laboratory between Ingredia and UMET (mainly with some members of the PIHM's team from INRAE) entitled as ***Proteinolab***, I also benefited from the support of this organization to pursue my work.

Organization of the document

The document is divided into three different chapters after the introduction, being:

– Review of literature – Chapter 2

– Results and discussion – Chapter 3

– General conclusion and Perspectives – Chapter 4

The specificities of the document are the following:

-**Chapter 3** of the document (i.e., **Results and Discussion**) was divided into four different subchapters (**subsections 3.1 to 3.4 of Chapter 3**) corresponding to 4 articles.

As a matter of fact, each subchapter constitutes “an article to submit” in order to constitute “a ready-to-submit manuscript.” Note that the first subchapter (article 1)

has been reviewed and is already accepted (accessible in line and in the course of the paper edition –August 2020). The reference is given later in the document in the preamble of Article 1.

At the beginning of each article, some additional context elements are given and/our objectives and scientific questions are reminded. At the end of each two articles (after articles 2 and 4), an intermediate conclusion is reported. This choice has been made since as previously underlined, the articles are representative of two different periods of study, which corresponds to the adopted strategies: i) the study of commercial powders (articles 1 and 2) and ii) the study of pilot-scale powders (articles 3 and 4).

For avoiding repetition and sake of concision, it was chosen that no traditional chapters devoted to **Material and Methods** in the document. All the material and methods are in the 4 articles. However, all the protocols associated with the methods developed in this Ph.D. Thesis have been written according to INRAE standard of archiving quality approach.

Each article also has its own bibliographic part. However, it was chosen to strengthen this aspect, which is also traditional in a Ph.D. thesis, with a chapter entitled **Review of literature (Chapter 2)**. This Bibliographic chapter was intentionally added to this thesis document to better identify state of the art concerning CMs structural organization before or after calcium-depletion and its impact on techno-functional properties. Indeed, the bibliographic section will describe some important points about the CMs, such as structural organization and their models, and the influence of some treatments (calcium-demineralization, heat treatments and, acidification). This bibliographic chapter was mainly focused on the unit operations, which are usually applied to produce ready-to-drink high protein beverages using casein-rich powders as a major source of proteins (rehydration and UHT treatments). At the end of the bibliographic chapter, we will present a compilation of the major identified lacks, which constitutes a bottleneck for the present study. Finally, we will present the principal scientific questions which came out from the bibliographic lacks and our strategy to partially answer these questions.

CHAPTER 2 - REVIEW OF LITERATURE

Review of literature – General aspects

The cow's milk is a complex dispersion containing different constituents that can be divided into two different phases, the so-called "colloidal phase" and the "soluble phase" (McCarthy & Singh, 2009). In the so-called "colloidal phase" is found the casein micelles, which are composed of 80% of the total milk proteins and associated with calcium and phosphorus and the fat globules. The casein micelles (CMs) are an assemblage of protein with an average size of 100–200 nm (Walstra, Wouters, & Geurts, 2006). The so-called "soluble phase" of milk is mostly composed of water, whey proteins, lactose, vitamins and, a vast number of salts such as calcium, phosphorus, magnesium, and potassium.

The present work has studied the dispersion of casein micelles, which is purer than the milk as getting rid of some constituents (lactose, fat, whey proteins). As a matter of fact, the fat globules are absent in the colloidal phase, and the amount of whey proteins and lactose is strongly reduced.

Casein micelles are formed by an association of four different variants, being three of them, α_{s1}, α_{s2}, β, capable of strongly bind calcium (Ca^{2+}) ions thanks to a group of several phosphoserines residues in their protein chains. The fourth one, κ casein, has only two phosphoserine residues and does not strongly bind calcium ions (Fennema, Damodaran, & Parkin, 2017).

Native casein, at the natural pH of milk (~6.7), is present as a disorderly and porous (*sponge-like*) supramolecular structure, denominated as casein micelles (CMs) (Figure 1). The size of native CMs is about 50 and 300 nm, with a predominance of around 150 nm (Rebouillat & Ortega-requena, 2015). The more phosphorylated casein fractions (α_{s1}, α_{s2}, β) are stabilized by the colloidal calcium phosphate (CCP) nanoclusters in the core of the CMs, while the κ is located on the CMs surface and play a major role in maintaining its stability in milk by electrostatic and steric repulsions (Andrews & Varley, 2005; De Kruif & Holt, 2003; Fox et al., 2015; Walstra et al., 2006). Figure 1 is a schematic representation of one model of the CMs, which shows the

distribution of the different casein fractions according to the study of Rebouillat & Ortega-requena (2015).

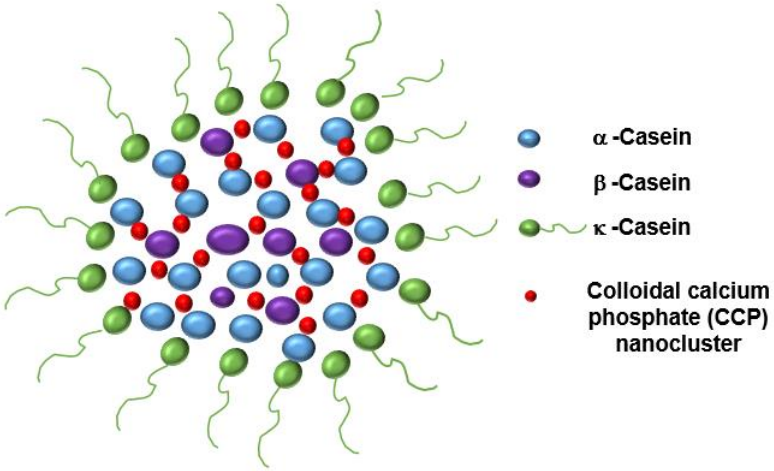

Figure 1 – Schematic representation of the structure of the casein micelles (CMs): cross-section.

2.1 Market demand for high-protein products

The caseins (milk) are widely used as a food ingredient for a large range of dairy products, such as cheeses (Fox et al., 2016), yogurt (Tamime & Robinson, 2000), ice-creams and foams (Chen et al., 2018; Jana, 2017; Sharma, Jana, & Chavan, 2012) and also used as a protein supplement for athletes (Wilborn et al., 2013).

The use of the caseins (native form or as caseinate form) as an ingredient is based on the technological and nutritional properties associated with these proteins, as an agent capable of modulating the texture of some dairy products by fat and water binding and also as an agent capable of emulsifying the fat (Britz & Robinson, 2008).

In the last years, the high protein applications are highlighted (Agarwal et al., 2015; International Euromonitor, 2018), especially with the increase of health and nutritional concerns. In fact, the World Health Organization (WHO) & Food and Agriculture (FAO) Organization preconize a consumption of a minimum amount of protein capable of maintaining the natural distribution of muscles in the human during the entire life (WHO and FAO, 2003).

As demonstrated in different studies and as recommended by the WHO, the consumption of the correct amount of proteins is important to keep the natural function of the body (WHO and FAO, 2003). The correct consumption of proteins is important, for both babies and adults, to increase the muscle growth (Furber et al., 2017), hormonal functions, cellular health and decrease the risk of obesity (Drummen et al., 2018) and diseases, such as diabetes (Yu et al., 2019), some types of cancer (Ho et al., 2011) and some diseases associated to the aging, such as Alzheimer (Vauzour et al., 2017). Indeed, for older people, a more severe problem is the sarcopenia, a disturbance associating with aging, low consumption of protein, and lack of physical activity (Frontera et al., 2012).

Sarcopenia is a disease associated with aging, which leads to a loss of muscle mass strength, muscle atrophy, and loss of functional capacity (Dhillon & Hasni, 2017). Sarcopenia had become a significant focus of research due to its impact on mobility, mortality, and the lack of pharmacological agents currently approved for its treatment.

In fact, the unique strategy that shows a decrease in the process of sarcopenia is the physical exercise (Yoo et al., 2018), being this approach difficult for older people that already have mobility difficulties. The second strategy is the increase in the dietary intake of proteins by using protein supplements (Lochlainn, Bowyer, & Steves, 2018).

In addition to the aforementioned groups (children and the elderly), another group that is involved in the high consumption of proteins in their diet are the athletes (Wilborn et al., 2013), which can be professional (people that use the sports as a professional carrier) or recreational (people that only incorporate some sportive activity in their lifestyle). Indeed, it is admitted among athletes that additional protein consumption would be responsible for increasing strength, improving performance, and muscular growth. However, some authors claimed that these beliefs having no scientific support (Menon & dos Santos, 2012) and may represent if consumed in exaggerated amounts, the risk for health, such as uric acid kidney stones and calcium kidney stones (Delimars, 2013).

For babies, the correct amount of protein requirement is of 1.12 g/kg/day at age six months (Millward, 1989). This value is essential to promote natural growth (Uauy et al., 2015). Furthermore, the protein requirement varies as a function of the physical activity of adults. For sedentary populations is about 0.8g/kg/day of body mass. On the other hand, and when we are looking for the dietary necessity for active adults following a recreational athlete lifestyle, the American College of Sports Medicine suggests an intake ranging from 1.2–2.0 g/kg/day, whether performing aerobic or resistance exercise (Egan, 2016).

However, the dietary intake of protein varies from 1 – 3.5 g/kg/day for professional athletes.

As an effect of this increase in the interest of the consumption of proteins, the industries of product associated to sports nutrition, especially food supplements, expect an increase in sales by 2021 to around 81 billion of dollars (an increase of ~7.9% between 2016-2021), especially for the population who lives in North America and western Europe. In this scenario, the “sports protein Ready-To-Drink (RTD)” beverages and especially the high-protein beverages (HP-beverages) remain within the best performing categories (International Euromonitor, 2018).

2.2 How the industrial sector reacts to the demand for high-protein products

As aforementioned in the previous section, the demand for high-protein products becomes a market trend in recent years. To fill that demand, the industrial sector responsible for producing that kind of food has been looking for alternatives and developing new products. In this context, several high-protein products have been studied, especially those based on milk, as a major source of proteins or foods that are not classified as dairy products.

The milk proteins, especially the whey proteins, represent an excellent source of protein for this application, due to its nutritional profile (essential amino acids), excellent digestibility properties, and pleasant taste. These properties have made the whey proteins as the most interesting protein ingredient to manufacture HP-beverages (Rittmanic, 2006).

However, the use of whey proteins in manufacturing HP-beverages presents an inconvenience related to their instability to heat treatments (Sadeghinezhad et al., 2013). Indeed, RTD HP-beverages are often treated with the ultra-high-temperature (UHT) to produce a shelf-stable product (Jordan, 1968). From the microbial point of view, the use of heating, and especially UHT, is necessary to produce shelf-stable dairy products (milk, RTD beverages, creams) , which can be stored for long periods.. The UHT treatment is based on bacteria destruction by increasing the temperature of heat treatment and reducing exposure time (Datta, 2018). However, its impacts on the structure and properties of high-protein dairy products are still under investigation.

Therefore, the caseins, as major proteins of cow's milk (Walstra et al., 2006) seem to be an excellent alternative to overcome the problem of heat-instability of proteins in the case of RTD HP-beverages. The most significant advantage associated with the use of the casein as a protein source for HP-beverages is related to its high heat-stability (Crowley et al., 2014). Indeed, as described in the literature (Beliciu, Sauer, & Moraru, 2012), the caseins as a native form of casein micelles (CMs) are resistant to heat-treatments that are usually applied at the industrial level to produce sterilized beverages.

In addition, the caseins are present in milk in a higher amount than whey protein (~80/20) (Walstra et al., 2006). From a nutritional and metabolic point of view, caseins also present an excellent digestibility and high amount of essential amino acids, such as leucine (Vickery & White, 1933).

On the other hand, the caseins are already widely used as a food ingredient for a large range of products, such as cheeses (Fox et al., 2016), yogurt (Tamime & Robinson, 2000), ice-creams and foams (Chen et al., 2018; Jana, 2017; Sharma et al., 2012) and also for producing protein supplements for athletes (Wilborn et al., 2013).

However, the CMs present a limiting factor to their use as an ingredient for HP-beverages, which is related to their high-viscosity in concentrated regimes (over 8% of protein) (Bouchoux et al., 2009; Dahbi et al. 2010). This property is related to the capacity of the CMs to bind water, which affects the apparent viscosity (Bouchoux et al., 2010).

To overcome the aforementioned inconveniences, some strategies have been applied to the CMs in order to induce structural changes and, as a consequence, to change their capacity to bind water and finally to improve the apparent viscosity of the casein dispersions in dense systems. Indeed, the study conducted by Pandalaneni et al., (2018) underlined that there is a relation between the modification of the structure of the CMs, by the addition of chelating agents such as SHMP, and the changes in both the apparent viscosity and heat stability of enteral high-protein beverages.

Overall, the industrial sector responds to consumer trends by developing high-protein products. In this context, the CMs represents one of the most interesting sources of proteins to RTD HP-beverages due to their high disponibility and heat-resistance. However, and in concentrated systems, they promote a high-viscosity for dispersions, being the apparent viscosity regulated by the native structure of the CMs. Therefore, some strategies can be applied to produce structural-changed CMs, which will result in dense dispersions with modified apparent viscosity, such as the calcium-demineralization. However, the impact of this structural change in the CMs structure is not yet deeply investigated, especially in the framework of RTD HP-beverages, which are submitted to UHT treatment.

2.3 Casein micelles structure and models of literature

From the late 1950s until today, several studies attempt to determine the real organization chart of the CMs, without adopting consensus about the edifice assemblage. The exception is for the distribution of the different casein fractions and their internal stabilization by CCP and dispersion stabilization by electrostatic and steric repulsions (Andrews & Varley, 2005; De Kruif & Holt, 2003; Fox et al., 2015; Walstra et al., 2006).

Therefore, several models have been proposed to describe the assembly and structure of the casein micelle. The first proposed model by Waugh, (1958) that was named as a “dual binding model” (figure 2a). This model was proposed and based on the observation of the self-assemblage of the caseins and the micellar structures, even in the absence of calcium. This model describes the importance of the CCP as a kind of glue that keeps the protein sub-unities (sub-micelles) together. After that, this model gave the bases for most recent models and namely that of the “casein sub-micelles” (figure 2b) proposed by de Kruif and Holt (2003) and then later used/extended by De Kruif et al., (2012).

In these two models (the “sub-micelle” and the “dual-binding” model) the CCP is described as being located at the periphery of the spheres (sub-micelle units) (Wouterse & Philipse, 2006). This approach is allowed only if the sub-micelles unities are much smaller than the CMs, as explained by De Kruif et al. (2012). Another model was proposed by Stieger et al. (2004) and used some microgel particles and micellar structures as a homogeneous poly-spheres and was based on the creation of the disordered models (figure 2c) (Rebouillat & Ortega-requena, 2015).

There are two other models found in the literature. The first one is the so-called “nanocluster model” (figure 2d), as described by De Kruif & Holt (2003). The nanocluster model is based on the idea the phosphorylated caseins are strongly-bonded by the nanoclusters of the CCP, which are associated with other proteins. These proteins are crosslinked together through a collection of weak interactions (hydrophobic, hydrogen and ions bonds, or electrostatic) responsible for forming a not uniformly bonded protein matrix (de Kruif et al., 2012).

The last model is one of the most recent models. According to this model, Bouchoux et al. (2010) described the CMs structure as an inhomogeneous sphere and proposed a “sponge-like” structural model of CMs (figure 2e). The Sponge-like inhomogeneous sphere model corresponds to CMs with different zones associated or not with the CCP nanoclusters, the “hard” and “soft” regions, respectively. These authors highlighted the presence of some empty zones, probably formed by the water channels and water cavities in the interior of the CMs structure. This model has been proposed based on the behavior of the CM structure compressed under strong osmotic pressure.

To resume, the casein micelles *are complex edifices and its organization chart is presently far from being wholly decrypted. The only consensus that we have in the bibliography is about the distribution of the casein fractions, the role of the colloidal calcium phosphate nanoclusters in the micellar stability.*

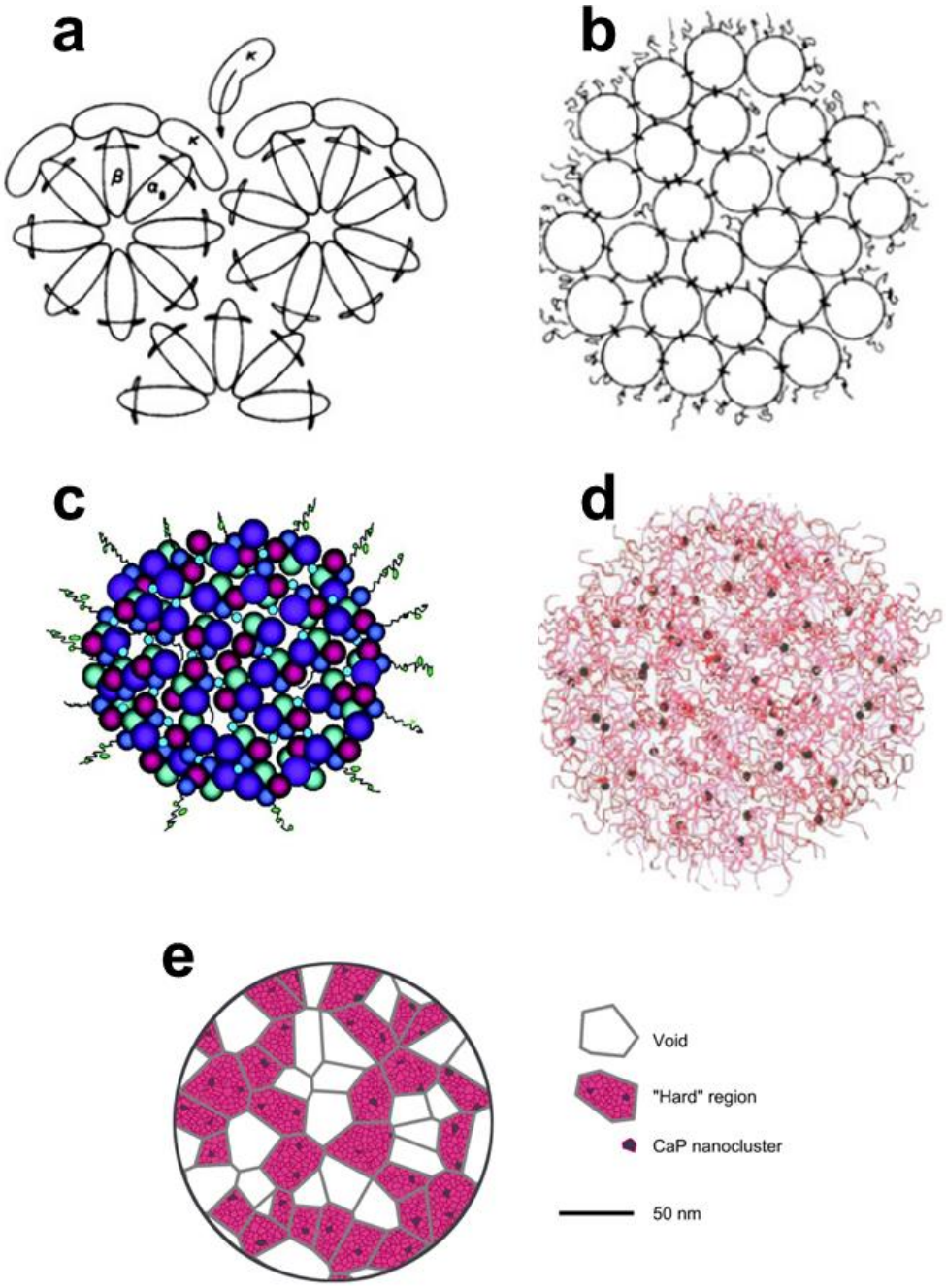

Figure 2 - Different models of the casein micelles (CMs) as described by the different authors. (**a**) Dual binding model (Waugh, 1958); (**b**) Sub-micelles model (Walstra et al., 2006); (**c**) Disordered model (Rebouillat & Ortega-requena, 2015); (**d**) Nanocluster model (De Kruif & Holt, 2003); (**e**) Sponge-like inhomogeneous sphere model (Bouchoux et al., 2010).

2.4 Production of casein-rich powders (from milk to powder) and powder rehydration properties

2.4.1 Production of casein-rich powders

The use of milk for human consumption, to produce dairy products or dairy ingredients, involves different processes that are able to transform the original fluid from the mammalians into a safe product, without microbiological risks, and with desirable properties.

To produce milk or milk ingredients with extended shelf-live, some processes are applied to raw milk, such as heat treatments or membrane separation treatments. Heat treatments are responsible for reducing the number of undesirable microorganisms and some natural enzymes responsible for milk deterioration. Thereafter, milk powders can be made by spray-drying a feed material that has been concentrated, usually by use of membrane separation prosses.

In this scenario, the most common process used to stabilize the milk microbiologically is heat treatments: pasteurization or sterilization, which results in the destruction of the pathogenic or spoilage bacteria (Walstra et al., 2006). However, such kind of treatments is also associated with some reactions between the different milk constituents, especially proteins and minerals, such as β-lactoglobulin denaturation and aggregation with the casein micelles (Zittle., et al. 1957).

To prevent such extensive reactions between the casein micelles and the whey proteins, heat treatments have to be minimized in the applied process to obtain the native phospho-casein (NPC); Consequently, the microorganisms are removed thought a microfiltration technique (Pierre, Fauquant, Graet, Piot, & Maubois, 1992). However, it has been reported that the chain of unit operations, applied to produce NPC powders (Schuck et al., 1994), also affects the native structure of the casein micelle, and these changes will be described below.

Firstly, to produce NPC, the milk needs to be skimmed before the membrane separation techniques to improve the efficiency of the separation (Hurt, Adams, &

Barbano, 2015). The most usual membrane techniques, used to produce NPC usually, involves two steps of microfiltration. The microfiltration processes are conducted using ceramic membranes with two different pores sizes. The first one with a pore size of 0.1 µm, which is capable of removing the constituents from the soluble phase, such as whey proteins, lactose, some minerals, and water. The second one with a pore size of 1.4 µm to remove the microorganisms which are present in the milk (Carvalho & Maubois, 2010).

As previously described, the microfiltration is responsible for decreasing the amount of the minerals and for producing more concentrate NPC dispersions, free of microorganisms. Some industrial practices still involve the use of the water (dialysis) during the process with the aim of producing NPC powders with a higher amount of caseins and/or with better rehydration properties (Eshpari et al., 2015).

Another consequence of microfiltration is the fact that this operation unit induces some changes in the calcium content of the colloidal phase of the CMs. Indeed, it was mentioned previously that the microfiltration is responsible for decreasing the amount of mineral in the initial solution. This phenomenon is unfortunately amplified when dialysis is applied. As a consequence, the CCP, which is responsible for stabilizing the internal structure of the CMs, will diffuse from the CMs to the soluble phase to maintain the mineral balance of calcium between the soluble and colloidal phase (Anema, 2009; Kim et al., 1990; Lucey & Fox, 1993; Ramasubramanian et al., 2013).

Spray drying is one of the oldest and the most common unit operations used to remove water and often used by commercial milk powder manufacturers. However, spray drying has generally been regarded as a relatively expensive process in terms of energy consumption, which is related to the high latent heat of water vaporization and to the inefficiency of using hot air as a heating fluid (Jin et al., 2010). In this context, and in order to reduce energy consumption, the dairy industry uses some techniques that can remove as much as possible the major quantity of water before the spray drying process. The most two common processes applied to remove the water in the dairy industry include the successively use of one or two membrane techniques

(ultrafiltration or reverse osmosis) (Carvalho & Maubois, 2010) and vacuum evaporation (Bylund, 1995).

It should be noted, however, that the membrane techniques will impact the CMs structure in the same way as described previously for the microfiltration. During the vacuum evaporation, water removal is facilitated by the decrease of the atmospheric pressure. Therefore, the evaporation can be conducted at temperatures below the ebullition temperature of the water at atmospheric pressure. Thus, the ebullition can be obtained in a temperature range varying from 40 to 70 °C (Bylund, 1995).

The final step NPC powder productions are the drying process through spray drying atomization (Schuck et al., 1994). The heat exposure effect of NPC during spray drying may vary considerably depending on the design of the drier equipment and the different applied operating conditions (Singh, 2007). After rehydration, the CMs return to their original size distribution (~200 nm) and their heat stability and “renneting characteristics” are not drastically affected, but the protein denaturation is completely dependent on the temperature achieved by the particle during the drying (Singh, 2007). As illustrated in the bibliography (Katie, 2000; Singh, 2007), the temperature of the particles is regulated by the temperature of the air used in the dying process.

Even though CMs are heat-stable, the application of heating process may impact its structure and this is well investigated by several research groups (Davies & White, 1966; Nicolai, & Durand, 2004; Sauer & Moraru, 2012; Nair, Dalgleish, & Corredig, 2013; Crowley et al., 2014; Panouill, Thomar & Nicolai, 2016). The effects of the different heat treatments on the CM organization and its impacts on techno-functional properties will be discussed later as a specific section is devoted to this aspect.

2.4.2 Rehydration of casein-rich powders

The casein-rich powders are produced to decrease the cost of storage, transportation, and increase the shelf-life of this product. The final product, when rehydrated, should maintain almost the same characteristics of the initial casein

concentrate (before the spray drying) or the spray-drying is also used to induce some structural modifications which will be responsible for some changes in some technofunctional properties of the powders.

In this scenario, the first desirable properties required for almost all kind of food powders is a correct “state of rehydration”, which is a hard notion of defining. The casein-rich powders, especially the NPC powders, usually are difficult to be rehydrated, especially to high solids/protein content dispersions (over 8% (w/w)). Poor wettability and longtime of rehydration of the casein powder are common problems described in the literature (Ji et al., 2016; Richard et al., 2013).

The possible causes for these rehydration problems are the hydrophobic interactions and the presence of non-micellar casein, which are formed during the production of the casein micelle powders (da Silva et al., 2018; Gaiani et al., 2006; Havea, 2006). Therefore, the rehydration time of CM powders has to be increased and this fact contributes to increasing the energetic cost of rehydration as it is generally performed at a temperature above ambient temperature. This poor rehydration properties make the handling of these NPC powders difficult at the industrial level (Hussain et al., 2011; Mimouni et al., 2010).

To achieve a faster rehydration, some strategies were proposed in the bibliography. Some of them are associated with changing the feature of powder particles, such as increasing the granulation of powders. In this case, powder particles contain large pores, and consequently, the contact area between powder the solvent is favored (Gaiani et al., 2005; Gaiani et al., 2007). Another approach suggested by Richard et al. (2013) is related to increasing temperature and/or agitation during the rehydrating of NPC powders, which results in a decrease of the rehydration time and a significant gain in energy. As demonstrated by the same authors (Richard et al., 2013), the rehydration kinetics is more affected by the temperature than by the stirring speed; for a given powder, the rehydration time is inversely proportional to agitator speed under the turbulent regime. Contrary to previous authors (Richard et al. 2013), it was, however, mentioned that the granulation is not always responsible for decreasing the rehydration time. This can be explained by the fact that the extent of

residence time in the spray drier can induce not only granulation but also some alterations of powder particles (Mujumdar, 2006).

Another parameter that shows a large influence on NPC powder rehydration is the ionic environment, which can be modified by the use of a mixture of distilled water, of sodium chloride (NaCl), and of calcium chloride (CaCl2) as a solvent to rehydrate the NPC powders (da Silva et al., 2018). Da Silva et al. (2018) suggested that increasing the ionic strength, by the addition of NaCl, may cause a decrease in the activity coefficients of the diffusible ions and an increase in the dissociation of ion pairs. The addition of $CaCl_2$ was shown to increase the rehydrate time of the NPC powder and even induce incomplete rehydration. This is probably due to the increases of the calcium-binding to the NPC powders and associated changes in the CM structure (Hussain et al., 2011; Hussain, Gaiani, & Scher, 2012)

As demonstrated by Crowley et al. (2015), the use of potassium chloride (KCl) or ultrafiltered permeate from milk (UFP) associated to heating was effective to produce a stable dispersion of casein after a short rehydration time (~ 90 min). This stability is probably associated with the restoration of the ionic strength, close to that of the initial milk.

Another recent strategy that has been used to produce powder of NPC with fast rehydration properties is based on the reduction of part of calcium by the action of chelate agents, such as Ethylenediaminetetraacetic acid (EDTA) (Tan, 2016), trisodium citrate (TSC) or sodium hexametaphosphate (SHMP) (McCarthy et al., 2017). As described by these authors, the addition of calcium chelating agents can significantly reduce the dissolution time by increasing hydration rate through micelle swelling and/ or dissociation and also producing dispersions with reduced turbidity; However, it was concluded that the added amount of TSC or SHMP has a significant impact on the apparent viscosity, becoming a limiting factor with the use of this strategy to produce fast rehydrated NPC powders and also the addition of these chelators agents goes in opposition with the demands for “clean lable” products, which preconize products with less addition of external adictives (Asioli et al., 2017).

As described in the bibliography (Broyard & Gaucheron, 2015), the chelating agents do not act directly on the CMs structure. However, they interact with the free

calcium ions present in the soluble phase, propitiating a decrease in the concentration of calcium in the soluble phase and inducing the diffusion of the calcium from the colloidal phase to the soluble one (Sikand, Tong, & Walker, 2013; Udabage, McKinnon, & Augustin, 2000).

Another strategy capable of removing the calcium from the interior of the CMs, as cited previously, is the demineralization. The demineralization can be achieved by associating two techniques, the acidification, which is responsible for releasing a part of the CCP from the CMs (Broyard & Gaucheron, 2015) and the dialysis during the microfiltration (Eshpari et al., 2015). That strategy (demineralization) has been found to be useful in the production of casein based-powders that exhibit properties similar to those obtained with the use of chelating agents, probably without the inconvenient of increasing the viscosity (Bienvenue et al., 2003; Meletharayil et al., 2018; Pandalaneni et al., 2018; Schkoda et al., 1999).

To resume, the various steps that are applied to produce the casein-rich powders affect an essential property of this powder, namely their rehydration; especially when the desirable final dispersion is composed of a high amount of protein. Different strategies have been applied to facilitate the rehydration of the casein-rich powder in high-protein dispersions, such as the demineralization by the addition of calcium-chelators. It should be noted that the mechanism underlying these techno-functional changes is not yet clear. The literature making a link between the structural changes of the CMs and some consequences on techno-functional properties will be presented in the next section.

2.5 CMs structural modification and its impact on techno-functional properties

Caseinate, a casein-rich powder composed of completely destructured casein micelles are widely used in the food industry as an agent capable of improving viscosity, texture, emulsifying, and foaming properties (Dickinson, 2006). In contrast, the techno-functional properties of dispersions made with CMs are less studied, which highlights the need to improve the current understanding of the relationship between the physicochemical conditions, the casein micelle structure, and the techno-functional properties. Such information is the key to propose new applications, to develop innovative dairy products or to improve some properties of the already existent CM products.

Figure 3 presents the major processes that could be envisaged in the dairy industry for modifying the structure of CMs.

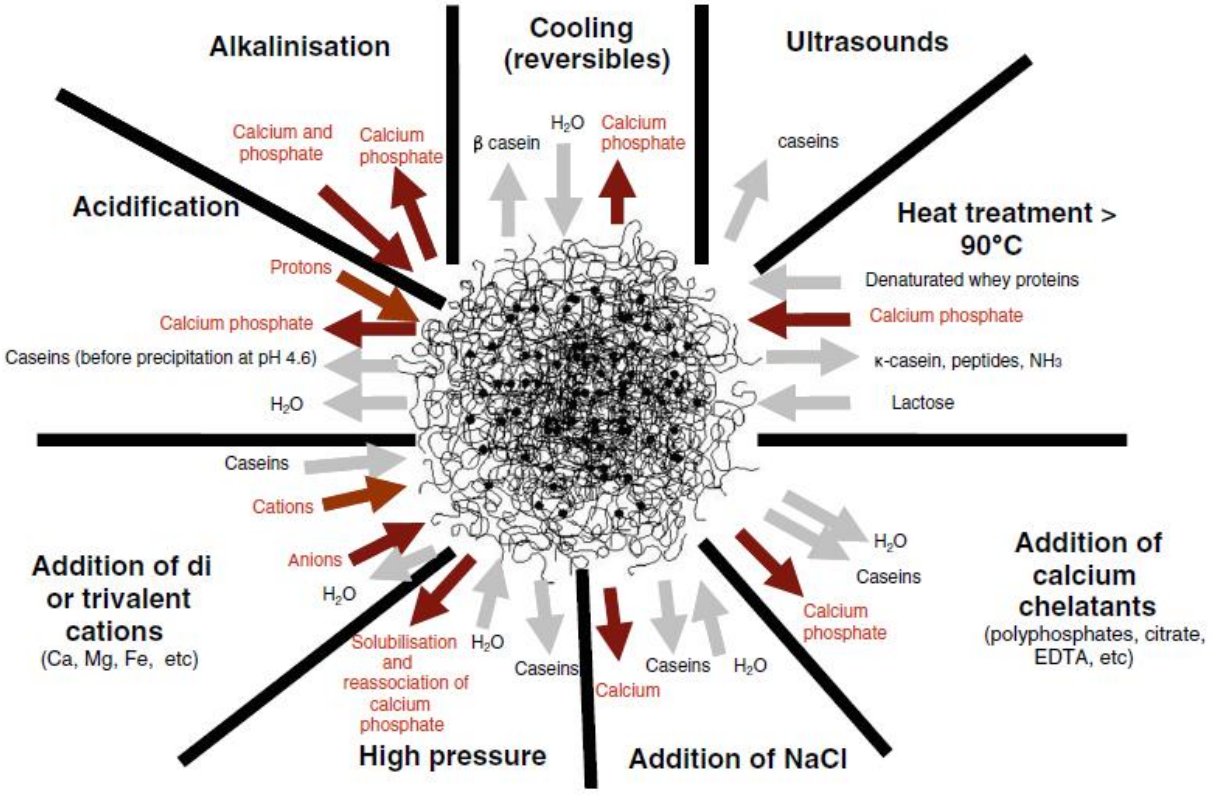

Figure 3 - Schematic representation of the changes of the CMs when submitted to different treatments according to (Broyard & Gaucheron, 2015).

The present section will be focused on the structural changes of the CMs present in casein-rich powders and in high-concentrated casein dispersions, such as the high-protein beverages. In this case, three levers/technological routes which are supposed to affect the techno-functional property of casein-rich dispersions will be discussed (the addition of calcium-chelators agents, the acidification and the heat treatments).

2.5.1 Impact of the addition of calcium-chelator agents in the CMs structure and properties

The calcium-chelator agents (EDTA, SHMP, and TSC) are responsible for chelating the ionic calcium, which is present in the soluble phase. Therefore, the calcium present in the CMs as a form of CCP is solubilized, resulting in structural modification of the CMs (Sikand et al., 2013; Udabage et al., 2000).

The addition of calcium-chelator agents to milk or casein dispersions is studied from the middle of the 1960s to today (Kort et al., 2011; Odagiri & Nickerson, 1965; Pandalaneni et al., 2018; Udabage et al., 2000). As described previously, this process induces changes in the structure of the CMs with repercussion on the techno-functional properties of casein dispersions, such as turbidity (Mizuno & Lucey, 2005), apparent viscosity (Kort et al., 2011; Pandalaneni et al., 2018) and powder rehydration properties (McCarthy et al., 2017) indirectly. These three properties are fundamental in the scenario of high-protein dispersions made of CMs from casein-rich powders.

As described in the previous section, some improvements of the casein-rich powder rehydration properties are necessary, especially when high-amount of proteins is needed (over 8g/100g) (Baldwin, 2010). It has been shown that the addition of calcium chelator agents is responsible for the improvement in the hydration rate (McCarthy et al., 2017). However, these agents also seem to increase the apparent viscosity of the casein (Kort et al., 2011; Pandalaneni et al., 2018). This increase of apparent viscosity could be explained by two hypotheses. The first one consists of

swelling of the CMs, which is a factor that increases the apparent viscosity (Doudiès et al., 2019). The second one is probably related to the creation of small casein aggregates by the disruption of the micellar structure which, results in a increase of the apparent viscoisty (Kort et al., 2011).

Another techno-functional property that is affected by the addition of calcium-chelator agents is the heat-stability. Indeed, as described in the literature (Kaliappan & Lucey, 2011; Pandalaneni et al., 2018), the addition of calcium-chelator agents is responsible for an increase of heat-stability of casein dispersions by the reduction of the free calcium ions, present in the soluble phase. This reduction of calcium ions is associated with a decrease in the capacity of the interaction of the ionic calcium with the calcium-sensitive regions of the CMs, which are responsible for micellar destabilization during heat treatments.

To resume, the addition of calcium-chelator agents affect three important properties that can impact the high-protein CMs beverages:

- The rehydration which is the initial step of the production of almost all protein beverages;
- The apparent viscosity which is one of limiting factors to the production of high-protein beverages made with CMs as a major protein source;
- The heat-stability which is a necessary property when we are searching for proteins that are stable to sterilization treatments, which will be used for the production of stable RTD protein beverages.

2.5.2 Impact of the heat treatments on the CMs structure and properties

From the initial milk to the final products, several heat treatments are applied to reach the microbiological and enzymatic stabilization (Britz & Robinson, 2008). Indeed, milk heat treatments have an objective to eliminate undesirable microorganisms (from natural origin or external contamination) (Marth & Steele, 2001) as well as to inactivate some enzymes that can affect the properties of the milk during the storage (Murphy et

al.,2016). Some “softer” heat treatments have as the objective to just decrease the number of vegetative pathogenic microorganisms, such as pasteurization. Other treatments look for the complete sterilization of milk, or milk products, through extreme heat treatment (over 100 °C), which are able to destroy both negative and spore-forming microorganisms (Melini et al., 2017).

The heat treatments also affect the different constituents of milk in different ways with some undesirable effects, such as fouling of heat exchanger during heat treatments (Daufin et al., 1987). The following section will be focused specifically on the impacts of the heat treatment on the structure of the CMs during the production of high-protein beverages by the commercial sterilization process. In literature, CMs have been described as being heat-stable (Beliciu et al., 2012). However, heat treatments often induce some structural modification leading to a modification of the techno-functional properties of the dairy products.

One of the most described side-effects of heat treatments is the decrease in calcium phosphate solubility. As described by Holt (1997), calcium phosphate becomes less soluble during heat treatment. Therefore, heat treatments induce the calcium precipitation, and the “internalization” of calcium and phosphate ions into the CM structure. Calcium “internalization” is responsible for an increase in the amount of calcium in the CMs, while calcium precipitation is responsible for a decrease in the amount of calcium in the soluble phase (Holt, 1997).

As described in section 2.2, the production of casein-rich powders composed by native phospho-caseins (NPC), intends to produce CM powders without extensive heat treatments and to produce CMs with almost the same properties of the initial CMs (i.e. the native ones) using microfiltration. Anyhow, the production of NCP powders presents, at minimum, two unitary operations that involve heating treatments at moderate temperatures (between 60 °C to 85 °C), the vacuum concentration, and the spray drying. Indeed, after the purification of the caseins by microfiltration and before the spray drying, the casein dispersions need to be concentrated by vacuum concentration to a solids content between 20% to 40% (w/w). This process aims to minimize the energetic cost of the water evaporation during the spray-drying, at temperatures between 60 °C and 80 °C (Bylund, 1995). In the same way, the particles

of powders, which are formed during the spray drying, could reach temperatures around 85 °C due to the contact with the hot air which is used to induce the evaporation of the water from the casein concentrates (Fang et al., 2012)

Literature shows that these mild temperatures can have a detectable impact in CM structure. As described in the bibliography (Anema & Li, 2003; Corredig & Dalgleish, 1996), heat treatments superior to 70 °C induce the interaction of the unfolded whey proteins, such as β-lactoglobulin, with the CMs. Another impact of heat treatment is the increase of their hydrodynamic size and changes in the surface organization (Anema & Li 2003). The attachment of whey proteins at CMs surface may have some important drawbacks for cheese production, which increases the time of gelation and a decrease of gel firmness (Anema, Lee, & Klostermeyer, 2007; Zittle et al., 1957). However, the increase of the interaction between the whey protein and CMs is also associated with an improvement of the gel firmness in the case of acid-induced gels (Koutina etal., 2014). For example, in the production of some acid-induced gels, such as yogurt, the interaction of whey proteins and CMs is desirable and necessary to produce a correct gelification of the products (Lee & Lucey, 2010; Lucey et al., 2001; Vasbinder et al., 2003).

The lactose that can be present in casein concentrates is also modified during heat treatments. This carbohydrate can be transformed into other compounds, such as organic acids and lactulose, which interact with aminoacid milk proteins (a mainly ε-amino group of lysine residue) to proceed Maillard and browning reactions (Olano & Calvo, 1989).

As described previously, severe heat treatments such as the pasteurization (85 °C/15 seconds) or commercial sterilization by ultra-high-temperature treatments (UHT) (120-140 °C from 4-2 seconds) will promote a calcium-precipitation that results in an ionic disequilibrium between the micellar (CMs) and soluble phases (Holt, 1997). This calcium-depletion is associated with heat instability of CMs to UHT treatments (O'Connell & Fox, 2003), and phase separation observed in UHT products (Dalgleish, 1992).

However, the impact of the UHT treatment on the internal organization of the CMs was not yet well investigated. The calcium-internalization and the calcium-

depletion should be responsible for an internal rearrangement of the CMs structure and especially affect the regions which are closely linked to these minerals in the CMs structure, the so-called “hard regions” as described in the literature (Bouchoux et al., 2010).

In the same way, there is no guideline on how to undertake the heat treatment of CMs in dense dispersions to modulate their viscosity.

2.5.3 Impact of the acidification in the CMs structure and properties

The acidification is responsible for changing the structure of the CMs, intentionally or not. Acidification is one of the most common processes which occur in milk due the lactose degradation into lactic acid by microorganisms (Marth & Steele, 2001). Indeed, these processes are suitable for some dairy products such as cheeses (Fox et al., 2016) and yogurts (Tamime & Robinson, 2000), where this lactose degradation also gives some desirable techno-functional properties, such as the gelation and the flavors of these products.

In parallel to the natural production of acid from lactose degradation by microorganism’s development, another way to acidify the milk corresponds to the external addition of acids, which is also the usual process utilized in the dairy industry to control the pH of their products. Indeed, in some processes, such as cheese production, lactic acid is added to the milk, with the intention of decreasing the natural pH of the milk to optimal pH values for rennet enzymes, which are around pH 6.0 (Nájera, De Renobales, & Barron, 2003).

In both situations, natural production of acid or external acid addition, the intent is to produce some structural changes of milk proteins to achieve some desirable properties in the final product. Of course, the industries, producing dairy-based protein ingredients, have also used the acidification to produce some ingredients with different techno-functional properties, such as different rehydration behavior (Marella et al., 2015). In the next section, we will report some impacts of acidification on the structure of the CMs and on techno-functional properties, as mentioned in the literature.

The structure and charge of CMs are dependent on the pH value. In fact, the decrease of the pH is responsible for some changes in intra- and intermolecular interaction (Broyard & Gaucheron, 2015). It has been reported that the acidification induces changes in the ionization of the phosphoseryl residues and carboxyl groups of the CMs; these changes are linked to the affinity of these groups for protons.

At the natural pH of the milk, pH around 6.7, the CMs presents negative charges, which is responsible for its electrostatic stabilization (Walstra et al., 2006). However, during the acidification, the charges of the CMs start to be neutralized by the protons from the acids, till complete charge neutralization on the isoelectric point at pH 4.6, which results in casein aggregation (Broyard & Gaucheron, 2015).

Besides of the charges of the CMs, the acidification also affects the interaction of the CMs with the CCP. Indeed, the acidification induces a protonation of the organic and inorganic phosphate, citrate, and carboxylic residues of the CMs. At the same time, the soluble phase becomes less saturated in calcium phosphate due to the dissociation of this salt, which results in a CCP dissolution from the CMs to the soluble phase (Dalgleish & Law, 1989).

As previously cited, the acidification induces CCP solubilization from the CMs, and in extreme conditions, the micellar structure can completely dissociate, because the interaction of the CMs with their salts is pH-dependent (Dalgleish & Law, 1988). At pH close to 5.2, a part of the micellar calcium and the totality of the phosphate is solubilized. The calcium is completely solubilized from the CMs to the soluble phase at pH around 3.5 (Le Graet & Brulé, 1993; Le Graët & Gaucheron, 1999).

This CCP solubilization from the CMs through acidification was used in the study of Silva et al. (2013). In this study, the authors have evaluated the impact of gradual CCP solubilization by using different pH values and dialysis during the membrane separation processes to achieve structure-changed CMs with different CCP levels. The results of this study suggest that the structural modification was responsible for a decrease in the foaming stability of CMs dispersions.

The partial demineralization was also conducted by injection of carbon dioxide CO_2) to the initial milk or during the membrane processes, being this process responsible for producing carbonic acid as a result of the interaction of CO_2 and water.

The result suggests that the addition of CO_2 results in an increase of CMs dissociation. The increase in the amount of nonmicellar casein was linked to an improvement in the acid gelation properties, resulting in gels with higher apparent viscosity (Meletharayil et al., 2018).

To sum up, the acidification is responsible for some structural modifications that induce some changes in the techno-functional properties, such as foaming and apparent viscosity. However, the structural characterization of these partially demineralized CMs by acidification was not conducted yet, and its impact on the apparent viscosity was not evaluated for concentrated systems, such as high-protein beverages.

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2.7 Sum up of the bibliography and scientific questions addressed

The ready-to-drink beverages with high protein content are market trends and bring together a product with nutritional advantages associated with a facility to consumption.

In this context, the whey proteins are still the major type of protein in use. However, casein micelle (CM), which is protein assembly with several colloidal calcium phosphate nanoclusters displaying many water cavities, seems to represent a better option for this application because of its natural abundance in milk and its unusual resistance to heat.

However, the use of CMs presents show drawbacks; CMs produce more viscous dispersions than whey proteins (at equal protein concentration) and display a slower rehydration time (after spray-drying).

It has been evoked in the literature that demineralization can be an alternative to change the viscosity CMs dispersions.

However, additional experiments are required:

i) To ascertain that this demineralization operated on casein-based concentrates before spray drying is still real at the molecular level and effectiveness after that, the modified casein-based powders have been re-dispersed at high protein content and subjected to UHT treatment, as it is the case at industrial practices to produce microbiologically stable beverages.

ii) To precise the underlying mechanisms involved explaining such a possible gain in apparent viscosity and its link with modifications.

Indeed the bibliographic study has clearly pointed out that some important scientific questions about the possible elaboration of HP dispersions with demineralized caseins powders with the desired functional properties are still open to debate:

1- How deep is the structural change in the casein micelle structure associated with the demineralization. Can the change of CM organization can be assessed by using

analysis tool? Could this change of CM organization be different whether the demineralization is performed into an industrial context instead of being carried out at a laboratory scale?

2- What will be the impact of the demineralization on the rehydration properties (particle size distribution and apparent viscosity) of the casein-based powders? What is the impact of the industrial rehydration practices on the properties of the NPC powders?

3- What will be the impact of the demineralization on the apparent viscosity after the UHT?

4- Will a gradual demineralization produce a gradual change in the CMs structure and/or in a rheological behavior? Are there some are there limits in terms of the degree of demineralization not to be exceeded? Is it possible to correlate the changes in the CMs originated from the demineralization and the rheological behavior?

The major objective of this thesis was to fill these lacks partially by addressing the above scientific questions.

The aim was clearly to improve the knowledge on the impact of the demineralization:

i) On the structure of the CMs in dense dispersions;

ii) On the rheological behavior of HP dispersions with casein-based powders at neutral pH, elaborated as in industrial practices.

It means that demineralized and native powders elaborated have been at least subjected to two steps for obtaining “a simple ready-to-drink beverage” (i.e. without any additives) at neutral pH:

i) Rehydration of the powders at the desired HP concentration (8%, 14%, 20% w/w) to obtain the dense dispersions

ii) A UHT treatment of the dense dispersions

The purpose is clearly to provide some guidelines to produce a low viscosity UHT casein model beverage at neutral pH.

The thesis process was articulated in 2 periods:

i) In the first period (which corresponds to subchapters 3.1 and 3.2 of the *Results and Discussion*), the study of two commercial powders used in existent HP beverage has been undertaken (a control powder and a demineralized one). The purpose of this part was to attest to the interest to demineralize casein powders. The changes in the casein micelle structure, related to demineralization, and the impacts caused by the rehydration temperature have been studied.

This part has allowed us to set a number of methodologies to analyze the structure of the casein (casein envelope, sub micellar.) deeply at different molecular levels (Small-Angle X-Ray Scattering - SAXS; Cryo-Fracture associated to Scanning electron microscopy - Cryo-F-TEM; NMR Spectroscopy; Fluorescence; electrophoresis;…) and the effect of rehydration at the different temperature on the particle size distribution and viscosity (Dynamic and Static Light Scattering –respectively DLS and SLS, Rheometry).

Analysis of the results has allowed us to identify some changes in the CMs structure associated with the demineralization (subchapter 1) and also to propose a rehydration protocol that mimics aggregates size distribution and apparent viscosity of native CM (subchapter 2).

ii) In a second period (which corresponds to sub-chapters 3.3 and 3.4 of the *Results and Discussion*), after having ascertained that the effect of such modifications is visible and significant on CM assembly, four different powders with different demineralization levels has been produced at the pilot-scale, and the impact of different demineralization degrees on the CM structure (using some of previously methodology developed) and its repercussion on apparent viscosity after rehydration and UHT treatment has been deeply investigated. The purpose of this second part was twofold: a) to find out whether changes in the CMs structure (subchapter 3) are gradual with the degree of demineralization of the powders. b) to evaluate (subchapter 4) whether it possible to correlate the changes in the CMs

(originated from the demineralization) and the rheological behavior after rehydration and UHT treatment?

The section entitled as "**Results and Discussion**" of the document (Chapter 3) were divided into 4 different subchapters (sections 3.1 to 3.4) corresponding to 4 articles. As a matter of fact, each subchapter constitutes "an article to submit" in order to constitute "a ready-to-submit manuscript." Note that the first subchapter (article 1) is already accepted and accessible in the web of science:

Marcio Henrique Nogueira, Salma Ben-Harb, Marc Schmutz, Bertrand Doumert, Sarah Nasser, Antoine Derensy, Romdhane Karoui, Guillaume Delaplace, Paulo De Sa Peixoto, Multiscale quantitative characterization of demineralized casein micelles: how the partial excision of nano-clusters leads to the aggregation during rehydration, ***Food Hydrocolloids****, Volume 105, August 2020, 105778 IF: 5.839, Q1*

CHAPTER 3 – RESULTS AND DISCUSSION

The first period of Ph.D. thesis: Study of a case of commercial casein-based powders

As underlined before, the first period of the thesis concerns investigations with two commercial casein-based powders. Precisely, two existent powders from Ingredia S.A. were used as a native one and 10% calcium demineralized casein powder. The changes in the casein micelle structure, related to demineralization, and the impacts caused by the rehydration temperature have been studied.

Two protocols of rehydration were evaluated, corresponding to a protocol commonly used in the dairy industry to initiate the rehydration of the casein-based powder and comparing with a control process. The two protocols correspond to rehydrate the powders at 50 °C/1 hour for the industrial-like protocol and 25 °C/24 hours for the control process, being the powders rehydrated to 8% (w/w) of solids content (article 1) or to (8%, 14%, and 20%) (w/w) of solids content with and without an additional homogenization step (article 2). This homogenization step is performed after the end of the initial rehydration, corresponding to a stirring of 10.000 rotation per minute of a rotor-stator homogenizer.

The objective was clearly to highlight the scientific question that was previously addressed (see section 2.5), which are:

1- Evaluate how deep is the structural change in the casein micelle structure associated with the demineralization. Can the change of CM organization can be assessed by using analysis tool? Could this change of CM organization be different whether the demineralization is performed into an industrial context instead of being carried out at the laboratory scale?

2- What will be the impact of the demineralization on the rehydration properties (particle size distribution and apparent viscosity) of the casein-based powders? What is the impact of the industrial rehydration practices on the properties of the NPC powders?

Article 1 (subchapter 3.1) reports the structural changes observed for the demineralized Casein micelle compared to the native one. The methodologies (Small-Angle X-Ray Scattering - SAXS; Cryo-Fracture associated to Scanning electron

microscopy - Cryo-F-TEM; NMR Spectroscopy; Fluorescence; electrophoresis;…) and the effect of rehydration at the different temperature on the particle size distribution and viscosity (Dynamic and Static Light Scattering –respectively DLS and SLS, Rheometry) to analyze the structure of the casein deeply at different molecular levels (casein envelope, submicellar..) are presented.
Article 1 also shows the impacts of the homogenization on the particle size distribution and its influence on the apparent viscosity.

Article 2 (subchapter 3.2) tackles the importance of a homogenization step for completing the rehydration protocol and shows that a fast rehydration protocol led at 50°C/1H, including an additional homogenization step, can mimic “the state of rehydration” of dense dispersion obtained for native micellar caseins.

Note that the first subchapter (article 1) is already accepted and in line. It will be edited soon:

Marcio Henrique Nogueira, Salma Ben-Harb, Marc Schmutz, Bertrand Doumert, Sarah Nasser, Antoine Derensy, Romdhane Karoui, Guillaume Delaplace, Paulo De Sa Peixoto, Multiscale quantitative characterization of demineralized casein micelles: how the partial excision of nano-clusters leads to the aggregation during rehydration, ***Food Hydrocolloids****, Volume 105, August 2020, 105778 IF: 5.839, Q1*

3.1 Multiscale quantitative characterization of demineralized casein micelles: how the partial excision of nano-clusters leads to the aggregation during rehydration

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ABSTRACT

The amount of colloidal calcium phosphate (CCP) nanoclusters modifies the structure and functional properties of the casein micelles. This study aims to analyze the structural changes in partially demineralized casein micelles (D-CMs) and compare them with native casein micelles (CMs) in order to provide new insights towards the subsequent changes in rehydration behavior. Two rehydration strategies were applied. First is the fast rehydration process (50 °C/1h), close to the industrial practice, while the second one (25 °C/24h), a slow rehydration process, used as control. The presence of large (>10 µm) and stirring resistant aggregates in D-CMs were evidenced by Static Light Scattering, being significantly higher for D-CMs rehydrated at 50 °C/1h. As evidence from the electrophoresis results, the non-covalent interactions play a major role in aggregate formation. The decrease in the CCP concentration (-15%) leads to a more loosely packed/porous structure of D-CMs (Transmission Electronic Microscopy/Cryo-Fracture), and (as observed using Nuclear Magnetic Resonance and Small Angle X-ray Scattering) the demineralization also contributes to increase disorder in the structure of the micelle. Fluorescence Spectroscopy data reveals the presence of casein-tryptophan residues in a more hydrophobic environment shedding light on the importance of hydrophobic interactions for the aggregation of D-CMs. In summary, the study sketches a clear picture of internal rearrangement of CMs structures, in partially demineralized conditions, followed by fast rehydration at high temperatures.

KEYWORDS: Casein micelles, demineralization, structure, aggregation, hydrophobic bonds.

1 INTRODUCTION

The concentration of colloidal calcium phosphate nanoclusters and their interaction with sub-micellar assemblies can modify the internal structure and packing. This aspect can be exploited to obtain differentiated functional and structural properties of casein-based milk derivatives (Broyard & Gaucheron, 2015; Philippe, Graët, & Gaucheron, 2005).

The modification of the CCP equilibrium in native casein micelle (demineralization) can nowadays be realized with various technological routes, such as pH changes (acidification or alkalization) (Gonzalez-Jordan, Thomar, Nicolai, & Dittmer, 2015; Perveen et al., 2015; Ye & Harte, 2013), phosphorus and calcium chelation (Kaliappan & Lucey, 2011; Kort et al., 2011; McCarthy et al., 2017; Pitkowski et al., 2008), membrane processes (Hurt, 2015; K. Muthukumarappan, 2013; Luo, Vasiljevic, & Ramchandran, 2016; Schuck et al., 1994), and is also performed at industrial scale.

Otherwise, the demineralization leads to gradual disaggregation of the structure of native casein micelles. This phenomena is associated to some changes of functional properties that impact food application, such as aggregation behavior [9–12], gelification (Lucey & Singh, 1997; Phadungath, 2005b; Vasbinder, Alting, et al., 2003; Vasbinder, Rollema, Bot, & de Kruif, 2003) emulsification (Lazzaro et al., 2017; Luo et al., 2016) and foaming (Chen et al., 2018; Silva et al., 2013). In particular, the decrease of the CCP in the CMs is associated to the formation of fragile rennet gels with consequent structural loss (Phadungath, 2005; Schkoda, Hechler, & Kessler, 1999) and syneresis on acid gels (Schkoda et al., 1999; Vasbinder, Rollema, et al., 2003), respectively observed in the cheese and yogurt manufacturing.

As far as the evolution of functional properties upon demineralization is concerned, an increase of soluble proteins dissociation in milk protein concentrate has been reported with decalcification (Pandalaneni et al., 2018; Xu, Liu, Yang, Zhang, & Liu, 2016). Finally, it was suggested that the depletion of the calcium in concentrated milk powders is associated with an increase in the heat stability of some high protein

content applications, such as in enteral dairy beverage formulations (Pandalaneni et al., 2018).

At the molecular level, the exact underlying role played by CCP concentration to change casein micelle's reactivity, and subsequent alterations in functional properties are far from being fully elucidated. The global charges of casein micelle are lowered upon demineralization and the reduced electrostatic repulsion alters micelle stability (Cornelis G. de Kruif et al., 2012; Horne, 2017; McMahon & Oommen, 2008; Tuinier & De Kruif, 2002). It is also established that the CCP concentration changes also contribute towards the equilibrium distribution of calcium and phosphorus between the micellar and the serum phases. These two mineral elements are known to be critical factors in maintaining heat-stability of micellar casein. It has been shown that these two mineral elements play an important role in aggregation/fouling by facilitating the binding of protein species (Joyce, Kelly, & O'Mahony, 2018; Visser & Jeurnink, 1997). In milk, calcium phosphate is one of the solid calcium-based agents implicated in the heat denaturation of the serum β-lactoglobulin. An open interconnected calcium phosphate-protein network was observed at temperatures around 80 ºC, Sadeghinezhad et al. (2013), Andritsos, Yiantsios & Karabelas (2002). The interactions between calcium phosphate and proteins might involve phosphoserine groups in milk caseins (Daufin et al., 1987).

In summary, The demineralization is related to decreasing the amount of the nanoclusters of CCP (Boiani, Fenelon, FitzGerald, & Kelly, 2018; Boiani, McLoughlin, Auty, FitzGerald, & Kelly, 2017; H S Rollema & Brinkhuis, 1989; Silva et al., 2013). The demineralization of native casein micelle and its influence on functional properties has been reported up to a certain extent in the past (Koutina, Knudsen, Andersen, & Skibsted, 2015; Lucey & Fox, 1993; Silva et al., 2013). However, in these previous studies, the change of functional properties induced by demineralization of native casein micelles was mainly evaluated by submitting modified casein-based powders to various reconstitution and heat treatment. With these applications, it is not possible to appropriately study and understand the effect of the partial demineralization at the end of the rehydration step, which precedes heat treatment. This prevents us from getting a clear view of the protein species population, before starting the heat process

and making a step forward in order to master the aggregation process for demineralized casein micelles. The objective of this study is to fill this pivotal gap in the process.

To achieve that, the demineralization of casein micelles has been carried out, followed by internal structural properties (amino acids, proteins and global structure of the casein micelle) of native (CM) and modified (D-CMs) casein micelles which have been characterized at the molecular level. Finally, the colloidal properties of the rehydrated CMs dispersion (native and modified) have been measured through particle size analysis and electrophoresis.

2 MATERIALS AND METHODS

2.1 Experimental approach

In this study, several techniques were associated to perform experimentations at different scales of the material, to understand the changes associated with the small fractions of the casein to the large effects associated with the CMs. Two levels were considered: (i) the large scales evaluation, corresponding to the properties of the CMs dispersion (aggregation and linking forces) and (ii) the characterization at very small scales, related to the effects at some specific regions of the CMs structure (amino acids, CCP, proteins, and structure of the CMs). The data presented in this study are the result of three independent repetitions (independent the sample preparation and a duplicate for each repetition, with the exception of nuclear magnetic resonance and cryo-fracture. For these last two methods, the specification will be described in their corresponding section.

2.2 Samples of casein micelles (CMs) powders

Native casein micelle rich powders with a total casein/total protein ratio of 0.92 with different levels of demineralization (calcium excision) were supplied by Ingredia S.A (Arras, France) are henceforth mentioned in this study as N-CMs for standard

native casein powder (Promilk® 872B for native non-demineralized), with calcium (Ca)/phosphorus (P) ratio of 1.8 (2.7 g and 1.5 g per 100g of powder), respectively and as D-CMs for demineralized casein micelles powder (Prodiet® 87 B Fluid) with calcium/phosphorus ratio of 1.66 (2.0 g and 1.2 g per 100g of powder) (data from Ingredia S.A). The composition of the powders is present in table 1 in supporting information.

The processing is the same at all points except for the part resulting in the demineralization. To be sure of the similarity between the chosen milk pools, the mineral composition and the structure of CMs have been controlled. As one can see, in supporting information, the mineral composition and the structure of the D-CMs and N-CMs from different runs (2017 and 2018) match very well indicating that, in our case, the method used to select the milk source to guarantee a minimal difference between milk pools. The desirable degree of demineralization was obtained by using a natural chelating agent (confidential information) that does not remain in solution. The samples have been produced through membrane processes (ultra and microfiltration). The analytical experiments have been done, at most, six months after the production of the powder. The powders were protected from humidity in hermetic plastic bags and protected from the light and heat at 4 °C.

2.2.1 Samples preparation

The samples rehydration was conducted based on the amount of powder and conducted using deionized water and adding 8.0 g (powder) x 100 g- 1 (water), which corresponds to a total of 6.64 g and 6.72 g (protein) in 100 mL of the dispersions for native and demineralized samples, respectively. Both powders were submitted to stirring at 500 r.p.m following two different protocols: 50 °C/1 hour corresponding to the industrial process of fast rehydration and control, 25 °C/24 hours of slow rehydration. To prevent microbiological growth, antimicrobial agent sodium azide added at 0.3 g L^{-1} (Sigma Aldrich, France). The NMR analysis performed on the samples hydrated in Deuterium oxide (D_2O) to 20 % (w/w), the D_2O was used to minimize the rapport signal/noise from the deionized water. The pH of the samples

were analyzed after rehydration, which corresponds to 6.7 ± 0.15 and 6.4 ± 0.18 to native and demineralized casein micelles dispersion respectively. As demonstrated in the bibliography (Heertje, Visser, & Smits, 1985) at the CMs pH range between 6.6 to 5.9 one does not observe casein aggregation.

2.3 Large scale characterization

2.3.1 Static Light Scattering (SLS)

Particle size distribution was determined by Static light scattering (SLS) as described in the bibliography (Richard et al., 2013) using a MasterSizer 1996, Malvern Instruments, Malvern, Worcestershire, UK) equipped with a 5 mW He-Ne laser operating at a wavelength of 633 nm. Samples were diluted into the Malvern cell (volume: 100 mL) with distilled water to reach appropriate obscuration (20 %) and refractive index for the solvent of 1.33 and the absorption index was set at 0.1. The SLS was also used to analyze the resistance of the aggregate to stirring.

For the physical aggregates resistance test, the powders were hydrated at 50 °C/1 hour to promote the formation of the aggregates then the samples were left under stirring at 500 r.p.m for 3 hours at 25 °C. Later on, samples were collected and analyzed every hour. The result is expressed in the % of the volume of the total particles and their relation in the different size range (0-1000µm). The aggregates resistance test also provides information about the linking forces of the aggregates.

2.3.2 Electrophoresis

The samples were diluted to 1:5 with deionized water and analyzed through the Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE) as described by Veloso et al (Veloso, Teixeira, & Ferreira, 2002) with some modifications. The analysis was performed at room temperature using a SE 600 Series vertical Slab Gel Unit (Hoefer Scientific instrument, San Francisco, US). The sample were prepared following the protocol from the bibliography (Pardo & Natalucci, 2002). The analysis

was performed at 30V until the samples had completely left the stacking gel and then the voltage was increased to 90 V until the tracking dye reached 4/5 of the gel. In this work, the slab gel consisted of 4% of Stacking gel, and two different separation gels, which are prepared to 10% and 12% acrylamide. Immediately after the protein migration, the gel was fixed for one hour with a solution of 10 % and 30% of acetic acid and ethanol respectively, colored (coomassie blue) and decolored, also as described in the literature (Pardo & Natalucci, 2002). The gels were scanned with a PC scanner and the scans were saved for further analysis. Densitograms was obtained using the gel analysis module of ImageJ Software (Hermanto, Sholaikah, & Mulyani, 2016) and were expressed in the intensity of the grey color (arbitrary value from 0-255) per molecular weight of each protein/aggregate band. The SDS-PAGE analysis was conducted in non-reducing and reducing conditions (2-mercaptoethanol), which promote the disruption of the disulfide bonds (Pardo & Natalucci, 2002; Veloso et al., 2002). This analysis was further applied to characterize the molecular weight of the proteins and and its aggregates. The linking forces was evaluated by addition of 2-mercaptoethanol and also to the SDS, which are associated to the decrease of the repulsive barrier caused by the hydrophilic C-terminal region of κ-casein, with consequent reduction of the electrostatic repulsion (Chakraborty & Basak, 2008; Gastaldi & Fuente, 1998; Lefebvre-Cases, Tarodo de la Fuente, & Cuq, 2001).

2.3.3 Cryo-Fracture associated to Scanning electron microscopy (Cryo-F-TEM)

The samples were rapidly immersed into liquid nitrogen slush (a mixture of solid and liquid nitrogen at its freezing point (-210 °C). The liquid nitrogen used was freshly filled to prevent the presence of particulates that may provide nucleation of ice crystals. To make the nitrogen slush, the liquid nitrogen was filled into a polystyrene cup that was then placed in the slushing chamber. The chamber was evacuated using a rotary pump until, through a loss of latent heat of evaporation, the nitrogen ceased to bubble and solidified. After 30 s, the vacuum was released to allow for the solidified nitrogen to melt. The process was repeated twice to ensure that the entire volume of the nitrogen was brought to its freezing temperature (-210 °C). The samples were then

rapidly immersed into the freshly prepared slush for 15 s. Following freezing, the frozen specimens were immediately transferred using the VTD into an attached Cryo preparation chamber. The VTD allows the sample to be transferred under vacuum (>10 $^{-4}$ Pa) to the sample preparation chamber to prevent ice recrystallization (the growth of smaller ice crystals to a larger size) and thus minimize any changes in microstructure. With the aid of an externally fitted binocular microscope, the sample was fractured using a chilled scalpel blade in the chamber which was maintained at - 140 °C under a high vacuum condition (>10^{-4} Pa). The specimen was then etched (facilitating the removal of ice from the surface of the fractured sample by vacuum sublimation) at -95 °C for 5 min, 15 min, 30 min, 45 min or 60 min and coated using a cold magnetron sputter coater with 300 V, 10 mA of sputtered gold/palladium alloy (60/40) for 60 s (w3 nm) or 120 s (w6 nm). It was then transferred under vacuum onto a nitrogen gas-cooled module, maintained at -140 °C.

The images obtained were transformed in 8-bit format and treated using the Fourier Transform (FTT) tool in the ImageJ software (Hermanto et al., 2016). The FTT profile was made from the complete image from the Cryo-F-TEM, which corresponds to approximately a result from about 400 micelles per image, and, in our study, the error bars come from an average of three images (about 1200 micelles) obtained after the Cryo-fracture. To measure the size of the internal cavities of the CMs, the first “shoulder” in the TEM FFT profile has been considered as a Guinier peak corresponding to the average radius of the CMs pores (Putnam, 2016). The relative difference between D-CM and N-CM radius has been given in the article instead of the absolute values. This is so because the actual size of the metal coating grains may mask and/or alter some of the measurement resulting in a bias in the absolute size of the pores. However, since the replica of the different samples has been produced in the same coating conditions, the bias added by coating should be reduced (or canceled) if one considers only the relative values.

2.4 Small scale characterization

2.4.1 Small angle X-ray scattering (SAXS)

SAXS measurements were performed at the French national synchrotron facility SOLEIL in Gif-sur-Yvette, France, on the beamline SWING operating at ~12 keV photon energy. Samples were hydrated at 8.0 g x 100 g^{-1} and placed in a watertight cell with a cylindrical cavity of diameter 4.5 mm and thickness 2 mm closed by flat mica windows. The scattered intensity was recorded on a detector placed at ~6.5 m from the sample. For each sample, data were first recorded at short exposure time (typically ~0.2 s) to avoid any radiation damage (aggregation) that could result in artifacts at low q values. Subsequently, data were recorded at long exposure times (typically ~15 s) using a larger beam stop to obtain a good signal/ noise ratio at high q values without damaging the detector. Intensities recorded at the two exposure times were then radially averaged and combined to get a scattering curve covering a q-range of 1.5 x 10^{3} to 1.4 x 10^{1} A^{1}. In some cases, artifacts due to sample radiation damage were visible in the low-q regions of the data recorded at long exposure times. The corresponding intensities were discarded before the merging procedure was carried out. For each sample, the intensity scattered from the solvent (Deionized water) in the same mica cell was measured and subtracted from the CMs sample pattern. The resulting corrected intensity is denoted by I(q). All measurements were performed at room temperature (~25 °C) and from samples of powder from 2017 and 2018 (figure 1 in supporting information).

2.4.2 NMR Spectroscopy

The ^{31}P spectra were obtained using a Bruker AVANCE I; 9.4T (1H: 400MHz; ^{31}P: 161.9MHz) spectrometer. The 4 mm probe heads were set with samples and submitted to 700 Hz of Magic Angle Spinning (MAS) speed. Quantitative experiments at 25 °C of ^{31}P were done with high power decoupling- recycle delay: 30s; 90° pulse; RF field (^{31}P): 65 kHz; ^{1}H decoupling (RF field: 60 kHz; SPINAL64); 1024 accumulations. The chemical shifts were given in parts per million (ppm) with the respect to the analysis of H_3PO_4 (85%) for ^{31}P NMR spectra at 0ppm. For the ^{1}H

analysis, we used one pulse experiment with a flip angle of 90°, a relaxation delay of 5s and RF field (^{1}H): 60 kHz. The analysis was set with a quantitative of 16 accumulations of inversion-recovery (t1ir): 90°-tau-180°. The chemical shifts were given in ppm with respect to TMS (0ppm) as an external reference for ^{1}H NMR spectra. In addition, the qualitative relation between ^{1}H-^{31}P are done through the Cross-Polarization (CP) experiments at 1 millisecond of contact time.
The different components of each spectrum of one pulse and CP and ^{31}P are simulated using the DMFIT software (De Sa Peixoto et al., 2017; Massiot et al., 2002) which permits to obtain an analysis of the signal of each phosphorus species. This method propitiates the quantification of the number of ^{31}P species with an error band of ± 1.5%.

2.4.3 Fluorescence measurements

Fluorescence spectra were recorded using a Fluoromax-4 spectrofluorometer (Jobin Yvon, Horiba, NJ, USA). The incidence angle of the excitation radiation was set at 60◦ to ensure that reflected light, scattered radiation, and depolarization phenomena were minimized. The spectrofluorometer was equipped with a thermostated cell and the temperature was controlled by a Haake A25 AC200 temperature controller (Thermo-Scientific, Courtaboeuf, France). CMs dispersions were poured into a 3 mL quartz cuvette and the emission spectra of tryptophan residues were recorded at 20 °C in the 305–450 nm, after excitation set at 290 nm. For each sample, three spectra were recorded.

2.5 Statistical treatments

Some different statistical treatments were applied to a different analysis. For the NMR results ANOVA followed by a T-test at $p<0.05$ was applied. To get statistical information about the internal organization of casein micelles from Cryo-fracture micrographs, Fourier transform (FTT) was done from three different images using ImageJ software. The FTT produces an average of frequencies corresponding to the

spatial distances between elements in the micrograph (Fig. 3A in the supporting information). This corresponds to an average of about 400 micelles by micrograph. To check the statistical difference of the micrograph by comparison of the FTT profile of three different images (Fig. 3B in the supporting information) with the same magnification (Fig. 3C and 3D in the supporting information). The match between different profiles was excellent. To further check the reproductively of the results, for the demineralized sample the Cryo-fracture and the micrograph were obtained from two different replicates.

A multivariate statistical analysis, such as principal component analysis (PCA) was applied (Saporta, 1990), making possible to extract information, from the fluorescence spectra, related to the structural changes in casein micelles, this statistical approach was used to investigate the basis of the observed spectral discrimination between the caseins sub-unities, the spectral patterns of tryptophan associated with the principal components (PCs). The spectral patterns associated with the PCs provide the characteristic wavelengths which are the most discriminating between spectra on the map.

3 Results

3.1 Impacts of the demineralization in the CMs dispersion

The aggregative properties of CMs samples (native and demineralized) were evaluated in this first part of the study, in order to understand the impact of demineralization and/or the rehydration condition.

3.1.1 Particle size characterization accessed through Dynamic and Static light scattering (DLS and SLS).

Figure 4 illustrates the results obtained by SLS after following the two rehydration procedures. The industrial rehydration procedure catalyzes the appearance of some large (>10 µm) aggregates for both samples (native and demineralized), resulting in a decrease in the intensity of the CMs characteristic peak between 0.15-0.25 nm.

As described in section 2.2.1, the control rehydration process (25 °C/24 h) (Fig. 4) was used to promote the complete dispersion of the casein micelles powders, without massive appearance of large aggregates as consequence of the heating during the rehydration process. But even at this condition, the D-CMs present some aggregates between 1-50 µm. The control rehydration protocol is impracticable for the industrial application, because potentially represent a risk of the development of microorganisms and enzymatic reactions which are undesirable, and it can represent technological problems, such as aggregation (de Kruif, 1998), later gelification in heat treated products (Baglinière, Jardin, Gaucheron, de Carvalho, & Vanetti, 2017) and others severe safety concern for food industries (Olivier, Jayarao, & Almeida, 2005)

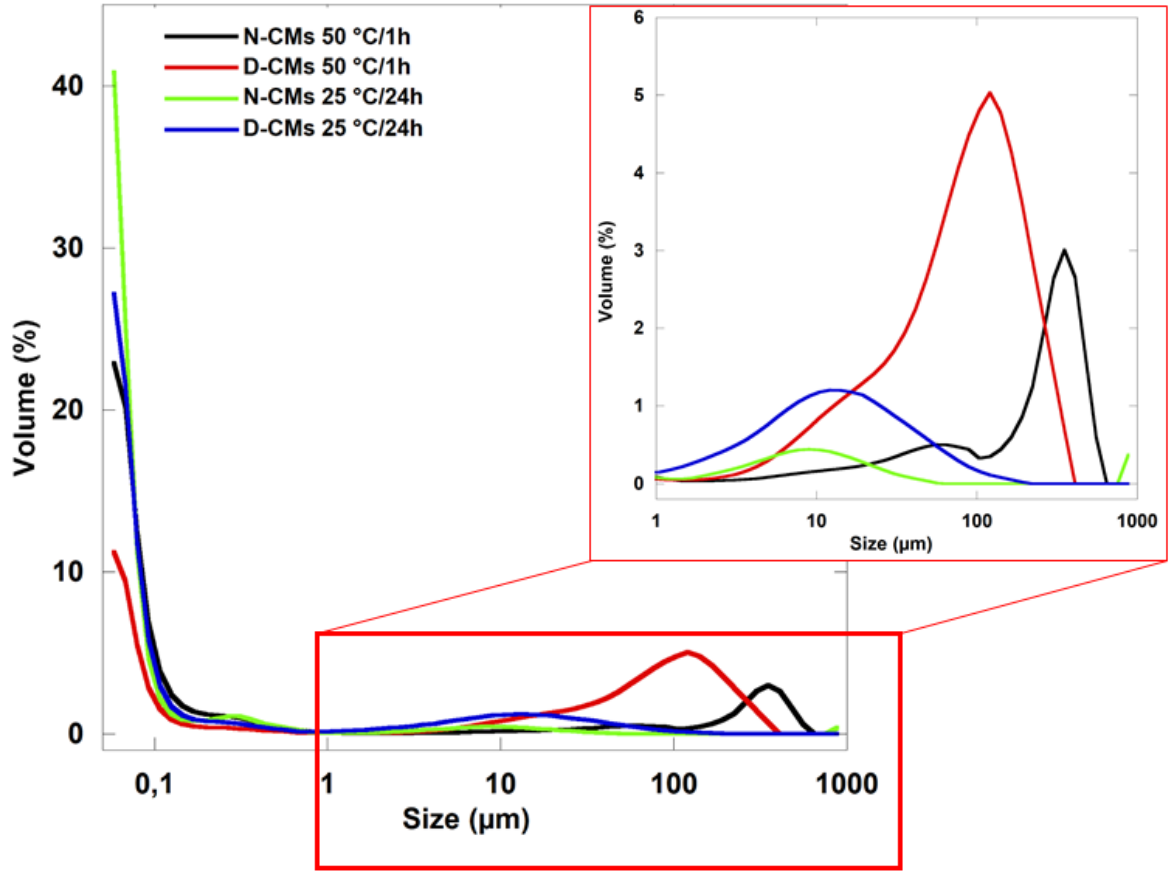


Figure 4 - Particle size distribution of the casein dispersion hydrated at the industrial (50 °C/1 h) and control procedure (25 °C/24 h).

As described by Richard, et al (2013) the optimal control of the rehydration conditions is fundamental to promote the correct dispersion of the milk powders. Temperature plays a significant role in this process, minimizing the mechanical energy necessary and promoting the complete dispersion.

To check characterize the molecular weight and the cohesive forces that form the aggregates (possible disulfide bonds), two different expriments have been done: A physical resistance test (stirring at 25 °C for 3 hours at 500 r.p.m) and SDS-PAGE (at reducing and not reducing condition see section 3.1.2).

The results for the native casein powder (Fig. 5) show that the supplementary stirring is capable to dissociate the large particles present in the dispersion (3 hours at 25 °C) with consequent release of particles equivalent to CMs particle size (result close

to the control rehydration process 25 °C/24 h), further demonstrating the importance of the temperature control during the rehydration [32,46-48].

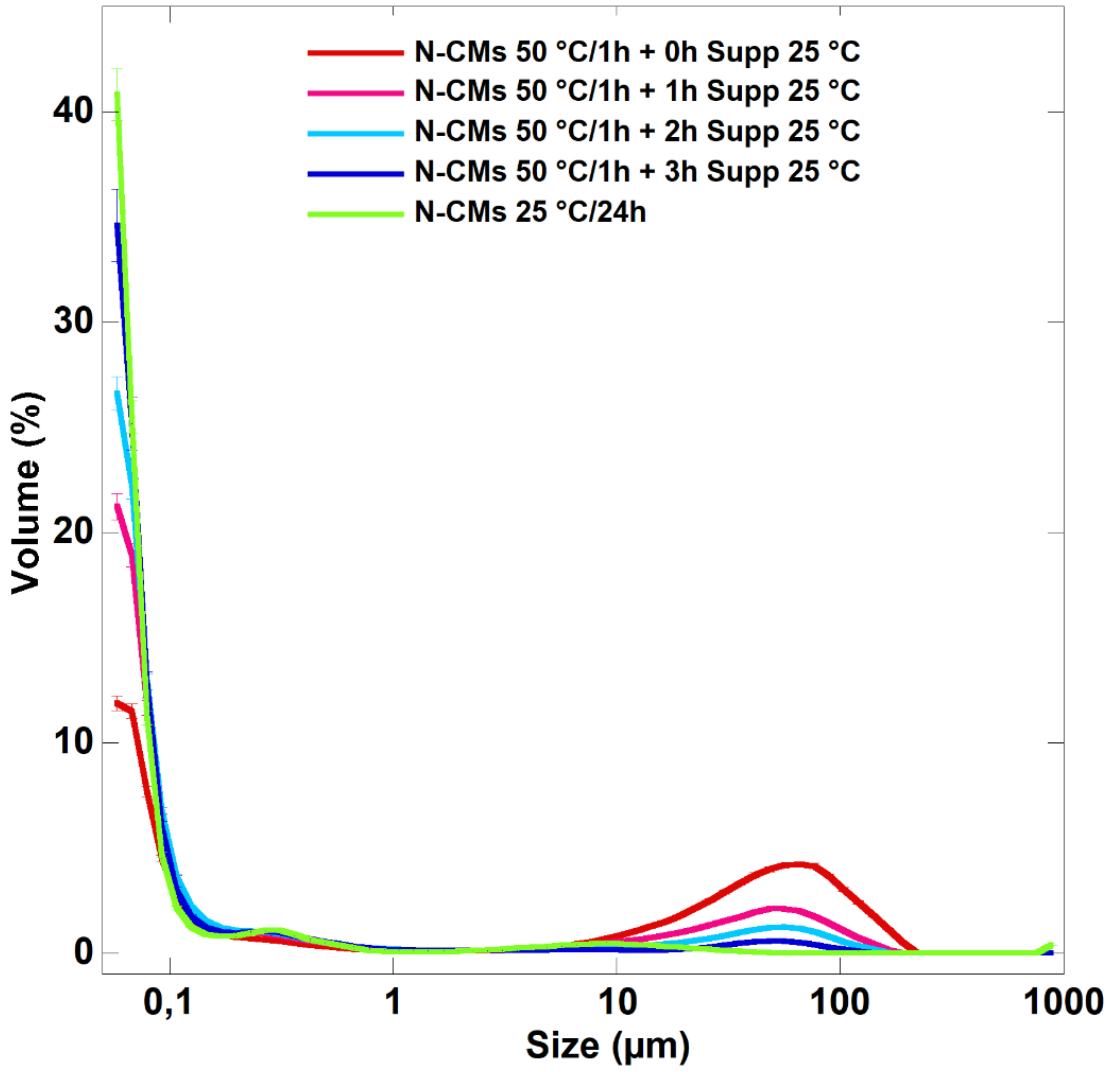


Figure 5 - Particle size distribution of native casein micelles (N-CMs) dispersions after supplementary stirring at 25 °C.

The demineralized casein (D-CMs) submitted to the same supplementary stirring at 25 °C/3 h (Fig. 6), did not present the same behavior. Compared to the native casein powders, the aggregates remain stable after 50 °C/1 h. This result shows that the modification of the structure of the casein micelles (demineralization) during the industrial rehydration process is responsible for the consolidation of the resistant aggregates.

Panouillé, et al. (2004), has shown that the demineralization of the CMs can be responsible for the dissociation of the micellar structure into smaller fragments or primary casein particles: When submitted to heating for a long time, these smaller fragments start an irreversible aggregation process. Same aggregation behavior was observed in our case for the demineralized casein powders (Fig. 6).

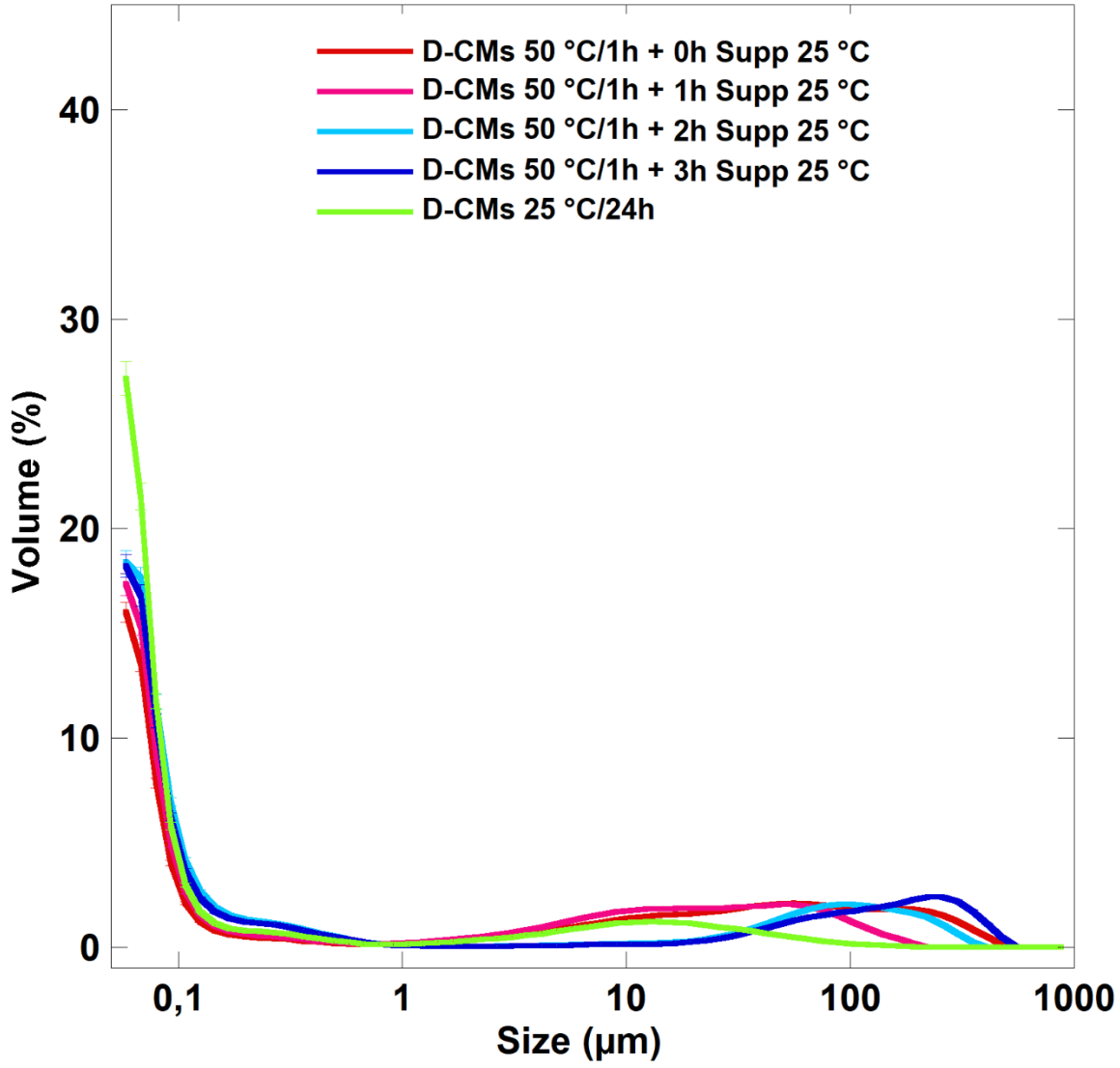


Figure 6 - Particle size distribution of demineralized casein micelles (D-CMs) dispersions after supplementary stirring at 25 °C.

3.1.2 Molecular weight of the CMs fractions and aggregates evaluated using the SDS-PAGE.

The molecular weight of the CMs fraction and its aggregates was evaluated using the SDS-PAGE analysis in reducing (2-mercaptoethanol) and non-reducing conditions.

In the non-reducing gel (Figure 7a) almost all the aggregates have been dissociated except a small amount of high molecular complexes (≥ 250 kDa), that are visible for all the samples. The fact that SDS was capable of disrupting the majority of the bounds responsible for the large aggregates formed in the dispersion, with the consequent release of the casein fractions, shows that the aggregates bounds are formed, mostly, by non-covalent forces.

The 2-mercaptoethanol (Figure 7b) disrupts the aggregates disulfide (high molecular-weight complexes). The heating induces the denaturation of the milk protein (especially the β-lactoglobulin) with an exposition of some sulfur segments, which are responsible for creating some disulfide bonds that form the high molecular-weight complexes, as demonstrated in the bibliography (Q. Fang et al., 2017) .

3.1.3 Size measurement by DLS

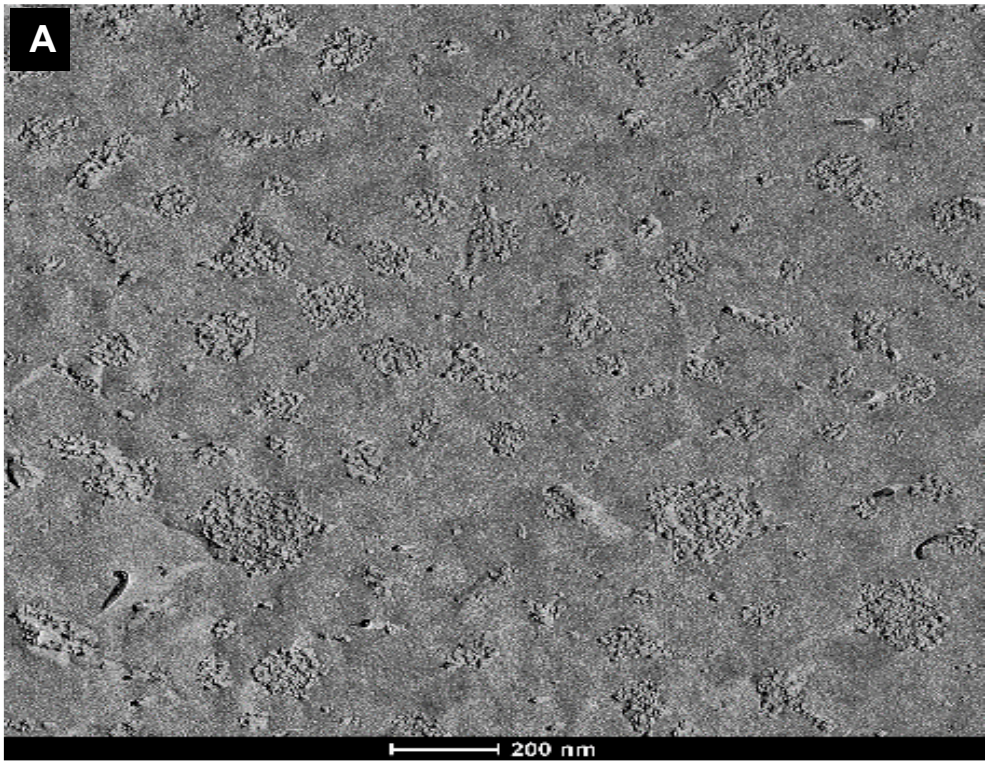
Since the typical size of the CMs is near to the detection limit of our SLS equipment, in the present study dynamic light scattering (DLS) has been used to well characterize the hydrodynamic diameter of the CMs. The description of the analysis procedure and the results are present in section 1, in Table 2 and in figure 2, respectively, in the supporting information. These data show that demineralization and the higher temperature favors a decrease in the micellar size.

3.1.4 Casein micelles rearrangements observed by Cryo-Fracture-TEM

To understand the impact of the demineralization on the global structure of the CMs, we associated the freeze-fracture to the transmission electron microscopy (Kamigaki, Ito, Nishino, & Miyazawa, 2018; Trejo, Dokland, Jurat-Fuertes, & Harte,

2011) to get access to the structure of the CMs. The statistical analysis (based on Fourier transform: See experimental section for details) was used to quantify the level of changes associated with the different levels of mineralization and the effect of the temperature.

In figure 7A, we present the micrographs of the native casein powder hydrated in control conditions (25 °C/24 h). The size of the individual CM goes from 40 nm to 220 nm. The casein micelles (Fig.7A) present a roughly surface as previously describe (Peixoto et al., 2015) with some dense parts (from 5 nm to 20 nm) which may be associated to internal water channels and water cavities (Peixoto et al., 2015; Trejo et al., 2011) (Antoine Bouchoux et al., 2010). The figures 4B and 5B present the micrographs of native and demineralized casein micelles dispersions hydrated using the industrial procedure (50 °C/1h).


A
200 nm

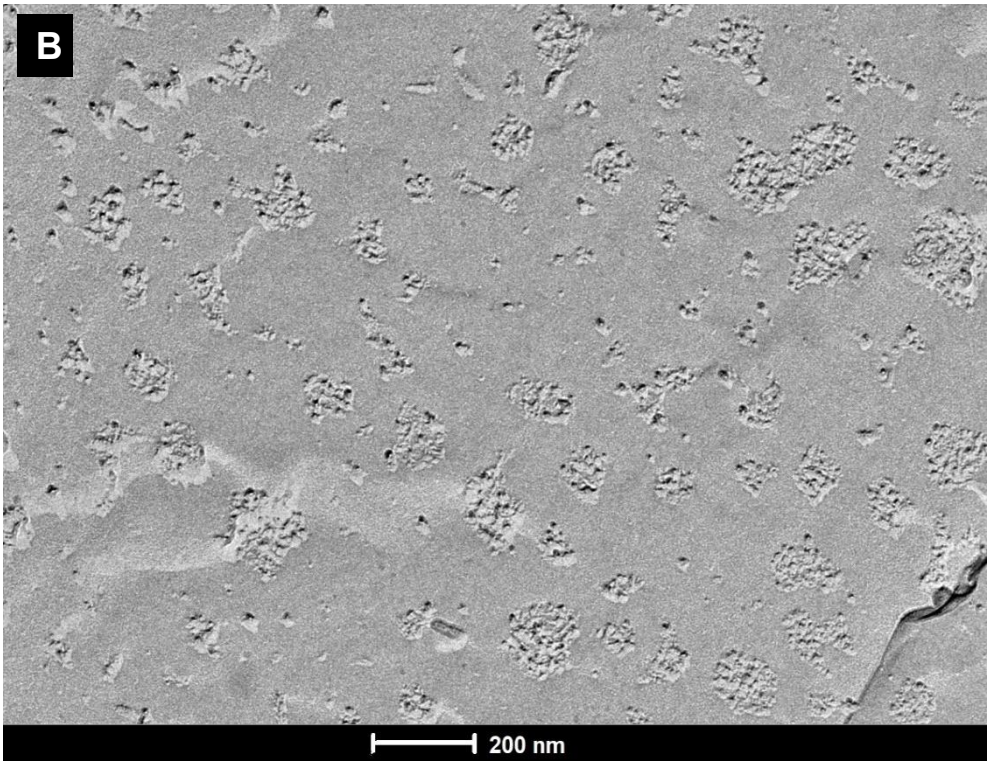

B
200 nm

Figure 7 - Freeze-Fracture electron microscopy for the native casein.
A= Native casein hydrated at the control process (25 °C/24 h); **B**= Native casein hydrated at the industrial process (50 °C/1 h). Bar corresponds to 200 nm.

The study of the internal organization of the N-CMs using FTT shows that there is no statistical difference (see SI for details) between the size of the internal water channels and water cavities for the control process (Fig. 7A) and the industrial process (Fig 7.B). However, between the native and the demineralized CMs, there is a remarkable difference. These differences are due to an internal micellar structure with internal elements displaying greater inter-distances. Based on the FTT the inter distance between internal elements for the demineralized casein micelles (Fig.8A) of the CMs structure increases about 30% compared to the pores present in the native casein micelle dispersions (which means that the demineralized present larger water-filled cavities).

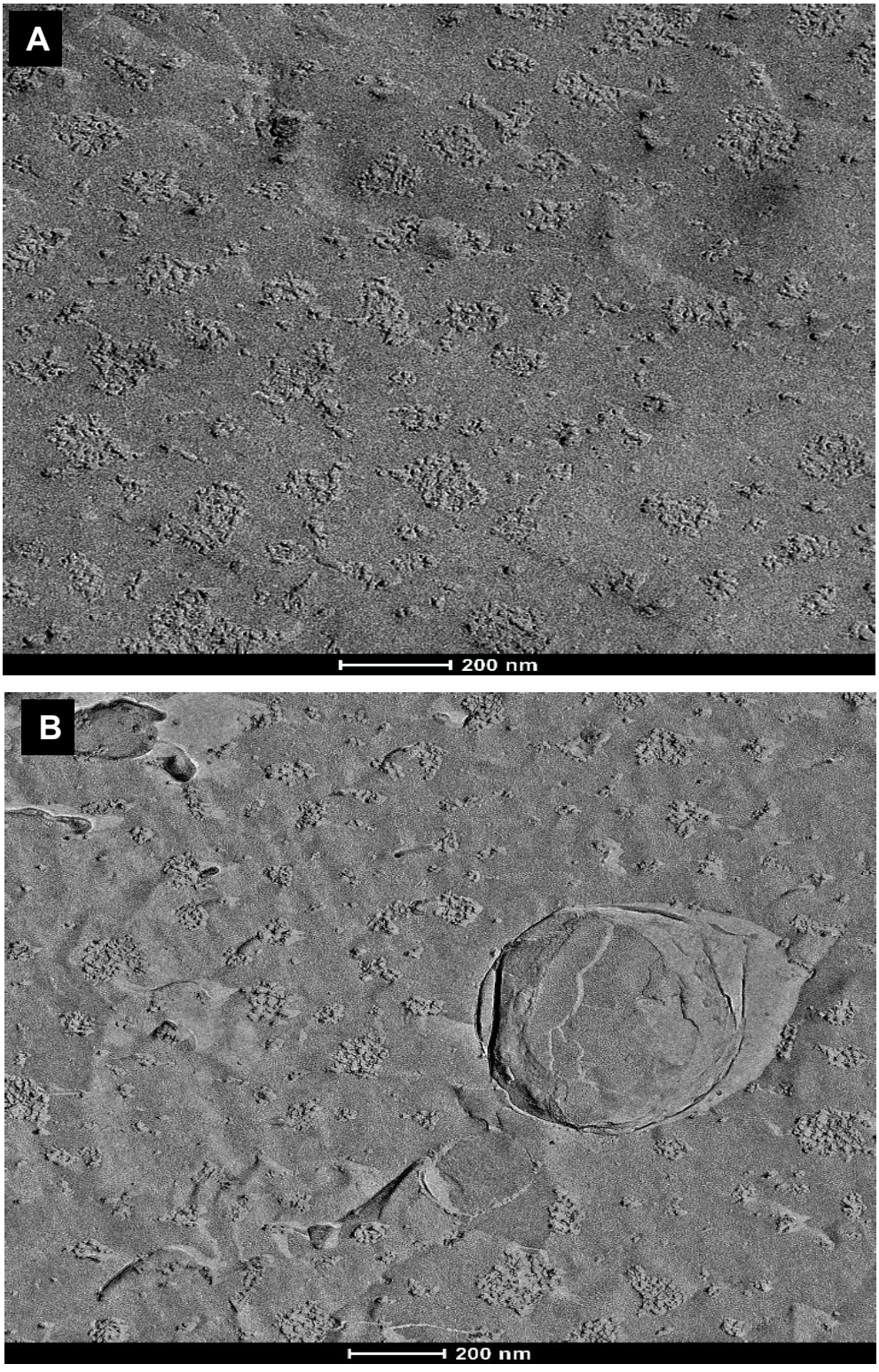


Figure 8 - Freeze-Fracture electron microscopy for the demineralized casein.
A= Demineralized casein hydrated at the control process (25 °C/24 h); **B**= Demineralized casein hydrated at the industrial process (50 °C/1 h). Bar corresponds to 200 nm.

As described by the bibliography (Panouill et al., 2004; Silva et al., 2013) the demineralization is responsible for a dissociation of the CMs structure, appearance of small casein particles, and the decrease of the free casein micelles amount. In this study, the observed opening of the micellar structure and the consequent increase of the size of the pores could be seen as an intermediary state between the native and the small casein particles described in literature.

3.2 Impacts in the casein micelle structure at the protein scale

This second part of the study had the objective to characterize the changes associated to the demineralization and the different rehydration processes at the protein scale, to understand the changes at the amino acids and minerals level, enabling us to provide some evidences that explains the mechanism involved in the aggregation observed previously (section 3.1).

3.2.1 Internal rearrangements observed by Small-Angle X-Ray Scattering (SAXS)

As describes in the literature (Bouchoux et al., 2010; Day et al., 2017; Xu et al., 2016), the SAXS spectra of the casein micelles present a “shoulder” at q ~0.080 nnm^{-1}, that can be described as “hard” regions attributed to parts of protein associated with CCP in the CMs. As proposed by Ingham et al., (2016) the SAXS spectra is formed by four different structures compounds at different scales,: (i) the casein envelope (around 100-200 nm in diameter,); (ii) an internal structure described by Bouchoux et al., (2010) as “hard” or “incompressible regions”, (around 20 nm in diameter); (iii) the CCP particles associated with proteins (around 5 nm), and (iv) protein inhomogeneities as describes by De Kruif, (2014). In our case the region iii (composed by CCP associated with proteins) was one of the most affected by the demineralization.

The changes in the amount of the CCP were observed between the control (N-CMs) and the demineralized casein micelles (D-CMs) with an evident decrease in the

"shoulder" intensity (Fig. 9). This confirms the bibliography hypothesis about the association of the "shoulder" in the SAXS spectra with the amount of the CCP assigned to CCP at q ~0.080 nm^{-1} (Bouchoux et al., 2010)), but no change in the shape or the positions of this "shoulder". This indicates that the elements producing this signal (composed by CCP and proteins) have been excised in the demineralized micelles. This also means that the remaining CCP regions, in contrast, do not show important structural changes. Indeed, if demineralization would have affected the sizes of the nanoclusters or the associated proteins, one should see some shift or change in the profile of this "shoulder" (and not only a decrease). Thus, it seems that demineralization displays a rather cooperative behavior since one does not observe many partially depleted sites. This result seems to agree with previous studies on partially depleted CMs where the shape and position of the "shoulder" do not display a greatly modification with CCP depletion (in contrast to the "shoulder" intensity" (Ingham et al., 2016).

In comparison to the rehydration control procedure, the industrial procedure also affected some proteins in the CMs structure (protein monomers). Indeed, a change in protein folding was observed through SAXS analysis at high Q regions (q 0.33 to 1.0 (Q/nm^-1), as a consequence of the unfolding of the proteins (Kikhney & Svergun, 2015). That the combination of temperature and demineralization increases the disorder in the internal structure of CMs. As described in the literature (Boiani et al., 2018; Rollema & Brinkhuis, 1989), heat treatments are responsible for some changes in the mineral levels of the CMs and consequently changes in the CMs structure (Boiani et al., 2018; H S Rollema & Brinkhuis, 1989; Sauer & Moraru, 2012).

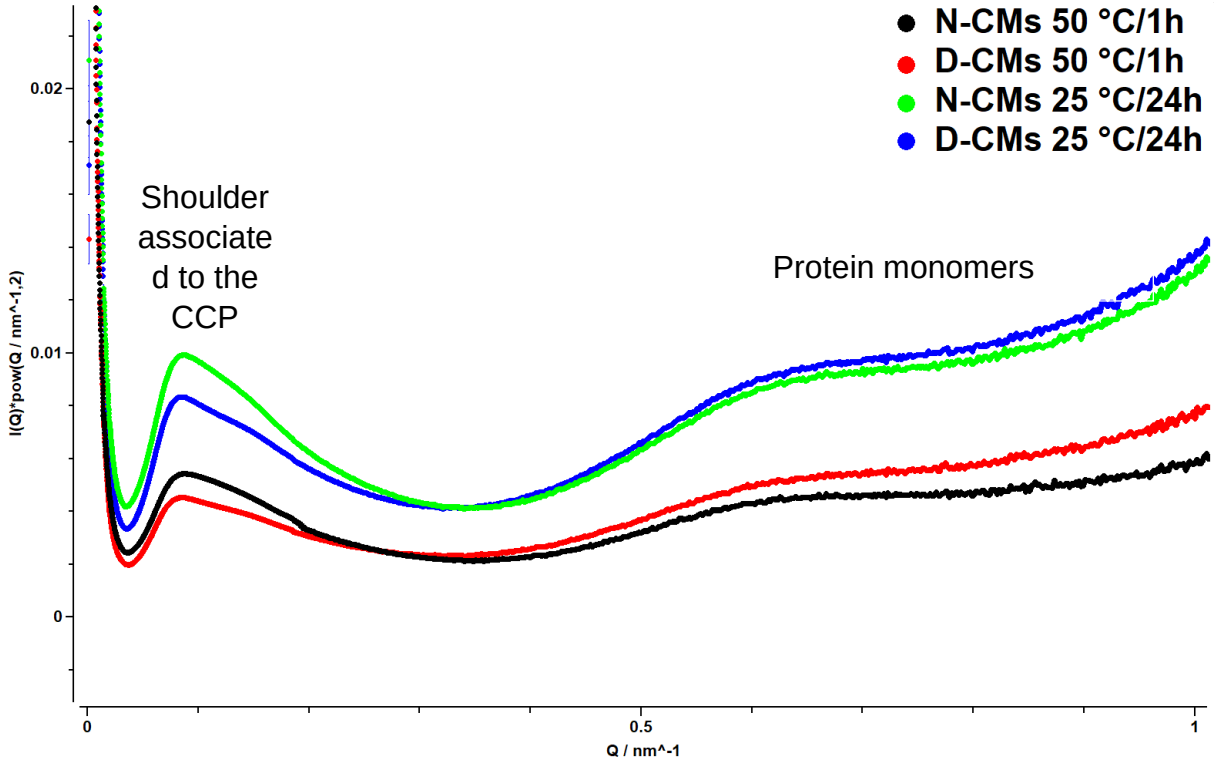


Figure 9 - SAXS profile of casein dispersion hydrated at 50 °C/1h (Industrial procedure) and 25 °C/24h (Control procedure).

3.2.2 Internal rearrangements observed by Nuclear Magnetic Resonance (NMR)

As demonstrated with SAXS experiments, the demineralization modifies the CMs internal structure, and the use of higher temperature during the rehydration promotes internal rearrangements. ^{31}P - NMR has been used to get further information about the quantity of free and bond CCP and phosphoserines.

Four types of phosphorus can be identified and quantified using the NMR, organic phosphorous from phosphoserine residues, organic phosphorus from serine to CCP, inorganic phosphorus in the CCP and free inorganic phosphorous in the bulk as describes in the literature (Boiani et al., 2018, 2017; De Sa Peixoto et al., 2017) and as observed in the results of this present study (Figure 6 in the supporting information). The major results of NMR analysis are compiled in figure 10 and represent, respectively, the results of the number of free phosphoserine residues(Fig. 10A), the amount of the phosphorus in the CCP nanocluster (Fig. 10B) and the amount of ionic

phosphorus (Fig. 10C). The differences were calculated based on the fitting of NMR spectra, the error bars correspond to the probability of fitting error, as explained in the experimental section. These figures show that no significant difference is observed in the amount of free phosphoserines for N-CMs and D-CMs rehydrated at 25°C. In contrast, the sample re-hydrated at 50°C displays a higher amount of free phosphoserines than N-CMs samples indicating, as it was described for SAXS data, that the combination of temperature and demineralization increases the disorder in the internal structure of CMs. Also in agreement with SAXS data one can observe by NMR that there is a decrease (around 15%) of the number of CCP in D-CMs in comparison with the number of N-CMs. There is no significant change in the number of free inorganic phosphorous between samples. However, this last result should be subject to caution since it is possible that temperature may induces the precipitation of some amount of phosphorous ions during the heating procedure (Broyard & Gaucheron, 2015) (before NMR analysis).

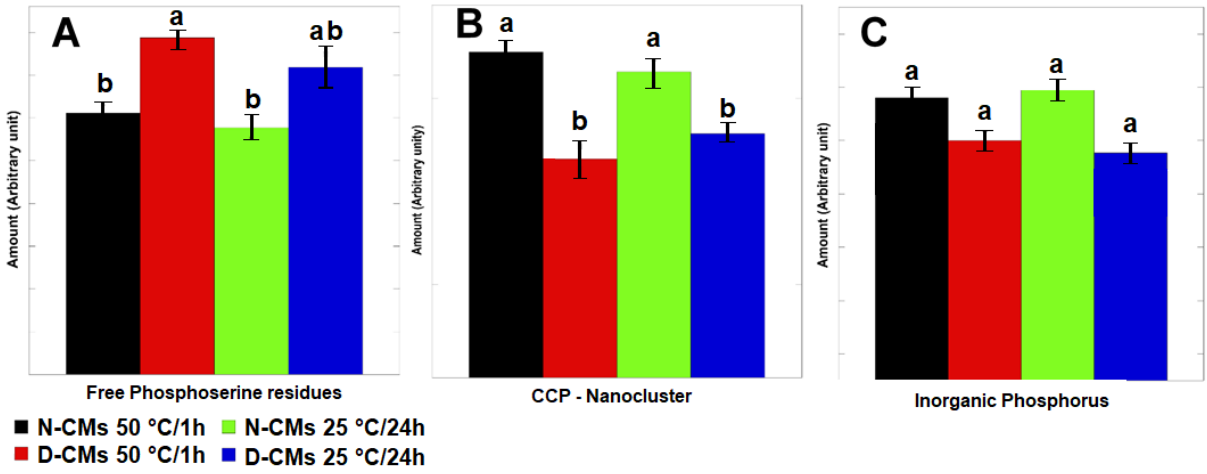


Figure 10 - Different phosphorus fractions obtained through NMR of native and demineralized casein hydrated at 50 °C/1h or 25 °C/24h. Different letters correspond to statistic differences at $p<0.05$.

NMR and SAXS show that demineralization plays an important role in the structural modification of the CMs which can be accentuated by the rehydration process at higher temperature (Industrial condition). The association of the results from SAXS and NMR confirms the assignment proposed by Bouchoux et al., (2010) that the “shoulder” observed in the SAXS spectra is related to the hard regions

associated with the CCP. In our case, the decrease in the CCP level, observed by NMR analysis can be related to a decrease in the intensity of these regions in the SAXS spectra. The specific structure of CMs, in the natural conditions of milk (pH and ionic environment), is a significant factor responsible for keeping the mineral equilibrium between colloidal calcium nano-clusters present in CMs and the ionic calcium and phosphate present in the soluble phase (S. G. Anema, 2009; Kim et al., 1990; Lucey & Fox, 1993; Ramasubramanian et al., 2013).

Previous studies (Pouliot, Boulet, & Paquin, 1989) have shown that that calcium phosphate solubility decreases at increasing temperatures inducing precipitation as well an internalization of calcium and phosphate ions within the CM structure. Such internalization may explain the small, although not significantly increased in ^{31}P in CCP from 25°C to 50°C suggesting that some internalization has occurred. (Pouliot et al., 1989) have estimate that between 40°C and 60°C a internalization aumont of phosphorous ions between 12% and 25% respectively which is somehow higher than the one observed in the present study. However, the their conditions in terms of casein and bulk ionic concentration are quite different from the present one which may explain this difference.

However, in the case of D-CM one does not observe such an increase between 25°C and 50°C but, otherwise, a decrease in CCP and, consequently, an increase of the amount of free phosphoserines. This difference in between the behavior of N-CMs and D-CMs, as respect to temperature, may indicate that the structural disorder induced by demineralization is not always reversible at any point of the demineralization process. Indeed, previous works show that native casein micelles are not thermodynamically stable at temperatures close to 50°C and, although first, some Ca++ and PI internalization occurs, after some hours there is a decrease in CM size followed by a later ions (Ca and PI) dissociation (Thachepan, Li, & Mann, 2010). Thus, in the case of D-CM, the previous demineralization of the samples combined with the temperature (50°C) could favor more the end of the reaction (observed in literature after long times of N-CM exposure) which explains the loss of nanoclusters and the liberation of free phosphoserines residues.

3.2.3 Internal rearrangements observed by Fluorescence Spectroscopy (FS)

To confirm further information about the nature of the non-covalent aggregate bonds, observed by SLS and Cryo-F-TEM and associated to the casein micelles structural rearrangements observed by SAXS and NMR, the fluorescence technique was used to identify the influence of the demineralization and the different rehydration conditions on the internal structure of the CMs.

Normalized emission spectra showed that the maximum of tryptophan shifted from 377 nm for the native casein micelles to 375 nm for the demineralized casein micelles (Fig. 8). In addition, the maximum fluorescence intensity was lower for the modified casein than for the native casein. Those features indicate a moderate but significant change in the tryptophan environment (Dalgleish, 1973). An explanation may arise from changes in the exposition of hydrophobic segments of the casein micelles indicating structural modifications of casein micelles because when a protein is partly disorganized, hydrophobic segments can be differently exposed causing different protein-protein and/or protein-water interactions (Philippe, Gaucheron, Le Graet, Michel, & Garem, 2003).

As the fluorescence spectra of the demineralized and native casein micelles exhibited slight differences, univariate analysis was not really appropriate for the study of a large number of data sets. Multivariate statistical analysis, such as Principal Component Analysis (PCA), makes it possible to extract information, from the fluorescence spectra, related to the structural changes in casein micelles.

The PCA was applied to the normalized tryptophan fluorescence spectra (Figure 7a in the Supporting information). The first two principal components (PCs) accounted for 94.2% of the total variance with a large predominance of the PC1 (84.8% of the variance). According to the PC1, a clear discrimination between the two groups was observed since demineralized casein micelles presented negative scores, while the native casein micelles showed positive values. From the obtained results, it could be concluded that the demineralization applied to the casein micelles induced specific modifications in the shape of the tryptophan fluorescence spectra. These differences could be explained by the change in the structure of the caseins micelles.

To investigate the basis of the observed spectral discrimination between the caseins micelles, the spectral patterns of tryptophan associated with the PCs were studied (Figure 7b in the Supporting information). The spectral patterns associated with the PCs provide the characteristic wavelengths, which are the most discriminating between spectra on the map. They are similar to spectra and may be used to derive structural information at the molecular level. The spectral pattern 1 showed a red shift of the native casein micelles indicating that samples were in hydrophilic environment and/or protein-water interactions. The spectral pattern 2 showed an opposition between a positive peaks located ~ 360 nm and a negative one observed ~ 340 nm. It appeared that tryptophan spectra reflect changes in the environment of the fluorophore and are indicative of structural modifications of the protein network.

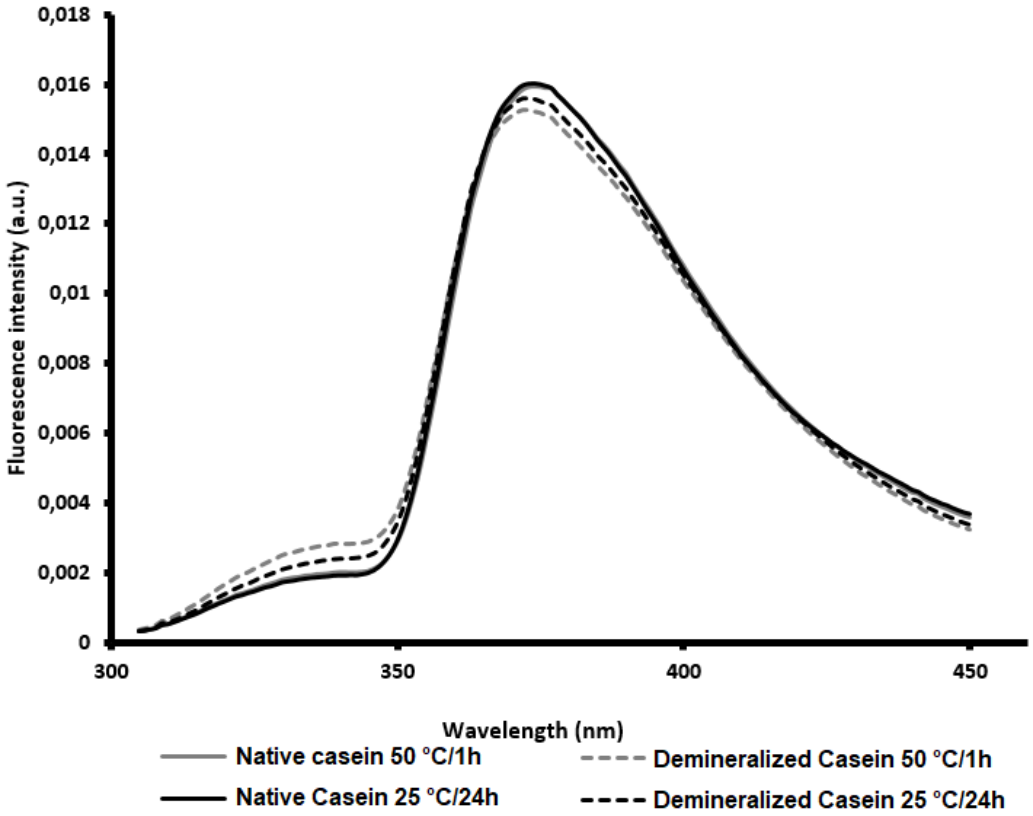


Figure 11 - Normalized tryptophan fluorescence spectra acquired after excitation set at 290 nm on casein dispersions, in two conditions of hydration: industrial (50 °C/1 h) and Control (25 °C/24 h).

The FS confirms the results observed by SAXS and NMR that show some structural rearrangements in the CMs structure as a consequence of the demineralization. With FS, it was possible to observe the tryptophan in a more

hydrophobic environment. This hydrophobicity increase can be related to an increase in the protein-protein interaction capacity, facilitating the aggregation, observed previously in section 3.1.

The results placed in evidence by FS confirm our previous assumptions, that the structure of the demineralized CMs is submitted to an internal rearrangement which is greater at higher re-hydration temperature. FS results show that demineralization and temperature favor stronger, likely non-native, the interaction between hydrophobic residues, in contrast to charged residues as phosphoserines, where it favors rather a liberation of those residues. This suggests that, at least part, of the stronger cohesiveness of the observed large aggregates observed by DLS at 50°C comes from hydrophobic interactions (although, one cannot exclude that some charged (as it is the case for phosphoserines) residues could, possibly, be capable to interact, facilitating the aggregation visible in the SLS, SDS-PAGE and Cryo-F-TEM analysis). Some studies have demonstrated that some hydrophobic interactions are involved in the decrease the solubility of casein micelles powders (Gaiani et al., 2006; Havea, 2006), or as referenced by Felix da Silva, Ahrné, Ipsen, & Hougaard, (2018) as micellar casein isolates, and in our case, the demineralization was responsible to promote an increase in the hydrophobicity, especially for the tryptophan, and this increase maybe was the response to the non-complete dissociation of the demineralized casein powder aggregates.

These results obtained also confirm the hypothesis postulated by Panouill, et al., (2004). In this work the authors suggest that aggregation occurs, initially via weak physical bonds, and that became stronger, as effect of the heat treatments on casein micelles fragments, being responsible to stabilize the casein aggregates.

4 Conclusion

The objective of this study was to evaluate the impact of the demineralization on the rehydration behavior on the casein powders. These data show that re-hydration of D-CM induces the formation of large (>10 µm) and stirring resistant non-dissolved aggregates held by non-covalent interactions. In the intent to better understand the underling mechanisms behind this behavior a multi-scale analysis have been made to quantitatively characterize: the CM size and substructure, the amount of the CCP, the dynamics of associated phosphoserines and the increase in the hydrophobicity (using the tryptophan as a probe). These data show that these two factors, demineralization and temperature, contribute to an internal rearrangement of CMs structures increasing the size of internal cavities of the micelle and increasing the disorder of the proteins. These data also indicate that hydrophobic interaction increases suggesting that this interaction plays a role in the formation of the observed aggregates.

Acknowledgments

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Supporting Information for

Multiscale quantitative characterization of demineralized casein micelles: how the partial excision of nano-clusters leads to the aggregation during rehydration

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Table 1 - Centesimal composition of a native (N-CMs) and a Demineralized (D-CMs) casein powders

Composition* (g/100g powder)	N-CMs powder	D-CMs powder
Moisture	5.0 ± 0.5	5.12 ± 0.33
Total protein	83.45 ± 0.6	84.69 ± 0.8
Casein/Total protein ratio	92	92
Fat	1.5 ± 0.8	1.5 ± 0.6
Lactose	2.0 ± 0.08	2.9 ± 0.15
Minerals	8.5 ± 0.08	6.56 ± 0.05
Calcium (Ca)	2.7 ± 0.08	2.25 ± 0.06
Phosphorus (P)	1.5 ± 0.04	1.22 ± 0.03
Ca/P ratio	1.8	1.6

* Data from the manufacturer's sheet donated by Ingredia dairy Experts from two powders from two different years (2017 and 2018), results were expressed as the average ± Standard Deviation.

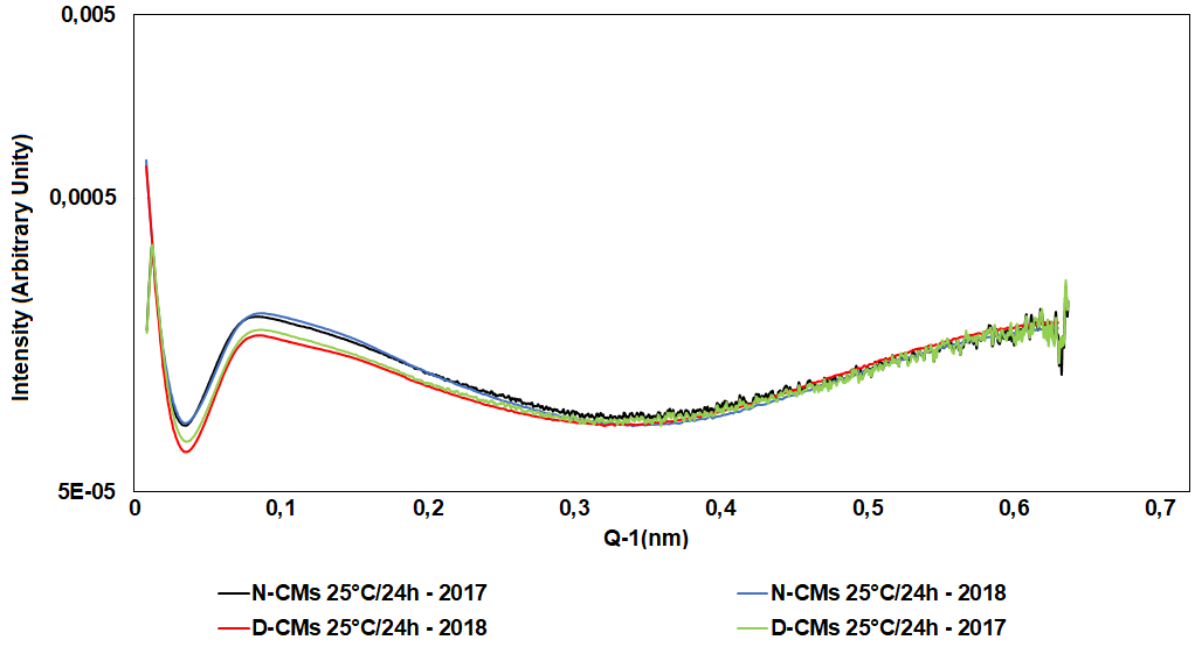

Figure 12 - SAXS from samples from different years (2017 and 2018).

N-CMs 25 °C/24h: Native casein powder hydrated for 24h/25 °C; **D-CMs 25 °C/24h**: Demineralized casein powder hydrated for 24h/25 °C.

1 Particles size characterization by Dynamic Light Scattering (DLS)

To evaluate the hydrodynamic diameter of the casein micelles (CMs), a DLS technique was used using a MasterSizer nano s (Malvern Instruments, Worcestershire, UK). The measuring was performed at the French National Synchrotron Facility SOLEIL in Gif-sur-Yvette, France. Measurements were carried out at a scattering angle of 173° and a wavelength of 633 nm. CMs dispersions were previously diluted to 1/100 with deionized water and left at 25 °C for 20 minutes before analysis. Experiments were running out during two minutes and were repeated six times for each one of the three repetitions and its duplicates.

Table 2 - Hydrodynamic diameter of CMs evaluated by Dynamic Light Scattering (DLS)

	N-CMs 50 °C/1h	D-CMs 50 °C/1h	N-CMs 25 °C/24h	D-CMs 25 °C/24h
Hydrodynamic diameter (nm)	251 ± 1.1	242 ± 3.7	273 ± 5.5	251 ± 4.9

Data from three repetitions and its duplicates, results were expressed as the average ± Standard Deviation. **N-CMs 50 °C/1h**: Native casein powder hydrated for 1h/50 °C; **D-CMs 50 °C/1h**: Demineralized casein powder hydrated for 1h/50 °C.; **N-CMs 25 °C/24h**: Native casein powder hydrated for 24h/25 °C; **D-CMs 25 °C/24h**: Demineralized casein powder hydrated for 24h/25 °C.

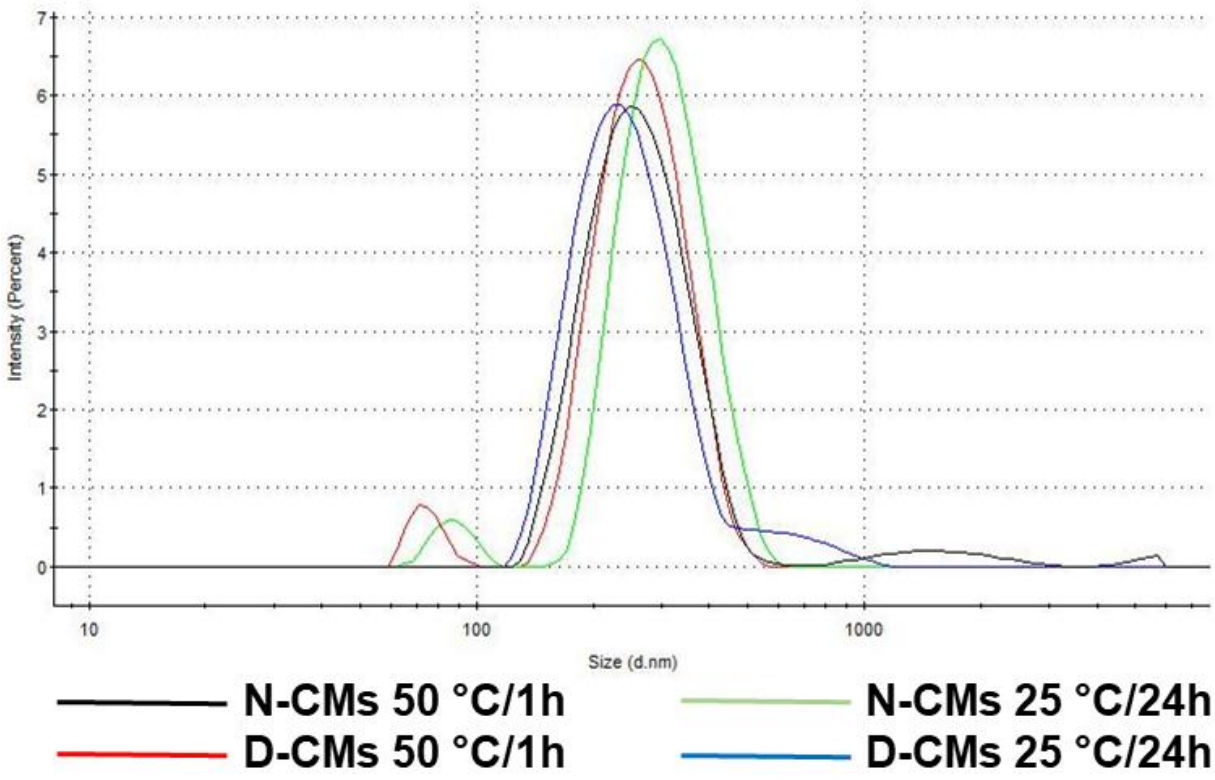

Figure 2 - Particles size characterization by dynamic light scattering of casein samples.
Data from three repetitions and its duplicates. **N-CMs 50 °C/1h**: Native casein powder hydrated for 1h/50 °C; **D-CMs 50 °C/1h**: Demineralized casein powder hydrated for 1h/50 °C.; **N-CMs 25 °C/24h**: Native casein powder hydrated for 24h/25 °C; **D-CMs 25 °C/24h**: Demineralized casein powder hydrated for 24h/25 °C.

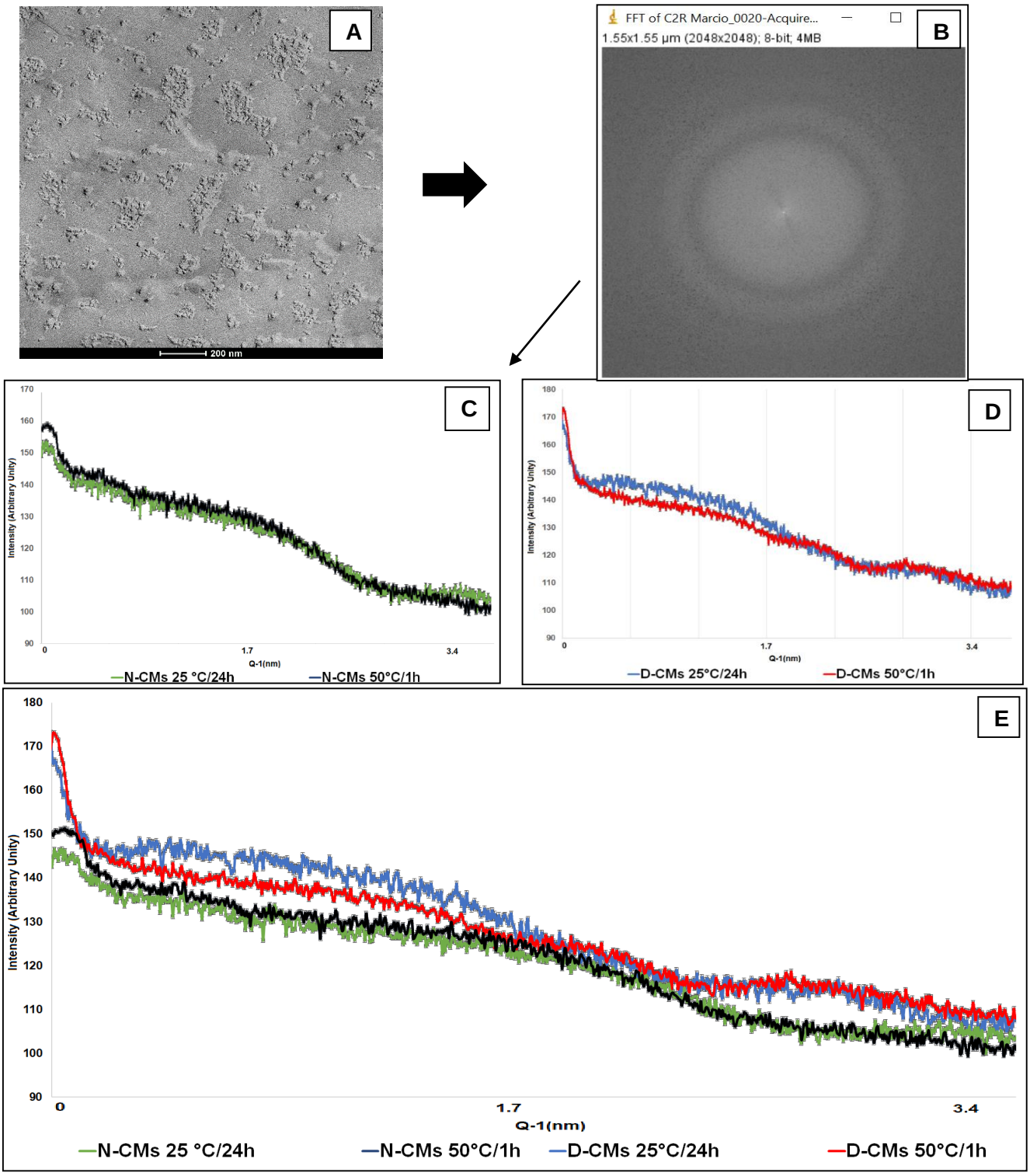

Figure 3 - Cryo-Fracture Transmission Electron Microscopy treatment process using Fourier Transform (FTT) tool in ImageJ Software. **A:** Transmission eletronic microscopic image obtained after cryo-fracture; **B:** FTT result; **C:** FTT graph of native samples; **D:** FTT graph of different casein samples; **E:** All the samples togheter. **N-CMs 25 °C/24h**: Native casein hydrated for 24h/25 °C; **D-CMs 25 °C/24h**: Demineralized casein hydrated for 24h/25 °C; **N-CMs 50 °C/1h**: Native casein hydrated for 1h/50 °C; **D-CMs 50 °C/1h**: Demineralized casein hydrated for 1h/50 °C.
Error bars correspond to a standard deviation obtained from measures from three different micrographs.

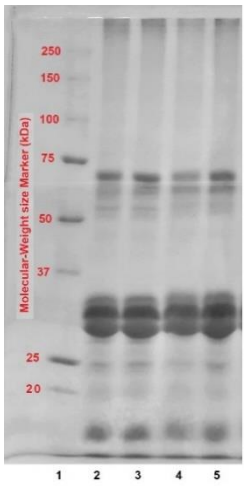

Figure 4 - Non-reducing SDS-PAGE of casein hydrated in different conditions **SD*** ***(1)***: Standard protein marker; **N-CMs 25 °C/24h** ***(2)***: Native casein hydrated for 24h/25 °C; **D-CMs 25 °C/24h** ***(3)***: Demineralized casein hydrated for 24h/25 °C; **N-CMs 50 °C/1h** ***(4)***: Native casein hydrated for 1h/50 °C; **D-CMs 50 °C/1h** ***(5)***: Demineralized casein hydrated for 1h/50 °C
* SD (1) Standard protein marker, corresponding from the bottom to the top, respectively: 20, 25, 37, 50, 75, 100, 150, 250 kDa.

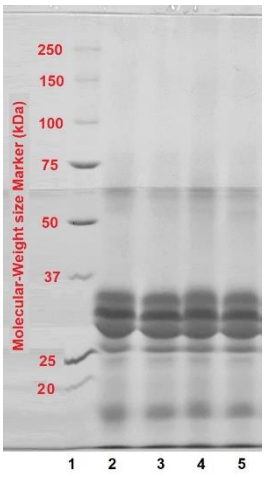

Figure 5 - Reducing SDS-PAGE of casein hydrated in different conditions **SD* (1)**: Standard protein marker; **N-CMs 25 °C/24h (2)**: Native casein hydrated for 24h/25 °C; **D-CMs 25 °C/24h (3)**: Demineralized casein hydrated for 24h/25 °C; **N-CMs 50 °C/1h (4)**: Native casein hydrated for 1h/50 °C; **D-CMs 50 °C/1h (5)**: Demineralized casein hydrated for 1h/50 °C
* SD (1) Standard protein marker, corresponding from the bottom to the top, respectively: 20, 25, 37, 50, 75, 100, 150, 250 kDa.

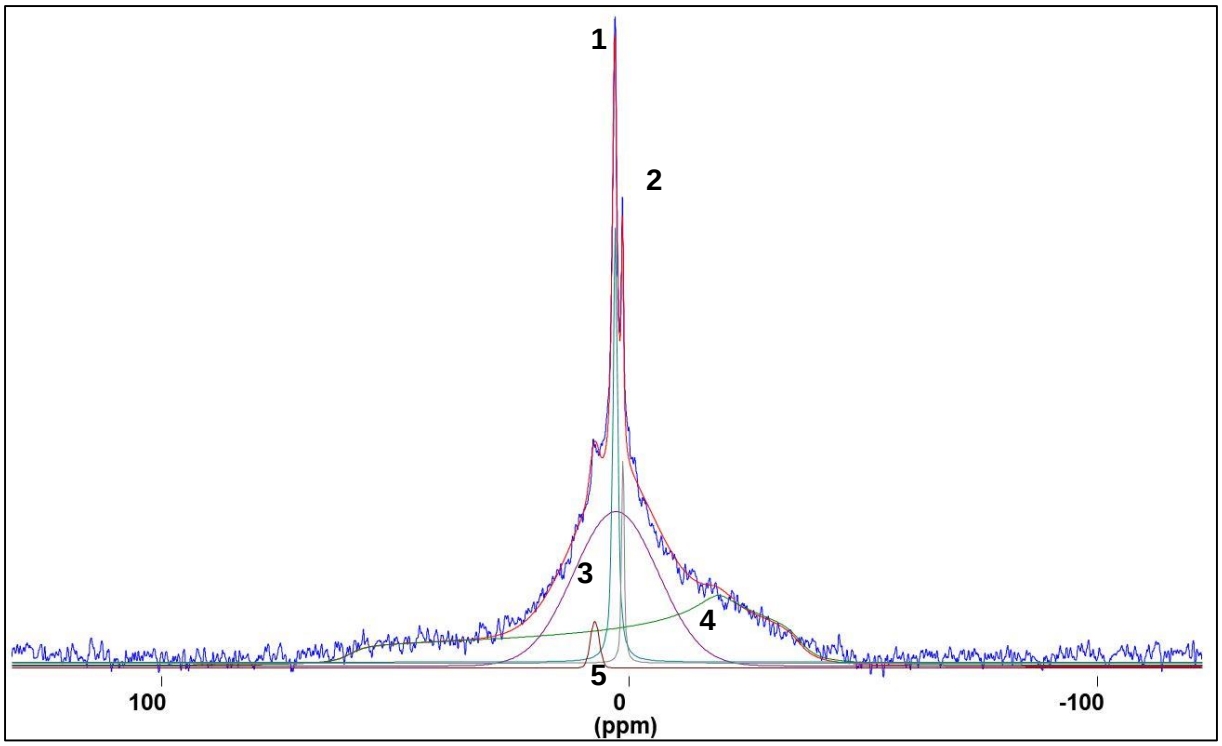

Figure 6 – Example of a nuclear magnetic resonance spectra of casein samples with its respective fit done with DMFIT software.
The number represent, respectively: 1 – Phosphorus from phosphoserine residues; 2 – Free Inorganic phosphorus; 3 – Inorganic phosphorus in the CCP; 4 – Organic phosphorus; 5 – Side band.

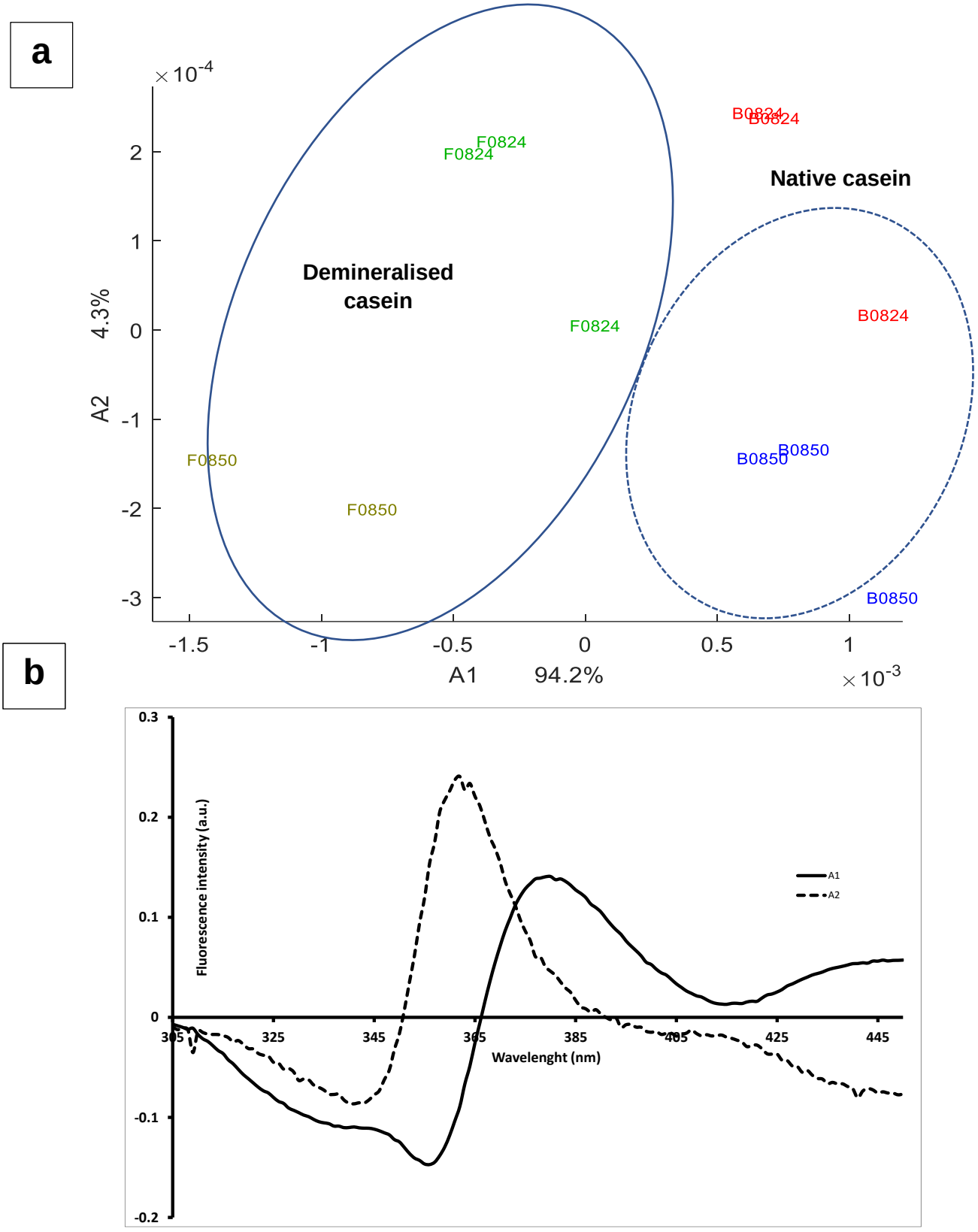

Figure 7. (a) Principal component analysis similarity map determined by principal components 1 (PC1) and 2 (PC2) of the tryptophan spectra recorded after excitation at 290 nm.

The two samples were identified as: (B): Native casein and (F) Demineralized casein, the numbers represent the rehydration condition (08) correspond to 8% of solids and (50) or (24) represent, respectively, the rehydration for 1 hour at 50 °C or 24 hours at 25 °C.

As demonstrated in the article 1 (section 3.1) the demineralization is responsible for internal changes in the CMs structure (the exposition of some phosphoserine residues, and the decrease of amount of CCPs in CMs) which results in hydrophobic interaction between the CMs resulting in aggregation during the industrial protocol of rehydration.

To trespass this drawback (the aggregation of CMs during rehydration) a protocol of homogenization is proposed and presented in the next section (sub chapter 3.2). The objective of this protocol is to facilitate the rehydration of casein-rich powders using industrial practices. The results presented in this section constitutes the article 2 and will be submitted to the journal entitled “Powder Technology”, as we think it represents innovative methods that can be used to rehydrate the casein-rich powder in almost one hour.

3.2 Influence of temperature and rotor-stator homogenization on the dispersion of native phospho casein powder and adapted protocol for fast rehydration of an ingredient used in hyper-protein beverages

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This present article will be submitted to the journal entitled as “Powder Technology”

ABSTRACT

Native phospho casein powders (NPC) very rich in casein micelles are valuables ingredients for the food industry. However, the hydration of these kinds of powders is difficult, especially at high solids content (>8 % (w/w)). In this study, a powder of NPC have been rehydrated at 8%, 14% or 20% (w/w) using an experimental, so-called “fast” protocol, that consist in a first step of letting the powder to be re-dispersed under soft stirring for 1 hour at 50°C and then, in a second step, the solutions are submitted to homogenization with a rotor-stator homogenizer. This fast protocol has been compared to a control protocol where the powder is re-dispersed under soft stirring for 24h, at room temperature, and then submitted to the same homogenization step. Static light scattering and rheological measurements were used to characterize the amount of remaining non-solubilized aggregates and the viscosity of the solutions, respectively, after each step of these protocols. For all concentrated solutions, the results of the fast protocol are almost as good and the control protocol in terms of the amount of re-disperse micelles and in keeping in the original fluid properties in terms of moderated-to-low viscosities. Thus, this data shows that the so-called fast protocol allows one to obtain quite fast rehydration of a relatively concentrated casein micelle powder.

Keywords: Native phospho casein, fast rehydration, protocol, particle size, apparent viscosity.

1 INTRODUCTION

The casein is known to be the major protein present in cow's milk (Walstra et al., 2006) and is important as a protein source for human consumption. That provides all the essential amino acids the body needs to keep its corrects functions (Egan, 2016). As a food source, casein also supplies carbohydrates, and two essential elements, calcium, and phosphorus. For its nutrition quotient, casein represents an important commodity for the food industry and a wide variety of uses, such as major components of cheese and even its use as a food ingredient additive as a powder form (Tamime, 2009).

This transformation enables to increase the shelf-life of casein protein, which facilitates their transport, storage, and their use as an ingredient in dairy derivative and other food products [2,3]. The casein-rich powders are used to facilitate the intake of casein in food products (Tamime, 2009)

The two main forms are hydrolyzed casein and micellar casein. The production of native micelle casein powders, which is our primary concern, involves several unit operations, from skimming, successive membrane separations techniques, such as ultrafiltration, microfiltration and reverse osmosis, concentration, and spray drying (Pierre et al., 1992; Schuck et al., 1994) [2]. To produce these kinds of powders, all the processes are applied with low heat treatments in order to minimize any substantial modifications of the casein structure. The target looking forward is to produce casein protein powder with characteristics as close as possible to the natural one present in milk [4].

The native casein micelles (CMs) present in these powders constitute an assemblage of different fractions of proteins and colloidal calcium phosphate nanoclusters (de Kruif et al., 2012) as illustrated by Schuck ., et al. (1994)(Schuck et al., 1994) and that is why the powders are also called native phospho casein (NPC) powders.

A significant obstacle to cross for these NPC powders is the rehydration stage, especially for high solid/protein content (over 8% (w/w)). Poor wettability and long duration of rehydration of the casein powder are common drawbacks mentioned in

some studies (Hussain et al., 2011; Mimouni et al., 2010), increasing the energetic cost of this unavoidable step of redispersion of casein (Afzal, Mahmood, Hussain, & Akhtar, 2011; Mimouni et al., 2010) substantially. Indeed, previous research has shown that it takes several hours (more than 10 hours) at room temperature to reach the complete rehydration of casein micelles powder (in this case, the addition of sodium azide is needed to prevent the bacteria development) (Schokker et al., 2011). However, at industrial scale, this rehydration protocol can be hardly considered, because long process represents a microbiological and enzymatic risk and can induce some technological drawbacks, such as acidification (de Kruif, 1998), aggregation (Panouillé, Durand, Nicolai, Larquet, & Boisset, 2005), instability to heat treatments (Baglinière et al., 2017) and health risk, such as foodborne diseases (Olivier et al., 2005).

This study aims to characterize and set-up an innovative and fast rehydration technique for the NPC powders, making it possible to obtain homogeneous casein dispersions, completely rehydrated after one hour of rehydration.

2 MATERIAL AND METHODS

2.1 Sample preparation

Powder of NPC with 87% (protein/100g) was provided by Ingredia S.A (Arras), being 92% of the total protein corresponding to caseins. The powders were rehydrated by dispersing it in deionized water at three different solids contents; 8.0 g 100 g- 1, 14.0 g 100 g-1 and 20.0 g 100 g-1, and submitted to two different protocols:

- The control protocol (**CP**): Stirring at 500 r.p.m at 25 °C/24 hours to obtain a reference rehydration level, close to being completed.
- Fast redispersion protocol (**FP**): Stirring at 500 r.p.m at 50 °C/1 hour.

To better understand the impact of the homogenization step, part of the samples rehydrated at 25 °C/24h and at 50 °C/1h were also submitted to further homogenization step. To perform that, the aqueous samples were subjected to a homogenization process at 10.000 rotations per minute (R.P.M) for 5 minutes using a rotor-stator homogenizer (Polytron® PTA 10-35). This protocol is based on a survey of industrial

practices. These protocols will be respectively referenced as CPH and FPH, whether they would have been preceded respectively by control or fast redispersion protocols. As described by the homogenizer manufacturer, the effect of homogenization is due to the spinning rotor that generates a vacuum inducing the samples to pass through the rotor/stator (shear gap). Consequently, it is supposed that the proteins are submitted to a high deceleration tangential and radial acceleration forces, which are able to break the large individual particles down and thus reduced the particles in size (Kinematica AG, 2013).

Finally, to prevents microbiological growth, sodium azide was added to 0.03 % (g/100g) (Sigma Aldrich, France).

2.2 Samples characterization after rehydration protocols

In this work, the particle size distribution was characterized to evaluate the presence or not of undissolved aggregates. It was carried out on aqueous samples using the static light scattering, after each protocol of re-dispersion of powders applied under stirring ([24h/25°C] or [1/50°C]) (corresponding respectively to samples referenced CP and FP) as well and after the subsequent homogenization step (corresponding respectively to samples referenced CPH and FPH).

The apparent viscosity of the dispersions was also determined to characterize the evolution of apparent viscosity with the shear rate of the rehydrated samples (CP and FPH).

2.2.1 Static light scattering (SLS)

The particle size distribution (volume-weighted distribution) was measured using a MasterSizer (Malvern Instruments, Worcestershire, UK) equipped with a 5 mW He-Ne laser operating at a wavelength of 633 nm. Samples were diluted into the Malvern cell (volume: 100 mL) with deionized water to reach the appropriate obscuration (25%±2). Refractive indexes for solvent, particle, and adsorption were

respectively 1.33, 1.3, and 0.1. These indexes are recommended by the manufacturer (Malvern).

2.2.2 Rheology

Apparent viscosity measurements were performed using an AR2000ex Rheometer (TA Instruments, Guyancourt, France) equipped with a coaxial cylinder (internal radius = 14 mm, external radius = 15 mm, height = 42 mm). The experiments were conducted at 20 °C, varying the shear rate from 10 to 500 s^{-1}.

2.2.3 Statistics treatments

The results in this present study come from two repetitions and its triplicates. For the data from the particle size characterization (D50), an ANOVA followed by a Tukey test at $p<0.05$ was applied. The same statistical test was also applied at $p<0.05$ for the rheological results. This test was led on the apparent viscosity value for which the apparent viscosity leveled, which corresponds to a shear rate range varying from 200 to 500 (s-1). The statistical treatment of the results is presented in table 1 in the supporting information.

3 RESULTS AND DISCUSSIONS

For the three solids contents investigated (8%, 14%, 20% w/w), the volume distribution in particle size obtained after rehydration at 25 °C during 24 hours, with and without subsequent homogenization step (respectively CP and CPH symbols) are displayed in figure 12.

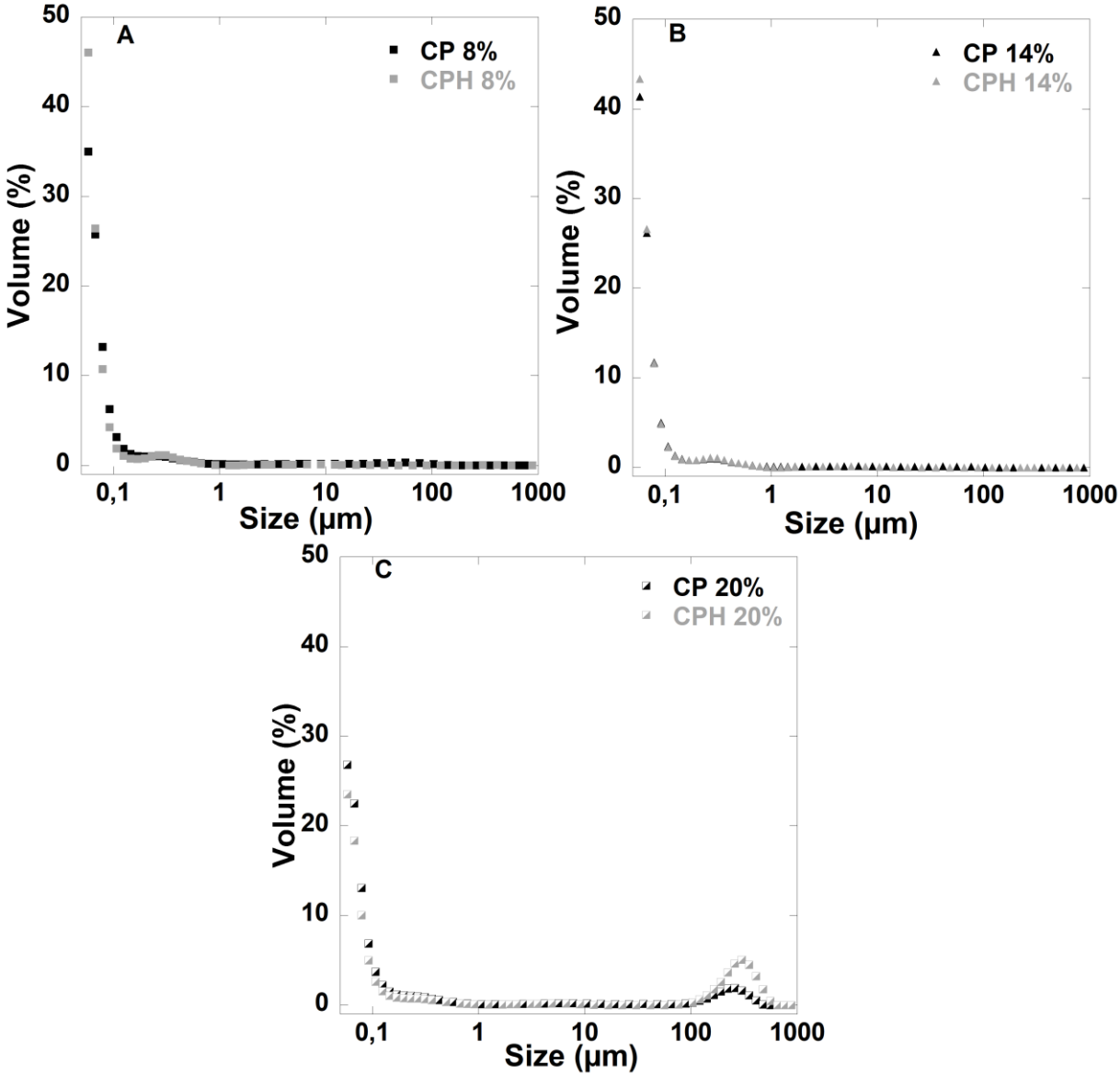

Figure 13 – Particle size distribution of native phospho casein using the control process (25 °C/24h) to three different concentrations: 8% (A), 14% (B) or 20% (C).
Black symbols represent control process without homogenization (CP), and Gray symbols represent control process followed by homogenization (CPH)
Errors bars are not represented in the graphs to facilitate the visualization of the results, but the error is 1.5%, 1.3%, and 1.1% of the average value for 8%, 14%, and 20%, respectively.

Figure 12A and 12B show that for the samples re-dispersed at 8% and 14% (w/w) after 24 hours of rehydration at 25 °C, no significant amount of aggregates is detected (a rough estimate is that aggregate represents less than 1% of the total number of proteins). These results are in agreement with the bibliography, which mentioned that for such temperature and solids contents, a satisfactory rehydration step for these NPC powders (Ji et al., 2016; Richard et al., 2013) is achieved after several hours.

As expected, figures 12A and 12B show that the samples at 8% and 14% (w/w) submitted to additional homogenization steps do not present aggregates either.

In contrast, figure 12C shows that the sample hydrated to 20% (w/w) for which the re-dispersion step is composed only of stirring (CP), presents some amount of aggregates. The volume of aggregate is about 10% of the total volume, and the size is about ~100-300 µm of size, which corresponds to the size of powder particles before the redispersion process.

Figure 12C also shows that, for the 20 % (w/w) of solids content, the homogenization step is not able to decrease the number of large aggregates.

In the same way as previously, figures 13A to 14C display the volume distribution in particle size for the three solids contents investigated (8%, 14%, 20% w/w). The difference with Figures 13A to 13C is that the powder has been re-dispersed by the fast process (50 °C/1 hour), with and without homogenization step (respectively FP and FPH symbols).

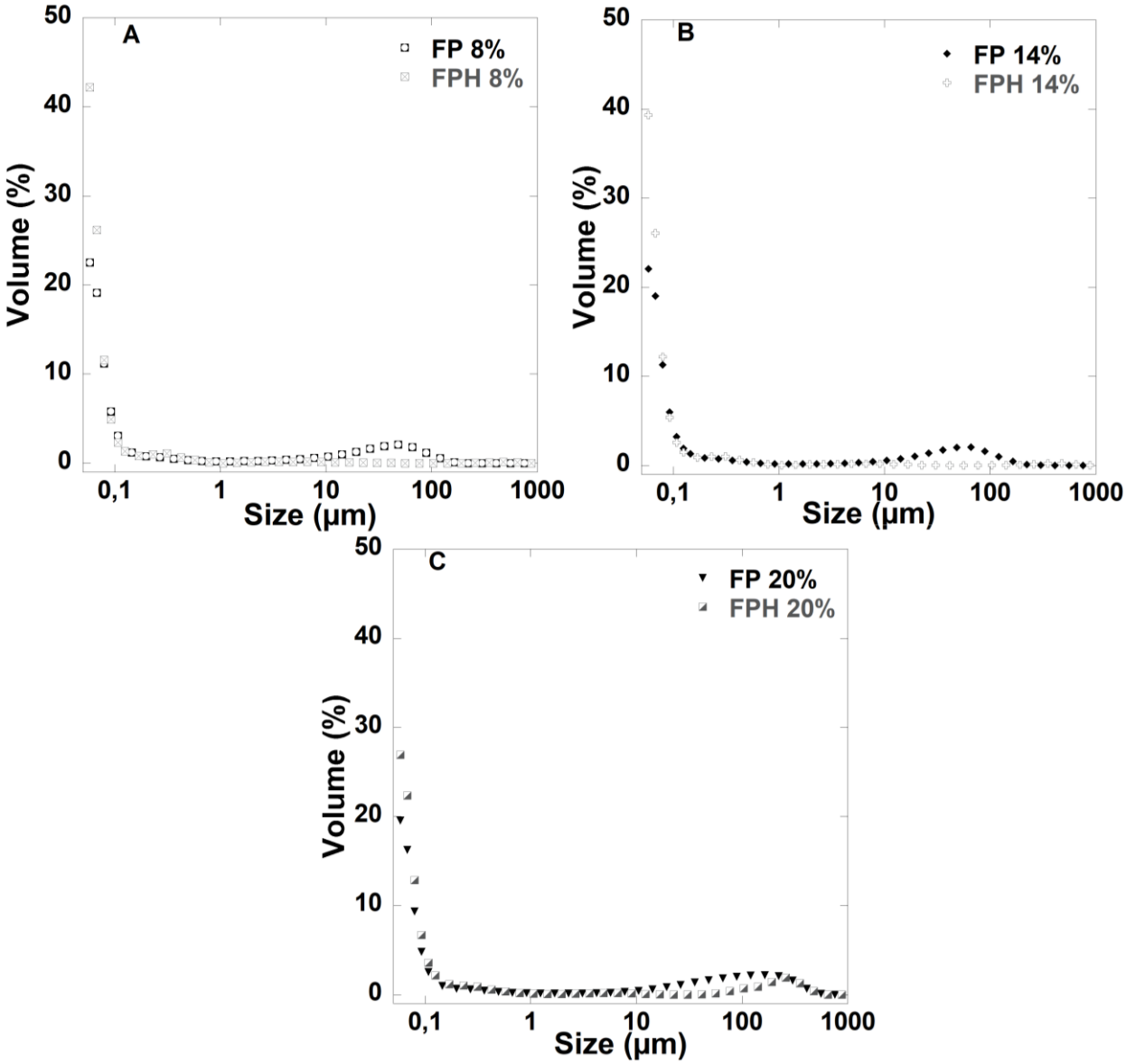

Figure 14 - Particle size distribution of native phospho casein using the fast process (50 °C/1h) to three different concentrations: 8% (A), 14% (B) or 20% (C).
Black symbols represent fast process without homogenization (FP), and Gray symbols represent fast process followed by homogenization (FPH)
Errors bars are not represented in the graphs to facilitate the visualization of the results, but the error is 1.0%, 1.2%, and 1.3% of the average value for 8%, 14%, and 20%, respectively.

Figures 13A to 13C show that, after one hour at 50 °C under stirring without homogenization step, all the samples (8%,14%, and 20% of solids) do display some amount of aggregates (about 10%) in contrast to the control protocol CP (25 °C/24h without homogenization step). These aggregates display a mean particle size ranging

from 80 to 200 µm and which increases with the solids content of the dispersion (in the range 8 to 20 % (w/w)).

Figures 13A to 13C also illustrate that the subsequent homogenization step can dissociate practically all the observed aggregates in the dispersions till 14% of solid content. On the contrary, aggregates are still persistent in the dispersion even after the homogenization step for the highest solid content sample at 20% (w/w). In this case, the homogenization is only able to disintegrate the aggregates within the range of particle size from 10 to 80 µm (figure 13C), while the aggregates with a mean particle size above 80 µm are undisrupted. These results are in agreement with the bibliography (Bouchoux et al., 2009), which reported an increase in the total amount of aggregates after rehydration when solid content increases. The comparison between the control process (25 °C/24h) without homogenization (CP) and the fast rehydration process (50 °C/1h) followed by the homogenization (FPH), is presented in figure 14.

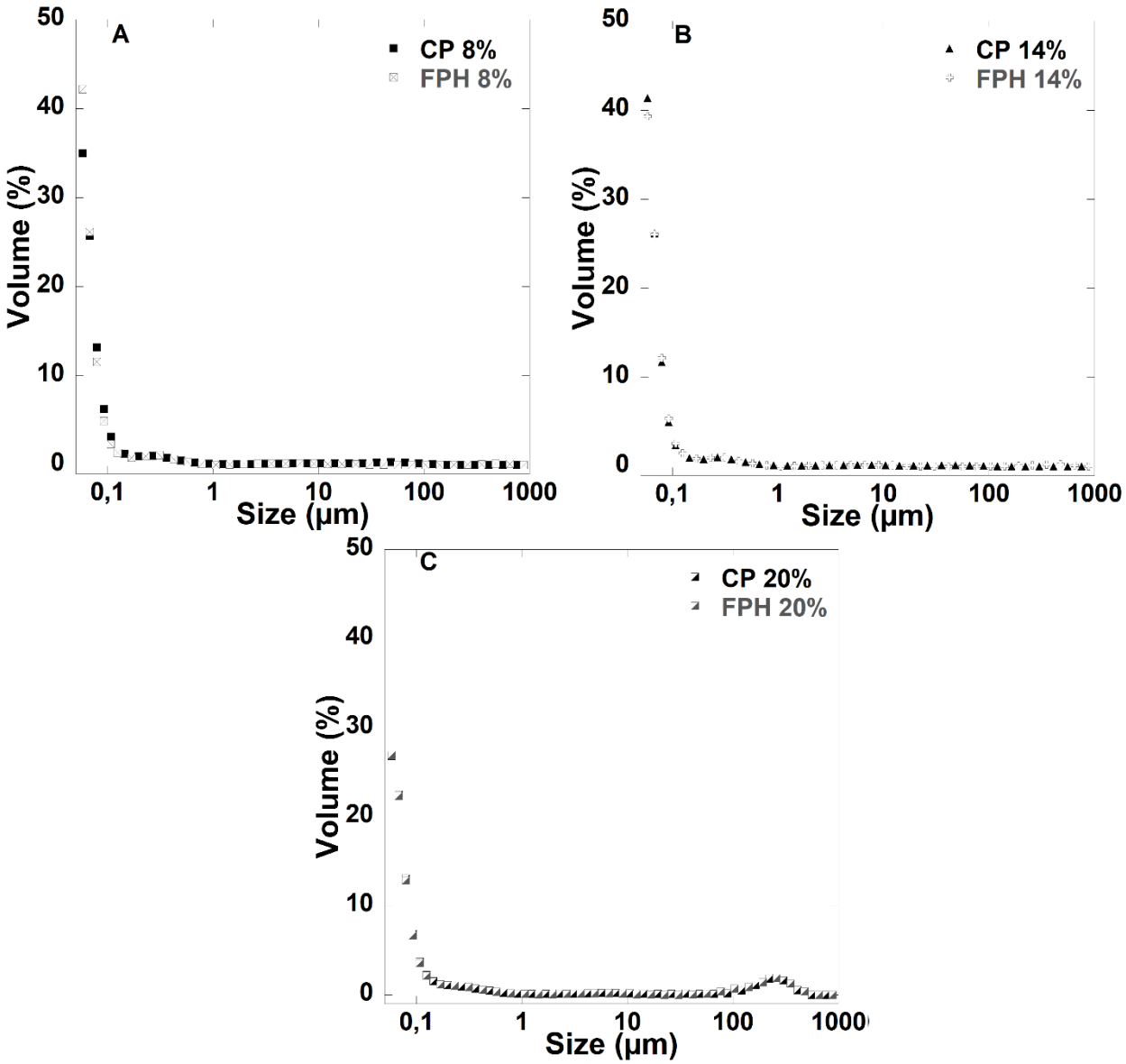

Figure 15 - Particle size distribution of native phospho casein using the control (25 °C/24h) and the fast process (50 °C/1h) to three different concentrations: 8% (A), 14% (B) or 20% (C).
Black symbols represent control process without homogenization (CP), and Gray symbols represent fast process followed by homogenization (FPH)
Errors bars are not represented in the graphs to facilitate the visualization of the results, but the error is 1.4%, 1.1%, and 1.0% of the average value for 8%, 14%, and 20%, respectively.

It can be observed in figures 14A to 14C that the volume distribution in particle size distribution after the fast protocol with homogenization step (FPH) and after the control process without homogenization (CP) are very close, whatever the solid content investigated (8%, 14%, 20% w/w).

This observation is still valid, whatever the existence (Figure 14C) or not (Figures 14A and 14B) of a class range of completely undisrupted aggregates. Moreover, it can be noted that this similarity between the CP and FPH redispersion protocols is also ascertained and reinforced by the statistical tests performed on the medians of the particle size (D50) for the CP and the FPH samples.

The homogenization represents a crucial unit operation for the rehydration of NPC powders, which is capable of limiting the formation of aggregates for all solid content that were investigated (8%, 14%, 20% w/w) in the present study. This process was able to ensure the disappearance of all aggregates for solids content up to 14% w/w.

These results also show that rehydration protocol FPH (which consists of rehydration for 1 hour at 50 °C followed by a homogenization step), enables to mimic the volume distribution in particle size of the NPC powder obtained after rehydration protocol CP (24 hours at 25 °C). In this case, the redispersion leading to near-complete rehydration process is faster than the control process usually performed at ambient temperature, and better adapted to minimize the contamination risks associated with long term rehydration.

As demonstrated previously, the volume distribution in size of the NPC particles rehydrated using the fast rehydration process (FPH) does not differ from the ones rehydrated using the control process (CP). However, it is not excluded that some other macro properties of the rehydrated samples, such as apparent viscosity, especially for these dense dispersions, are kept unchanged. To address this issue, in the next section, we are going to present the apparent viscosity evolutions obtained for the samples rehydrated by CP and FPH protocols.

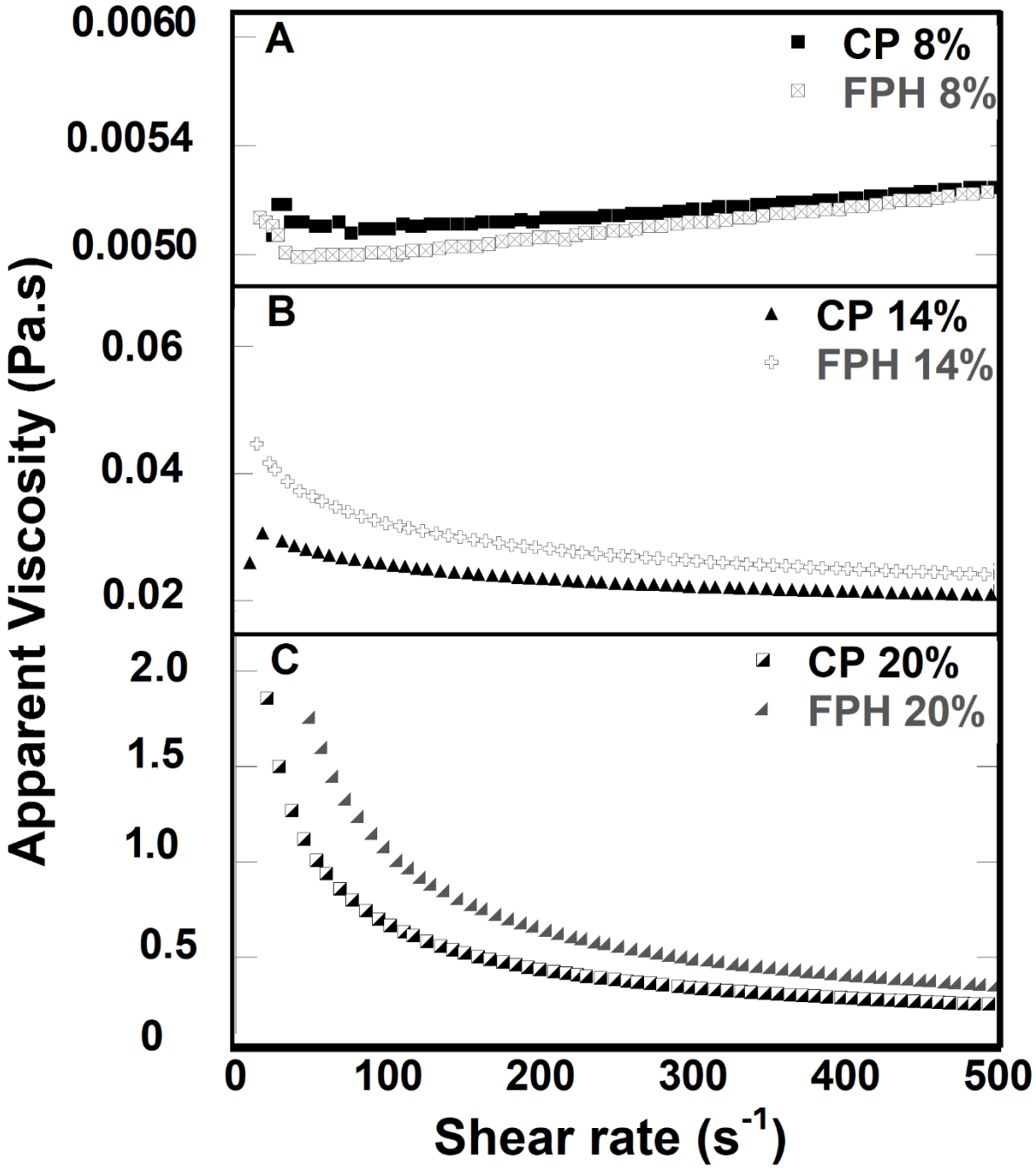

Figure 16 - Apparent viscosity of native phospho casein using the control (25 °C/24h) and the fast process (50 °C/1h) to three different concentrations: 8% (A), 14% (B) or 20% (C).
Black symbols represent control process without homogenization (CP), and Gray symbols represent fast process followed by homogenization (FPH)
Errors bars are not represented in the graphs to facilitate the visualization of the results, but the error is 1.3%, 2.0%, and 1.6% of the average value for 8%, 14%, and 20%, respectively.

The evolution of the apparent viscosity (Pa.s) of rehydrated NPC samples as a function of the shear rate (s^1) is shown in figure 15A to 15C. Each plot corresponds to

a solid content for NPC powder (8%, 14%, or 20%). On each graph, two symbols appear corresponding to the various rehydration protocols adopted (namely CP and FPH).

Comparing the values of the ordinate axis of figures 15A to 15C, one can observe a strong non-linear increase of viscosity as the solid content goes up. This observation is still valid under different rehydration protocols applied (CP or FPH). These results are in agreement with the bibliography (Bouchoux et al., 2009), which reported an increase in the apparent viscosity when solid content goes up.

As observed previously for the particle size distribution, it can be observed, whatever the solid content, that the value of apparent viscosity at 300 s-1 is very close for the samples subjected to control (CP) or Fast rehydration protocol followed by a homogenization step. Indeed, the mean standard deviation is respectively 8%, 15%, and 45% for respectively solid content of 8%, 14%, 20% (w/w). Moreover, the statistical test captures the same trends. For instance, it was possible to affirm that the apparent viscosity of the samples hydrated to 8% does not statistically differ ($p<0.05$) when the samples are hydrated using the control protocol (CP) or using the fast protocol (FPH). The increase of 15% of the apparent viscosity observed for the sample with 14% of solids (FPH in comparison with the CP) is not statistically similar but is not tremendous and acceptable for industrial case.

On the contrary, for the sample hydrated to 20% of solids, the increase of 45% is significant, but some applications with such protein content are not still elaborated on the market of hyper-protein beverages, making necessary the use of a proteins mix, which contains both native and hydrolyzed caseins to reach this massive amount of protein (~20%). Presently, on this date, the standard upper limit of protein content for hyper-protein beverages is 8-10% and few market players developed solutions at 14%. Above this limit, the industry suffers from difficulty in elaborating and stabilizing such an application with native casein protein.

4 CONCLUSION

The objective of this work was to compare the fast rehydration protocol (FPH) (corresponding to a redispersion of powder at 50 °C for 1 hour under stirring followed by a step of homogenization) (FPH) to a protocol of rehydration at ambient temperature (24h at 25°C), on a powder of native phospho casein at higher solids contents (from 8% to 20% (w/w).

Particle size analysis and apparent viscosity measurements show that, even at the highest solid content (20%), it is shown that the fast protocol guarantees the same distribution in particle size and achieves complete rehydration. Moreover, it is shown that till 14% of solid content, without minor and non-significant changes in viscosity are measured. To conclude, the so-called fast protocol could be applied in the field of hyper-protein beverages, where the application requires the use of solid protein content minimum of 6% and manufacture process start with a rehydration unit operation. The application of such protocol is able to reduce the time necessary to rehydrate the NPC powders, being possibly responsible for decreasing energy consumption, microbiological risks, and enzymatic issues.

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Study of commercial casein-based powders: intermediate conclusion

Article 1 (section 3.1) had the objective to evaluate the impact of the demineralization in the structure of the CMs during two different rehydration protocols. It was found that the demineralization associated to high-temperatures during rehydration (50 °C/1h) was responsible for internal changes in the CMs structure which results in aggregation during the rehydration process.

It has been confirmed that the effect of such modifications is real and visible on CM assembly. It was also found that demineralization, during rehydration, is responsible for liberating some reactive chemical groups (hydrophilic and/or charged ones) that favors the formation of hard-to-dissolve particles. It was shown that the effect could be stronger (the particles formed are harder to dissolve) whether rehydration temperature is higher.

To disrupt the aggregates formed during the rehydration, a fast homogenization protocol (1h/50°C) have been proposed and evaluated in Article 2 (section 3.2).
Article 2 demonstrated that the adding of a homogenization step allow a complete rehydration of the casein-rich demineralized powders, resulting into i) dispersions with an homogeneous distribution of particles; ii) CMs sharing almost the same functional properties (viscosity and particles size distribution) than the native casein-rich powder when homogenization step is not undertaken and rehydration made at ambient temperature.

However, the results of these two articles were only obtained from commercial casein-rich powders, which does not allow to have an overview of how the degree of demineralization affects and whether industrial manufacture led to similar results.

To go further in the understanding of the effect of the demineralization in the CMs structure, four casein-rich powders were elaborated with different degree of demineralization at pilot-scale and the results will be presented in the next two subchapters of the results (corresponding to articles 3 and 4).

Second period of PhD thesis: Study of a case of pilot-scale casein-based powders

The second period of PhD thesis concerns the study of four pilot-scale casein-based powders with different demineralization degrees. Precisely, the dispersions studied in this part of the study are: a native casein-based one, which is used as a control, and three calcium demineralized casein-based dispersions, which corresponds to: a 5%, a 10%, and a 25% calcium-depleted.

For this period, the fast protocol of rehydration (see section 3.2) was applied to rehydrate quickly the casein-based powder to produce a dispersion with 14% of protein (% of protein/100g).

The changes in the casein micelle structure that can be related to the calcium depletion are the core subject of article 3

The objective was clearly to highlight the scientific question that was previously addressed (see section 2.6), and precisely to find out whether changes in the CMs structure (article 3) are gradual with the degree of demineralization of the powders.

Article 4 concerns the structural and rheological changes of the 14% casein dispersions submitted to the UHT process. In Article 4, the same samples used in article 3 were handled, only adding the UHT treatment step.

The UHT treatment used in the present study was performed at a laboratory scale, corresponding to a sterilization treatment of 140 °C/10 seconds. The UHT is used in the production of ready-to-drink beverages to produce microbiologically stable beverages.

The objective to article 4 was to whether it possible to correlate the changes in the CMs (originated from the demineralization) and the rheological behavior after rehydration and UHT treatment?

1

3.3 The heterogeneous substructure of casein micelles: how demineralization trigger internal structural modifications of the micelle

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ABSTRACT

The casein micelle (CM) is a sponge-like protein assembly held together by protein mineral interactions between phosphoryl groups from the protein, and several internal calcium phosphate nanoclusters (CCPs). In the present work, the structure of a native and three different demineralized CMs samples with less; 5% (DM-05), 10% (DM-10), and 25% (DM-25) of calcium in relation withthe native, have been analyzed. In the less demineralized sample (DM-05the number of CCPs is significantly reduced (-15%) in relation to the native, but only very few changes in the CM internal structure have been detected. In the more demineralized samples (DM-10), and (DM-25) the amount of CCPs is only moderately reduced (~--2% and -10% in relation to DM-05), but strong internal structural modifications have been detected. These data reveal that the first CCPs removed from the CM are weak attached and play little role in keeping this protein assembly. In contrast, in more demineralized samples, the CCPs removed are strongly attached to the CM and play a major role in keeping the cohesiveness of the CM. Finally, in agreement with all the observations, an improvement in the actual CMs sponge-like model of Bouchoux (2010) is proposed. It is suggested that not all the CCP are located in the interior of the "hard" structures of the CMs, but some of them are located in their borders (in less dense regions of the micelle), facilitating their

detachment and explaining the varying internal structure modification observed with course of demineralization. **Keywords**: Casein micelle nanostructure, demineralization, calcium phosphate clusters, NMR.

1 INTRODUCTION

In recent years, a drastic increase in the market of high-proteins beverages (HP-beverages) has been observed (International Euromonitor, 2018), corresponding to a wider audience. Both clinics and sports nutritions drive the growing interest in HP-beverages. Indeed, on the one hand, patients recovering from malnutrition and/or affected by cancer or by muscle degenerations need a low-volume dietary treatment that contains plenty of high-quality protein. On the other hand, athletes of all age groups encompassing a large variety of lifestyles, are looking for easy, high-quality nutrition products (Jäger et al., 2017).

In this context, the milk proteins, especially the whey proteins, represent an excellent protein source due to its nutritional profile (essential amino acids), excellent digestibility properties, and pleasant taste, justifying to be the principal protein ingredient to the manufacture of HP-beverages (Rittmanic, 2006). However, the use of the whey proteins for HP-beverages presents an inconvenience: their instability to heat treatments (Sadeghinezhad et al., 2013).

Indeed, in the case of HP-beverages, the ultra-high-temperature (UHT) is usually applied to produce shelf-stable ready-to-drink beverages that are microbiologically stabilized by the commercial sterilization provided by the UHT treatment (Jordan, 1968).

To overcome the problem of heat-instability, the caseins, major protein present in the cow's milk (Walstra et al., 2006), can be considered an alternative source of protein to the production of the HP-beverages. Indeed, the caseins represent an excellent alternative to produce HP-beverages. The caseins are present in milk in a higher amount when compared to the whey protein (~80/20) (Walstra et al., 2006). In the nutritional aspect, they also present excellent digestibility, high amount of amino acids, especially leucine (Vickery & White, 1933). The most significant advantage associated with the use of the casein for HP-protein is related to its high heat-stability (Crowley et al., 2014), compared to the whey proteins. Indeed, as described in the literature (Beliciu et al., 2012), the caseins as a form of casein micelle (CMs) are

resistant to heat-treatments that are usually applied at the industrial level to produce sterilized beverages.

Unfortunately, in the case of the CMs, a limiting factor to their use as an ingredient for HP-beverages is associated with their high-viscosity in concentrated regimes (over 8% of protein) (Bouchoux et al., 2009; Dahbi et al. 2010). It is known that the structure of CM regulates the apparent viscosity of the dispersions since the CMs naturally present as a high-hydrated sponge-like polymer (Bouchoux et al., 2010).

A lever often suggested in the literature for changing the native structure of the CMs is the demineralization (Boiani et al., 2017). Indeed, The demineralization changes the structure of the CMs as a result of the removal of the colloidal calcium phosphate nanocluster (CCP), which promotes a mineral equilibrium change between the disperse phase (CMs) and soluble phase (solvent) (Boiani et al., 2018). The native structure of the CMs and its mineral equilibrium based on the sponge-like polymer described by Bouchoux et al. (2010) is represented in figure 16.

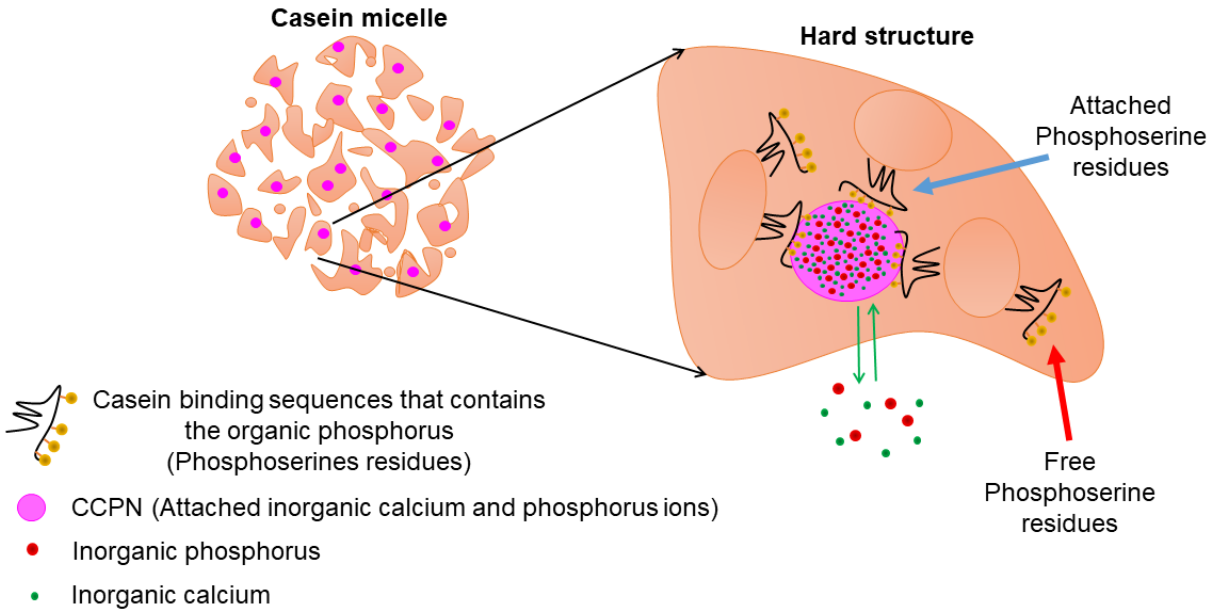

Figure 17 – (Left) Schematic representation of a CM with hard structures (protein assembly) containing all nanoclusters as proposed by Bouchoux et al. (2010). (Right) Zoom into a so-called “hard structure” representing casein micelles binding sequences (phosphoserine residues) interaction with calcium phosphate nanoclusters (CCP) through organic phosphorous from phosphoserine residues. Green arrows represent the equilibrium between calcium and phosphorous ions between the CCP (Colloidal phase) and the soluble phase. One can notice that all nanoclusters (CCP) are “enveloped” by proteins represented in brown (which constitutes the “hard” structures).

The influence of the demineralization on the CMs structure has been reported in several previous publications (Kort et al., 2011; McCarthy et al., 2017). Unfortunately, in these studies, the demineralization is made by the addition of calcium-chelators agents, which are responsible for binding the soluble calcium, resulting in a CCP excision from the CMs and consequently causing undoubtedly a specific CMs structural change (Ramchandran, Luo, & Vasiljevic, 2017). Moreover, there is a crescent demand on the production of clean-label products in the food industry (Asioli et al., 2017), justifying the search for alternatives to the production of this kind of calcium-demineralized CMs-rich powders.

One of the sole examples of studies that do not use calcium-chelators agents to demineralize the CMs was conducted by Silva et al. (2013). Indeed, the authors in their study have associated acidification to several steps of dialyzes (membrane separation techniques) to ensure the demineralization from the CMs.

As described by Broyard & Gaucheron (2015), at more acid pH, the CCP is progressively released from the colloidal phase to the soluble phase. As a consequence, increasing rates of calcium and inorganic phosphate are present in the soluble phase altering the structures of the CMs and, as a consequence, its technological functionalities.

The study of Silva et al. (2013) has emphasized mainly to illustrate that a link exists between the demineralization and foam stability; even if the authors suggest that these results were associated with changes in the CMs structure, this group does not investigate in-depth the effect of the process of demineralization on the CMs structure.

Another lack in the pioneering work of Silva et al. (2013) is the fact that the powders were produced by lyophilization. In the dairy industry, the most usual method used to produce casein-rich powders is spray-drying (Tamime, 2009). It is not evident that this spray drying unit operation, which involves much more severe transport phenomena for heat, has a negligible impact on the CMs structure and that the suggested modification of the CMs structure is still present after rehydration at high protein concentration, such as HP-beverages.

This work has for main objective to partially fill the gaps aforementioned and to better illustrate how the evolution of CMs structure happens with the demineralization level.

In the present study, the CMs concentrates have been firstly demineralized, such as described by Silva et al. (2013), and the casein-rich powders were produced by spray drying, following the industrial practices. Then the CMs rich-powders ingredients were rehydrated to 140g/L, protein concentration close to the demands of the market for HP-beverages. Finally, different analytical techniques (Nuclear magnetic resonance, Small-angle x-ray scattering, Fluorescence spectroscopy) were performed to identify the change of CMs structure associated with the different levels of demineralization.

2 MATERIALS AND METHODS

2.1.1 Samples identification

The casein-based powders used in the present study were provided by Ingredia S.A (Arras, France). The powders were produced into the industrial pilot plant of Ingreda S.A using the traditional drying conditions, which can be found described in the literature (Pierre et al., 1992; Schuck et al., 1994).

All the powders contained over 82% (w/w) of protein and were mostly composed of caseins, representing over 90% of the total nitrogen from the powders (see table 1 supporting information for details). Four powders were used in the present study, being:

- A native casein-based powder (Native) was used as control.
- A 4.47% calcium-demineralized powder (DM-05).
- A 9.16% calcium-demineralized powder (DM-10).
- A 25.73% calcium-demineralized powder (DM-25).

The four powders were identified as described previously as the next entire value closest to the real demineralization value.

2.1.2 Demineralization and powder production

The calcium-demineralized CMs-rich powders start from skimmed milk, which was acidified to three different pHs (6.4; 6.2; and, 5.9) by the addition of lactic acid. This process permits the excision of part of the calcium from the CMs, as described by the literature (Broyard & Gaucheron, 2015).

The protocol that was carried out in this present study is close to the one that was described by Silva et al., (2013), which describes a decrease of about 6%, 14%, and 23% of the total calcium present in the CMs concentrate for calcium-demineralized CMs dispersions prepared by acidification with hydrochloric acid to the three pHs values (6.4, 6.1 and, 5.8). Then the acidified skimmed milk is left at 10° C/10 hours (maturation time) to ensure that the complete equilibrium is achieved.

After the maturation time, the milk is submitted to a protein concentration step, which consists of an ultrafiltration process, with a membrane cut-off of 10 kDa. This ultrafiltration process is responsible for concentrating the proteins present in the milk by remotion of the water and soluble salts (Carvalho & Maubois, 2010).

The concentered of proteins are then submitted to separation membrane techniques, consisting in two subsequent microfiltration unit operation. The first microfiltration was conducted with a membrane cut-off of 1.4 µm pore, which is capable of removing bacteria. The second microfiltration was performed using a 0.1 µm pore size membrane, aiming at removing constituents of the soluble phase (whey proteins, lactose, soluble minerals) and simultaneously at concentrating the CMs (Pierre et al., 1992).

2.1.3 Powder rehydration

The casein-rich powders were rehydrated with deionized water to 14.0 g (protein) x 100 g^{-1} (water) submitted to stirring at 500 r.p.m at 50 °C/1 hour. Three drops of antifoam silicon solution were added to each powder dispersion at the beginning of the rehydration to prevent the foam formation. The pH was adjusted to 7.0 with NaOH 1M. After pH adjustment, the samples were homogenized at 10000 r.p.m for 5 minutes using a rotor-stator homogenizer, Polytron PT 10-35a (Kinematica, France). Antimicrobial agent sodium azide was added to 0.3 g L^{-1} (Sigma Aldrich, France) to prevent microbiological growth.

2.1.4 Fluorescence measurements

Fluorescence spectroscopy (using tryptophan as a probe) has been used to measure the change in the number of hydrophobic interactions in the CM. The Fluorescence analysis method was the same as in a previous paper of our group (Nogueira et al., 2020) using a Fluoromax-4 spectrofluorometer (Jobin Yvon, Horiba, NJ, USA). The analysis was conducted with an angle of the excitation radiation set at 60°, being the temperature controlled by Haake A25 AC200 temperature controller

(Thermo-Scientific, Courtaboeuf, France) set to 20 C. Samples were poured into a 3 mL quartz cuvette and the emission spectra of tryptophan residues in a wavelength from 305 to 450 nm after excitation set at 290 nm.

2.1.5 CMs structure organization observed by Small-angle X-ray scattering (SAXS)

In the present study, the SAXS was applied to investigate the structure of the CMs at different levels. As described in the literature (Bouchoux et al. 2010) the SAXS spectra of the CMs is representative of three levels which correspond to different structures forming the micelle: i) the level 0 (zero) which is the micellar envelope (of about 100 nm of diameter) corresponding to a Q range 0.0065 to 0.0010 nm^{-1} and, in the present concentration, to the inter distance between micelle; ii) the level 1 (one) which corresponds to smaller structures (20 nm of diameter) described as “hard,” which are related incompressible structures (Ingham et al., 2016), once submitted to osmotic pressure, within the micelle corresponding to a Q range of 0.042 to 0.27 nm^{-1}; iii) the level 2 (two) which corresponds to the structure of the CCP, and proteins associated (about 5 nm of diameter), assigned to an apparent “shoulder” at a Q range between 0.042 to 0.27 nm^{1}. Bouchoux et al. (2010) have represented the so-called “hard” (~20 nm) structures as filled regions containing several CCP (~5 nm).

The SAXS measurements were conducted as described in (Nogueira et al., 2020). All SAXS acquisitions were performed at room temperature (~25 °C) at the French national synchrotron facility SOLEIL in Gif-sur-Yvette, France, on the SWING beamline operating at ~12 keV of photon energy. The SAXS intensities were recorded on a detector placed at ~0.5 m and 6.5 m from the sample. For each sample, data were first recorded at short exposure time (typically ~0.2 s) to prevent any radiation damage.

2.1.6 Nuclear Magnetic Resonance (NMR) Spectroscopy quantification of attached phosphoserines and nanoclusters phosphorous

In the present study, the NMR was utilized to characterize the four different species of phosphorus present in the CMs dispersions (see figure 1): Organic phosphorus from phosphoserine residues that are attached to the CCP, organic phosphorus from the phosphoserine residues that are "free" (displaying higher dynamics since not attached to a CCP); inorganic phosphorus present in the soluble phase and inorganic phosphorous forming (or attached) to a CCP.

The NMR analysis was conducted as described by Nogueira et al., (2020) using a Bruker AVANCE I; 9.4T (1H: 400 MHz; ^{31}P: 161.9 MHz) spectrometer which was used to measure the ^{31}P spectra, proton (^{1}H), cross-polarization (CP) and the t1 (direct correlation between ^{31}P and ^{1}H).

More specifically, Bruker AVANCE I; 9.4T (1H: 400 MHz; ^{31}P: 161.9 MHz) spectrometer was used to measure the ^{31}P spectra, proton (^{1}H), cross-polarization (CP) and the t1 (direct correlation between ^{31}P and ^{1}H). The 4 mm probe heads were set with samples and submitted to 700 Hz of Magic Angle Spinning (MAS) speed. Quantitative experiments at 25°C of ^{31}P were done with high power decoupling- recycle delay: 30s; 90° pulse; RF field (^{31}P): 65 kHz; ^{1}H decoupling (RF field: 60 kHz; SPINAL64); 1024 accumulations. The chemical shifts were given in parts per million (ppm) concerning the analysis of H_3PO_4 (85%) for ^{31}P NMR spectra at 0ppm.

In the quantitative spectra, the area of the different peaks in the spectrum corresponds to the relative abundance of each phosphorus species obtained from the 31P-NMR analysis). The NMR spectra were simulated using the DMFIT software (Peixoto et al., 2017; Massiot et al., 2002) to access the area of each peak that forms the ^{31}P-NMR spectra and are correspondent to the different species of phosphorus present the CMs dispersions. The peaks decomposition was presented in figure 1 in supporting information. The signal from the native sample used as representative of the 100% of the signal for the attached phosphoserine and CCP and was used as a control for the demineralized samples.

Concomitantly, a NMR ^{1}H -^{31}P cross-polarization experiment have been made to study the calcium/phosphorous concentration in the CCP by studying the NMR signal of the organic phosphorous from the phosphoserines residues, attached to the CCP. The principle is that, in our conditions organic phosphorous from the

phosphoserines residues display a chemical shift anisotropy (CSA) signal and the CSA is sensitive to the ionic environment of the phosphorous (Gardiennet-Doucet, Assfeld, Henry, & Tekely, 2006). Thus, ^{1}H- ^{31}P cross-polarization experiments strengthen specifically the chemical shift anisotropy (CSA) signal of the attached phosphoserines phosphorous (Peixoto et al., 2017) and, by doing so, allows a better access the CSA parameter of the organic phosphorous.

2.1.7 Statistical analysis

The data present in this study were analyzed using an analysis of variance (ANOVA) followed by a Tukey test at $p<0.05$ and a principal component analysis was performed for the data from the fluorescence spectroscopy.

3 RESULTS and DISCUSSION

3.1 Demineralization impacts on the internal organization of the CMs structure

3.1.1 The stability of hydrophobic interactions evaluated through fluorescence spectroscopy

In the present study, no significant difference of fluorescence intensity, or λ-max (the wavelength in which is the tryptophan of the sample presents the maximum of its absorbance), has been observed between the native CMs and the three calcium-demineralized CMs samples (figure 3 in supporting information).

These results demonstrate that the molecular environment of the tryptophan did not display a significant change, even for the most demineralized samples (DM-25) in comparison to the native samples. These results prove that demineralization did not disturb hydrophobic interactions between proteins.

As described in the literature (Walstra et al., 2006), the CCP are attached to the hydrophilic or charged structures of the CMs (manly composed by phosphoserine and also with some glutamate residues). In the case of the present study, the demineralization that induce depletion of CCP from the CMs should be responsible for changes in the hydrophilic interactions between the internal proteins that form the CMs, being responsible, indirectly, for creating new hydrophobic protein-protein interactions (Horne, 2017).

3.1.2 Organic and inorganic ^{31}P distribution and the environment by NMR

The decrease in the amount of CCP is an essential factor governing the CMs structure since CCPs act as cross-linking centers in casein (De Kruif & Holt, 2003). Calcium and phosphorous ions within the CCPs are in equilibrium with the ions in the soluble phase, and this equilibrium depends on the chelating properties of the local structure of the micelle, for example, the number of organic phosphorus attached to

the phosphoserines residues from the proteins attached to the cluster (see figure 16) (Bijl et al., 2019).

Figure 17 presents the ^{31}P-NMR spectra correspondent to the organic phosphorus from the free phosphoserine residues and inorganic phosphorus present in the soluble phase. As observed in figure 17, the more demineralized is the sample (DM-25) the greater is the amount of free phosphoserine residues.

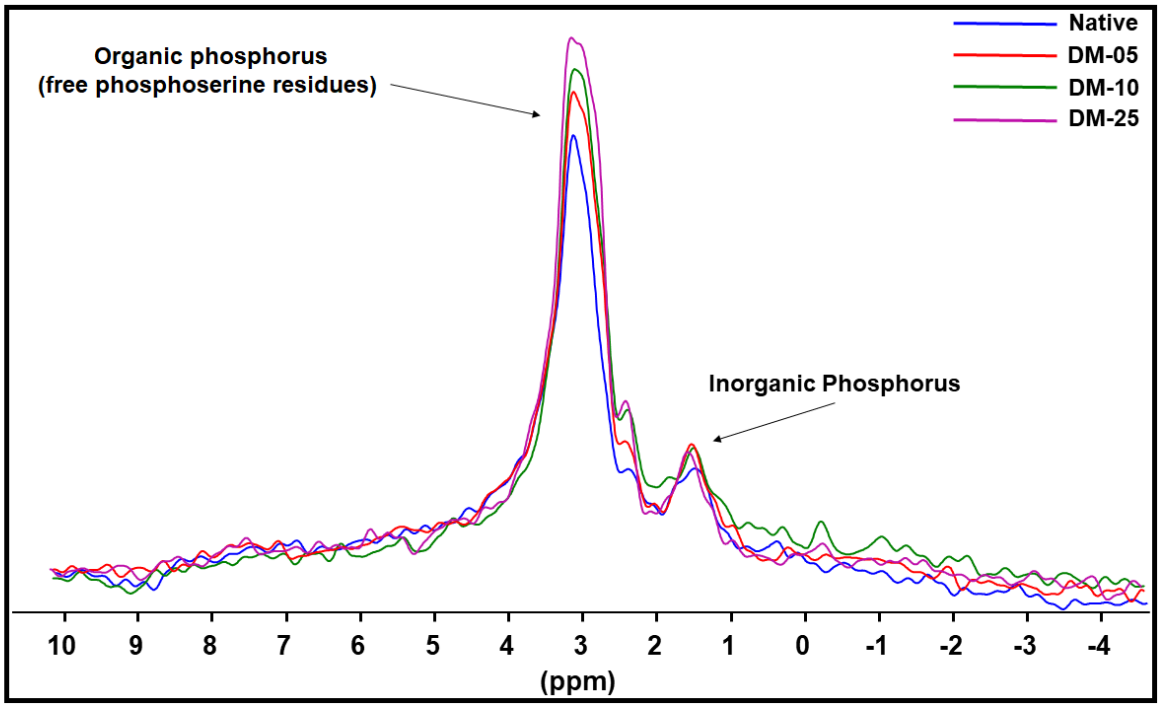

Figure 17 – ^{31}P spectra obtained using the nuclear magnetic resonance (NMR) spectroscopy from casein samples with different demineralization levels, being: (Native) = Native casein; (DM-05) = 4.47% demineralized casein; (DM-10) = 9.16% demineralized casein; (DM-25-25) = 25.73% demineralized casein.

Concomitantly to the increase in the number of free phosphoserine residues as observed represented in figure 17, for the demineralized samples, a decrease in the number of attached phosphoserine residues (figure 18) and attached inorganic phosphorous was measured.

The decrease in the number of attached phosphoserines residues has been reported to be related to the loss of phosphoserine residues attached to the CCPs as a result of CM demineralization (Famelart et al., 2009).

In accordance with the literature, in figure 18, it can be observed that the amount of inorganic attached phosphorus displays the same trends as the CCP.

Quantitatively, the results from ^{31}P-NMR (figure 18) reveal that the decrease of attached phosphoserines and attached CCP as a function of demineralization display a two steps relation: there is a more substantial loss of attached species (phosphoserine residues and CCP) for the less demineralized samples (between native and DM-05) than for the most demineralized samples (between DM-05, DM-10, and DM-25) as a function of the demineralization level. Indeed, the difference in the total calcium content between the native and DM-05 is only 5%, but this represents more than 15-17% of the loss in the attached inorganic phosphorus. In contrast between DM-10 and DM-25, there is only a loss of 10% in the total calcium content corresponding to a loss of 10% of the attached inorganic phosphorus (CCP).

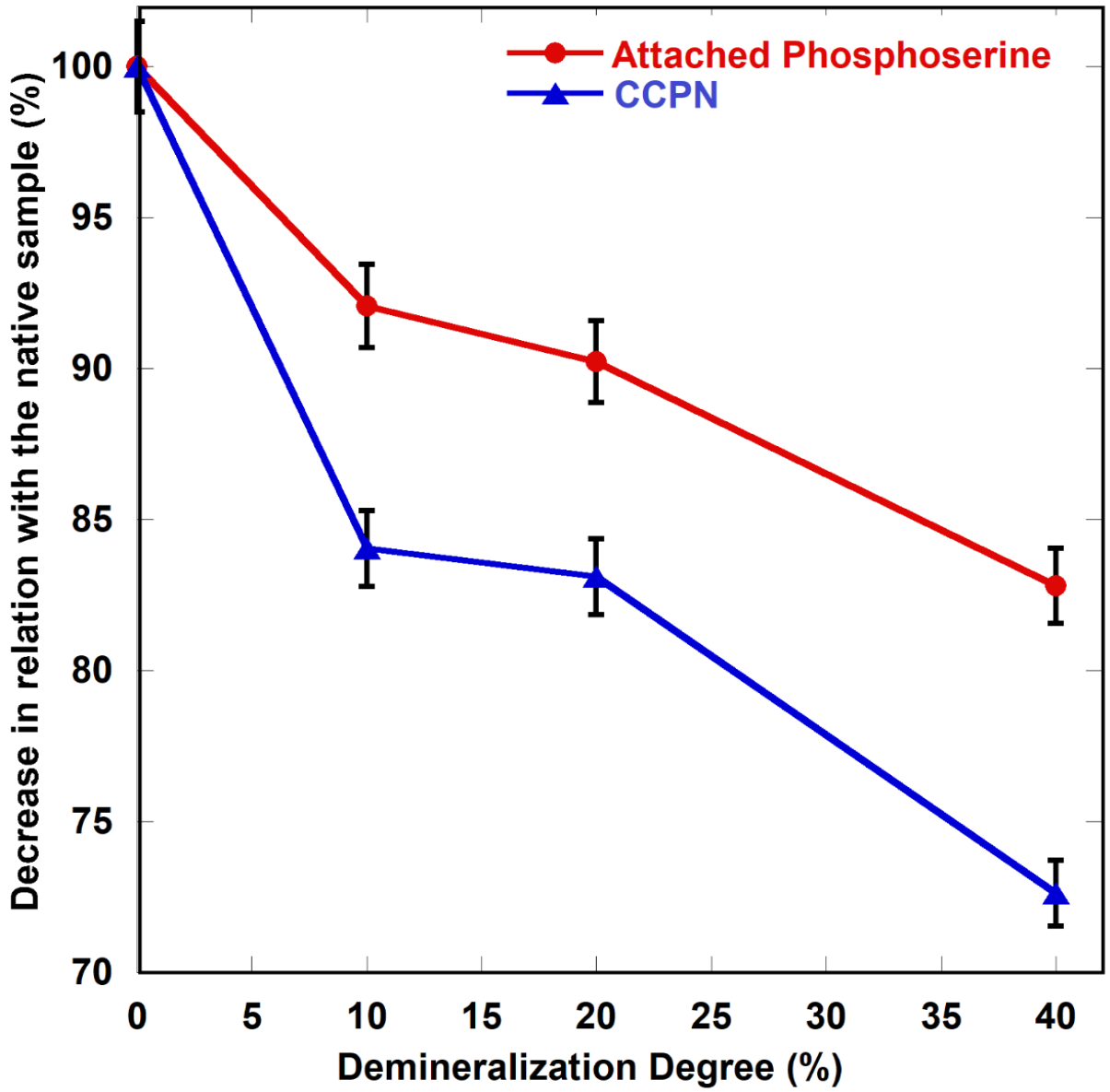

Figure 18 - Decrease in the number of attached phosphoserine (P-Ser) residues (●) and CCP (▲) as a function of the different demineralization degrees of casein dispersions. These results were obtained from the differences obtained from the fitting of the ^{31}P-NMR of a Native casein micelle dispersion and three different degrees of demineralization (DM-05) 04.47% less calcium; (DM-10) 9.16% less calcium and (DM-25) 25.73% less calcium.

The signal of the attached phosphorus from the phosphoserine residues, in this work, also displays a clear CSA (chemical shift anisotropy) shape (Figure 1 in supporting information). As explained in the experimental section, the shape of the

CSA is informative about the proximity of cations around phosphorous from the phosphoroserine (Gardiennet-Doucet et al., 2006). The present data (Figure 1 in supporting information) show that demineralization induces only small changes in the CSA shape of attached phosphoserines suggesting that, even in strong demineralized samples, most of the remaining CCP cluster keeps a near-native composition in terms of Ca/P ratio.

Since this result indicates that even in the most calcium-demineralized sample (DM-25), the Ca/P ratio in the CCP remains close to the one in the native sample, the observed loss of inorganic phosphorus is likely to correspond to instead to equal loss of calcium from the clusters.

3.1.3 Internal structures reorganization evaluated through SAXS:

In this section, SAXS data have been used to observe the impact of demineralization over the cluster size, as well as over CM structures (~5 to 50 nm). Each “shoulder” of the SAXS profile (figure 19) can be assigned to a characteristic structure of the micelle (see details in the experimental section).

As it concerns CCP, one can be noticed that demineralization induces some decrease in intensity of the corresponded “shoulder” (figure 19a corresponding to level two), but there is no detectable shift. This lack of shift in the shoulder position means that there is a decrease in the amount of CCP structures giving origin to this signal but no critical change in the size of the cluster or shape for the remaining clusters (Bouchoux et al. 2010). One can notice that such a relative decrease in the “shoulder” intensity correlates quite well with the relative decrease in the amount of phosphorous in the CCP clusters detected by NMR as a function of the demineralization level. Indeed, as it is the case for the amount of organic and inorganic phosphorus measured in figure 18, from the Native sample to DM-05, there is a significant loss of SAXS intensity for this “shoulder”, but not a loss as strong as for the other demineralized samples (DM-10 or DM-25).

The results of the present study (figure 19) show only a decrease of intensity for the shoulder that is correlated to the CCP. These observations indicate that

demineralization does not reduce the size of the clusters but, instead, remove the clusters entirely (the more unstable ones first) in a cooperative like-way.

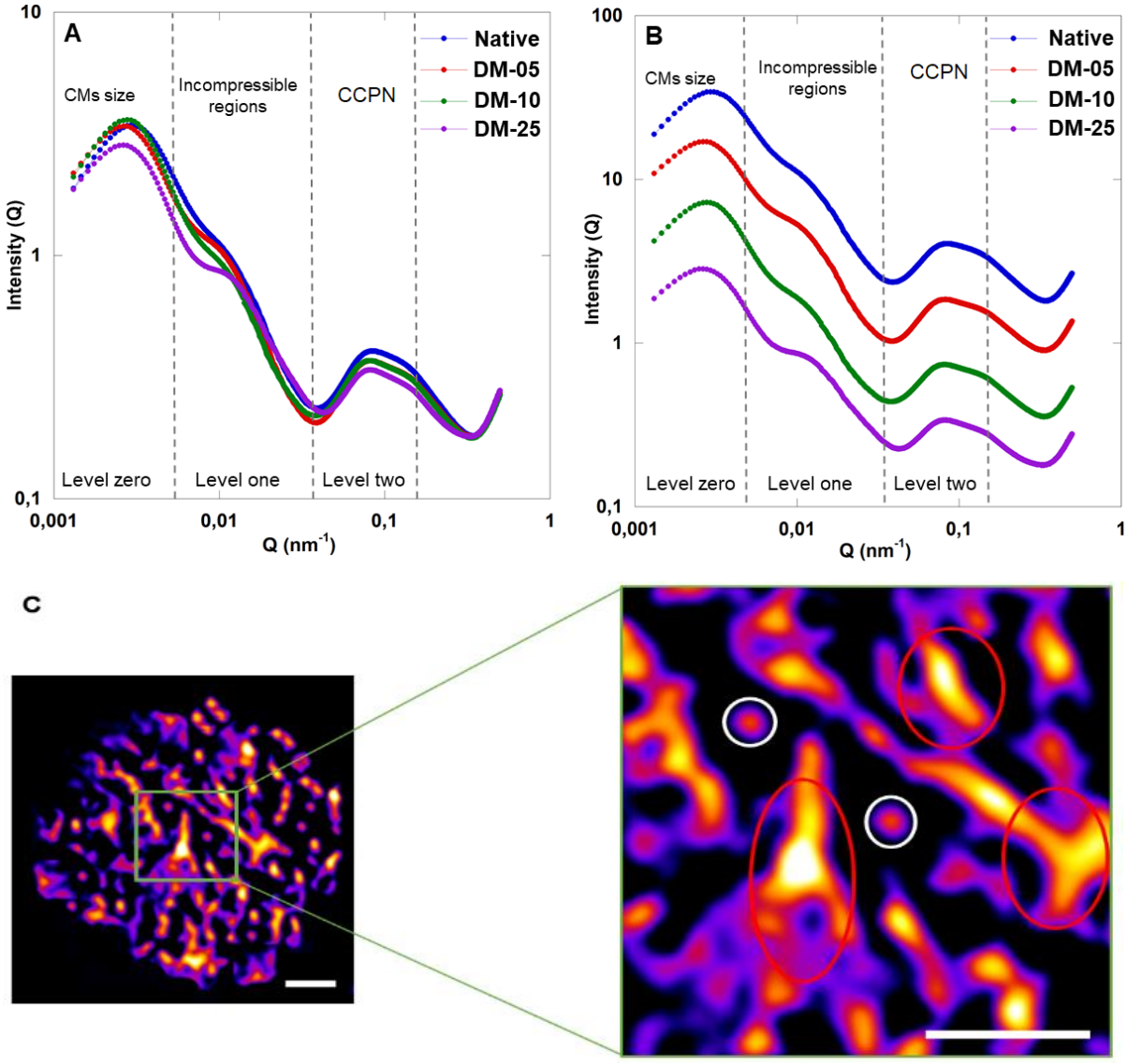

Figure 19 – Internal characterization obtained through Small Angle X-Ray Scattering (SAXS) of casein samples with different demineralization levels, being: (Native) = Native casein; (DM-05) = 4.47% demineralized casein; (DM-10) = 9.16% demineralized casein; (DM-25) = 25.73% demineralized casein.

(A) = SAXS spectra from all the samples, Results from an average of three repetitions and its duplicates.
(B) = SAXS spectra from the separated samples*;
* To obtain this graphic representation, the intensities were multiplied by a factor only to present samples separately.
(C) Schematic representation of an internal structure of a CMs, being the red circles representing the «hard» structures that contain the CCP and the white circles the CCP present in the "void" regions, scale bar correspond to 50 nm.

The three levels are represented in figure 5A, being: Level zero, which corresponds to the CMs size; Level one, which represents the "hard" incompressible structures, and level two that are characteristic of the CCP region.

Looking now to the second "shoulder," the one associated with the so-called "Hard" structures (~10 to 40 nm) in the CM (Bouchoux et al. 2010), the detected decrease in intensity of these regions seems to be uncorrelated to the decrease in the intensity of the CCP "shoulder." Indeed, comparing the Native sample with DM-05, one can notice no remarkable changes in the intensity or the shape of the profile of the "Hard" structures. It seems that the substantial loss of CCPs detected by SAXS and NMR did not affect much the structures of the micelle at this scale range. In contrast, passing from DM-05 to DM-10 and, subsequently, to DM-25, there is a small and progressive shift of the "shoulder" of these "Hard" structures to larger Q. These shifts indicate that demineralization induces a reorganization of these "Hard" structures in the strongest demineralized samples.

Noting that the interpretation concerning the last "shoulder," the one at the largest scale and the lowest Q, is not possible from our data alone since this signal represents mixture of signals coming from the CM gyration radius (the form factor of the CM) as well a change in the average inter distances between CM in solution (the structure factor). This last factor is susceptible to change with a change in the size of the micelle (which will decrease the average CM inter distance in solution) as well with a change in stickiness and repulsiveness between the CMs (which will affect the distribution of inter distances between CM in the solution).

4. Discussion

In the present study, the NMR and SAXS analysis of the three calcium-demineralized CMs samples reveal that the CCP removal from the CMs structure is not linearly related to the amount of calcium that is depleted. In the first demineralization level (DM-05) the demineralization the excised CCPs are the ones that are not strongly attached to the internal protein structures. In this first level of demineralization, there is no significant modification of the internal CM structures (the

"Hard" structures). However, In the subsequent demineralization levels (DM-10 and, DM-25), the calcium and phosphorous ions are stronger attached to the CCPs and the demineralization has a stronger impact on the internal structures of CM (the "Hard" structures).

As preconized in the study conducted by Bouchoux et al. (2010), the internal proteins that form the CMs are organized as species of incompressible structures under osmotic stress (the so called "Hard" structures). The authors propose that all the CCPs are present inside of these hard incompressible structures (as represented in figure 1). The results in the present study are better explained if there are some CCP that are not within these "hard" structures but in their borders (as displayed in figure 5). Indeed, if some CCPs (about 15%) are located in the borders of such "hard" structures it will explain why no significant change in the "hard" structures is detected while removing these CCPs. In contrast, the removal of the CCPs located within the "hard" structures (in DM-10 and DM-25) will have a stronger impact in this lasts (as displayed in figure 20).

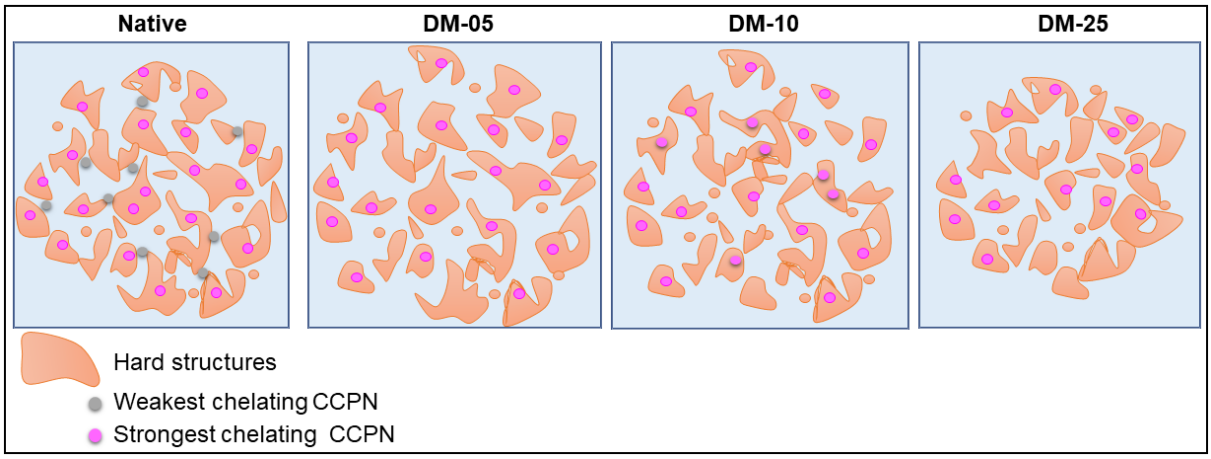

Figure 20 - Schematic representation of the changes observed in the internal structure of the CMs as a demineralization consequence.

This schematic representation represents the four different samples that were used in the present study, being: (Native) = Native casein; (DM-05) = 4.47% demineralized casein; (DM-10) = 9.16% demineralized casein; (DM-25) = 25.73% demineralized casein.

This proposition also explains why it is harder to remove ions in the lasts levels of demineralization (DM-10 and DM-25) than at the first levels (native and DM-05). It is reasonable to think that CCPs located in the borders of the "hard" structures are

likely to display fewer protein-CCPs interactions than the ones in the core of the “hard structures”, as displayed in figure 21. CCPs with fewer protein stabilizing residues (as phosphoserines residues) interacting with the CCP ions should decrease the CCP stability (Bijl et al., 2019; Cross et al., 2005).

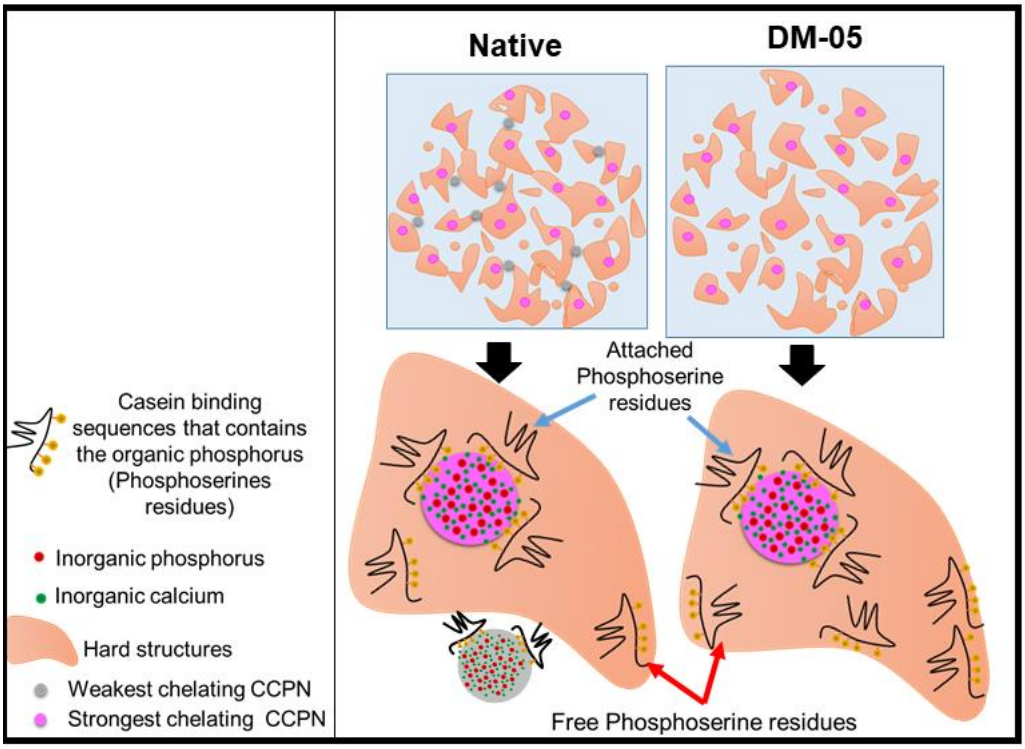

Figure 21 – Schematic representation of the CMs formed by a conjunct of hard structures made by protein assembly with the CCP presenting weakest chelating properties (gray circles) and strongest chelating properties (lavender circles). Blue arrows are pointed to the attached phosphoserine residues; Red arrows are pointed to free phosphoserine residues.

5 CONCLUSION

This study aimed to evaluate the impact of the demineralization on the internal organization of the CMs structure of a dense CM dispersion. The present data indicate that demineralization does not induce a change in hydrophobicity of the micelle, suggesting that no substantial protein-protein reorganization occurs. It shows demineralization first induces the loss of some CCPs, which are not strongly attached

to the protein structures (so-called ‘”hard structures”) and rather are located in less dense regions of the micelle. Subsequently, in more demineralized samples, the loss of CCPs induces stronger internal structure modification of the CM. This data brings more clarity about the role of the nanoclusters in keeping the structure of the micelle and represent an improvement in the sponge-like model described by Bouchoux et al., (2010) showing that not all the calcium phosphate nanoclusters are located in the interior of the “hard” structures of the CMs or that exist some clusters that are strongest linked to these structures than others.

Acknowledgments

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Supporting Information for

The heterogeneous substructure of casein micelles: how demineralization trigger internal structural modifications of the micelle

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Table 1 - Centesimal composition of casein powders with different demineralization levels.

Composition* (g/100g of powder)	Native	DM-05	DM-10	DM-25
Moisture	6.25	4.73	3.99	4.96
Total protein	82.16	83.81	84.94	85.97
Lactose	3.13	3.95	2.98	3.23
Minerals	7.68	7.17	7.11	7.03
Calcium	2.35	2.24	2.16	1.81
Calcium demineralization (%)*	0	4.47	9.16	25.73

* Data from the manufacturer's sheet donated by Ingredia dairy Experts obtained from the internal analysis of the powders.
(Native) = Native casein; (DM-05) = 05% demineralized casein; (DM-10) = 10% demineralized casein; (DM-25) = 25% demineralized casein
The amount of fat was not expressed in the table because it represents less than 1g/100g of powder
* The calcium demineralization (%) was calculated taking into consideration the amount of calcium that was reduced from the native casein sample

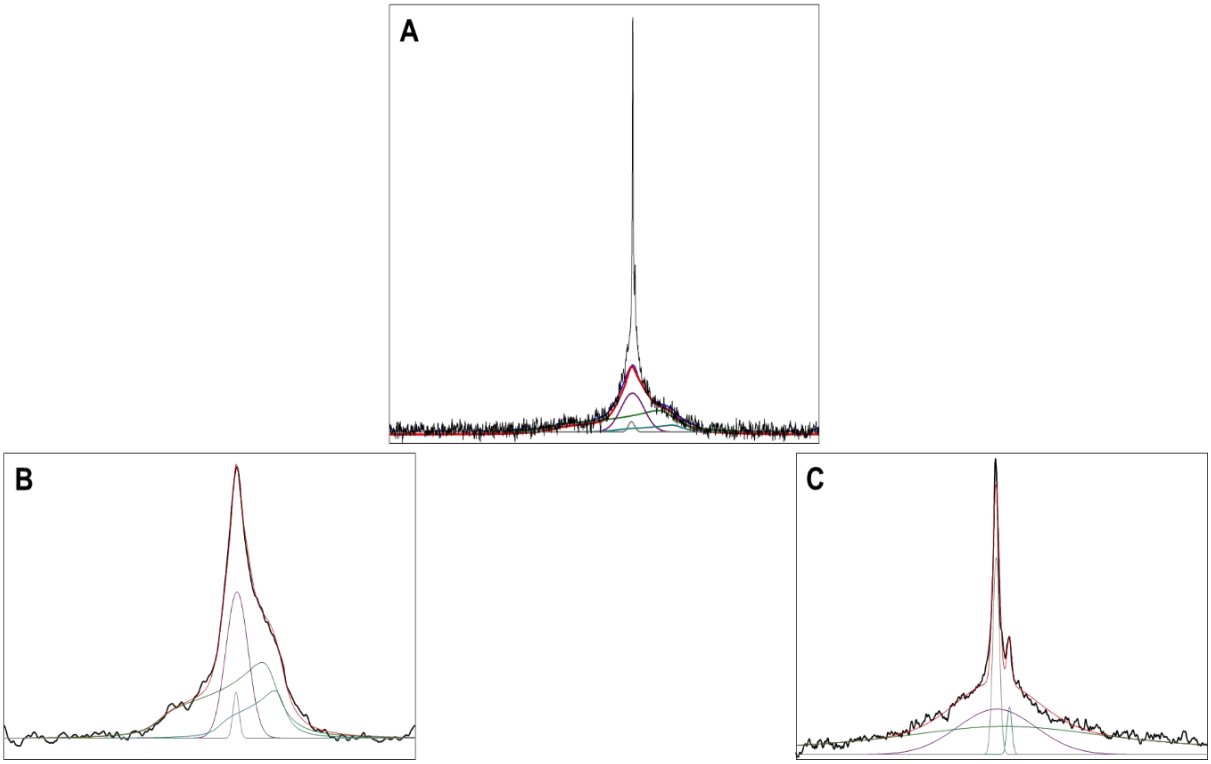

Figure 1 - NMR fit decomposition using DMFIT software. Black lines correspond to experimental data obtained from a casein dispersion; Red lines correspond to the fit obtained from the decomposition of different peaks; other colors (green, violet, blue and gray) corresponding to the different peaks which were added to create the fit line.
(A) ^{31}P spectra with the cross-polarization (CP) fit; (B) CP fit with different Chemical Shift Anisotropy (CSA) peaks (blue and green curves), which have been used to produce the fitting line; (C) ^{31}P fit using two fine peaks to produce the fitting of the phosphoserine and the ionic phosphorus

Table 2 - CSA parameters obtained from the CP analysis of NMR

	δ11 (ppm)	δ22 (ppm)	δ33 (ppm)
Native	-32.43	-20.45	63.40
DM-05	-34.86	-23.31	57.59
DM-10	-37.89	-19.92	60.93
DM-25	-36.55	-18.25	64.98

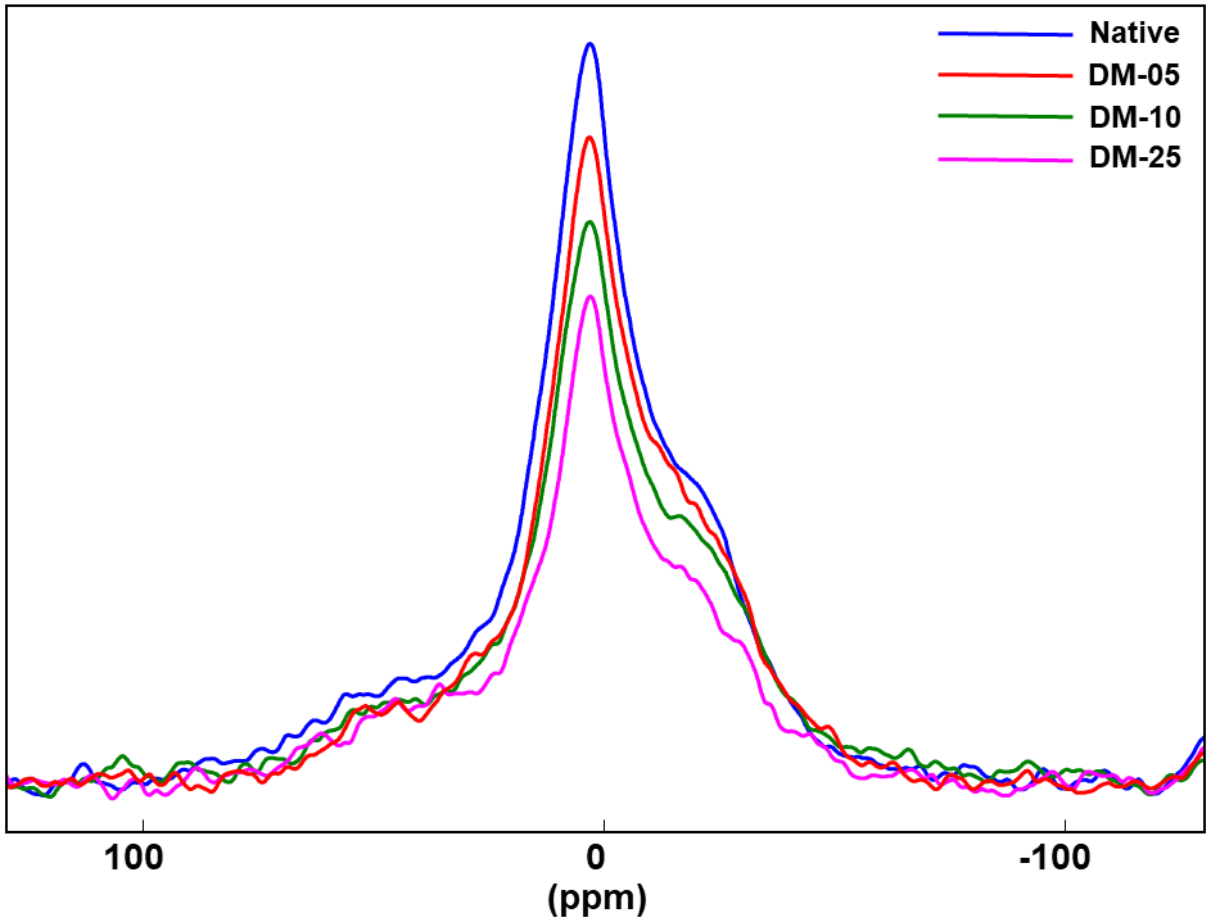

Figure 2 - Cross-polarization (CP) results from the NMR analysis of casein with different degree of demineralization, being: (Native) = Native casein; (DM-05) = 05% demineralized casein; (DM-10) = 10% demineralized casein; (DM-25) = 25% demineralized casein.

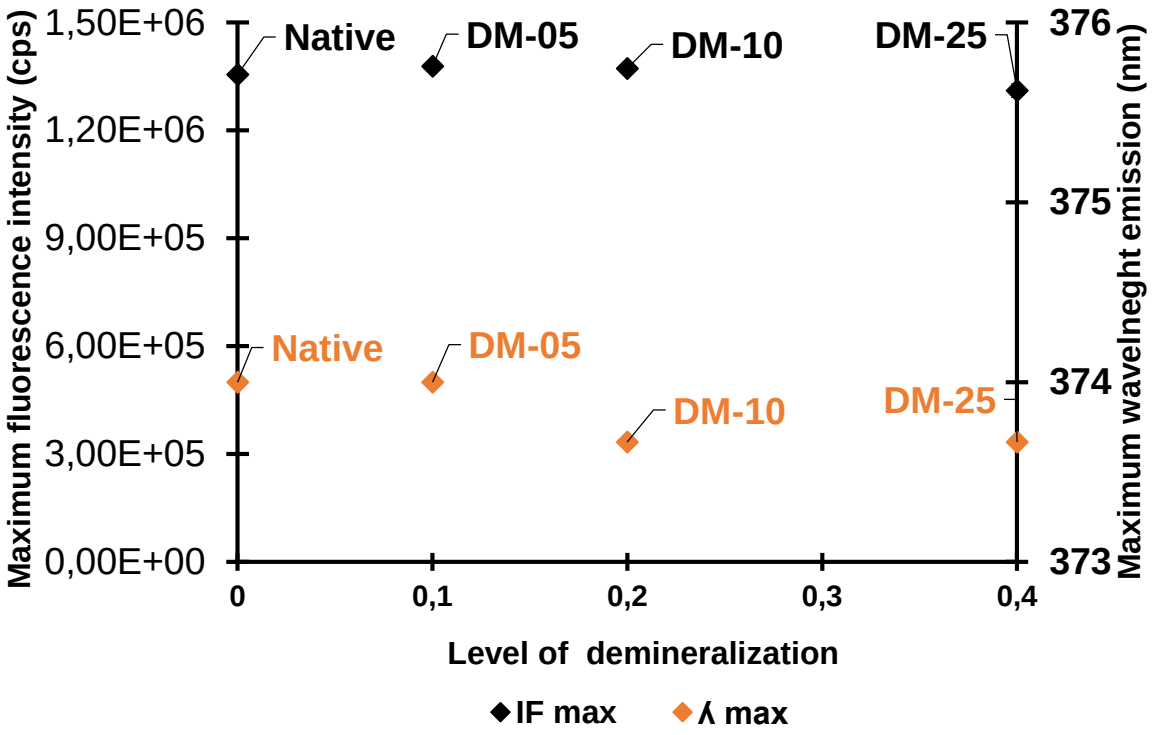

Figure 3 - Fluorescence results of CMs dispersions with different demineralization degrees. Results from three repetitions and its duplicates. Being (Native) native casein micelle without demineralization, (DM-05) 05% less calcium; (DM-10) 10% less calcium and (DM-25) 25% less calcium.

IF max corresponds to the maximum intensity of fluorescence and λ-max as the wavelength which is the sample present the maximum of its absorbance

4

It has been demonstrated in the third part of the results (section 3.3) that the demineralization is responsible for a non-linear change of the CMs structure in dense dispersions. This result is representative to an initial stage of production of ready-to-drink protein beverages, before UHT. As described in the bibliographic review section, the UHT could induce some changes in the CMs structure. However the impact of this heat-treatment on an already modified CMs (demineralized) it has not yet been evaluated. To fill this gap, the results present in this last section of the results (3.2.2) had an objective to evaluate the impact of these two sources of variation, the heat-treatment (UHT) and the demineralization on dense dispersions of casein-rich powders.

3.4 - Impact of ultra-high-temperature (UHT) on native and demineralized casein dense dispersions: structural and rheological changes

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This present article is under final revision

ABSTRACT

The aim of this work is to evaluate the impact of UHT over the structure and the relation with the apparent viscosity of partially demineralized dense casein micelles (CMs) dispersions (14% protein over the total liquid mass). Three calcium-demineralized CMs with less 5% (DM-05), 10% (DM-10), and 25% (DM-25) were studied and compared with a control sample composed by native CMs (Native). The present data indicate that for all samples with the exception of the most demineralized one (DM-25), UHT induces no loss of casein micelles nano-clusters. It also shows that UHT induces the "fixation" of soluble calcium and phosphorous ions by the phosphoserine residues of the CMs. The amount of this ion fixation depends on the level of demineralization: DM-05 and DM-10 CMs display a more significant amount of internalized ions after UHT than the native sample. This dependency is linked to the higher amount of "free" phosphoserines (not attached to a nanocluster) available in demineralized samples. DM-05 and DM-10 ions fixation also had an interesting side effect of decreasing solution viscosity. SAXS and DLS results indicate that this change is probably linked to a modification in the surface of the CMs that becomes less sticky. For DM-25, in contrast to other samples, UHT induces a loss of native-like nanoclusters, indicating that these strong demineralized samples are more sensitive to heat than the others. Moreover, for these samples, UHT induces an increase in the viscosity. Thus, these data show that the impact of heat treatment in CM structure and viscosity is greatly

dependent on the degree of demineralization of the CMs, and one has to carefully target a given demineralization degree to obtain an optimal viscosity.

Keywords: Casein micelles, demineralization, dense dispersions, ultra-high-temperature, viscosity.

Introduction

In recent years, a significant boom in demands for high protein application has been noted and forced the food industry to innovate in order to propose innovative products, such as high protein bars, high protein beverages, and high protein yogurts. This crescent demand is addressed to the consumers that are looking for benefits (for weight loss, for muscle building) and also with a view in the clinical nutrition market (patients recovery) (Henchion et al., 2017). In this context, the ready-to-drink (RTD) high-protein beverages have been identified as an excellent application that brings together the high-amount of proteins to the ease of consumption (Rittmanic, 2006).

In the panorama of RTD high-protein beverages, milk proteins are more often encountered compared to plant proteins, and among them, the milk proteins are the main species of protein in reason of their excellent overall properties (Rittmanic, 2006). Indeed, these proteins associate an excellent nutritional profile (essential amino acids), good digestibility ability and offer a wide range of possibilities to develop pleasant taste (Chavan, Shraddha, Kumar, & Nalawade, 2015). However, in some cases, the use of the whey proteins for RTD beverages is not the best option, especially when this product needs to be submitted to high-temperature treatments (Sadeghinezhad et al., 2013).

The RTD beverages are generally produced through an industrial process that involves the rehydration of milk powder Ingredients and a sterilization treatment on the dense protein dispersions. The ultra-high-temperature (UHT) treatment is widely applied in the industry as a sterilization process, which associates the binomial high-temperature and short time, necessary to produce extended shelf-life products without several changes in the nutritional and sensorial aspects (Jordan, 1968).

In this case, for RTD beverages produced by UHT treatment, the whey proteins are not necessary the best option as a major protein sources due to its heat-instability (Sadeghinezhad et al., 2013). An alternative to the whey proteins for improving heat treatment resistance is to incorporate another milk protein, the caseins, which are

naturally present in the cow's milk as a form of casein micelles (CMs) (Walstra et al., 2006).

The CMs represent a suitable source of proteins to RTD beverages submitted to UHT treatment as they are heat-stable (Beliciu et al., 2012), are in higher amount in milk, in comparison to the whey proteins (CMs represents about 80% of the total proteins from milk. Moreover, the CMs have the same nutritional and sensorial advantages than the whey proteins, amino acid complexity (Vickery & White, 1933), good digestibility properties, and no strange taste, which are common in other sources of proteins, such as plants (Damodaran & Arora, 2013).

Despite their advantages of use, as aforementioned, the CMs also present an inconvenience to the production of high-protein beverages: its high-viscosity in dense systems (above 8% of protein) (Bouchoux et al., 2009). This property is essential to some products, such as cheese (Fox et al., 2016) and yogurt (Tamime & Robinson, 2000), but can represent a problem to RTD beverages, which should be consumed as a liquid form.

A strategy that can modify the apparent viscosity of these high-protein beverages is based on the use of chelators to reduce the calcium ion activity of CMs, as highlighted by Kort et al. (2011). Indeed, Investigating the effect of various phosphates and citrate on the physical changes of casein micelles in concentrated micellar casein solutions (9% (w/v)), Kort et al. (2011) reported that an increase in viscosity could be observed after addition of certain calcium chelators, due to swelling of the casein micelles.

Recently, Pandalaneni et al. (2018) have also used varying levels of calcium chelating salts to study the change of viscosity in enteral beverage formulation containing CMs (8% (w/w)). They have also highlighted that apparent viscosity increased because of calcium chelation, which is supposed to induce casein micelle dissociation.

These observations clearly provide new ideas for controlling functional properties the viscosity of concentrated dairy systems by reduced the calcium ion activity in MCs dense system. Although partial demineralization through ultrafiltration can also be a way to promote a mineral disequilibrium between CMs and soluble phase (solvent) by

excision of the colloidal calcium phosphate nanocluster (CCP), the impact on casein demineralization on apparent viscosity was not studied in depth.

In this context, the present study has an objective to investigate the impact of the demineralization on the structure, and in the apparent viscosity of dense systems (14%) constituted exclusively by CMs and water submitted to the same industrial protocol utilized to produce RTD beverages, such as powder rehydration, homogenization and UHT treatment.

2 MATERIALS AND METHODS

2.1 Samples of casein powders

Four casein-based powders with different amounts of calcium were provided by Ingredia S.A (Arras, France) (the composition of the powders is present in table 1 in supporting information). The procedure responsible for reducing the calcium of the CMs dispersions, before spray-drying, was based on the amount of calcium that is removed during the ultrafiltration process after initial acidification such as described in the literature (Silva et al., 2013).

The four different powders of the present study were referenced as described below.

- A native casein-based powder (Native) was used as control.
- A 4.47% calcium-demineralized powder (DM-05).
- A 9.16% calcium-demineralized powder (DM-10).
- A 25.73% calcium-demineralized powder (DM-25).

The value, which appears in the bracket corresponding to the next entire value closest to the real demineralization value.

2.1.1 Powder rehydration

The powders were rehydrated under stirring of 500 rotation per minute (r.p.m) for one hour, three drops of antifoam silicon solution 426R (Prolabo, France) were

added at the beginning of the rehydration to prevent the foam formation, after one hour, the pH was adjusted to 7.0 with NaOH 1M (Sigma Aldrich, France). After pH adjustment, the samples were homogenized at 10000 r.p.m for 5 minutes using a rotor-stator homogenizer, Polytron PT 10-35a (Kinematica, France). This homogenization process was applied with an objective of break all the remained aggregates (Kinematica AG, 2013).

The powders were hydrated to a final concentration of 14.0 g (protein) x 100 g^{-1} (deionized water). To prevent microbiological growth, sodium azide was added to a proportion of 0.3 g L^{-1} (Sigma Aldrich, France). All the dispersions (liquid samples) studied in this work have been submitted to homogenization.

The samples will be posteriorly referenced as follows:

- **Native:** Native samples before the UHT.
- **Native UHT:** Native samples after the UHT.
- **DM-05:** 5% calcium-depleted sample before the UHT.
- **DM-05 UHT:** 5% calcium-depleted sample after the UHT.
- **DM-10:** 10% calcium-depleted sample before the UHT.
- **DM-10 UHT:** 10% calcium-depleted sample after the UHT.
- **DM-25:** 25% calcium-depleted sample before the UHT.
- **DM-25 UHT:** 25% calcium-depleted sample after the UHT.

2.1.2 Ionic calcium (Ca^{2+}) quantification by ion-selective electrode

A quantification of the amount of Ca^{2+} using an ion-selective electrode was made using a PASCO CI-6738 ISE (Ion Selective Electrode) Amplifier and following the protocol described by the manufacturer (Pasco-Scientific, 1997) with some modification. The quantification was conducted at 20 °C with the samples at least the time used to equilibrate the temperature of the samples was not inferior to two hours and the samples were kept under stirring of 200 rotations per minute. An eight points calibration curve was prepared using calcium chloride ($CaCl_2$) range from 5 to 100 mg/L. These values for a calibration curve have been chosen after the preliminary test

with the samples at the same CMs concentration that are used in the present study (data not showed).

2.1.3 Particles size characterization by Dynamic Light Scattering (DLS)

To evaluate the average size of the CMs, a DLS technique was used using a MasterSizer nano s (Malvern Instruments, Worcestershire, UK). The measuring was performed at the French national synchrotron facility SOLEIL in Gif-sur-Yvette, France.

Measurements were made at a scattering angle of 173° and a wavelength of 633 nm. Suspensions were previously diluted 1/100 in deionized water and left at 25 °C for 20 min before analysis. Experiment duration was set to 2 min and each experiment was repeated six times for each one of the three repetitions and its duplicates.

2.1.4 CM structural organization evaluated by Small-angle X-ray scattering (SAXS)

The SAXS measurements were performed at 20 °C at the French national synchrotron facility SOLEIL in Gif-sur-Yvette, France, on the SWING beamline operating at ~12 keV of photon energy. Samples were placed in a watertight cell with a cylindrical cavity with 4.5 mm of diameter and 2 mm of thickness, closed by flat mica windows. The scattered intensity was recorded on a detector placed at ~0.5 m and 6.5 m from the sample. For each sample, data were first recorded at short exposure time (typically ~0.2 s) to avoid any radiation damage (aggregation) that could result in artifacts at low q values. Subsequently, data were recorded at long exposure times (typically ~15 s) using a larger beam stop to obtain a good signal/noise ratio at high q values without damaging the detector. Intensities recorded at the two exposure times were then radially averaged and combined to get a scattering curve covering a q-range of 1.5×10^{-3} to 1.4×10^{-1} A ^{1}. In some cases, artifacts due to sample radiation damage were visible in the low-q regions of the data recorded at long exposure times. The corresponding intensities were discarded before the merging procedure was carried out. For each sample, the intensity scattered from the solvent (deionized water) in the

same mica cell was measured and subtracted from the casein sample pattern. The resulting corrected intensity is denoted by I (q).

In the present study, the regions in the SAXS spectra were assigned in according with the bibliography (Bouchoux et al., 2010; Ingham et al., 2016) as three types of structures that forming the micelle which are represented in different levels of the SAXS spectra as: the level 0 (zero) which correspond to the micellar envelope (of about 100 nm of diameter) corresponding to a Q range 0.0065 to 0.0010 nm^{-1} and, in the present concentration, the inter distance between CMs; the level 1 (one) which corresponds to smaller structures (20 nm of diameter) described as “hard”, which are related incompressible structures (Ingham et al., 2016), once submitted to osmotic pressure, within the micelle corresponding to a Q range of 0.042 to 0.27 nm^{-1} and, finally, the level 2 (two) which corresponds to the structure of the CCP nanoclusters, and proteins associated (about 5 nm of diameter), assigned to a clear “shoulder” at a Q range between 0.042 to 0.27 nm^{-1}. Bouchoux et al. (2010) have represented the so-called “hard” (~20 nm) structures as filled regions containing several CCP nanoclusters (~5 nm).

2.1.5 Different phosphorus species quantified by Nuclear Magnetic Resonance Spectroscopy (NMR)

The number of different species of phosphorus (i.e. inorganic soluble phosphorous in the bulk, inorganic fixed phosphorus in the CM, organic phosphorous from the phosphoserines residue in a fixed state or in a mobile “free” state), can be measured in the quantitative spectra through decomposition of the NMR spectra into three different types of peaks: The first one corresponding to the chemical shift anisotropy (CSA) which correspond to the fixed phosphoserine residues (BP-Ser) (Gardiennet-Doucet et al., 2006); the Gaussian peak, which correspond to the BI-Phosphorus; and the fine peaks which correspond to the FP-Ser and FI-Phosphorus as described in the literature (Boiani et al., 2017). Actually phosphoserine residues of the CMs play a crucial role in binding the CCP nanoclusters (McSweeney & Fox, 2009) . NMR is also capable of quantifying specifically the organic (from phosphoserines

residues), and inorganic ^{31}P (from the clusters) in their “fixed” (fixed to cluster) or “free” states as as shown by (Boiani et al., 2018). Noticed that in the case of phosphoserines, the residues considered “free” are the ones displaying strong dynamics. Such strong dynamics indicates that the organic phosphorous belonging to the phosphoserines residues are not fixed to the CCP (although the residue can belong to a protein still fixed to the CM).

The different components of each spectrum of the ^{31}P were simulated using the DMFIT software (De Sa Peixoto et al., 2017; Massiot et al., 2002) which permits to obtain results about the signal from each phosphorus species. This method propitiates the quantification of the number of ^{31}P species with an error band of ± 1.5%. The peaks decomposition was present in figure 2 in supporting information. The number of fixed phosphoserine residues and CCP was calculated based on the peaks decomposition, and the intensity of the peaks was compared. The native sample was considered as a control for the demineralized samples with 100% of the signal of the fixed phosphorous from phosphoserine and phosphorous from CCP.

In addition, the cross-polarization analysis between the ^{31}P and ^{1}H allow one to better access to the CSA shape of the fixed phosphorous from phosphoserines residues (BP-Ser) view of accessing information about the changes in the ionic environment close to these residues. Actually, such information is indirectly derived from the analysis of the chemical shift anisotropy (CSA) of the phosphoserines in the CP-NMR spectra (Peixoto et al., 2015) (figure 1 and table 2 in supporting information). Indeed, CSA is a spin interaction in NMR (Hou et al., 2013), which can a wealth of information about the ratio of calcium ions around the phosphoserines (Gardiennet-Doucet et al., 2006).

A Bruker AVANCE I; 9.4T (1H: 400 MHz; ^{31}P: 161.9 MHz) spectrometer used to measure the ^{31}P spectra, proton (^{1}H), cross-polarization (CP) and the t1 (direct correlation between ^{31}P and ^{1}H). The 4 mm probe heads was set with samples and submitted to 700 Hz of Magic Angle Spinning (MAS) speed. Quantitative experiments at 25 °C of ^{31}P were done with high power decoupling- recycle delay: 30s; 90° pulse; RF field (^{31}P): 65 kHz; ^{1}H decoupling (RF field: 60 kHz; SPINAL64); 1024 accumulations. The chemical shifts were given in parts per million (ppm) with and the

respective analysis of H_3PO_4 (85%) for ^{31}P NMR spectra at 0ppm. For the ^{1}H analysis, we used one pulse experiment with a flip angle of 90°, a relaxation delay of 5s and RF field (^{1}H): 60 kHz. The analysis was set with a quantitative of 16 accumulations of inversion-recovery (t1ir): 90°-tau-180°. The chemical shifts were given in ppm with respect to TMS (0ppm) as an external reference for ^{1}H NMR spectra. In addition, the qualitative relation between ^{1}H-^{31}P is done through the Cross-Polarization (CP) experiments at 1 millisecond of contact time (Figure 1 in supporting information).

2.1.7 Rheological measures

The apparent viscosity was measured using a Couette geometry, using an AR2000ex Rheometer (TA Instruments, Guyancourt, France) equipped with a coaxial cylinder (internal radius = 14 mm, external radius = 15 mm, cylinder height = 42 mm). The experiments were conducted at 20 °C varying the shear rate from 10 to 500 s^{-1}.

2.1.8 Experimental planning and Statistical treatments

The present study was conducted using the experimental plan of 4x3x2, being four samples, rehydrated in triplicate and each analysis was conducted two times for each replicate. The results were analyzed using a one-way analysis of variance (ANOVA) followed by a Tukey test at $p<0.05$ for the data from the particle size characterization, NMR, and apparent viscosity, for the stable region, from a shear rate of 300 to 500 (s-1). For the data from fluorescence a principal component analysis (PCA) was done.

3 RESULTS AND DISCUSSIONS

As described previously; the present study was focused on the impact of the demineralization on the structure and apparent viscosity of dense dispersions prepared with CMs submitted to a UHT treatment such as utilized to produce RTD beverages. In this case the results are characteristic from the samples just before the UHT treatment (after the homogenization step) and after the UHT treatment. The results in the present study are divided into two parts: The first part corresponds to the structural study after UHT for the four demineralized dispersions. The second part concerns the impact of UHT on the apparent viscosity of these samples.

3.1 Effect of UHT in the structure and calcium phosphate ion equilibrium:

3.1.1 Ca^{2+} quantification in the soluble phase

As described in the literature (Walstra et al., 2006), the CCP within the CMs are in equilibrium with calcium and phosphorous ions in the soluble phase. The Ca^{2+} quantification in the soluble phase allows one to have some information about the evolution of this equilibrium before and after UHT as a function of the demineralization level of the sample. In figure 22 are displayed the results for the CMs with different demineralization levels before and after UHT.

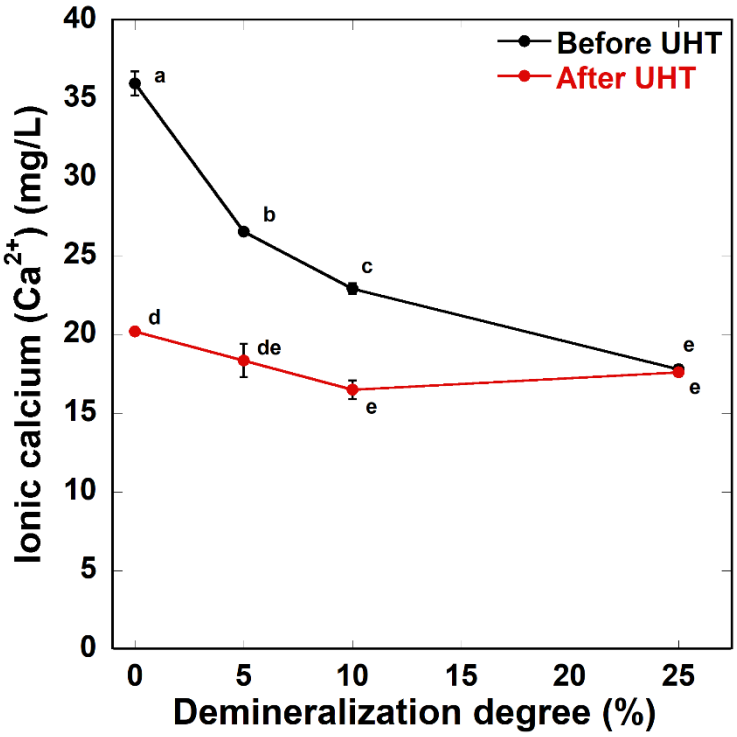

Figure 22 - Ca^{2+} quantification of CMs with different demineralization level, before UHT (dark blue lines) and after UHT (red lines). Results are from three repetitions and its duplicates, error bars corresponding to the standard variation. Different letters between the curves represent a statistical difference between samples (at $p<0.05$).

Figure 22 shows a significant decrease in the amount of the Ca^{2+} as a function of the demineralization level before the UHT treatment (blue line).

Looking now to the UHT impact in each sample, a remarkable decrease of the soluble calcium concentration has been detected for the native, DM-05, and DM-10 after UHT. In contrast, the most demineralized sample, DM-25, does not display a significant change in the amount of soluble Ca^{2+} after UHT.

Concerning the effect of demineralization on the samples which have been subjected to UHT, one can notice a small decrease in soluble calcium with increasing demineralization degree, passing form the native sample to the DM-05 one (red line). In contrast, no statistical change was observed for soluble calcium content is noticeable between the DM-05 UHT sample and all the other samples.

For native CMs, such a decrease in the soluble calcium content after UHT has been reported in the literature (Beliciu et al., 2012; Broyard & Gaucheron, 2015). The reason for that is in line with the decrease of solubility of phosphorous and calcium

ions with the increase of temperature (Bell, 1925). Thus, as a consequence of the UHT treatment, such a change in ionic equilibrium favors the “fixation” of calcium and phosphorous ions into the native CMs as well as their precipitation in the form of large clusters (Beliciu et al., 2012; Bell, 1925; Broyard & Gaucheron, 2015). Nothing that no work has specifically looked at the impact of UHT in demineralized CMs. The present data seem to indicate that demineralized CMs are also capable of fixe calcium ions, although the exact quantity cannot be quantified by this analysis alone.

3.1.2 Structural changes of the CMs structure observed using ^{31}P NMR

To indirectly access the actual amount of calcium ions fixed by the CMs after UHT, NMR has been used. Indeed, NMR allows the quantification of the amount of organic (from the phosphoserines residues of the caseins) and inorganic (triangles in figure 23) phosphorous “fixed” or “free” in bulk. Thus, if UHT induces fixation of calcium ions within the CMs-one is likely to observe the decrease of “free” phosphorous from phosphoserines and an equivalent increase of fixed ^{31}P from phosphoserines. In contrast, if the UHT induces the precipitation of calcium ions (without interaction with proteins), one should not observe a strong difference in the amount of fixed or “free” ^{31}P from phosphoserine residues.

Figure 23 presents the results obtained from the NMR spectra of CMs dispersion with four different demineralization levels (Native to 25%) before and after the UHT process.

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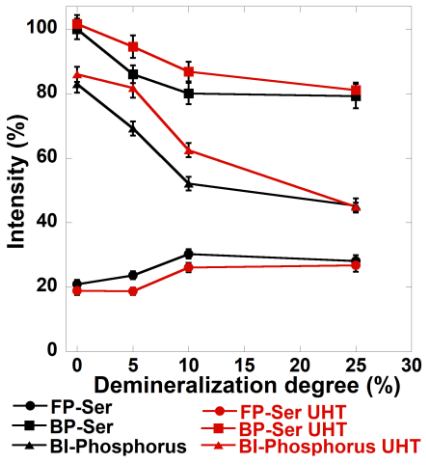

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Figure 23 -Results obtained from the NMR analysis of CMs dispersions with different demineralization degrees.

Black symbols represent the samples before the UHT treatment, and Red symbols represent the samples after UHT treatment. Circles represent the free phosphoserine residues (FP-Ser); squares represent the fixed phosphoserine residues (BP-Ser); triangles represent the fixed inorganic phosphorus (BI-Phosphorus from CCP)

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Firstly, the black curves, which show the impact of demineralization before UHT, will be analyzed. One can see that the more the sample is demineralized, the lower is the amount of fixed inorganic phosphorus (triangles in figure 23). In the same way, the more the sample is demineralized, the higher is the amount of fixed phosphorus from the phosphoserines (squares symbols in figure 23) and, symmetrically, the amount of ”free” phosphoserines (circles in figure 23) increases. These data support previous results in the literature (Nogueira et al., 2020; Boiani, Fenelon, FitzGerald, & Kelly, 2018)) showing a relation between the loss of ^{31}P from the CCPs (by demineralization) and the relative increase in the amount of “free” phosphoserines residues. An exception is the sample denominated as DM-25, which displays values quite close to the ones of the DM-10 sample.

Looking now for the impact of UHT in each sample (the reds curves in comparison to the black curves): all samples, with the exception of the most demineralized one, DM-25, displays an increase in fixed inorganic phosphorous (triangles in figure 23), as well an increase of organic phosphorus (^{31}P) (from phosphoserines residues from casein, squares in figure 23) and a decrease of organic ^{31}P (circles in figure 23, from phosphoserines residues from casein). One can notice in figure 23 that UHT has a greater impact on the demineralized samples, DM-05, and DM-10 than at the native sample. Thus, it seems that UHT favors a greater ion fixation in demineralized samples than in the native ones. This supports a picture where the demineralized samples display more sites to calcium fixation; these free sites are likely to be “free” phosphoserines, capable of recapturing ions. This recapture UHT-dependent is stronger for the demineralized samples. In the case of the most demineralized sample, DM-25, no significant apparent change in the amount of fixed and free species occurs. It seems that the structure of CM in DM-25 is not able to recapture calcium ions during UHT, probably due to too deeper change in CMs structure.

^{1}H-^{31}P cross-polarization experiments (CP) have been used to observe a change in the average concentration of calcium ions close fixed phosphoserine induced by UHT. The CSA parameter (table 2 in supporting information) indicates that UHT induces an increase in the calcium concentration in the close environment ^{31}P of

phosphoserines for the native, DM-05, and DM-10. In contrast, the CSA parameter of the organic ^{31}P of the DM-25 indicates the poorest environment in calcium near the fixed phosphoserines. These data indicate that UHT favors a stronger recapture of calcium ions than phosphorous ions as a function of the demineralization level of the CMs at the exception of the most demineralized sample, DM-25.

3.1.3 UHT effect on the CMs size distribution

The changes in the amount of phosphorous and Ca^{2+} ions are directly related to a change in the internal structure of the CM, at least in the level of the CCP sites. The question now is how the fixation of calcium ions alters this structure of the CM in a large scale. A DLS analysis has been performed to evaluate the impact of UHT over the hydrodynamic diameter of the CMs.

The evolution of the hydrodynamic diameter of the CMs samples with different demineralization degrees (0 to 25%), before or after UHT treatment, is presented in figure 24.

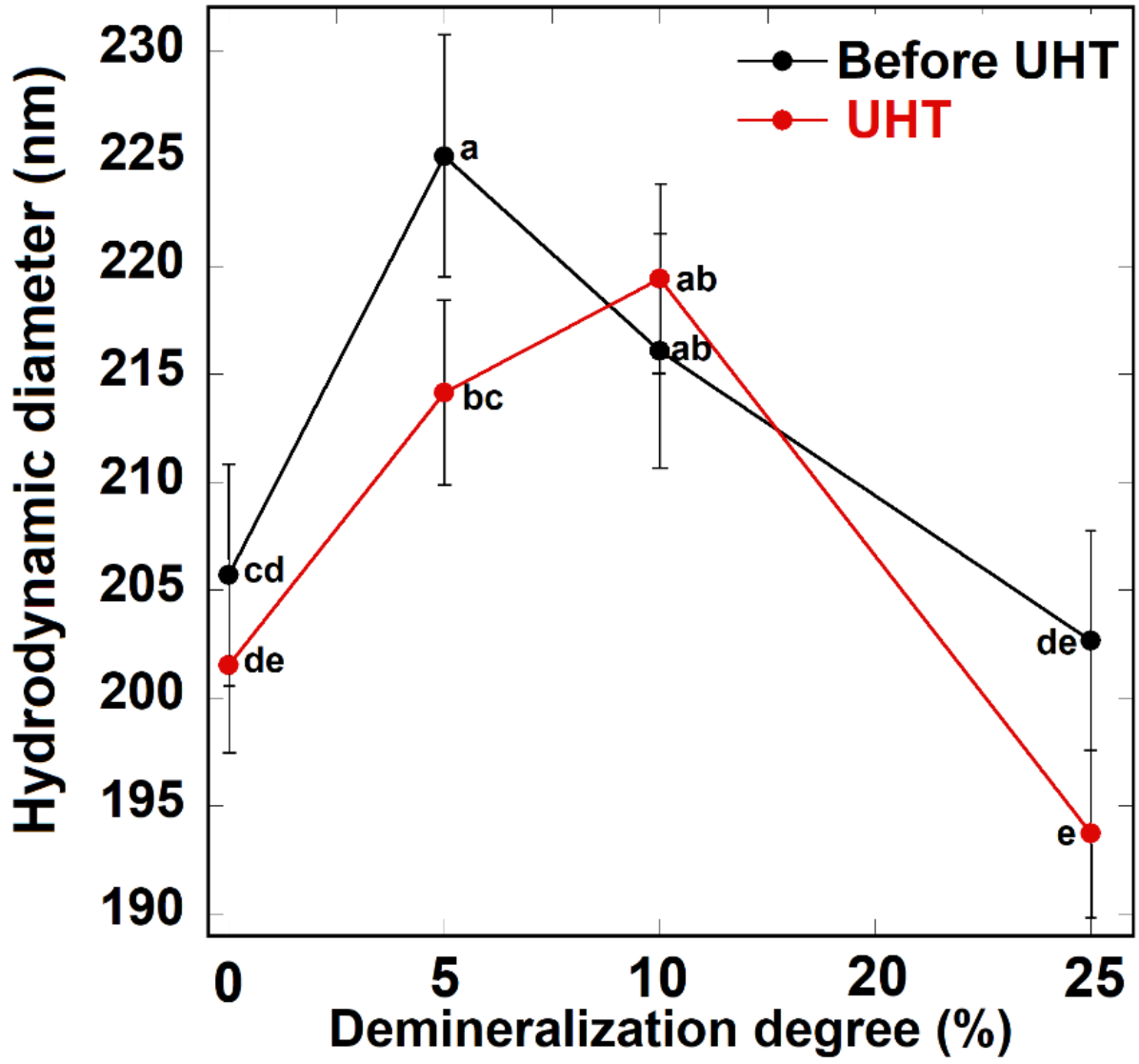

Figure 24 – Hydrodynamic diameter of CMs with different demineralization level, before UHT (black circles) and after UHT (red circles).
Different letters between the curves represent a statistical difference at p<0.05

For the samples before the UHT treatment (black symbols), the native sample presents an average diameter quite compatible with the average size reported in the literature (Dalgleish, 2011) around ~200 nm. The UHT treatment is responsible for a small but significant decrease in the CMs size for the DM-05 UHT sample. For the other samples, Native, DM-10, and DM-25, the UHT does not induce a significant change in size.

3.1.4 Structural changes of the CMs structure observed using the SAXS

The SAXS was used to investigate the structural changes of the CMs from the large to the nanoscale level. Figure 25 displays the superposition SAXS profiles after UHT (figure 25A) and for the sake of clarity a superposition of profiles before and after the UHT for each one of the four different demineralization levels (figure 25B to E).

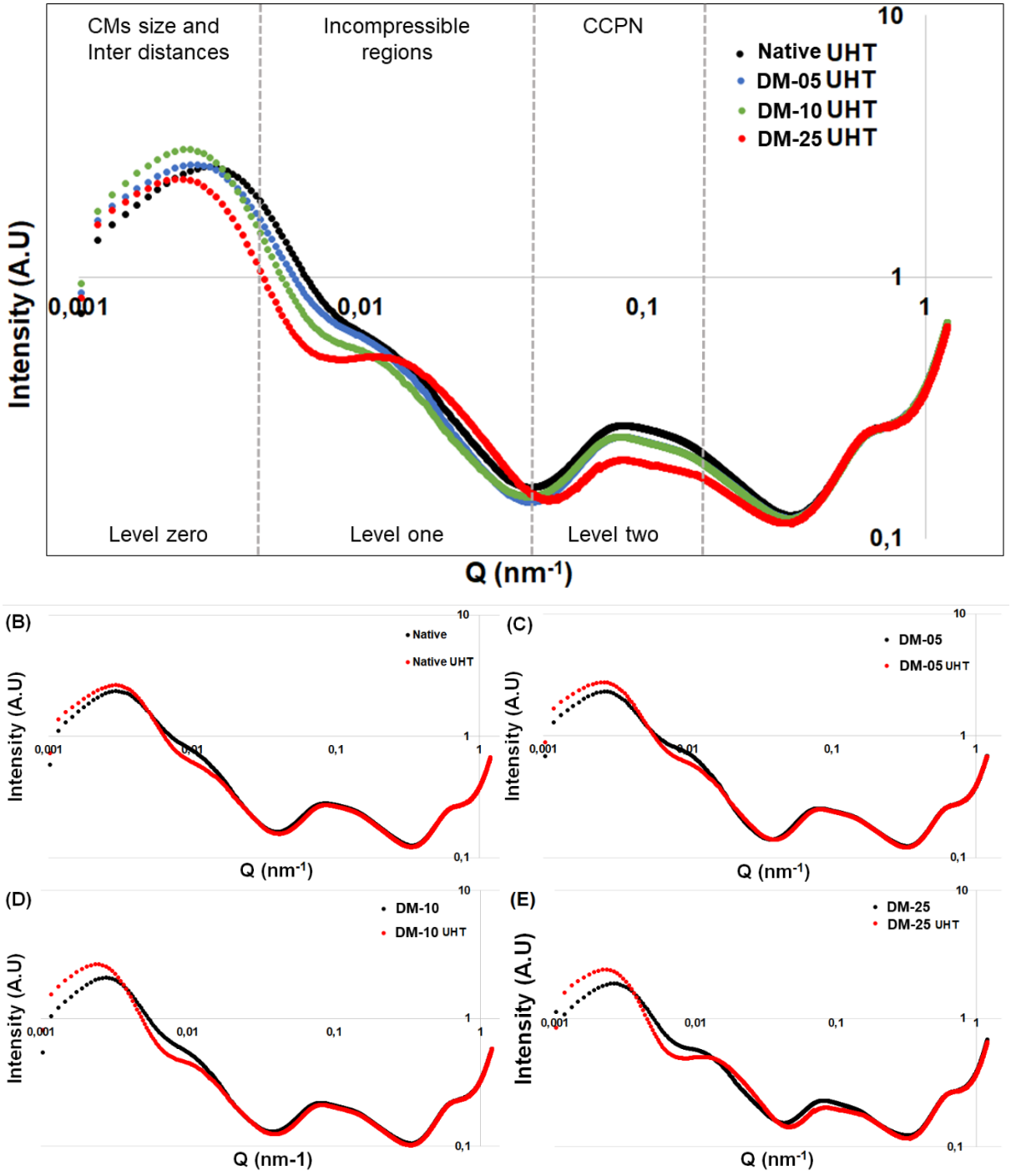

Figure 25 - SAXS profiles of CMs with different demineralization level

(a) All samples together after UHT treatment and (B-E) representing the samples before UHT (black circles) and after UHT (red circles).

Being Native CMs without changes in the natural calcium amount (B); (C) a 05% calcium demineralized CMs; (D) a 10% calcium demineralized CMs; (E) a 25% calcium demineralized CMs.

In figure 25A, one can see that all samples display SAXS profiles with the typical "shoulders" corresponding to the different substructures of the micelles (see figure 26). Moreover, one can observe that the "shoulders" position for level 1 (the large, 30 nm, "Hard" incompressible structures) and level 2 (the CCP of about 5 nm) display a decrease in intensity but do not display an important shift in position for almost all samples (native, DM-05 and DM-10). A decrease in intensity without a shift in the position of the "shoulder" indicates changes in the number of physical structures of the CM, giving origin to these SAXS signals (Bouchoux et al., 2010). The SAXS shoulder representative for level one, the so-called "hard" structures (figure 26) shows a progressive decrease for the native, DM-05 and DM-10 in the "shoulder" scattering intensity, indicating that UHT has depleted part of those structures.

Thus, demineralization and UHT had a consequence the depletion of some those structures form the CM, but the remaining ones display roughly constant features in terms of shape and density. In contrast, for DM-25, level 1 displays a definite shift in the shoulder. This indicates that the physical structures giving origin to these signals, the so-called "Hard" structures are quite different from the native one.

Looking now to the impact of UHT treatment on the individual samples, figures 25B, C, D, the effect of UHT is stronger for the most demineralized micelles.

The level two (CCP and proteins associated) does not change as a consequence of the UHT treatment for almost all samples except for DM-25. If the calcium UHT dependent fixation (observed by NMR) would create "new native-like nanocluster" sites, one should expect an increase in these SAXS "shoulders". Rather than that, these data indicate that such fixation forms new sites with quite different structures in terms of size and density. It also indicates that UHT does not disturb the remaining native-like sites in the micelle.

In contrast, in the case of DM-25 there is a decrease in the intensity of the "shoulder" after UHT. A decrease in intensity without a shift in the position indicates a decrease in the amount of the native nanoclusters. Since NMR and calcium titration does not show that DM-25 liberates ions after UHT, one must conclude that the ions lost in these nanoclusters in DM-25 have been re-internalized by the CM somewhere else forming non-native like interactions with phosphoserines. This further

corroborates NMR cross polarization data showing that the environment of phosphoserines displays a quite different ionic environment after heat treatment.

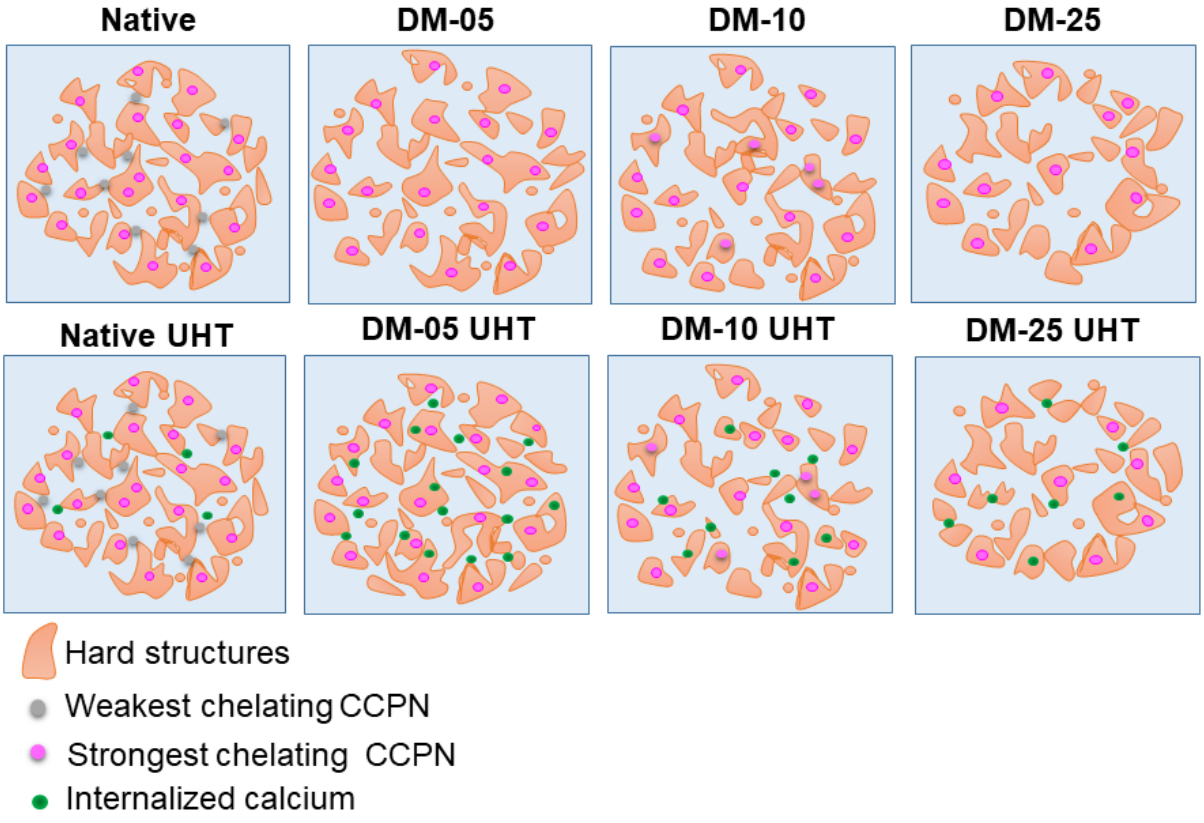

Figure 26 -Schematic representation of the changes in the internal structure of the different demineralized CMs before UHT (Above) and after UHT (below).

Being Native CMs without changes in the natural calcium amount (B); (C) a 05% calcium demineralized CMs; (D) a 10% calcium demineralized CMs; (E) a 25% calcium demineralized CMs.

For level zero (affected by the gyration radius and the inter distances between different CMs), there is an increase in intensity and a small shift to lower Q values after UHT for all samples. The more the sample is demineralized, the stronger is the increase in the intensity of the UHT as well stronger is the shift. In principle, such changes can be due to an increase in the global size/shape of the CM (the gyration radius or the form factor of the CM) and/or the interaction between micelles are more sticky (a change in the structure factor) (Goldenberg & Argyle (2014). Since DLS results show that there is not strong change in hydrodynamic radius, one must conclude that these changes must be related to a change in casein shape and/or a change in the interaction between casein micelles in solution.

3.1.5 Apparent viscosity modulated by the CMs structure and interaction after UHT treatment

The apparent viscosity of the four CMs dispersions presents a pseudoplastic behavior such as preconized by Bouchoux et al., (2009) at this protein concentration. The complete curves of apparent viscosity in relation to a shear rate are presented in the supporting information (figure 3 in SI), and in the present section the results are characteristic from the plateau obtained at a shear rate between 300 to 500 (s^{-1})

Below (figure 27) are presented the results of apparent viscosity for the three calcium demineralized CMs samples and the native CMs before and after UHT treatment.

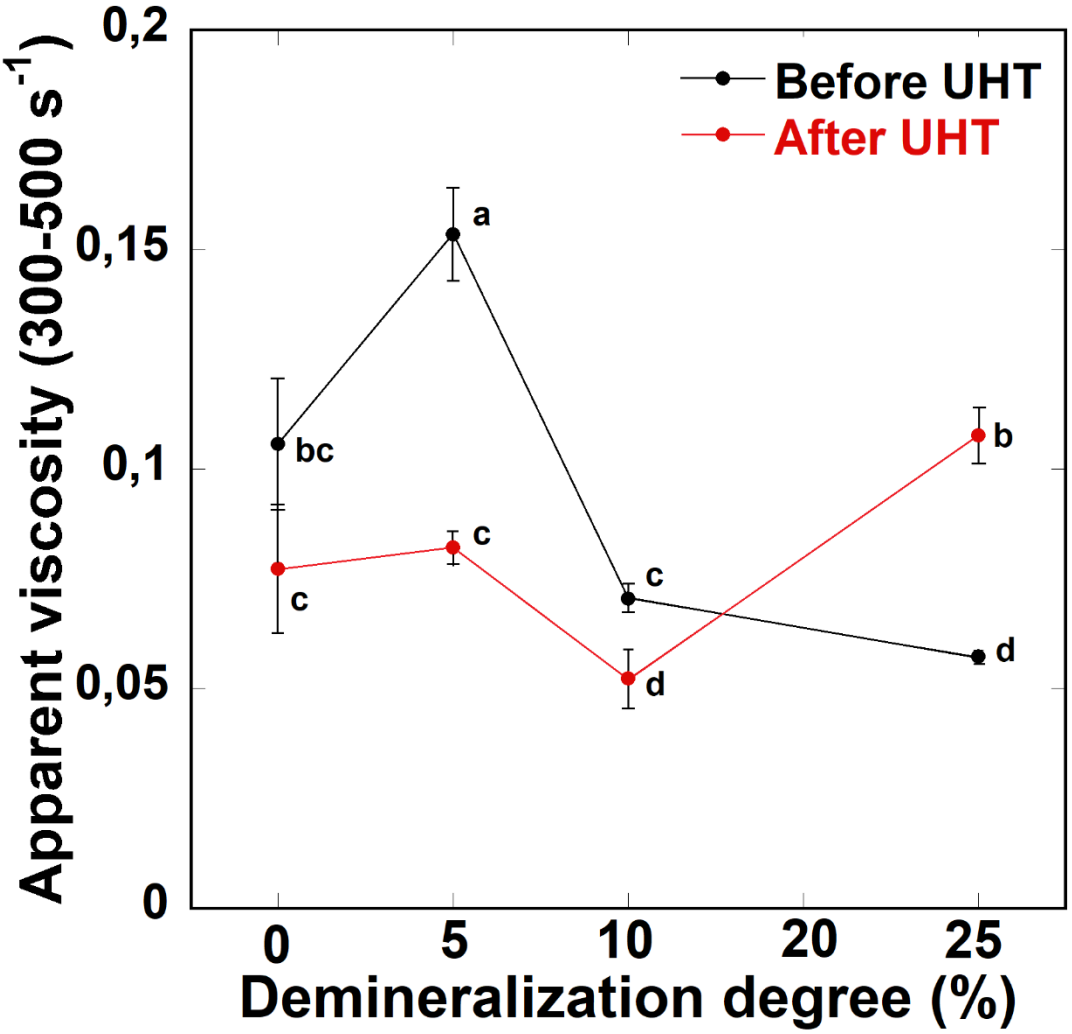

Figure 27 – Apparent viscosity of CMs with different demineralization level, before UHT (black circles) and after UHT (red circles).
Error bars correspond to standard deviation; different letters represent statistical difference at $p<0.05$.

As illustrated in figure 27, three of our samples (Native UHT, DM-05 UHT, and DM-10 UHT) show the same tendency, a decrease of apparent viscosity after the UHT treatment. For the native sample, the UHT promotes a non-significant decrease in the apparent viscosity.

For the two intermediary demineralized samples (DM-05 UHT and DM-10 UHT), a significant decrease in the apparent viscosity was observed as a consequence of the UHT treatment.

For the most demineralized sample (DM-25) the UHT treatment was responsible for increasing its viscosity significantly. This viscosity increase was also reported in the

bibliography (Pandalaneni et al., 2018) for milk protein concentrates with a modified calcium content by the addition of calcium chelators. These changes are associated with the changes in the structure and interaction of the CMs that can be observed in the SAXS results.

In principle, the apparent viscosity of dense dispersions of demineralized CMs is correlated with the CMs size (in terms of hydrodynamic size) and/or the potential of interaction between micelles in terms of repulsiveness/stickiness (Rueb & Zukoski, 1998). A direct correlation between viscosity and CMs hydrodynamic diameter, at least for the DM-10 and DM-25, is not found after the UHT treatment (based in DLS data), indicating that in this case, the interaction between the CMs micelles is an essential element that governs the apparent viscosity and contrary to some results described in the literature (Kort et al., 2011; Pandalaneni et al., 2018) the demineralization also can induce a decrease in the apparent viscosity of dispersions produced with CMs.

For almost all samples (exception for the DM-25 UHT), the internal changes go in the same sense, which is the calcium diffusing to the interior of the CMs as a result of its decrease of solubility caused by the UHT treatment, as confirmed by the Ca^{2+} quantification and NMR analysis. However, this calcium does not form native clusters, and the structure of the incompressible regions was more and more affected as a consequence of demineralization and UHT treatment, from DM-05 UHT to DM-10 UHT, as evidenced by the SAXS results.

This calcium fixation to a non-native seems to form a kind of “armor” for the structure of the CMs as a consequence of the UHT treatment, resulting in CMs with less capacity to interact, thus decreasing the viscosity.

For the DM-25 UHT, the results were completely different from the other samples in terms of viscosity and structure. As evidenced in the Ca^{2+} quantification and confirmed by NMR and SAXS analysis, the CMs of this sample lose part of its CCP as a consequence of the UHT treatment affecting its incompressible regions drastically.

As observed by the SAXS results, the structure of the CMs was utterly changed as an effect of the UHT treatment, including an increase the intensity for the regions associated with the CMs size and inter distances. As illustrated by the DLS results, its

hydrodynamic diameter decrease after UHT treatment, which means that the CMs interaction has changed.

In the case of the other three samples (Native, DM-05, and DM-10), it seems like the calcium present in the soluble phase goes to the interior of the CMs and acts as a protector of its structure, resulting in a less sticky protein. However, the DM-25 presents more sticky CMs as a consequence of an insufficient amount of calcium and the most disordered CMs, which will produce the most viscous dispersion after the UHT treatment. These viscosity changes observed for the three demineralized samples (DM-05 UHT, DM-10 UHT, and DM-25 UHT) could be the results of the evolution of the sticky properties, explaining the decrease of apparent viscosity for the two intermediary demineralization degrees (DM-05 UHT and DM-10 UHT) and the increase in viscosity for the DM-25 UHT.

4 – General conclusion

This study has evaluated the structural and rheological changes observed in dense CMs dispersions (14% protein) submitted to a sterilization treatment (UHT). Three calcium-demineralized were utilized and compared with a native CMs, DM-05, -10, and DM-25 for samples with 5%, 10%, and 25% less calcium ions than the native control sample. It has been demonstrated that UHT has a greater impact on the structure of the demineralized CM than the native one. Actually, UHT favors the "capture" of calcium and phosphorous ion by the micelle preferentially in the "old" CCP-depleted sites. This ionic "recapture" induces a small change in CM size and a change in the interaction between CMs (stickiness). These changes affect the apparent viscosity of the respective casein-based dispersions significantly, indicating a non-monotonous relation between demineralization and apparent viscosity for dense casein dispersions submitted to UHT, just as the ready-to-drink high-protein beverages. The present data allows a better understanding of the impact of UHT over CMs structure and an overview of the apparent viscosity evolution that could be expected after the demineralization practices. This work presents an innovative point of view in order to select the most appropriate casein-rich protein ingredients for high-protein ready-to-drink beverages.

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Supporting information for:

Impact of ultra-high-temperature (UHT) on native and demineralized casein dense dispersions: structural and rheological changes

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Table 3 - General composition of the powders utilized in the present study.

%	Native	DM-05	DM-10	DM-25
Moisture	6.25	4.73	3.99	4.96
Protein	82.16	83.81	84.94	85.97
Lactose	3.13	3.95	2.98	3.23
Ash	7.68	7.17	7.11	7.03
Calcium	2.35	2.24	2.16	1.81
Phosphorus	1.53	1.48	1.40	

The constituents represented in table 1 are representative of the four different powders, with different demineralization degree as Native for the no-modified calcium content micellar casein powder; DM-05 for the 05% calcium demineralized powder; DM-10 for the 10% calcium demineralized powder; DM-25 for the 25% calcium demineralized powder.
The results were expressed as % (g/100g of powder)

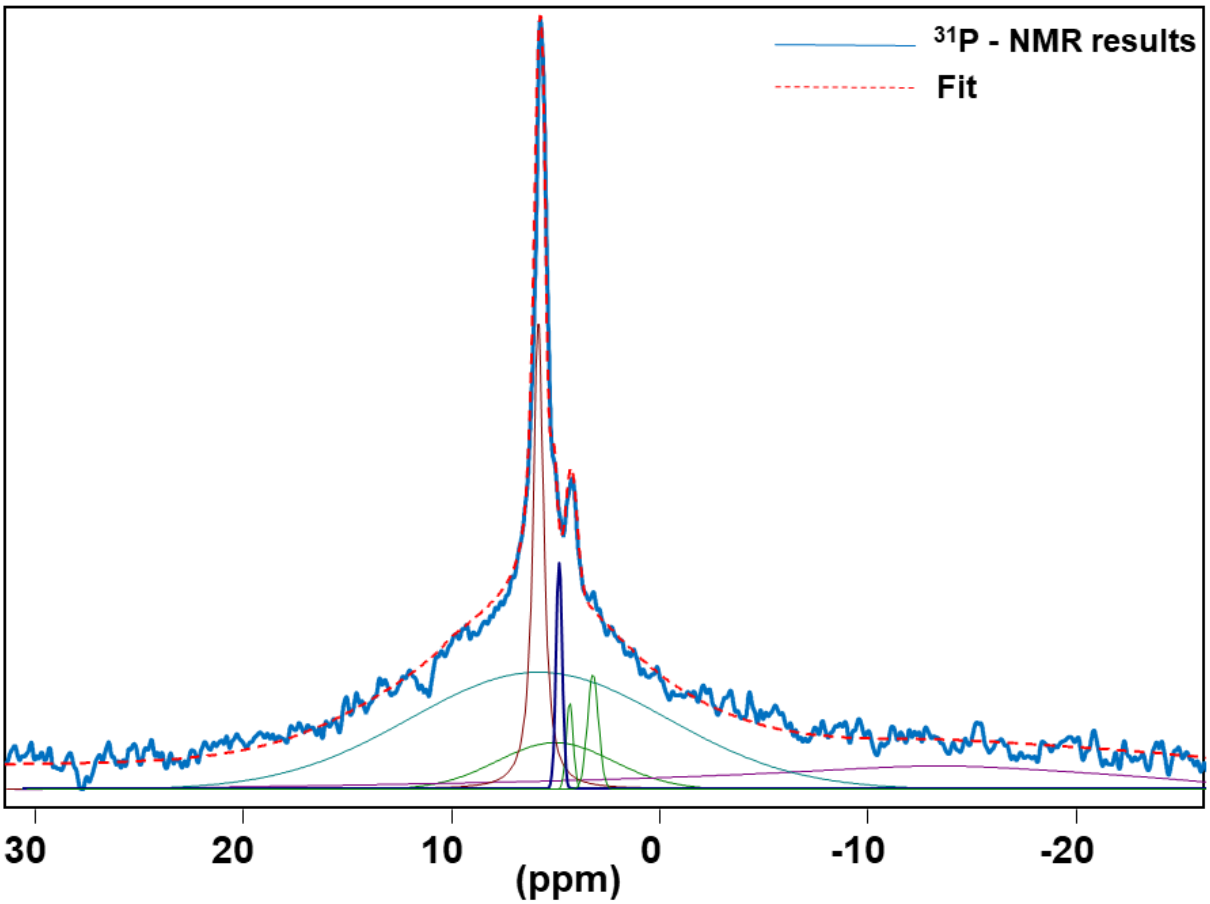

Figure 18 - ^{31}P - NMR spectra (blue) and its perspective fitting (red).
The fitting line is a results of a decomposition of different peaks, which are represented in the figure as the peaks with different colors.

Table 4 – CSA parameters obtained from the Cross-polarization (CP) analysis of NMR of four CMs powders with different amounts of calcium before and after UHT treatment.

	δ11 (ppm)	δ22 (ppm)	δ33 (ppm)
Native	-32.43	-20.45	63.40
DM-05	-34.86	-23.31	57.59
DM-10	-37.89	-19.92	60.93
DM-25	-36.55	-18.25	64.98
Native UHT	-34.85	-19.44	65.34
DM-05 UHT	-36.55	-17.74	66.92
DM-10 UHT	-36.75	-18.25	64.98
DM-25 UHT	-32.96	-21.33	60.05

Being: (Native) representing the native CMs without changes in the calcium amount; (b) (DM-05) representing a 05% calcium demineralized casein samples; (DM-10) representing a 10% calcium demineralized casein sample; (DM-25) representing a 25% calcium demineralized casein sample

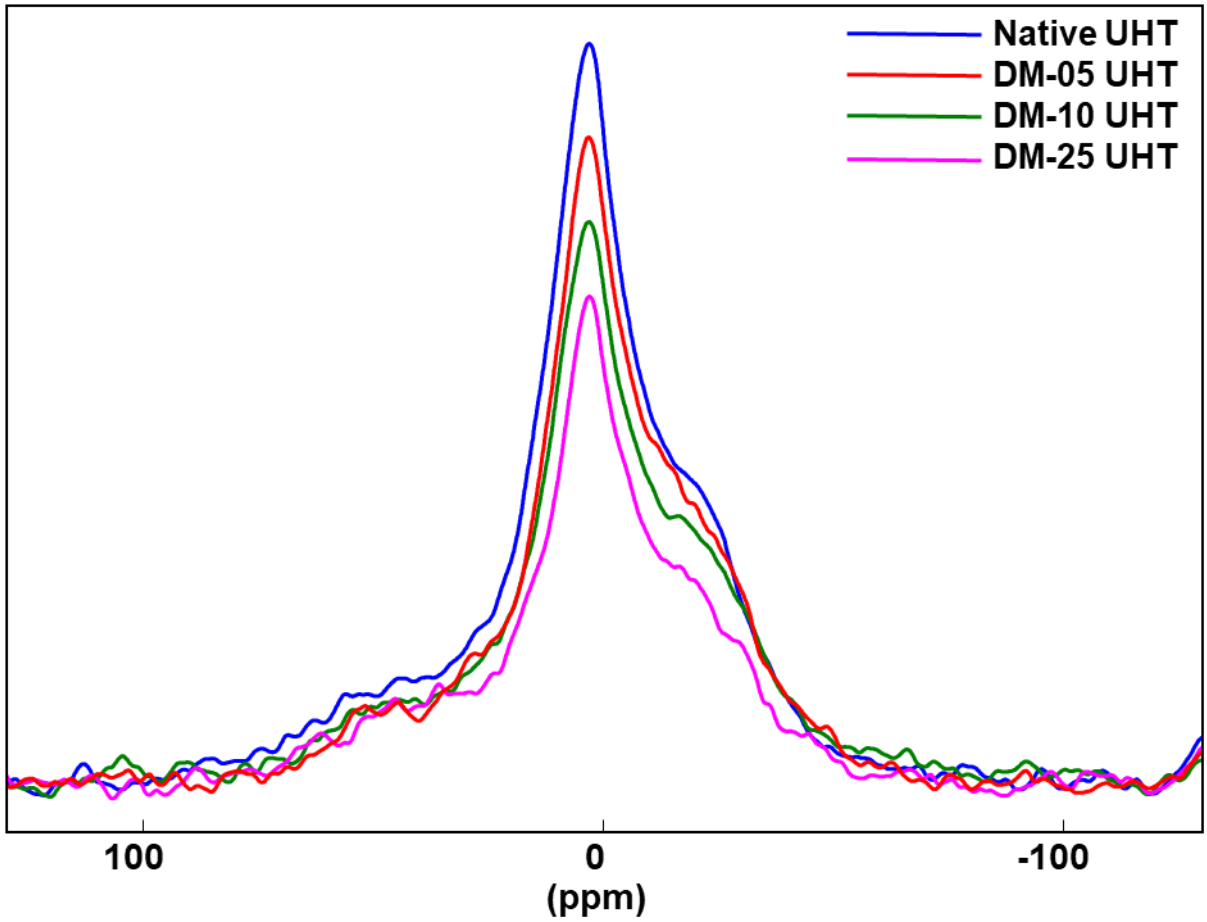

Figura 219 - Cross-polarization (CP) spectra from the NMR analysis of CMs with different amounts of calcium submitted to the UHT treatment.
Being: (Native) representing the native CMs without changes in the calcium amount; (b) (DM-05) representing a 05% calcium demineralized casein samples; (DM-10) representing a 10% calcium demineralized casein sample; (DM-25) representing a 25% calcium demineralized casein sample

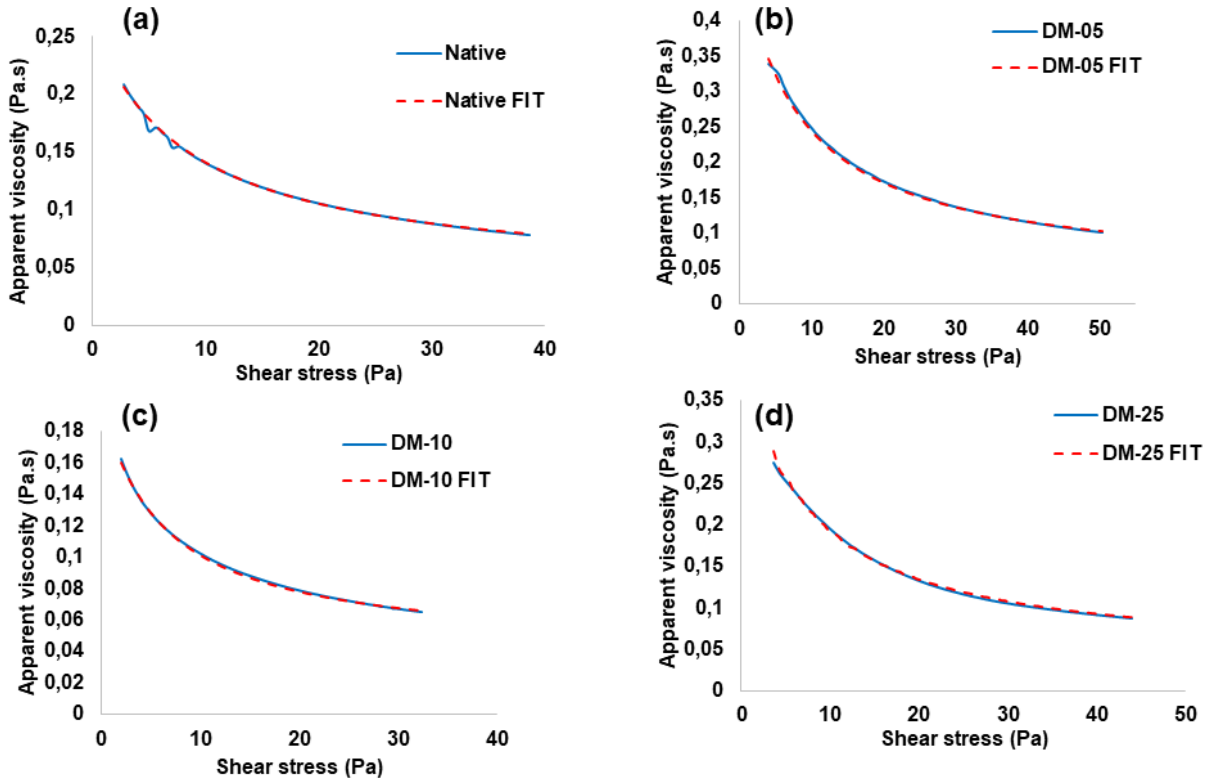

Figure 3 - Apparent viscosity results (blue lines) for the four different CMs powders with its respective fits (red lines) after the UHT treatment.

(a) Native casein sample; (b) 05% calcium demineralized casein samples (DM-05); (c) 10% calcium demineralized casein sample (DM-10); (d) 25% calcium demineralized casein sample (DM-25)

Study of pilot-scale casein-based powders: intermediate conclusion

It has been established that the demineralization is responsible for a non-linear change in the CMs structure. Both the colloidal calcium phosphate (CCP) nanoclusters and the structure of the proteins associated to the CCPs are affected but differently according to the demineralization degree; the CCPs are more affected in the less-to-moderate demineralized CMs and, in contrast, the so-called “hard structures” are more affected in the most demineralized CM. This indicates that there are, at least, two types of nanoclusters present in the CM structure; nanoclusters in the core of the so-called “hard regions” (the lasts to be removed) and nanoclusters at the border of the “hard structures” in contact to the water cavities (so-called “void regions”) composed exclusively by the solvent (the firsts to be removed). The removal of the first type of cluster has little impact on the CMs structure, and the removal of the last type induces a greater disorder in the CM nano-structure.

It has been shown that UHT has a varying impact on the CM structure according to the demineralization degree. However, It has been that the evolution of apparent viscosity with a demineralization degree, after rehydration and after UHT treatment do not follow monotonous trends and the same shape; in particular, the shape of these curves becomes different for a high degree of demineralization.

It has been observed that, for the samples with weak-to-moderate degrees of demineralization, the main impact of UHT on the CM structure is the internalization of calcium phosphate ion by the CM. After UHT, the concentration of calcium and phosphate ions decreases in the soluble phase, and it increases within CMs. This effect is greater in weak-to-moderate demineralized samples than in the native one. In contrast, for the most demineralized sample, UHT induces a greater structural change and a loss of nanoclusters. Moreover, for samples displaying weak-to-moderate degrees of demineralization, UHT is responsible for producing the less viscous dispersions. It is likely that calcium internalization during UHT neutralizes the reactive chemical groups (hydrophilic and charged sites), on the surface of the CMs which are likely to favors a sticky interaction between. In contrast, for the most demineralized

sample, UHT induces an important increase in viscosity. In this case, it is likely that the further increase in disorder induced by UHT favors a further exposition of reactive sticky chemical groups. Thus, the UHT dependent liberation of these groups favor the sticky interaction between CMs resulting in the increase of the viscosity of the dispersion.

CHAPTER 4 – GENERAL CONCLUSIONS AND PERSPECTIVES

4.1 - General conclusions

The market of high-protein ready-to-drink beverages become a trend in recent years, especially with products produced with whey proteins. However, casein micelles, represents an excellent alternative source of proteins to this application, due to its abundance in milk, its excellent nutritional properties and its resistance to heat treatments.

A drawback in the use of CM dispersions for this application is its high apparent viscosity. To decrease the apparent viscosity, a structural modification by demineralization can be used. However, the impact of these structural changes has not yet been the object of a profound scientific investigation.

To fill this gap, this thesis has analyzed the structural and techno-functional changes in CMs dense dispersions produced with different degrees of demineralization. The casein-rich dispersions were submitted to almost the same procedure that is needed to produce ready-to-drink beverages, from powder rehydration to UHT treatment.

It has been demonstrated that the CMs structure evolves as a function of the demineralization degree. The impact of the demineralization is visible until the rehydration behavior on the casein powders. It was found that the demineralization associated to high-temperatures during rehydration (50 °C/1h) was responsible for internal changes in the CMs structure, which results in aggregation. It was also proved that demineralization, during rehydration, is responsible for liberating some reactive chemical groups (hydrophilic and/or charged ones) that favors the formation of hard-to-dissolve particles.

These data bring more clarity about the role of the nanoclusters in maintaining the structure of the micelle and the impact of the demineralization on the casein micelle organization. The data obtained in this field represent an improvement in the sponge-like model described by Bouchoux et al., (2010) showing that not all the calcium phosphate nanoclusters are located in the interior of the “hard” structures of the CMs or that exist some clusters that are strongest linked to these structures than others.

This study also brings important information about the evolution of the structure and the organization of CM dispersions after being submitted to UHT. It is clearly shown that there is no existence of monotonous trends linking decrease of apparent viscosity versus increase of demineralization degree, suggesting us that demineralization degree have to be finely tuned. It has been revealed that a low-to-moderate degree of demineralization (between 5 to 10% less calcium ions) is the ideal to produce less viscous dispersions.

4.2 - Perspectives

This work investigated the structural and techno-functional properties of demineralized CMs dispersions in the context of a high protein ready-to-drink beverage. The structural changes generated from the demineralization were responsible (in some cases) for producing dispersions with reduced viscosity.

Before this study, it has never been suggested that the CM demineralization, associated with UHT, could be responsible for a decrease in the apparent viscosity of dispersions of CMs. These data can be used in the optimization of demineralized CM powders.

The behavior of this “new” CMs under other conditions, such as pH, it is not evaluated in the present study, but also represent a perspective of study, especially in the field of fermented products, such as yogurt.

Another possibility will be to evaluate the impact of these degrees of demineralization on the properties of the powder. As well known, the rehydration of casein-rich powders become more difficult with the increase of the storage time and temperature, but the impact of the storage conditions of these demineralized powders was not investigated