Evaluation by rhéométry and microscopy of the role of calcium on fouling mechanisms in mixtures of milk proteins

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EVALUATION BY RHEOMETRY AND MICROSCOPY OF THE ROLE OF CALCIUM ON FOULING MECHANISMS IN MIXTURES OF MILK PROTEINS

Internship Report

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01/02/2022 - 29/07/2022

Abstract

Fouling is the formation of undesirable layers composed mostly of proteins and minerals on heating surfaces, generally made of stainless steel. This phenomenon significantly affects the performance of operations and the quality of products in the dairy industry. The formation dynamics of these protein-based deposits is linked to the denaturation and aggregation of whey proteins by reactions in the liquid flow and on the surface of equipment. Furthermore, the growth of solid layers is favored by the presence of minerals in the initial solution serving as bridges between activated aggregates. However, the studies available in the literature are based on an "off-line" analysis of solid deposits, therefore fail to shed light on the specific role of each component on the different stages of fouling. Whey protein isolate (WPI) is able to aggregate and by studying the kinetics of denaturation of whey proteins β-lactoglobulin that will allow to understand the mechanisms of thermal fouling in falling film evaporators.

Key words: Evaporators, Thermal fouling, Whey proteins, Denaturation, Rheology, Shear rate, Calcium, Microscopy.

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Brief description of INRAE and STLO research unit

The National Research Institute for Agriculture, Food and the Environment (INRAE). INRAE was created in the 1^{st} of January 2020. It results from the merge of INRA: National Institute of Agronomic Research and ISTREA: National Institute for Research in Science and Technology for the Environment and Agriculture, they are two french public research institutes. INRAE is now the word's leading research organisation specialising in agriculture, food and environment phase to the major global concerns such as : climate change, biodiversity loss, ressources scarcity and growing world population.
INRAE's main goals is to develop solution that involve multi-performance agriculture, high quality food and sustainable management of resources and ecosystems. The institute carried out targeted research on these topics. Just some figures about the institute: it consists of a community of about 12.000 people that are in 268 research and experimental units. They are located in 18 research centers and the institute is divided in 14 scientific departments specialising in various fields (biology, genetics, digital ...).
Concerning the research unit : Science and Technology of Milk and Eggs (STLO). It is located in Britany in the western of France. The two study objects are milk and eggs that are explored in the disciplinary areas of biochemistry, processing and microbiology. The specificity of the lab is that it conducts research according to a multidisciplinary and multiscale approaches. It has two high calibres facilities:

1. The dairy platform, which is an experimental facility at pilot scale
2. Biological resource center that is dedicated to food bacteria (CIRM-BIA).

Chapter 1

General Introduction

Thermal fouling is defined as the accumulation of solute components during flow on heating surfaces generally made of stainless steel, due to both thermal gradient and flow [10, 11]. This phenomena is observed during heat treatment and vacuum evaporation in dairy industry. This accumulation involves a significant problem during milk thermal processing [12] that results in : higher pressure drop, lowered thermal exchange and pipe blockage that may stop the production cycle. Accumulation of these fouling layers favour the development of microorganisms (biofilms, bacteria, spores) that can cause product spoilage and a number of human diseases. As a consequence, costly cleaning steps (time, water, energy and cleaning products) are daily required to restore surfaces to their original cleanliness and to ensure human health safety, storage and export. For this reason, understanding thermal fouling mechanisms is an absolute priority to increase operations efficiency in dairy industry.

Several theoretical and experimental studies have been carried out on this subject leading to development of various theoretical models but these results remain contradictory and based mainly on an "off-line" analysis of solid deposits. Most of these scientific research is focused on the formation of deposits in plate heat exchangers but there is not enough information or results on thermal fouling dynamics in falling film evaporators. The latter is in several aspects a heat exchanger but it is often incorrectly assumed that fouling is similar in both equipment. Indeed, most studies on thermal fouling in heat exchangers are often carried out at temperatures above $70°C$ whereas for an evaporator mostly operates in a range of temperatures ($T = 45 - 80$°C) and shear rate ($\approx 100s^{-1}$) typical of evaporators. Therefore, it is unlikely to predict the same fouling mechanisms in evaporators. However, this equipment is playing an increasingly essential role due to the considerable growth in the production of infant powders, whereas in the past it was used almost exclusively for the production of basic products with low added value (e.g. whole or skimmed milk powder).

In conclusion, despite the technological and economic interest of the subject, the physico-chemical mechanisms of thermal fouling remain to be clarified. Especially, in the case of evaporators due to the complexity of the dairy products processed and the impact of the environmental conditions and the materials used.

1.1 Research hypotheses

1. The unfolding or thermal denaturation of whey proteins in bulk solution is the governing reason for fouling deposit. Unfolded species are responsible for deposition growth.
2. Fouling occur when protein aggregates attach to the stainless steel surface.
3. Deposit formation is due to both aggregates and unfolded proteins during thermal processing.
4. Surface characteristics: roughness and surface energy may participates to the formation of fouling deposit layers.

1.2 Objectives of the study

In this study we aim at:

1. Investigating the effect of temperature and shear rate on the thermal denaturation mechanisms of whey proteins.
2. Determining the impact of protein concentration on the denaturation rate of whey proteins.
3. Understanding the influence of calcium content on the denaturation and aggregation of whey proteins.

1.3 Structure of the internship

This report contains the following chapters:

Chapter 2: —Literature review about the impact of heating and shearing on the physico-chemical characteristics of whey proteins.

Chapter 3: —Materials and Methods to present and describe the equipment and analytical methods used during this internship.

Chapter 4: —Presentation and discussion of results that we obtained during the experimental study.

Chapter 5: —General conclusion about the overall chapters mentioned above and give future perspectives that can be developed in this field of research and may open further directions for the understanding and possible applications.

Experimental strategy

1 → ○ **Impact of heat treatment on whey protein denaturation.**

- ➢ Measure of native protein concentration in the treated samples and its evolution with:
  - ▪ Time (t=0-4h) for T=50°C, 65°C, and 80°C.
  - ▪ Initial protein concentration: [WPI] = 5-10-15-20 %(wt).
- ➢ Average particle size distribution.

2 → ○ **Additional influence of the shear rate.**

➢ Similar experimental conditions than evaporators: $\dot{\gamma}$=100s^{-1} , T=50°C, 65°C, t=0-5-10-20 min, [WPI]= 5-10-15-20 %(wt).

Bulk

- ➢ Rheometry: Couette geometry (constant shear rate).
- ➢ Characterization of protein denaturation and particle size distribution.
- ➢ Optical microscopy (Bright field):
  - ▪ Drying of heat treated and sheared droplets: to get indirect information about denaturation according to the morphology of droplets. « Carreon *et al.* 2018 ».

Surface

- ➢ Rheometry: parallel-plates geometry (constant rotation rate):
  - ▪ Observation of the deposit of labelled whey proteins (aggregates anchored on the surface) by optical microscopy in fluorescence mode.
  - ▪ Observation of the deposit of whey proteins aggregates on glass surface disc.

Chapter 2

Literature review

2.1 Falling film evaporators

Concentration by evaporation consists of placing a liquid under conditions of temperature and pressure that allow vaporisation of solvent (usually water in food processes). The non-volatile elements of the treated product will be concentrated. Evaporators are equipment consisting of long tubes of $5\ cm$ to $25\ cm$ diameter. In the dairy industry, falling film evaporators are the most commonly used, they consist of three main components: an evaporator system, a separator and a condenser. Liquid flows from the top inside the tubes by gravity. To get uniform distribution of the liquid distributors are used to minimise a non uniform distribution of the liquid. Typically, resident time in a falling film evaporator is ($\approx$ 20 minutes and the shear rate value is ($\approx 100 s^{-1}$) in a range of temperature between: ($T = 45 - 80°C$).
Falling film evaporators are susceptible to fouling because of:

—Film break of the product results on the incomplete wetting of the wall.

—Low shear forces on the film, as the film flows only by gravity which facilitate the adhesion of particles to the wall of the tubes.

Multiple effect evaporators : In a multiple effect evaporator (figure 2.1), the steam fed into the first effect produces steam at low pressure. This steam is then used in a second effect as heating steam. Furthermore, the temperature along the evaporator decreases from $70°C$ in the first effect to $45°C$ in the last. The temperature drop from one effect to the next is ($\geq 5°C$).

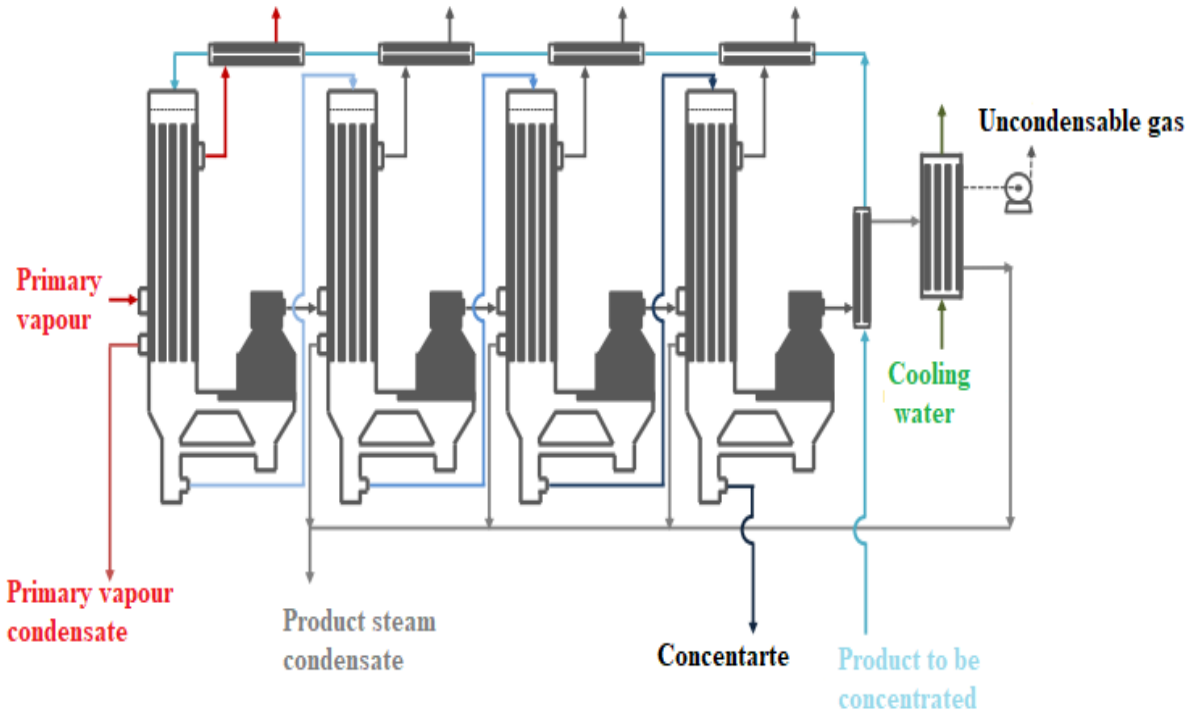

Figure 2.1: Multiple effect evaporator.[1]

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2.2 Dairy fouling

The deposition of fouling layers on the wall of stainless steel surface may be classified into three groups depending on the component material [13], as it will be mentioned below:

—**Microbiologic deposit**: accumulation of micro-organisms on surfaces in the form of biofilms.

—**Mineral deposit**: came from products or water used in the manufacturing process.

—**Organic deposit**: accumulation of products such as: proteins, fats...This type of deposit that occur often in the dairy industry.

It should be noted that the above mechanisms can occur simultaneously, resulting in a composite deposit (Figure2.2).

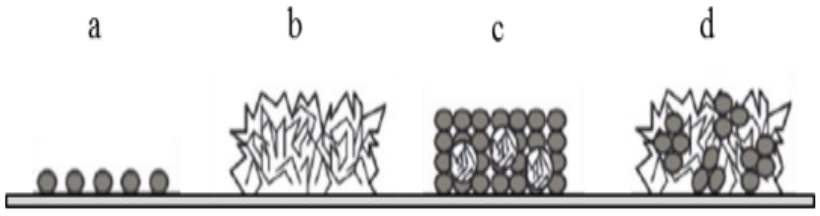


Figure 2.2: Example of deposits following a thermal deposition treatment: a) adsorption of a protein monolayer, b) crystallisation, c) protein deposit with mineral inclusion, d) mineral deposit with protein inclusion. [2]

2.2.1 Chemical composition of fouling deposits

Generally, fouling deposits have been classified into two types: type A and B during thermal processing.
—**Type A** (protein deposit): it is formed at temperatures between $75°C$ and $100°C$. It is spongy, dense white colour. This type of deposit is constituted mainly of $(50\% - 60\%)$ of proteins (whey proteins in particular β-Lactoglobulin) and 5% of lipids.
—**Type B** (mineral deposit): appears at temperatures higher than $100°C$. It is gray, compact, granular and hard. This type contains 75% of minerals (mainly calcium phosphate), $(15\% - 20\%)$ of proteins and 5% of lipids.
The figure (2.5) presents types A and B fouling deposit that occur during heat treatment, depending on the applied temperature.

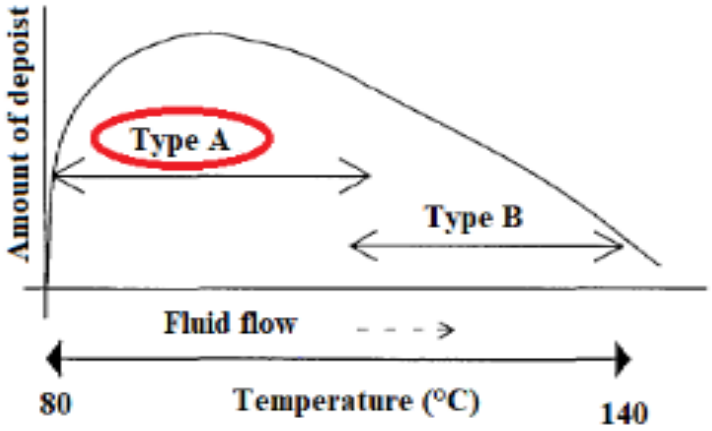


Figure 2.3: Distribution of A and B fouling deposit type with temperature along flow path .[3]

2.3 Chemical composition of milk

The chemical composition of milk and all dairy products is very complex and it is unique for each specie. The percentage of proteins in cow milk is 3.3%(wt) and they are two compounds. First, caseins with a percentage of 78% comprising four major components: $\alpha s1$, $\alpha s2$, β, κ (at 40, 10, 35 and 12 $\%$ respectively). These caseins are organised in form of micelles as spherical colloids 92% of which are proteins and 8% inorganic minerals such as calcium phosphate. Secondly, whey proteins are the soluble part in serum after caseins precipitation [14].

2.4 Whey proteins

0.7% of raw bovine milk corresponds to whey proteins. The four major types of whey proteins are β-Lactoglobulin (β-LG), α-Lactalbumin (α-LAC), bovine serum albumin (BSA) and Immunoglubulins (Ig) with approximate percentage of 57%, 27%, 6% and 10% respectively [15, 16]. The main characteristic

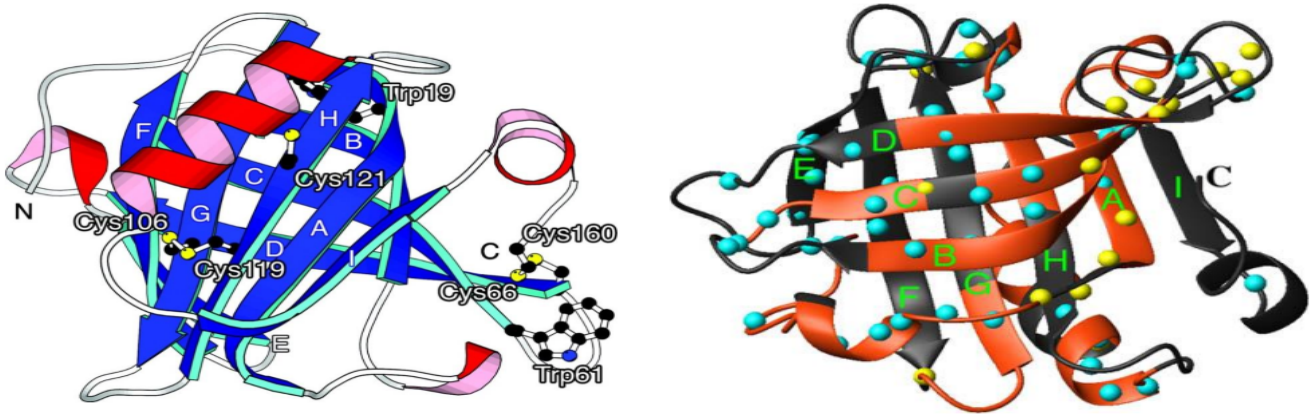
of these proteins is that they have high hydrophobicity and are densely folded peptide chains which are sensitive to denaturation upon $60°C$ [17, 18]. These proteins are classified in terms of heat sensitivity as follow: Ig $>$ BSA $>$ β-LG $>$ α-LAC [19, 20]. The table (3.1) shows some characteristics of whey proteins: MW : is the Molecular Weight (kda).

Whey protein	MW (kda)	Concentration in milk (g.kg^{-1})	Disulphide bonds	Amino Acide residues
β-LG	18.4	3.3	2	162
α-LAC	14.2	1.2	4	123
BSA	66.3	0.4	17	582
Ig	150-900	0.7	21	

Table 2.1: Properties of major whey protein in milk[9]

2.4.1 β-Lactoglobulin

β-LG consists of 162 amino acids with a molecular weight of 18.4 kda [21] and size of about 3mm [22]. Its isoelectric point is $pH_I = 5.3$ [23] and its temperature of denaturation is estimated to be $77°C$ [24, 25]. β-LG is a globular protein with a negative charge at neutral pH, which forms a dimer of two monomers linked with non-covalant bonding [18]. It contains two disulfide bridges (Cys100- Cys119 and Cys66-Cys160) and free thiol group (Cys121) hidden inside the molecule at the native state, figure (2.4a).
This β sheet protein contains nine β-strands and one α-helix at C-terminal end of the molecule and between the hydrophobic residues of this helix and the β-strands is situated the reactive thiol group [26], figure (2.4b).



(a) (b)

Figure 2.4: (a) β-LG structure indicating disulfide bonds and thiol group[4], (b) β sheet protein[5].

2.4.2 α-Lactalbumin

α-Lac present 20% of total whey proteins. It contains 123 amino acids with a molecular weight of 14.3 kda. α-Lac is a monometric globular protein consisting of four disulphide bonds (Cys6-Cys120, Cys28-Cys111, Cys61-Cys77 and Cys73-Cys91)[27]. It is more sensitive to heat treatment than β-Lactoglobulin.

2.4.3 Whey protein denaturation

Under thermal or thermo-mechanical treatment, whey proteins denature and then aggregate[28, 29]. Upon heating, the denaturation mechanism of β-Lg starts with the dissociation of the dimeric structure of the molecule under the conditions of pH and concentration of milk. The disruption of the quaternary structure and the modifications of the tertiary and secondary structures of β-Lg lead to two major events: (1) the exposure of the sulphydryl group of the protein which leads to exchanges of disulphide bridges within the molecule itself or with a neighbouring protein; (2) the unfolding of the protein which leads to an increase in the exposure of its internal hydrophobic zones and, subsequently, to the association of several hydrophobic zones with each other. These two events will eventually lead to the formation of aggregates [30]. According to the literature denaturation and aggregation change the structure of whey proteins in solution and consequently their interfacial properties [31].

2.4.4 Simplified kinetic model to describe thermal denaturation of β-Lg

To give a clear view of the thermal denaturation of β-Lg at temperatures between $60°C - 100°C$, (Oldfield *et al.*, 2005) [32] , have proposed a simplified model of this denaturation mechanism. This model summarises the heat denaturation of β-Lg as a succession of two reactions: $\mathrm{N} \rightleftharpoons \mathrm{U} \rightarrow \mathrm{A}$
Unfolding occurs during heat treatment when the native β-Lg (N) unfolds to form the reversible unfolded β-Lg (U). The U species then reacts with the native β-Lg or other unfolded proteins, via disulphide exchange or thiol oxidation to form irreversibly denatured β-Lg or so called aggregates (A). Each step of the denaturation reaction is governed by kinetics.

2.4.5 Factors influencing whey protein denaturation

2.4.5.1 Effect of temperature

Heating induced destabilisation of whey proteins and also precipitation of calcium phosphate that are the main drivers for component deposit during thermal processing [33]. The β-Lg is crucial during thermal processing because:

1. It is the most abundant whey protein in bovine milk.
2. Contains thiol groups that can interact with other proteins through $-SH$ group.

Therefore, β-Lg molecule can be activated by increasing temperature above $70°C$ result in weakening of hydrophobic bonding and intiating thiol-disulphide interchange reactions [34].

2.4.5.2 Effect of shear rate

Application of mechanical shear also known as velocity gradient or activation energy on the protein molecules will result in destabilisation of native protein structures leading to denaturation and aggregation [35, 36]. A good understanding of this phenomena will lead to the most appropriate design and conditions for high quality products and innovation in dairy industry. For Newtonian liquids, the mathematical formula between the shear stress τ (Pa) and the shear rate $\dot{\gamma}$ (s^{-1}) can be expressed as follow:

$$\tau = \eta \times \dot{\gamma} \tag{2.1}$$

Where η is fluid viscosity $(Pa.s)$.
According to (Wolz *et al.*, 2016) [37] shearing has an effect on the degree of denaturation at different protein concentrations. This study carry out experiments at $80°C$ and shear rates between: 100 and 1400 s^{-1}
—**At high whey protein concentration of** 30%**wt**: after a short heating time of about 10 seconds the denaturation reaction is finished: all whey proteins are denatured (degrre of denaturation $> 95\%$) in this case shear rate has lower impact compared to the lower protein concentration of 5%.
—**At low protein concentration of** 5%**wt**: increasing the shear rate will increase the degree of denaturation. Due to the higher probability of collisions between proteins. These collisions increase also the aggregation rate of proteins.
The combined effect of both temperature and shear can induce a denaturation and aggregation rate more and more important in proteins. The effect of both heat treatment and shear has been studied by several authors: (Steventon *et al.*, 1994) [38] and (Walkenström *et al.*, 1998 (a) and (b)) [39, 40].
Steventon *et al.*, studied this effect at range of temperature between $(75 - 90°C)$ and shear rate value up to $1480s^{-1}$ for a maximum treatment time of 60 minutes. Walkenström *et al.* studied heat and shear effect at temperatures from $20°C$ to $82°C$ at constant rates between 0.5 and 126 s^{-1} for 20 to 240 seconds.

2.4.5.3 Effect of concentration

The concentration of native β-Lg in whey protein solutions has a very important effect on protein denaturation mechanisms and aggregation [41] as there will be a large amount of matter interacting under heat treatment and shear forces. With increasing whey protein concentration the shape and morphology of the formed aggregates will change from irregular particles to spherical ones smaller, denser and more compact [37]. High protein concentration resulted in significantly more fouling [42].

2.4.5.4 Effect of pH

pH is one of the main factors that affect the denaturation kinetics of whey protein during thermal processing. Each whey protein acts differently under pH degree, for example: at $pH = 6.7$ β-Lg has the lower stability while BSA has the highest. It has been demonstrated that when pH is reduced from 7 to 4.5 the β-Lg denaturation decreases. Besides, α-La denaturation is independent of pH values[22].

2.4.5.5 Effect of minerals

The composition of the minerals, mainly the calcium concentration plays an important role in the denaturation of β-Lg especially during aggregation. Researchers have also shown that it is possible to lower the denaturation temperature from $83°C$ to $75°C$ of whey proteins by increasing the calcium chloride ($CaCl_2$) concentration from (5 to 10 mmol) [43]. In the aggregation zone, the denaturation kinetic constant of β-Lg increases with increasing calcium content. Calcium catalyses the aggregation reaction by charge exchange [44]. Furthermore, Phan-Xuan *et al.*, 2013 [45] have shown that calcium favours the formation of large aggregates ($\approx 300nm$).
In addition, analysis of the fouling deposit layers reveals that the deposit formed with low calcium content has a spongy and soft texture whereas the deposit formed with high calcium content is more dense and elastic[46].

2.5 Growth mechanisms of fouling deposits

Whatever the type of deposit, the following mechanisms participate to the formation of deposits on stainless steel surface [3] :
—Bulk chemical reactions that transform chemical constituents on one or more species able to be deposited on surface.
—The transport of initial constituents of precursors of fouling to the surface.
—Adsorption of the first fouling constituents derived from the product into the surface to form the first fouling layer deposition.
—Deposit growth by deposition of several layers on the first one.
—Characteristics and properties of surface material.
The figure(2.5) describes the mode of adsorption of β-Lg molecules onto the stainless steel surface. At $25°C$ native β-Lg molecules are adsorbed to the stainless steel surface to form a monolayer. At $75°C$, this temperature denature the β-Lg molecule by activated the thiol group. At this stage, denatured β-Lg aggregates occurred at the surface that increases the amount of deposit. It has to be mentioned that the formation of β-Lg aggregates molecules occur also in bulk solution but they are hardly adsorbed into the surface. β-Lg denatured molecules are thermally unstable so that the adsorption can occur according to these two ways:

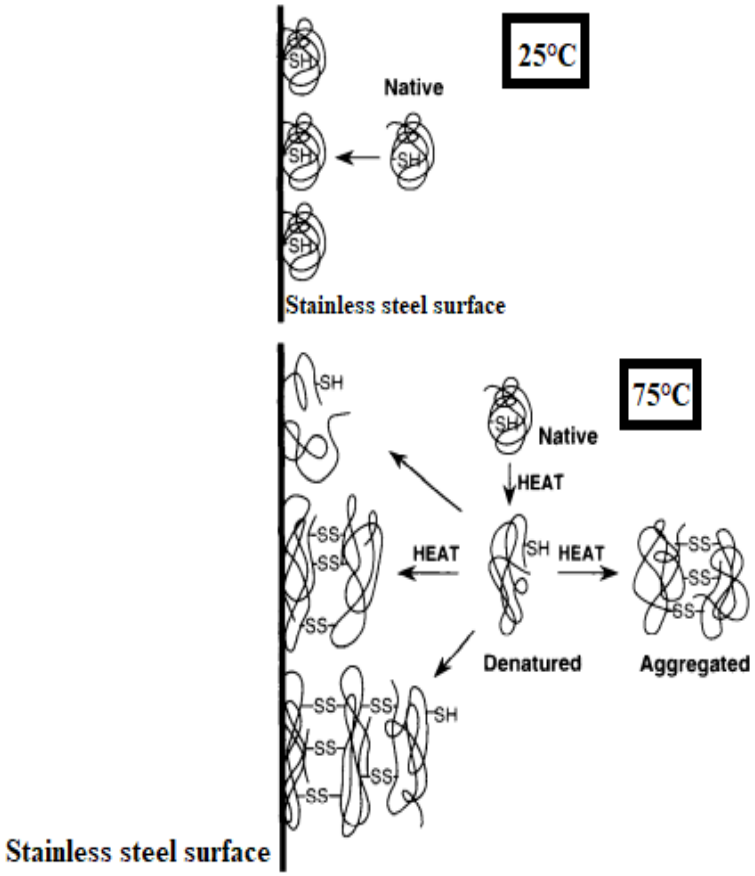


Figure 2.5: Adhesion mechanisms of β-Lg onto the stainless steel surface. [6]

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1. β-Lg molecules already adhered to the surface form disulphide bridges with other molecules near wall surface.

2. Formation of aggregates due to the disulphide interactions between molecules in the bulk solution or migrate to the surface that increases the amount of deposit.

This protein-surface interactions depend on several factors such as: the pH, temperature, physico-chemical properties of the protein molecule and the properties of the surface material.

2.6 Characterization techniques

2.6.1 Methods for measurement of denaturation of proteins

Among several characterisation techniques that are used to measure the rate of denaturation of proteins is: High Performance Liquid Chromatography with Diode Array Detection "HPLC- DAD", based on retention indices and UV spectra. It is a general analytical technique used to separate the different components of a mixture. This method allows to determine the amount of native whey proteins after precipitation and

separation of the denatured and aggregated whey proteins at $pH = 4.6$ [41].
The major whey protein affecting denaturation kinetics is β-Lactoglobulin since it represents over 50% of the total whey protein in bovine milk. The denaturation degree presents the remaining native protein concentration before and after the thermal or the thermo-mechanical treatment in relation to the native protein concentration before the treatment.

2.6.1.1 How does the method work?

The compounds are first dissolved in a solvant. This mixture is introduced into the liquid mobile phase. This mobile phase passes through the pump, after the injector which is the sample containing the compounds of interest (analytes, impurities, solvent). These different molecules will be separated at the level of the column and in this column there is what is called "stationary phase" which will allow the different molecules to be separated according to their affinity to this phase. At the exit of this column, there is a detector, which will allow us to detect the molecules of interest, this detection can be done by different types of detectors radiation (UV-visible, fluorescence, mass spectrometry ...). The separated compounds, transported by the mobile phase are recorded as signal peaks by the detector. The total amount of all peaks is called chromatogram (figure 2.6).

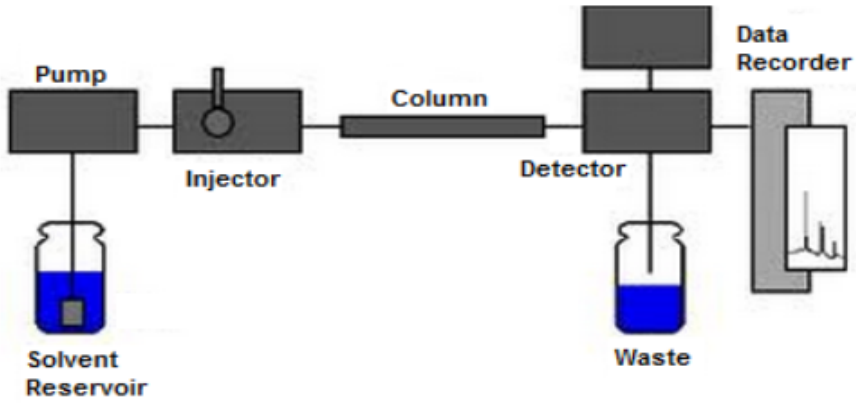


Figure 2.6: Operating principle of HPLC. [7]

2.6.2 Dynamic Light Scattering: (DLS)

DLS is an optical technique used to analyse dynamic properties and particle size distribution: colloidal particles, bubbles, droplets or molecules. DLS measurements allow to:
—Determine the size of particles in suspension where the particles move in random manner due to Brownian motion.
—Measure the velocity of the particles to obtain the diffusion coefficient. There is a dependence between the velocity and particle size: small particle diffuse faster, larger particles diffuse slower.

2.6.2.1 Experimental configuration

The sample is illuminated by a monochromatic laser beam then particles scattered the light. The interference of these scattered waves generates a scattered light intensity signal.It should be mentioned that the Brownian motion causes fluctuations in the scattered light intensity. After that, the correlator takes very fast snapshots of the scattered light, these changes in light intensity with time will allow the correlation function to be calculated according to Stokes-Einstein law:

$$D_H = \frac{K_B \times T}{3 \times \pi \times \eta \times D_C} \tag{2.2}$$

Knowing that :
D_H: is the hydrodynamic diameter [m]. Particles are assimilated to hard spheres that scatter light at the same speed as the particle being measured.
D_C : Correlation coefficient [m^2/s].
T : Temperature [K] .
K_B : Boltzmann constant [$m^2 kg/Ks^2$].
η : Viscosity [Pa.s]
By this technique, size distribution may be presented by:

1. **Number distribution**: particles have an equal weight: particles of 5nm size are equivalent to 50nm particle size for number distribution.
2. **Volume distribution**: particle size of 50nm has 1000 times more volume than the 5nm particle.
3. **Size distribution by intensity**: the intensity is proportional to to the assumed radius of a particle at power six (r^6), this technique is therefore very sensitive to the presence of large particles or aggregates.

Conclusion

State of the art highlighted in this literature review about thermal fouling in dairy products show that this phenomenon is complex to understand and to model because it is governed by several factors. This master internship is a preliminary study on the mechanisms of thermal denaturation of whey proteins especially β-Lactoglobulin. Experimental conditions typical of an evaporator will be considered such as: temperature (50, 65 and $80°C$), neutral pH, different concentrations of whey protein isolate (WPI) (5, 10, 15 and 20 $\%$ (wt)), shear rate of $100s^{-1}$ and calcium content at different treatment times. Surface deposition after treatment is characterized by optical microscope in fluorescence and bright field mode.

Chapter 3

Material and Methods

3.1 Preparation of WPI solution

Whey protein isolates (WPI) with protein content equal to 86%(wt), were dispersed in deionized water containing 0.02% of sodium azide (NaN_3) as a bacteriostatic agent and the fnal mass concentration was adjusted to: 5%, 10%, 15% and 20% (wt).

Mass concentration (wt$\%$)	Mass of the powder (g)	Mass of deionized water ($+0.02\%$ of azide)
5	5	95
10	10	90
15	15	85
20	20	80

Table 3.1: Mass concentration of WPI solutions.

For complete dissolution of WPI powder, the solution was gently stirred with rotation velocity of 360 (rad/s) for about 24h at a temperature of $20°$C.

3.2 Heat treatment

The heat treatment was performed for three different temperatures of $50°$C, $65°$C and $80°$C. For each temperature, the heating ramp time (ie. the time to reach the target temperature) was controlled with a Thermometer inserted in a reference tube. The ramp time was equal two minutes for all heated samples. The unfiltered stock solution of WPI was devided into 15 glass capillary tubes (8mm inner diameter and 40 mm length, Waters, USA) containing 1ml of the solution (Figure 3.2a), were placed in a thermal controlled Fisherbrand ^{TM} Isotemp ^{TM} water bath, Thermo Fisher Scientific, Newington, USA) (Figure 3.1) .

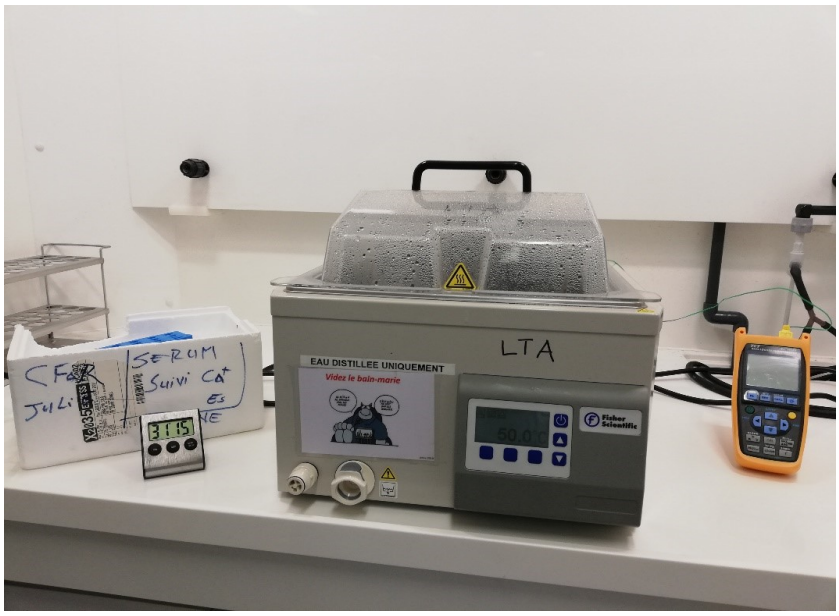
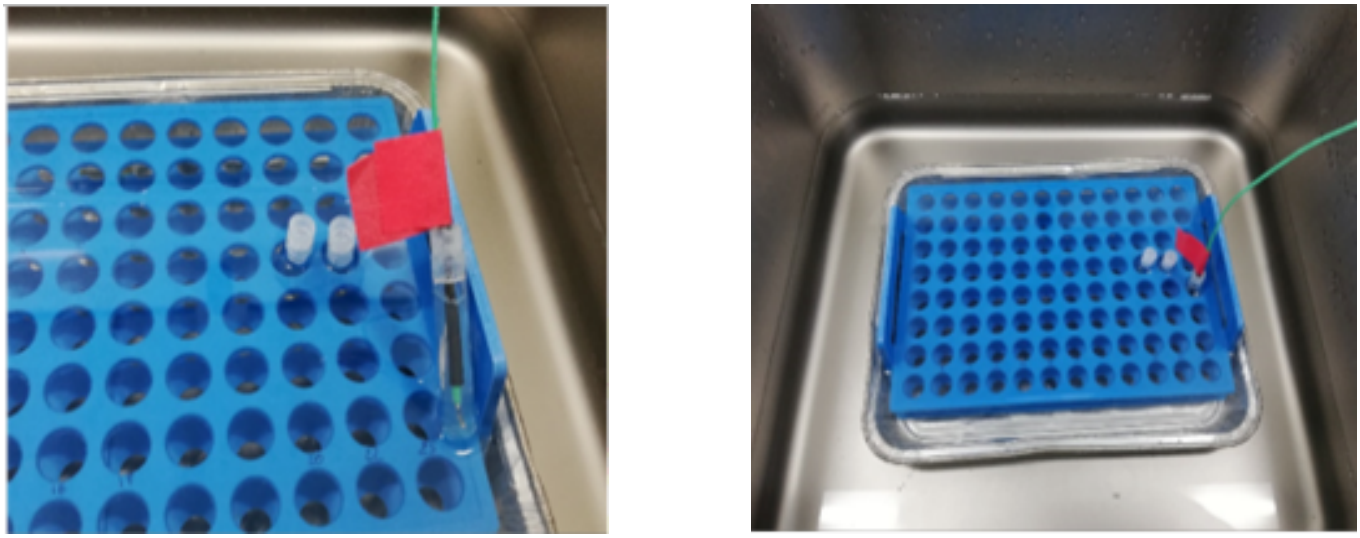


Figure 3.1: Experimental device for thermal treatment.



(a) (b)

Figure 3.2: (a) Glass capillary tube. (b) Tubes holder.

Depending on the heat treatment time : $0, 3, 5, 10, 20, 40, 60, 120, 180$ and 240 minutes, the tubes are taken and immersed directly into ice water to stop the chemical reaction of denaturation or aggregation of the whey proteins. The tubes that have not undergone this heat treatment will be used as a reference to determine the initial level of native proteins present in WPI stock solutions.
Three independent thermal experiment have been performed for these three temperatures : $50°C$, $65°C$ and $80°C$ taking into account the same heat treatment times for these temperatures.

We notice from the colour of the samples that : at $80°C$ all proteins are gelled after just few minutes of treatment. We will not taking into account this temperature for the following experiments we have just consider the temperatures of $50°C$ and $65°C$.

3.2.1 For filtered stock solution

Stock solution is filtered through a $0.45\mu m$ nalgene filter to remove large aggregates that affect the results of the particle size distribution by (DLS). To verify that filtration did not change the initial protein concentration compared to the unfiltered WPI solutions. Spectrometry analyses were performed and the measured of absorbance values "A" were evaluated. Results confirm that concentration doesn't change before and after filtration.
During the tests in the water bath and whatever the temperature of treatment ($50°$C or $65°$C) at ($0, 3, 5, 10, 20, 40$ and 60 minutes, we noticed a little difference of about $0.6°$C between the temperature displayed by the recording thermometer immersed in one of the samples and the temperature displayed by the water bath.

3.2.2 Concentration of native whey proteins by HPLC - DAD

After cooling, in Eppendorf tube: 60µl of an 0.5M acetic acid and 5M acetate buffer was added to 600µl of the sample to lower the pH to 4.6. We mixed the resulting solution with a vortex then place this mixture in a water bath at $30°$C for 5min.By centrifuging at 13,000rad/min for 10min at $25°$C using Eppendorf tubes and in pH 4.6 the fractions of soluble proteins will be separated from the precipitate.
With 1ml pipette, the supernatant is recovered from the samples and transferred in other Eppendorf tubes.

3.2.2.1 Putting in vials

For analysis by high-performance liquid chromatography (HPLC), the supernatant was diluted using TpA (water$+0.106\%$ of Trefluoroacetic acid (TFA)). TFA is a chemical compound with a formula CF_3COOH frequently used as an ion-pairing agent in liquid chromatography for the separation of organic compounds such as : peptides and proteins.
We put the resulting mixture (supernatant + TpA) into 1ml vials after:

1. Well mixing the solution with a vortex for few seconds.
2. Withdrawal the solution from the Eppendorf tubes with 2.5ml syringe and pump through 0.45µm filters into the vials.

3.2.2.2 HPLC equipment

Column : PLRP-S 300A 8m 150∗2.1mm (LTA 38)
Dimensions : 150∗2.1mm
Catalogue number : PLRP-S
Serial number : 0006140396-1
The detection wavelengths are 214 and 280 nm which correspond to the adsorption wavelengths of proteins. For data processing in HPLC analysis is at 214nm.

3.2.3 Particle size measurements

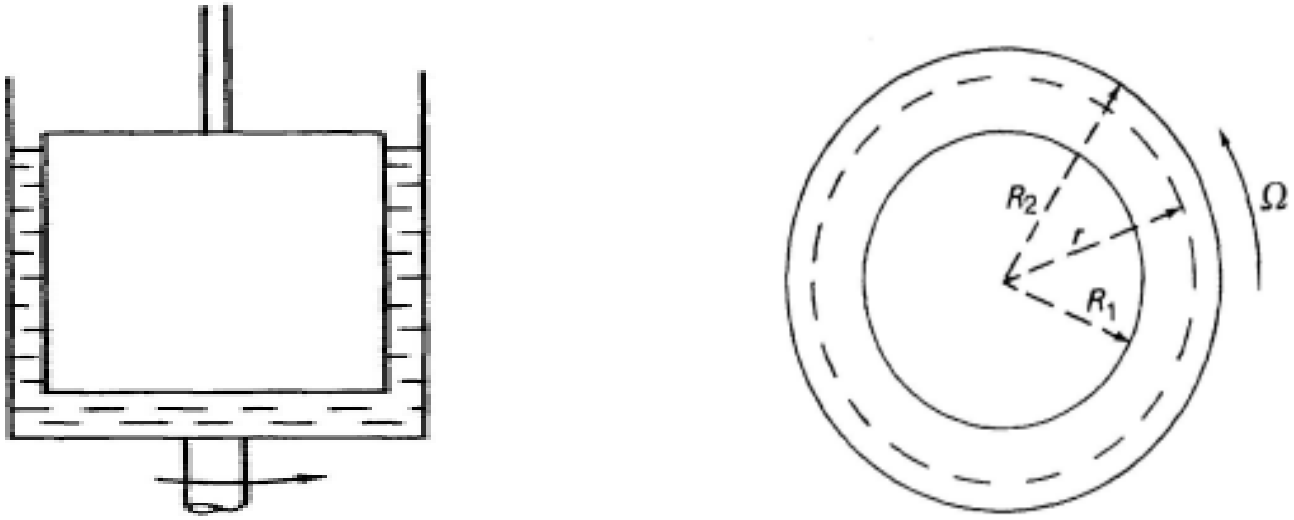
To get the distribution size and the average particle size for the aggregates that are present in our heat treated samples at neutral pH and temperature of $23°C$ at various: concentration and time treatment, we carried out a (DLS) analysis by Zetasizer (Nano Z.S, Malvern, Instruments, Malvern, UK) [47] based on the dynamic light scattering technique (DLS). This measure is used to get particle size with average diameter between: 0.3nm to 10µm for an ideal volume of 1ml of the sample. Samples should be transparent, if they are opaque it will be necessary to dilute it. For our samples we adapt the dilution rate according to the samples because they become more and more opaque aver the time of heating.
Typically, a 5% native WPI solution and lightly heat-treated samples are diluted $1/10$ (100µl of the sample in 900µl of osmosis water), while heat-treated samples are more diluted $1/100$ (10µl of the sample in 990µl of osmosis water).
Each sub-measure (i.e run) lasts 10 seconds. 12 successive sub-measures were performed, so that one whole measure lasts 2 minutes. Every analysis is repeated 3 times and the results are presented as an average of these three runs.

3.3 Shear rate treatment

The whey protein isolate (WPI) solution was sheared at a constant shear rate of $100s^{-1}$ and at two different temperatures of: $50°C$ and $65°C$ for 5min, 10min and 20min for each temperature, in order to apply the same operating conditions of an evaporator. The rheometer that is used to apply shear in the present study is Anton Paar MCR 301 (Figure 3.3) equipped with couette or concentric cylinders (CC) (Figure 3.4a) cell with reference CC17 which corresponds to an air gap of 0.71mm and a cell height of 30mm [48].

Figure 3.3: Rheometer (Physica MCR 301 series: Anton Paar GmbH).

The main advantage of couette geometry is that it allows applying uniform shear rate in continuous regime to understand the shear rate effect on the samples.



(a) (b)

Figure 3.4: The concentric cylinder rheometer (side view on left and top view right) [8]

.

The mathematical equation of couette geometry is: (Figure 3.4b)

$$\dot{\gamma}(r) = \frac{r \times d\omega}{dr} = \Omega \times \frac{r}{R_e - R_i} \tag{3.1}$$

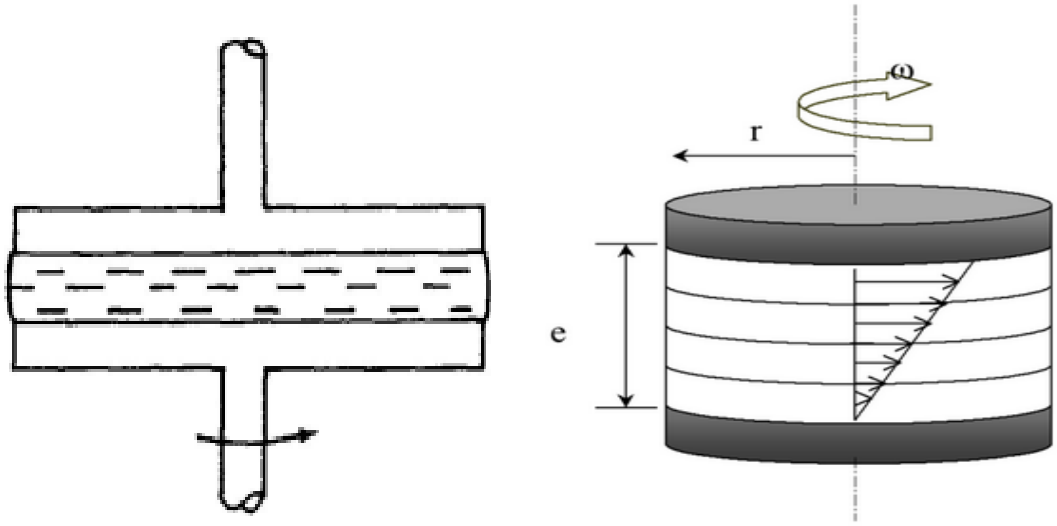
$\dot{\gamma}$: Shear rate (s^{-1}).
Ω : Angular velocity (rad/s).
r : Radius (cm)
R_e : Outer radius (cm).
R_i : Inner radius (cm).
In our manipulations with couette geometry for bulk tests, we have proceed as follow:

1. In the instrument software we fix the shear rate value of $100s^{-1}$, the temperature of $50°C$ or $65°C$ at different shearing times (5min, 10min and 20 min). With a 5ml pipette a volume of 5ml of filtered WPI 5% solution is taken and poured into the cylinder of the couette geometry.

2. The stirrer is dipped into the narrow gap coaxial cylinder which contains the solution and to start applying the shear rate. Two minutes is the average time to reach the target temperature.

3. Pick up the sheared sample with 5ml pipette and pour this volume into glass tubes and place them directly into ice water to stop the chemical reaction of denaturation or aggregation of whey proteins.

Parallel-plates (PP) geometry (Figure 3.5a) has been used also to characterize surface anchored aggregates on the lower plate of this geometry by optical microscopy.
The main characteristic for Parallel-plates geometry is that the shear rate varies from zero at the centre to a maximum at the outer edge.



(a) (b)

Figure 3.5: Parallel-plates rheometer [8].

The mathematical equation of parallel-plates geometry is: (Figure 3.5b)

$$d\gamma = \frac{r \times \omega \times dt}{e} \rightarrow \dot{\gamma} = \frac{\omega \times r}{e} \tag{3.2}$$

$\dot{\gamma}$: Shear rate (s^{-1}).
ω : Angular velocity (rad/s).
r : Radius of the parallel disk of the geometry (cm).
e : Sample gap height (cm).
In our manipulations for parallel-plates, we have proceed as follow:

1. For the first part: in the instrument software we fix the angular velocity value at $4s^{-1}$, the temperature of $65°C$ at different shearing times (5, 10 and 20 minutes). With a 5ml pipette a volume of 2.5ml of labelled WPI 5% (by RITC labelling) solution is taken and poured into the lower plate of the rheometer.

2. For the second part with the same experimental conditions like the first part. A glass disc with the same diameter as the lower plate of the rheometer is glued with a double-sided skotch. The aggregates anchored on the surface of this disc is observed by optical microscopy for WPI and WPI with calcium content samples.

3.4 Optical microscopy

An optical microscope (Olympus upright microscopes, BX51) offers excellent observations for bright field and fluorescence mode. In our case it is used to characterize the morphology of the droplets crack of WPI samples and surface-anchored aggregates by fluorescence.

3.4.1 Drying droplets

The droplets of heated and sheared WPI samples were placed onto clean glass coverlips using a syringe for sessile configuration. The volume of the droplets was 0.5µl. The droplets were evaporated under controlled conditions: $T = 20°\text{C}$ and relative humidity (RH) of 40%. Several tests ($\approx ten$) were carried out on the same sample for bulk tests.

3.4.2 Aggregates observation

The lower plate surface of the parallel-plates geometry of the rheometer has been characterised and it shows some roughness that will hepl us to understand the effect of surface characteristics on the aggregates deposition mechanisms. WPI stock solution was labelled by RITC-labelling to facilitate the detection of labelled protein aggregates anchored to the surface of the lower plate of the rheometer after heating and shearing.
Same experiment without labelling was carried out to characterize the effect of calcium content in WPI samples on the deposition of aggregates on the glass disk surface. These samples are compared to the ones without calcium content.

Chapter 4

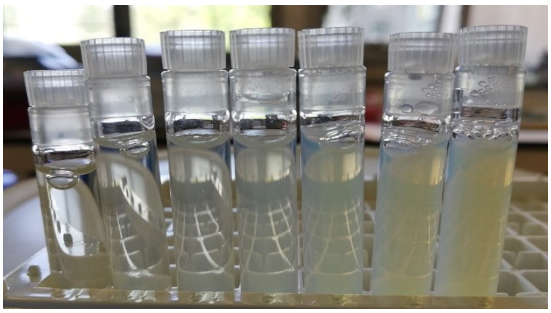
Results and discussion

4.1 Influence of heating on the stability of whey proteins

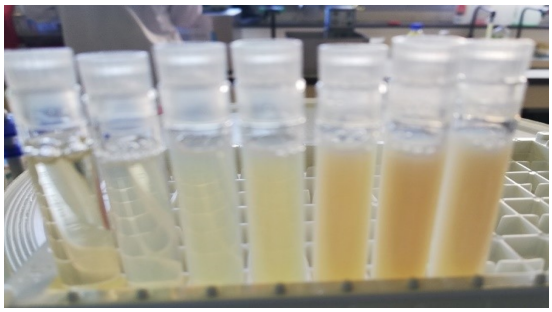
4.1.1 Influence of heating temperature

In the following, the results are presented for the following treatment times: 0, 3, 5, 10, 20, 40 and 60 minutes. The figure (4.1) shows the appearance of the samples after heating of : 5%, 10%, 15% and 20% WPI samples at $65°C$. Qualitatively, we can see the change of the colour in the WPI samples as a function of heating time and also as a function of the concentration.

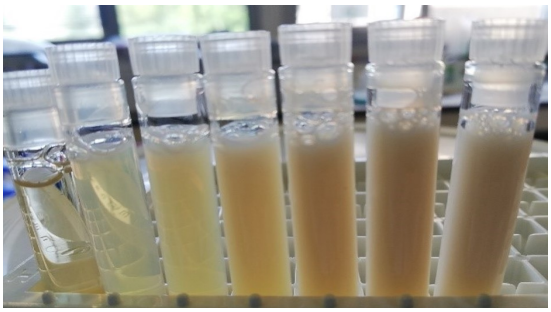
(a): 5% WPI



(b): 10% WPI



(c): 15% WPI



(d): 20% WPI

Figure 4.1: Appearance of $5\%wt$, $10\%wt$, $15\%wt$ and $20\%wt$ WPI samples treated at $65°C$ from 0 to 60 minutes.

In the other hand, the appearance of 5%, 10%, 15% and 20% WPI samples at 50°C for heating times of: 0, 3, 5, 10, 20, 40 and 60 minutes doesn't show any change in the colour of the samples.
Figure (4.2) shows the percentage of native proteins remaining in the solution after different heating times. Results are obtained by High performance Liquid Chromatography : HPLC analysis.

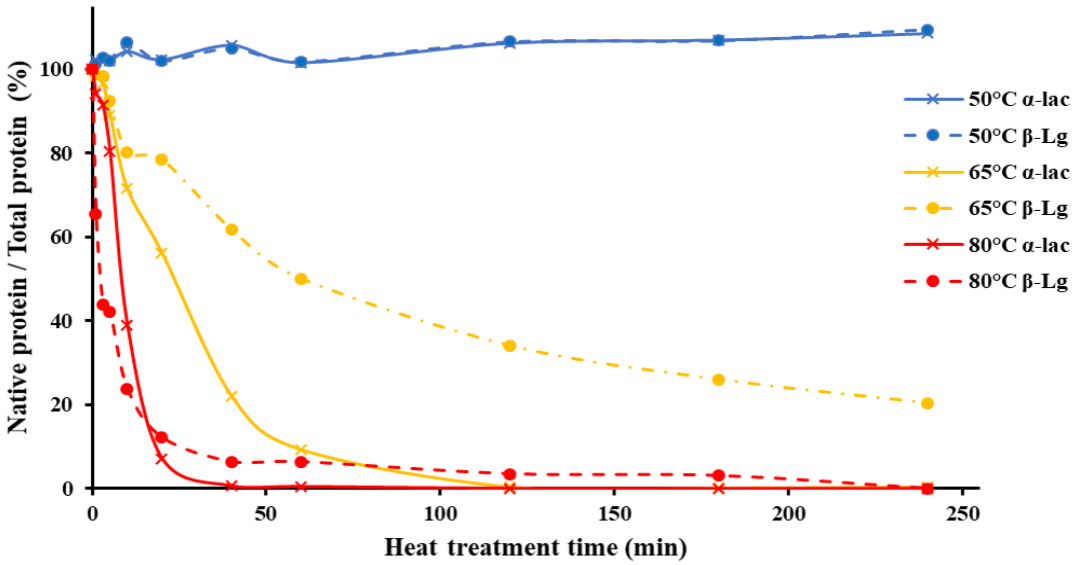


Figure 4.2: Evolution of whey proteins level as a function of heating time for three different heating temperature: 50°C, 65°C and 80°C.

Denaturation of whey proteins is known to occur mainly between 65°C and 80°C [49]. Denaturation is immediate at 80°C, but there are no denaturation kinetics at 50°C. It can also be seen that in addition to temperature, the duration of heating has an effect on protein denaturation because the concentration of natives decreases more and more with time.
Figures (4.3, 4.4) show the concentration of native whey proteins compared to the total concentration of proteins present in the samples at different heating times: 0, 3, 5 and 20 minutes. The temperature of 65°C induces denaturation of β-lactoglobulin according to (figure 4.3). It has strong effect when the total protein content increases. Moreover, at 15% and 20% protein we observe a gelation of the samples after treatment times of 10 and 5 minutes respectively. It should be noted that α-lactalbumin is denatured more quickly (figure 4.4) than β-lactoglobulin for equivalent heating times.

Regardless of the protein content, from $5\%wt$ to $20\%wt$ protein, a heating temperature of 50°C does not affect the denaturation kinetics of the samples. So that, the denaturation rate of the whey proteins (α-lactalbumin and β-lactoglobulin) is a constant as a function of heating time.

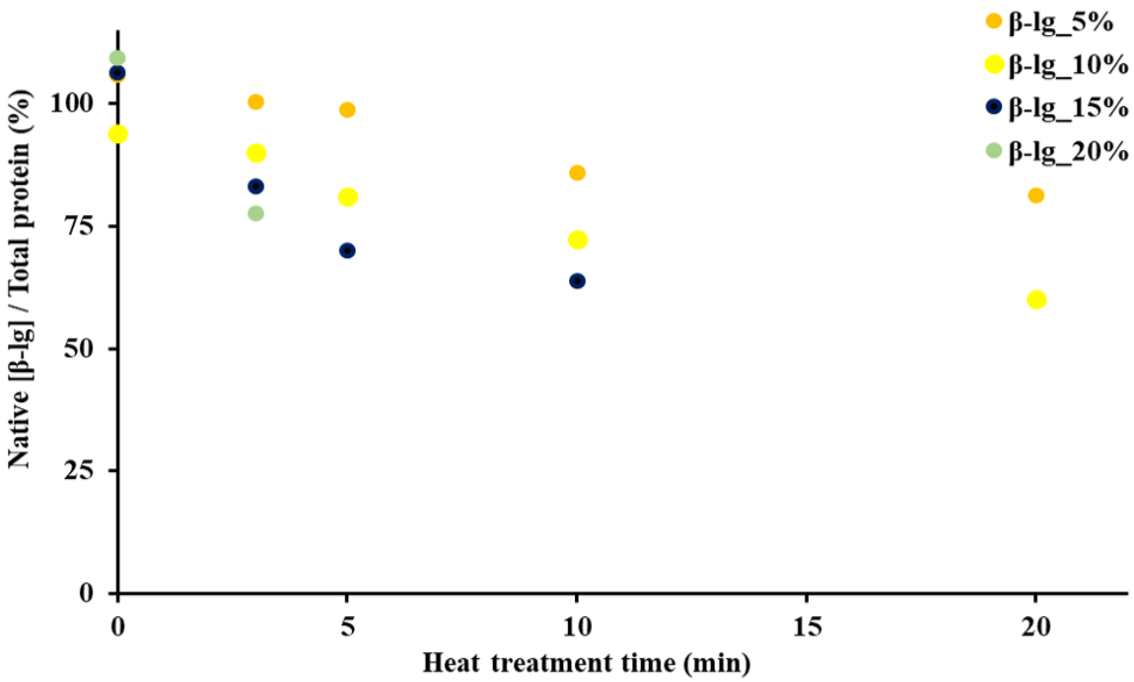


Figure 4.3: Evolution of native β-lg concentration as a function of heating time at a heating temperature of $65°C$.

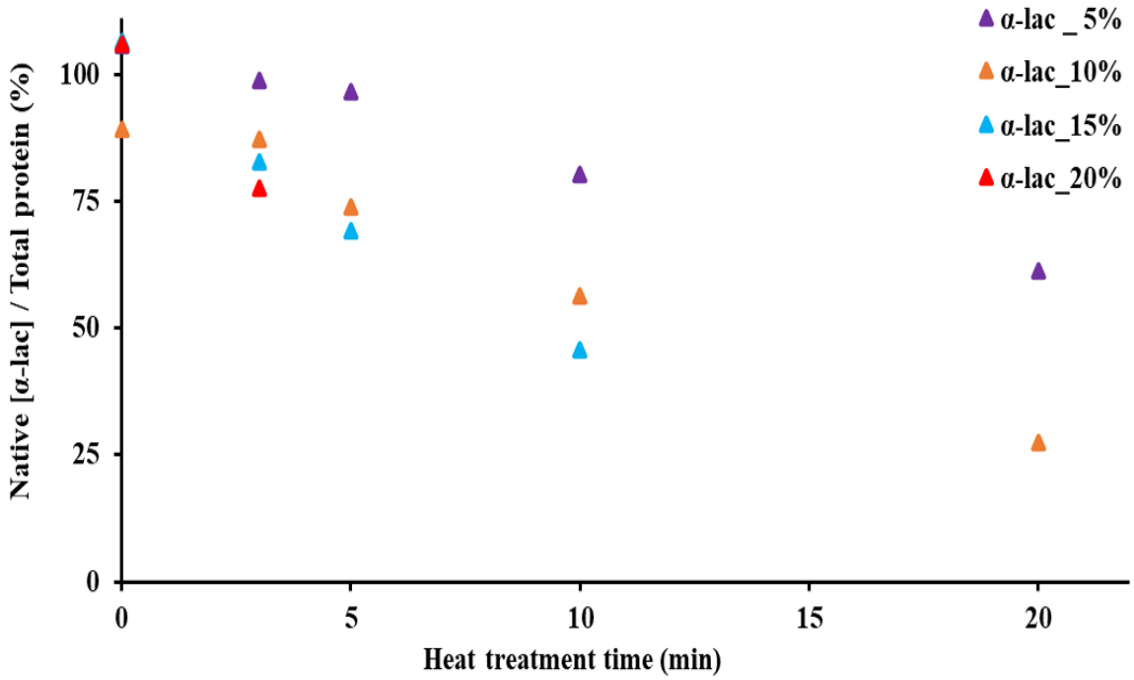


Figure 4.4: Evolution of native α-lac concentration as a function of heating time at a heating temperature of $65°C$.

4.1.2 Does the denaturation correspond to a change in terms of particle size distribution?

These heating samples will then undergo a size distribution analysis by DLS to estimate the size of the particles or aggregates present in these samples, which will also allow us to explain the effect of heating on the size distribution. The results are presented below for temperatures of $50°C$ and $65°C$ for: 5, 10 and 20 minutes. We are taking into account the same heating time as the shear rate experiments in the rheometer in order to make a comparison and see if the shear rate has an additional effect on whey protein denaturation for the same experimental conditions as the heat treatment.
By increasing the temperature to $65°C$ of the $5\%wt$ and $20\%wt$ WPI solutions (figures4.5, 4.6) respectively. The size distribution curves show the appearance of a single peak that presents one population of aggregates with a more monodispersity for a size around $100nm$. The peaks in figure (4.5) overlap so there is no effect of the heating time on the peaks for samples treated at $50°C$. In figure (4.6) show peaks that shift more and more as a function of heating time towards the large aggregates around $1000nm$ of size. At a temperature of $65°C$ and $20\%wt$ of WPI concentration the solutions are gelled from 5 minutes of treatment so we have presented only the peaks that correspond to 0, 3 and 5 minutes of heat treatment.

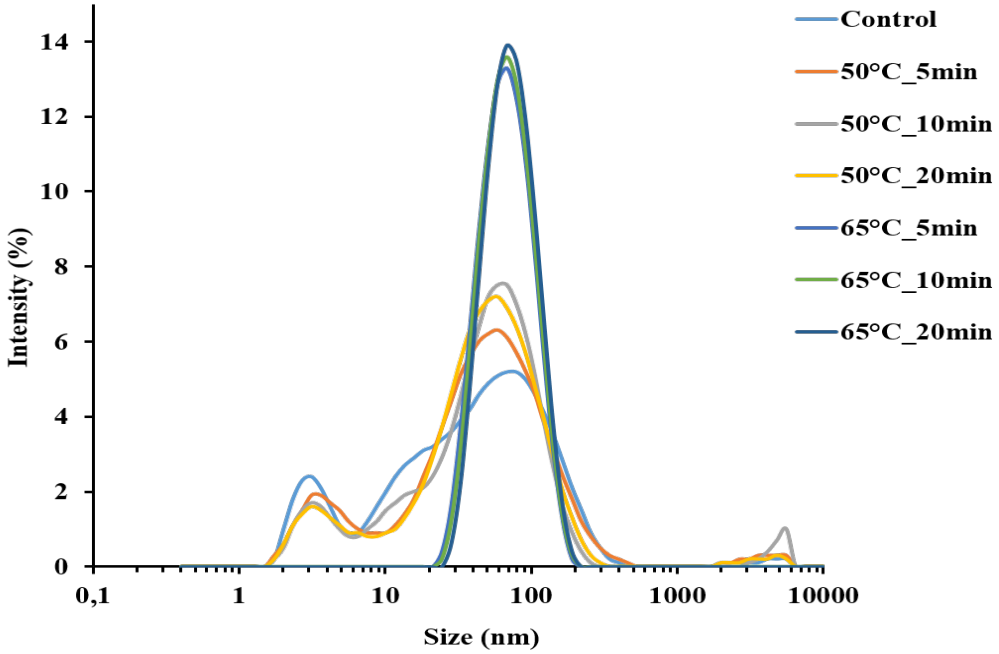


Figure 4.5: Particle size distribution of $5\%wt$ WPI at: $50°C$ and $65°C$ and different heating time of 5, 10 and 20 minutes.

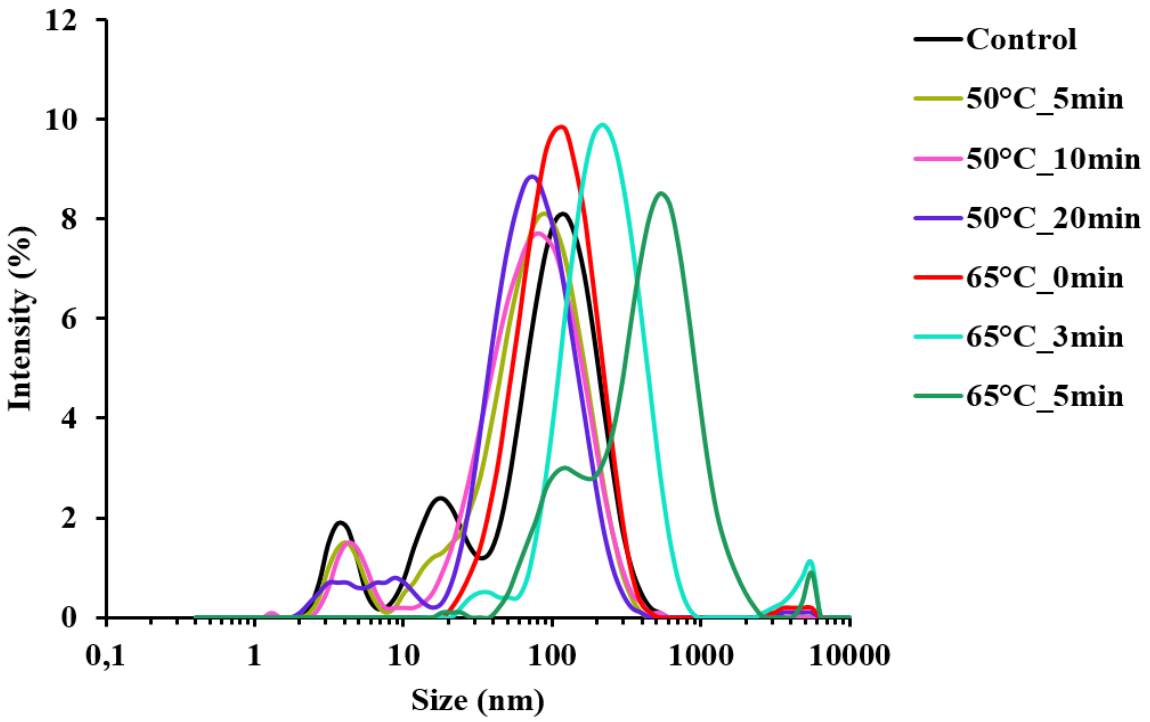


Figure 4.6: Particle size distribution of $20\%wt$ WPI at: $50°C$ and $65°C$ and different heating time of 5, 10 and 20 minutes.

Reaching the denaturation temperature of whey proteins of $65°C$ and a concentration of $20\%wt$ increase the kinetic energy of the reagent protein particles. This will increase the amount of effective shocks and the speed of reactions. These chemical reactions leads to an increasingly large particle size.

4.2 Influence of shear rate on the stability of whey proteins

4.2.1 In the bulk : Influence of heating and shearing in the rheometer

Three important parameter are taken into account for these experiments:
—**Temperatures :** $50°C$ **or** $65°C$;
—**Shear rate :** $100s^{-1}$.
—**Heating time** : 5, 10 and 20 minutes.

4.2.1.1 Temperature of $50°C$

For heat treated samples at $50°C$ (these samples were previously treated in the water bath), no outstanding denaturation of whey proteins (α-lactalbumin and β-lactoglobulin) was observed because the denaturation temperature has not reached yet. The same behaviour was observed for heated and sheared samples. Native protein concentration of (α-lactalbumin and β-lactoglobulin) is constant as a function of treatment

times of: 5, 10 and 20 minutes. We conclude that there is no additional effect of shear rate at these fixed experimental conditions.
In figure (4.7) we represent the evolution of whey protein denaturation level at $50°C$ for :low concentration of $5\%wt$ WPI and high concentration of $20\%wt$ WPI. Increasing concentration at a temperature of $50°C$ and shear rate of $\dot{\gamma} = 100^{-1}$ don't affect the denaturation kinetics of whey proteins for : 5 , 10 and 20minutes.

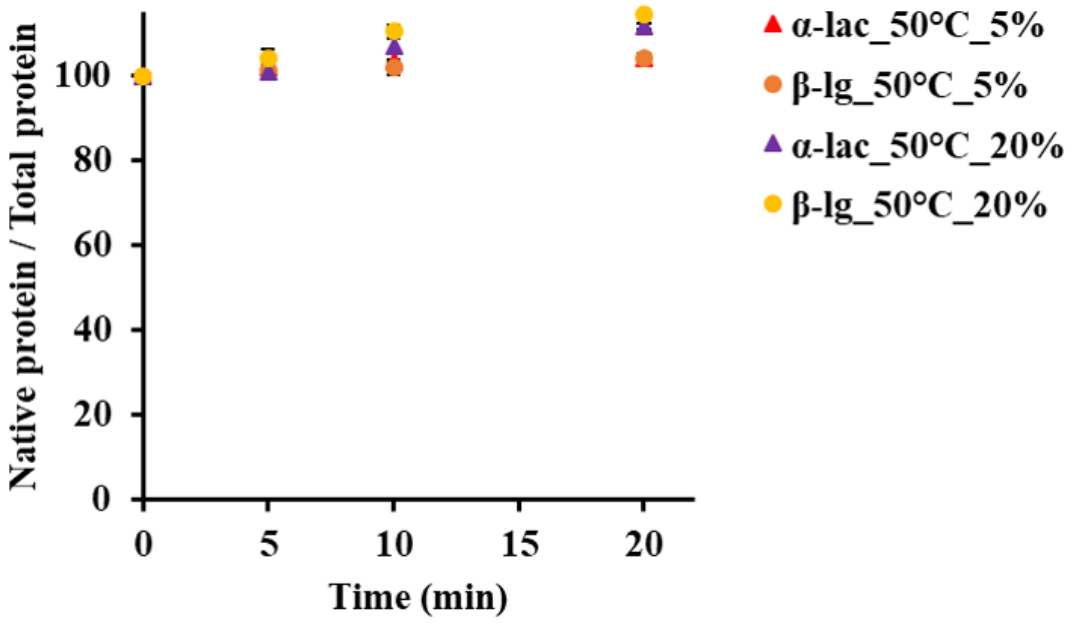
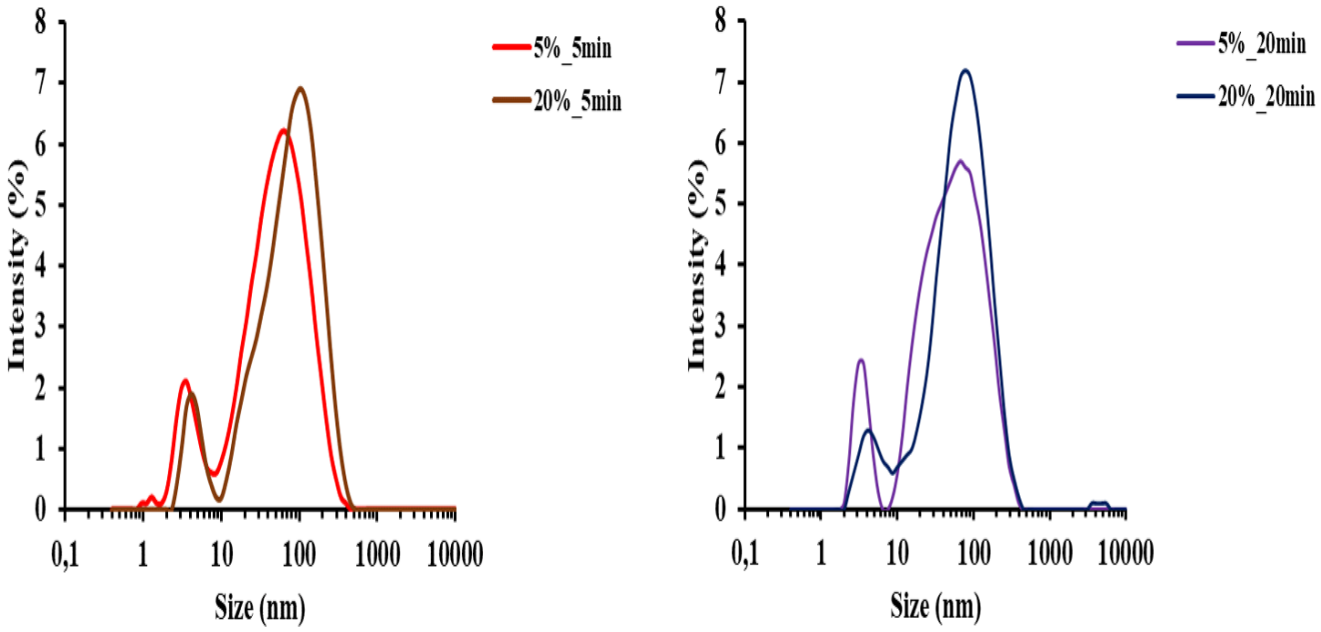


Figure 4.7: Evolution of native whey protein level for heating temperature at $50°C$ and sheared samples at $\dot{\gamma} = 100^{-1}$ for different concentrations : $5\%wt$ and $20\%wt$ of WPI.

For average size measurements (figure 4.8), we notice that by increasing concentration of the WPI solution until $20\%wt$ the size distribution peaks are narrower arround 100nm size than the peaks at $5\%wt$ WPI and shift a little towards the large aggregates. The period of the heating time has no effect as there is no difference between the peaks at 5 and 20minutes.



(a) (b)

Figure 4.8: Comparison of particle size distribution between heated and sheared samples at $50°C$ and $\dot{\gamma}$ = 100^{-1} during **5minutes** with different concentrations of WPI $5\%wt$ and $20\%wt$. (b) Comparison of particle size distribution between heated and sheared samples at $50°C$ during **20 minutes** with different concentrations of WPI $5\%wt$ and $20\%wt$.

4.2.1.2 Temperature of $65°C$

Denaturation rate of α-lactalbumin and β-lactoglobulin is more pronounced for samples that are heat treated in a water bath conditions (see figures 4.3 and 4.4). On the one hand, there is not a such effect of thermal denaturation already observed in figure (4.2) on heated and sheared samples in the rheometer conditions figure (4.9). The temperature of the solution in the out surface of the rheometer is almost reached with ($\approx$2°C) less than $65°C$.

In the other hand, dynamic light scattering results figure (4.10) show a difference in the size distribution of aggregates. At higher temperature of $65°C$ the peaks are more narrow and show more homogeneous size distribution around 100nm size. Adding to that the peak of native aggregates around 10nm disappear for the samples that are heated at $65°C$ and sheared at $\dot{\gamma}$ = 100^{-1}. Shear rate with these experimental conditions brings an effect on the homogeneity and uniformity of the aggregate size distribution.

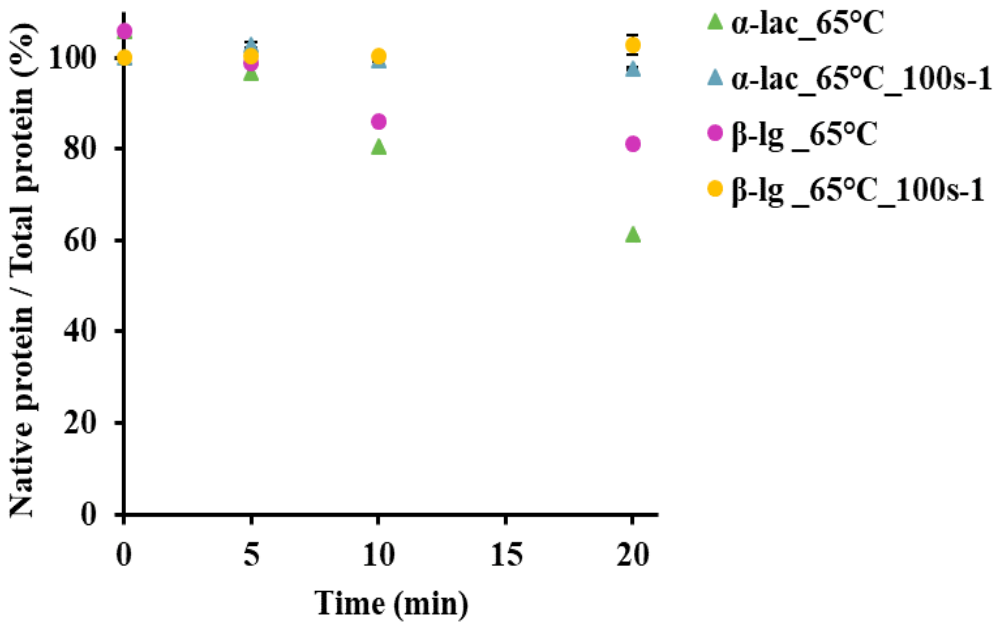


Figure 4.9: Evolution comparison of native α-lac and β-lg concentrations between heated samples at $65°C$ and heated and sheared samples at $65°C$ and $\dot{\gamma} = 100^{-1}$ of $5\%wt$ WPI concentration for : 5, 10, and 20 minutes.

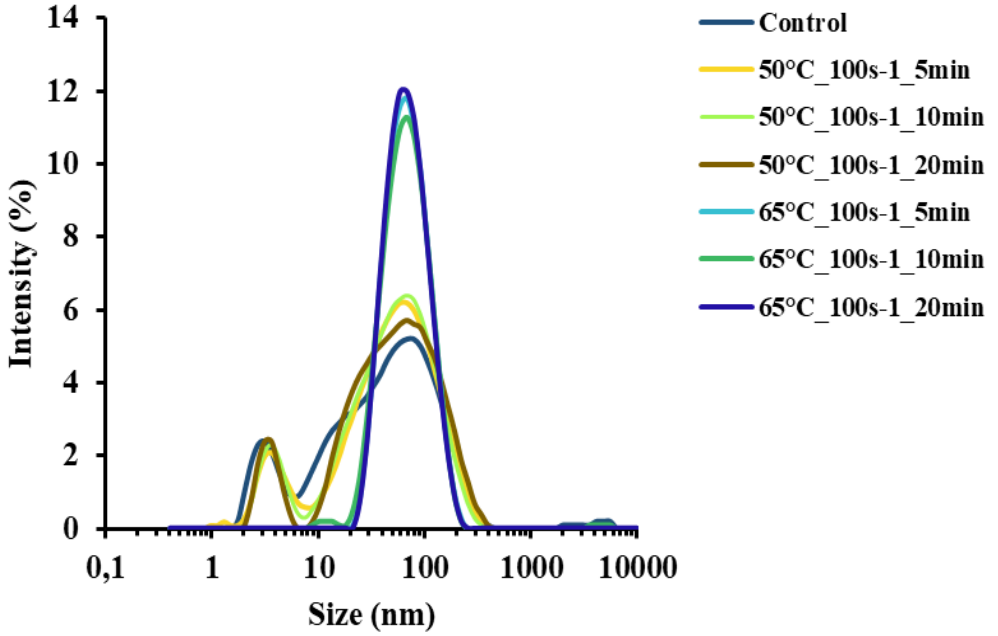


Figure 4.10: Size distribution of whey protein isolate particles for heated and heated and sheared samples at various temperatures of $50°C$ and $65°C$ for: 5, 10 and 20 minutes

.

Explication concerning HPLC results : From the results of High Performance Liquid Chromatography that show no denaturation kinetics in all samples that are heated and sheared at $50°C$ for the reason that the denaturation temperature is not reached yet. The same behaviour was found even at $65°C$ that is

known as the temperature of denaturation of whey proteins (see figure4.2). While the Dynamic light scattering curves we obtained show a different size distribution at $65°C$ compared to that obtained at $50°C$ with the same shear conditions at $\dot{\gamma} = 100^{-1}$. That is may be due to the experimental protocol applied during the preparation of vials for HPLC that can be improved or to the analysis machine in our laboratory. These repeated results of HPLC led us to look for another alternative method that will allow us to investigate the denaturation rate in whey protein isolate solutions. This method will be developed in the next section.

4.3 Influence of Calcium content on the stability of whey protein

In this section we have investigated the effect of calcium chloride ($CaCl_2$) content on the denaturation of soluble proteins, aggregate size distribution and surface mass deposition. For this $0.5g$ of ($CaCl_2$) was added to 200ml of $5\%wt$ WPI solution. The solution was recovered after stirred for 24hours at a temperature of $20°C$.
Samples were heat treated and sheared at $65°C$ and $\dot{\gamma} = 100^{-1}$ for: 5 , 10 and 20minutes. HPLC analysis were carried out in order to measure the concentration of the remaining native protein in the heated and sheared samples with this amount of calcium content. No denaturation of native proteins (α-lactalbumin and β-lactoglobulin) is observed even with the addition of calcium. The concentration of native proteins in the solutions remains constant as a function of time.
In the second part, particle size measurement show more larger aggregates than samples without calcium content (see figure 4.11). These particles were monodispersed with a microscale size ($\approx 100\mu m$). According to figure (4.11), the peaks of particle size distribution shift as a function of time toward more larger aggregates. Calcium ions caused further aggregates of whey proteins. [50]

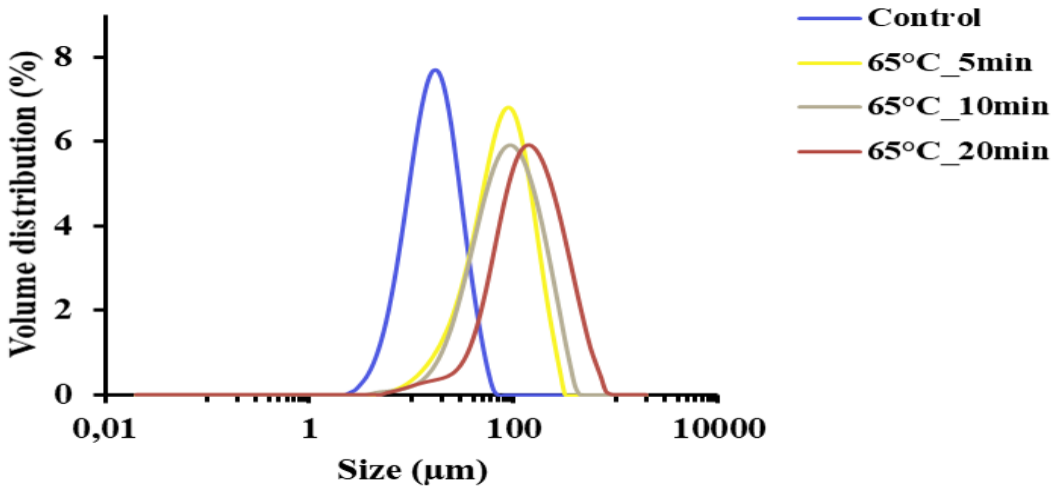


Figure 4.11: Particle size distribution of whey protein isolate for heated and sheared samples of WPI $5\%wt$ with $CaCl_2$ content at $65°C$ and $\dot{\gamma} = 100^{-1}$ for various treatment time of: 5, 10 and 20 minutes.

4.4 Optical microscopy : Drying droplets

Droplet evaporation is one of the crucial element to characterize the morphology of the crack droplets as well as the deposit of patterns. In our case drying droplets technique is used as an alternative method to estimate the denaturation and aggregation rate of WPI and WPI with calcium content samples after heating and shearing. The deposition of the materials in the droplet is formed after evaporation of this one. The final morphology of a deposit depends on the transport mechanisms and the aggregation processes of the colloidal particles. The competition between continuity-driven capillary flows and Marangoni flows driven by surface tension gradients determines mass transport within a droplet [51].
The native $5\%wt$ WPI solution shows crack in the droplet (see figure4.12). This figure is a reference allow us to compare changes in the morphology of the crack with other samples.

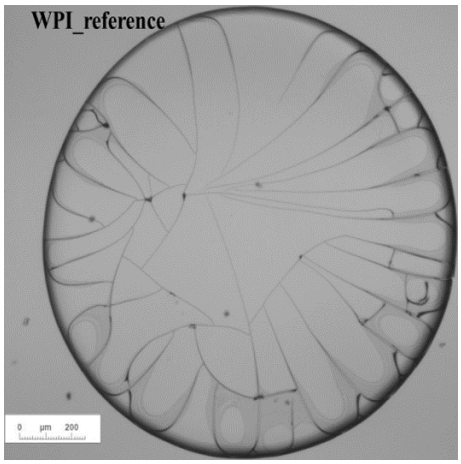
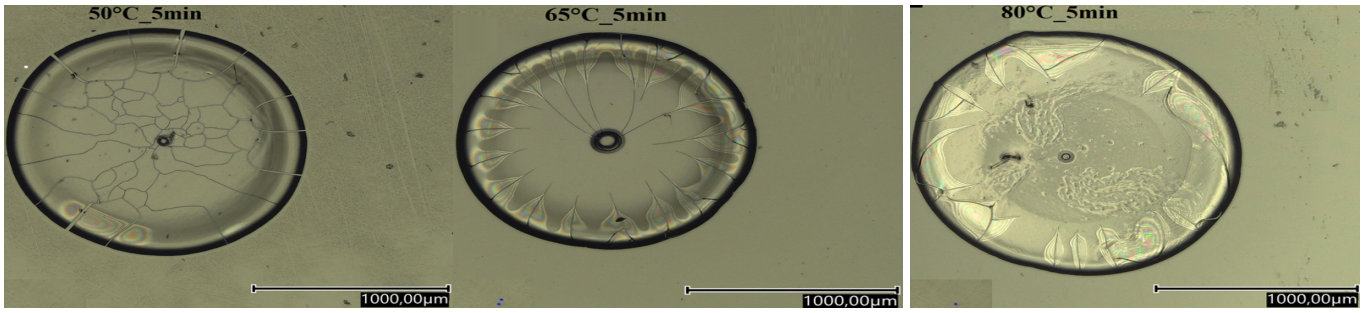


Figure 4.12: Droplet crack of WPI $5\%wt$ stock solution.

Here we present the evaporation of WPI $5\%wt$ droplet during 5minutes of heating temperature of $50°C$, $65°C$ and $80°C$. According to Figure (4.13) below, we notice that the droplets crack is started to be more and more complex by increasing temperature from $50°C$ to $80°C$. Small circular structures inside the protein deposit has been observed [52], for the temperature of $80°C$. This result is in agreement with HPLC result in figure (4.2).



(a) (b) (c)

Figure 4.13: Droplet crack from heated samples for 5minutes at: (a) $50°C$, (b) $65°C$ and (c) $80°C$.

Dried droplets for heated and sheared samples at $50°C$ and $65°C$ figure (4.14). These figures show

that the crack of the droplets is more uniform and directional by increasing temperature for samples that are heated and sheared after 5 minutes of treatment time. After 20minutes at $65°C$ the crack shows a different structure in the centre of the drop which means appearance of protein deposit that can be related to the denaturation and aggregation of whey proteins.

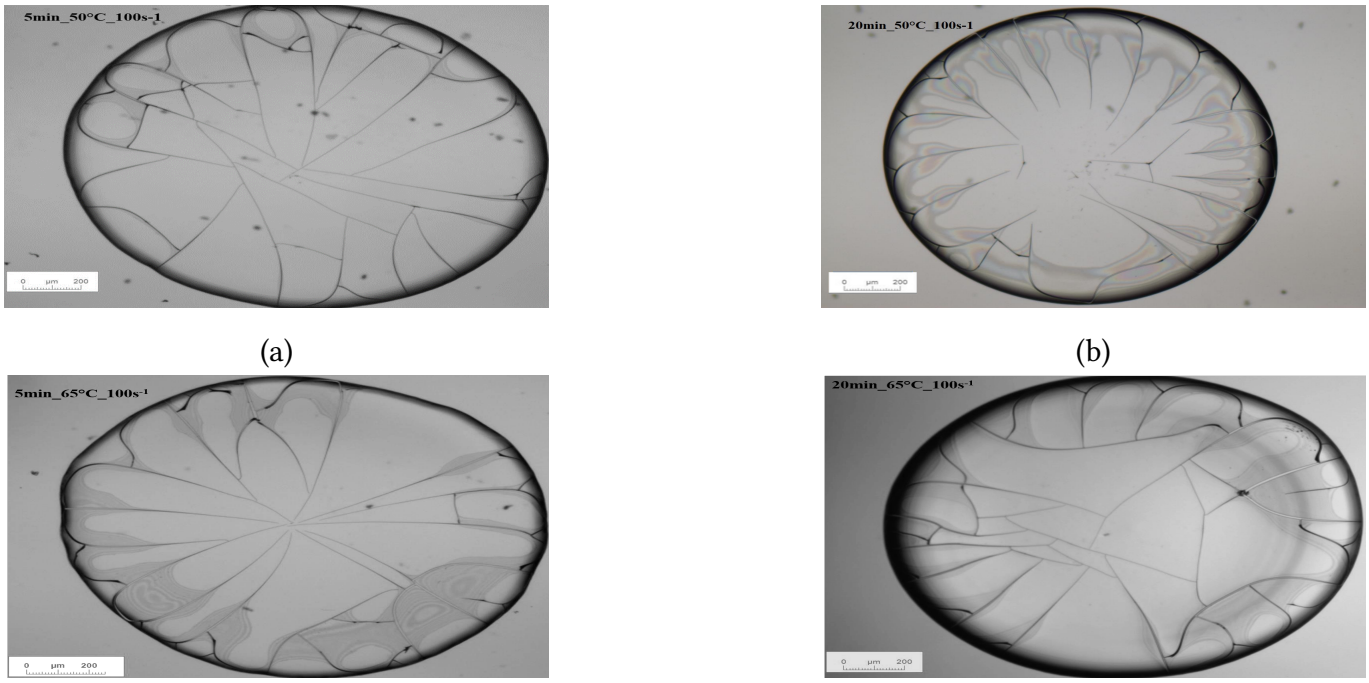


Figure 4.14: Droplet crack from heated and sheared samples at $\dot{\gamma} = 100^{-1}$: (a) $50°C$ for 5 minutes, (b) $50°C$ for 20 minutes, (c) $65°C$ for 5 minutes and (d) $65°C$ for 20 minutes

Droplet crack decrease when WPI solution contain calcium with the appearance of pattern deposit at the middle of the droplet. This deposit is more compact and dense over treatment time (see figure 4.16c).

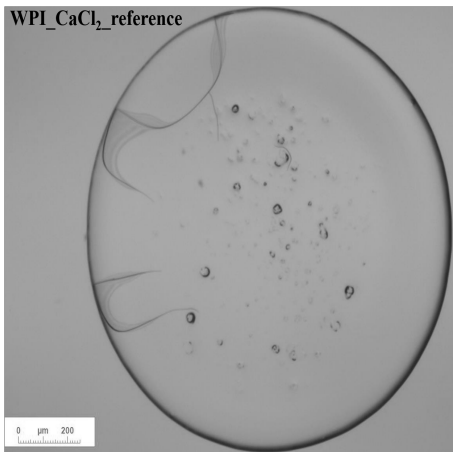
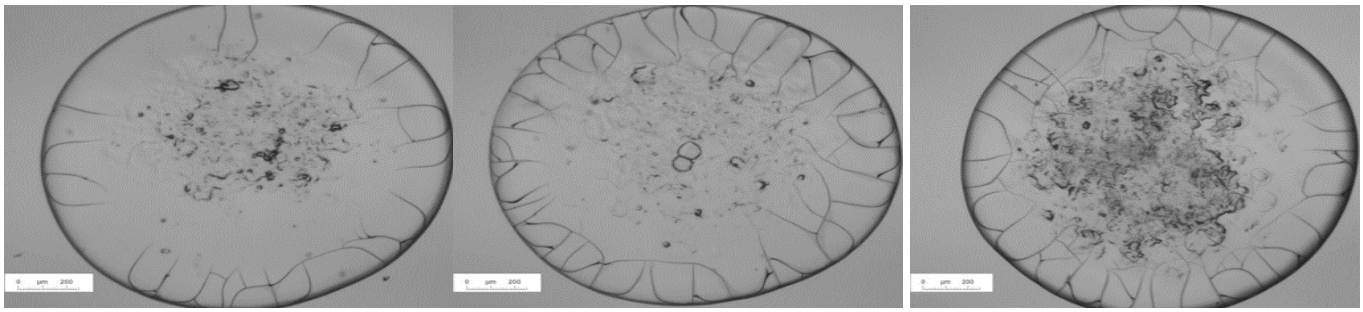


Figure 4.15: Droplet crack of WPI $5\%wt$ with calcium content stock solution.

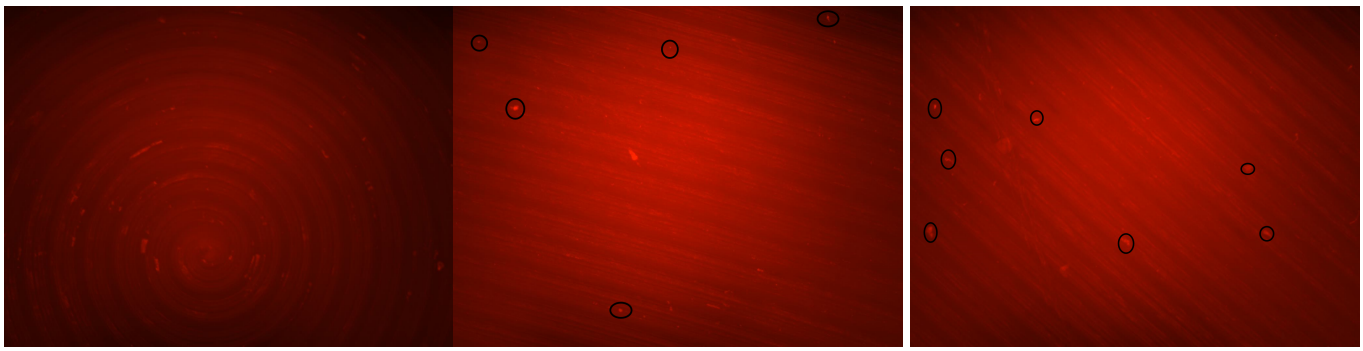


(a) (b) (c)

Figure 4.16: Droplet crack from heated and sheared samples of WPI 5% with calcium content at $65°$C and $\dot{\gamma} = 100s^{-1}$ for : (a) 5 minutes , (b) 10 minutes and (c) 20 minutes.

4.4.1 Surface characterization

Parallel plates geometry of the rheometer is used to characterize the anchored aggregates on the surface of the lower plate of this geometry which shows some roughness 4.17. More red dots or labelled aggregates at the edges of the plates surface compared to the number of aggregates at the centre and the middle. This observation confirm the characteristic of the parallel plate geometry is that the shear rate is zero at the centre and maximum at the periphery.

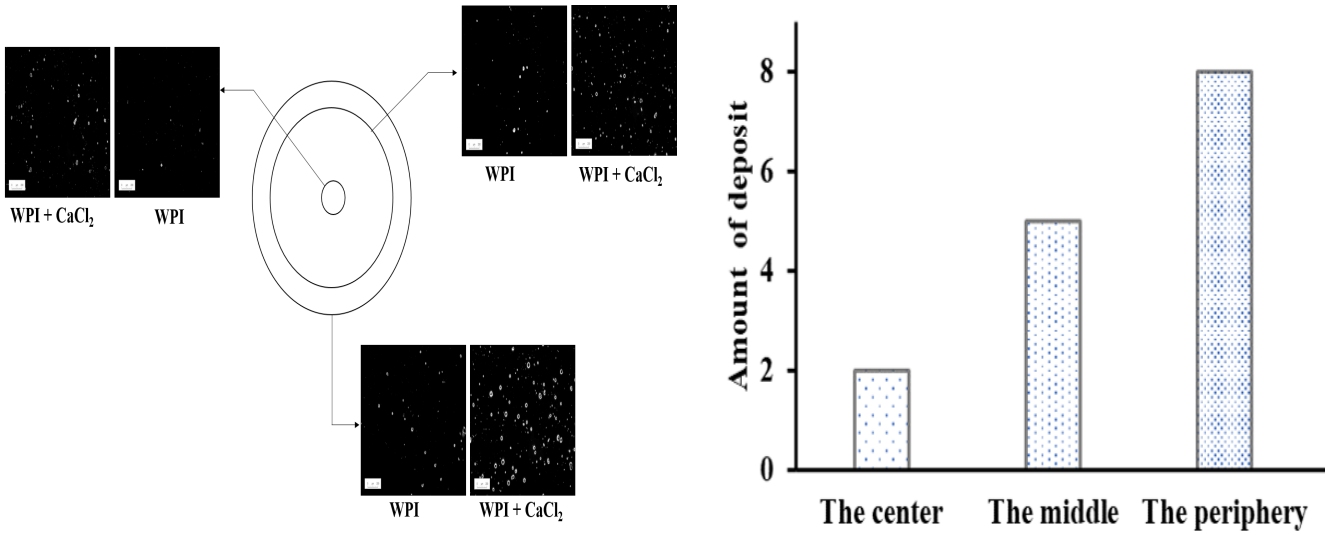


(a) (b) (c)

Figure 4.17: Observation by fluorescence of the lower plate of the rheometer : (a) in the centre , (b) in the middle and (c) at the periphery.

We report here the characterization of glass surface by optical microscope of heated and sheared samples of WPI $5\%wt$ and heated and sheared samples of WPI $5\%wt$ with calcium content at $65°$C and Ω = $4s^{-1}$ for 20 minutes.

From these observations figure (4.18) we conclude that whey protein samples with a calcium content present more amount of deposit on the glass surface than samples with just whey protein isolate.



(a) (b)

Figure 4.18: Characterization of the glass disc surface for WPI and WPI with calcium content samples : in centre, in the middle and at the periphery : (a) Optical microscope observation and (b) Representative diagram on the amount of aggregates at the different places of the glass disc surface.

Conclusion

In the first part of this section we studied the influence of heating on the denaturation mechanisms of whey proteins, in particular β-lactoglobulin. This allowed us to determine the denaturation kinetics at different temperatures and whey protein isolate concentrations. In a second step we wanted to understand if there is an additional effect of shear rate on the denaturation mechanisms of whey proteins by using the rheometer with couette geometry. The effect of shear was observed for the average particle size distribution but not on the denaturation of whey proteins as the microscopic drying droplets technique showed change in the morphology of these sheared sample droplets as a function of treatment time. Finally, we conclude this part of the results by the impact of calcium content on the surface mass deposition and thus its link with thermal denaturation reactions of β-lactoglobulin.

Chapter 5

General conclusion and perspectives

During this master's internship we have studied thermal denaturation of whey protein and its influence on fouling phenomena in dairy industry. Our equipment of investigation are falling film evaporators so that experimental conditions (temperature, whey protein concentration and shear rate) are fixed to be similar as the working conditions in evaporators. This experimental work is based : **On one side about bulk analysis** of the thermal denaturation of whey proteins under the effect of heating at various temperatures: $50°C$, $65°C$ and $80°C$ and various concentration of WPI : $5\%wt$, $10\%wt$, $15\%wt$ and $20\%wt$ as function of heating times. Then, the additional effect of shear rate to heating has been evaluated by applying a shear rate of $\dot{\gamma}$ = 100^{-1} and temperatures of $50°C$ and $65°C$ in the rheometer with couette geometry for : 5, 10 and 20 minutes. The characterisation techniques that were used for this are: High Performance liquid Chromatography (HPLC) to determine the change in native whey proteins concentration specially β-lactoglobulin. Added to that dynamic light scattering technique (DLS) for average particle size distribution in the heated and heated and sheared samples. Absence of denaturation kinetics in all the samples that are heated and sheared led us to search for another alternative technique to investigate the denaturation rate indirectly. Analysis of dried droplets of these samples by optical microscope show different morphologies of the droplets as function of treatment time. **On the other side about surface deposit characterization** of the labelled whey protein aggregates that are anchored on the surface of the lower plate of the parallel plate geometry. Observations of the deposit of particles on glass disc surface have been carried out for heated and sheared samples of WOI at $65°C$ and $\dot{\gamma}$ = 100^{-1}. Same experiment for heated and sheared samples of WPI with calcium content show an increase of the amount of deposit and the size of aggregates that are deposited on the glass surface. The results obtained will be valuable in estimating the relative importance of different parameters such as solution composition, temperature and shear rate in the falling film evaporators. Some short-term scientific perspectives:

1. Direct observation of thermal fouling in falling film evaporators by microfluidics and microscopic approach.

2. Characterize the different steps of the fouling accumulation by reproducing the environmental and flow characteristics typical of evaporators in microchannels with variable geometries.

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