



# Stabilisation des émulsions laitières aux cours des traitements technologiques : action combinée des agrégats de protéines de lactosérum et des caséines.

Marie Chevallier

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# AGRO CAMPUS

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## OUEST

**Marie CHEVALLIER • 10 mars 2017**

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sous le label de l'Université Bretagne Loire  
pour obtenir le grade de  
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Spécialité Science de l'aliment

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LABORATOIRE D'ACCUEIL • UMR INRA - AGROCAMPUS OUEST  
Science et Technologie du Lait et de l'Œuf (STLO)

**Stabilisation des émulsions  
laitières aux cours des  
traitements technologiques :  
action combinée des agrégats  
de protéines de lactosérum  
et des caséines.**

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Presented by:

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Rennes, 10 March 2017

Combined effect of the whey protein aggregates and caseins on dairy emulsions stability during technological treatments.

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« L'obstination est le chemin de la réussite. »

Charlie Chaplin



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---

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## Thesis Outputs

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### Peer-reviewed papers

- **Chevallier, M.**, Riaublanc, A., Lopez, C., Hamon, P., Rousseau, F., & Croguennec, T. (2016). Aggregated whey proteins and trace of caseins synergistically improve the heat stability of whey protein-rich emulsions. *Food Hydrocolloids*, 61, 487–495.  
<https://doi.org/10.1016/j.foodhyd.2016.06.009>
- **Chevallier, M.**, Riaublanc, A., Lopez, C., Hamon, P., Rousseau, F., Thevenot, J., & Croguennec, T. (2016). Increasing the heat stability of whey protein-rich emulsions by combining the functional role of WPM and caseins. *Food Hydrocolloids*. In press.  
<https://doi.org/10.1016/j.foodhyd.2016.12.014>
- Norwood, E.-A., **Chevallier, M.**, Floch-Fouéré, C. L., Schuck, P., Jeantet, R., & Croguennec, T. (2016). Heat-Induced Aggregation Properties of Whey Proteins as Affected by Storage Conditions of Whey Protein Isolate Powders. *Food and Bioprocess Technology*, 9(6), 993–1001. <https://doi.org/10.1007/s11947-016-1686-1>

### Oral communications in international conferences

- Marie Chevallier, Alain Riaublanc, Christelle Lopez, Pascaline Hamon, Florence Rousseau, Thomas Croguennec. Whey protein aggregates modulate the heat stability of whey protein-stabilized emulsions. 2nd Food Structure and Functionality Forum Symposium, Singapore, from 28 February to 2 March 2016
- Marie Chevallier, Alain Riaublanc, Christelle Lopez, Pascaline Hamon, Florence Rousseau, Thomas Croguennec. Whey protein aggregation modulates the heat stability of emulsions made with industrial whey proteins. 13th International Hydrocolloids Conference, Guelph, Canada, from 16 to 20 May 2016.
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- Marie Chevallier, Alain Riaublanc, Christelle Lopez, Pascaline Hamon, Florence Rousseau, Chantal Cauty, Maryvonne Pasco, Thomas Croguennec. Improving the heat stability of whey protein microgel emulsion with a small quantity of casein. IDF WDS 2016, Rotterdam, The Netherlands, from 16 to 21 October 2016.

## Table of contents

Acknowledgement	i
Thesis Outputs	iii
List of abbreviations	xi
General Introduction	1
Chapter 1: Literature review	5
<b>1.1 Whey protein aggregates</b>	<b>7</b>
1.1.1 Milk proteins	7
1.1.2 Characteristics of the whey proteins aggregates	8
1.1.2.1 Fibrils	8
1.1.2.2 Strand-like aggregates	10
1.1.2.3 Microgels	12
1.1.3 Functionalities of the whey proteins aggregates	14
1.1.3.1 Physical stability	14
1.1.3.2 Interfacial properties	15
<b>1.2 Emulsions and whey proteins aggregates</b>	<b>16</b>
1.2.1 Emulsion definitions	16
1.2.2 Emulsion formation	17
1.2.3 Destabilization mechanisms of the emulsions during technological treatments	18
1.2.3.1 Flocculation	19
1.2.3.2 Creaming	20
1.2.3.3 Partial coalescence	21
1.2.3.4 Coalescence	22
1.2.4 Pickering and Pickering-like emulsions	22
1.2.5 Whey protein aggregate emulsions	26
1.2.6 Emulsions containing whey proteins <i>versus</i> whey protein aggregates: stability during technological treatments	27

## Table of contents

<b>1.3</b>	<b>Whey protein and casein emulsions .....</b>	<b>30</b>
1.3.1	Whey proteins and caseins competitive: adsorption for the oil droplet surface _____	30
1.3.2	Emulsions containing caseins <i>versus</i> a mixture of whey proteins and caseins: stability during technological treatments _____	31
<b>1.4</b>	<b>Conclusion.....</b>	<b>32</b>
<b>Chapter 2: Aims and strategy of the PhD _____</b>		<b>35</b>
<b>2.1</b>	<b>Context of the PhD .....</b>	<b>37</b>
<b>2.2</b>	<b>Aim, objective and strategy .....</b>	<b>40</b>
<b>2.3</b>	<b>Originality of the research project .....</b>	<b>42</b>
<b>CHAPTER 3: Materials and methods _____</b>		<b>45</b>
<b>3.1</b>	<b>Materials .....</b>	<b>47</b>
<b>3.2</b>	<b>Protein suspensions and emulsion preparation .....</b>	<b>48</b>
3.2.1	Whey protein, sodium caseinate and micellar casein suspensions _____	48
3.2.2	Strand-like aggregate suspensions _____	48
3.2.3	Whey protein microgel suspensions _____	49
3.2.4	Preparation of protein-coated emulsions _____	50
<b>3.3</b>	<b>Stability of protein-coated emulsions and protein suspensions during technological treatments.....</b>	<b>50</b>
3.3.1	Heat stability _____	50
3.3.2	Freeze/thaw stability _____	50
<b>3.4</b>	<b>Protein particle characterization .....</b>	<b>51</b>
3.4.1	Size distribution of the protein particles _____	51
3.4.2	Gyration radius and molar mass of protein particles _____	51
3.4.3	$\xi$ -potential of the protein particles _____	52
3.4.4	Morphology of the protein particles _____	52
3.4.5	Composition of the strand-like aggregate suspensions and the whey protein microgel suspensions _____	52
3.4.5.1	Gel filtration _____	53



## Table of contents

3.4.5.2	SDS-PAGE	53
3.4.6	Interfacial properties of the protein particles	54
<b>3.5</b>	<b>Emulsion characterization</b>	<b>54</b>
3.5.1	Fat droplet size distribution	54
3.5.2	Microstructure	54
3.5.2.1	Confocal laser scanning microscopy	54
3.5.2.2	Transmission electron microscopy	56
3.5.3	Quantification and composition of the proteins adsorbed at fat droplet surface	57
3.5.3.2	Protein quantification	57
3.5.3.3	Protein composition	58
<b>CHAPTER 4:</b>	<b>Impact of whey proteins powder ageing on the structure of heat-induced aggregates.</b>	<b>59</b>
<b>4.1</b>	<b>Introduction</b>	<b>61</b>
<b>4.2</b>	<b>Materials and methods</b>	<b>62</b>
4.2.1	Whey protein powder	62
4.2.2	Whey protein powder storage conditions	62
4.2.3	Powder composition	62
4.2.4	Browning Index	63
4.2.5	Level of $\beta$ -Lg lactosylation	63
4.2.6	Heat treatment of whey protein suspensions	63
4.2.7	Analysis of the heat-induced aggregates	63
<b>4.3</b>	<b>Powder state after storage</b>	<b>64</b>
<b>4.4</b>	<b>Heat-induced aggregation</b>	<b>68</b>
<b>4.5</b>	<b>Conclusion</b>	<b>72</b>
<b>CHAPTER 5:</b>	<b>Selection, composition and characterization of the structure and functional properties of whey protein aggregates.</b>	<b>73</b>
<b>5.1</b>	<b>Introduction</b>	<b>75</b>
<b>5.2</b>	<b>Selection and characterization of the whey protein aggregates</b>	<b>75</b>

## Table of contents

5.2.1	Selection of the whey protein aggregates: potentialities of the strand-like aggregates and the microgels	75
5.2.2	Aggregate screening based on their ability to stabilize emulsions during technological treatments	77
5.2.3	Composition and characterization of the selected aggregate suspensions	78
5.2.3.1	Composition of the aggregate suspensions	78
5.2.3.2	Structural characterization of the aggregates	82
<b>5.3</b>	<b>Functional properties of the aggregate suspensions.....</b>	<b>85</b>
5.3.1	Heat stability and freeze/thaw stability of the aggregate suspensions	85
5.3.1.1	Heat stability of the aggregate suspensions	86
5.3.1.2	Freeze/thaw stability of the proteins suspensions	88
5.3.2	Emulsifying capacity of the aggregate suspensions	88
5.3.2.1	Interfacial properties of the protein suspensions at the air-water interface	89
5.3.2.2	Emulsifying capacity of the protein suspensions	90
<b>5.4</b>	<b>Impact of the whey proteins powder composition on the structure of the aggregates .....</b>	<b>91</b>
5.4.1	Casein effect	92
5.4.2	Calcium effect	93
<b>5.5</b>	<b>Conclusion.....</b>	<b>94</b>
<b>CHAPTER 6: Stability of emulsions containing whey protein microgels revealing the beneficial impact of caseins</b>		<b>95</b>
<b>6.1</b>	<b>Introduction .....</b>	<b>97</b>
<b>6.2</b>	<b>Heat stability of the emulsions .....</b>	<b>98</b>
6.2.1	Characteristics of the unheated emulsions prepared with WPM A and WPM AC suspensions	98
6.2.2	Characteristics of the heated emulsions prepared with WPM A and WPM AC suspensions	101
6.2.3	Influence of fat droplet surface composition	104
6.2.4	Mechanism of emulsion stabilization	106
6.2.4.1	Influence of the adsorbed proteins on the heat stability of the emulsions	106

## Table of contents

6.2.4.2	Capacity of the WPM to bridge fat droplet surface .....	112
6.2.5	Design of heat-stable emulsions containing WPM .....	115
6.3	Freeze/thaw stability of the emulsions .....	117
6.4	Conclusion.....	120
CHAPTER 7: Impact of the whey protein aggregate morphology and the presence of caseins on the emulsion stability .....		123
7.1	Introduction .....	125
7.2	Stability of the SA <sub>4</sub> emulsions during technological treatments.....	126
7.2.1	Design of heat-stable SA <sub>4</sub> emulsions .....	126
7.2.2	Heat stability of SA <sub>4</sub> emulsions at various protein concentrations but at constant casein/SA <sub>4</sub> ratio .....	127
7.2.2.1	Emulsion characteristics before heat treatment .....	128
7.2.2.1.2	Fat droplet surface composition .....	129
7.2.2.2	Emulsion stability to heat treatment .....	132
7.2.2.3	Heat stability of emulsions <i>versus</i> proteins in suspension .....	136
7.2.2.4	Discussion .....	137
7.2.3	Heat stability of the SA <sub>4</sub> emulsions at various casein/SA <sub>4</sub> ratio .....	141
7.2.4	Freeze/thaw stability of the SA <sub>4</sub> emulsions and casein/SA <sub>4</sub> emulsions .....	143
7.3	Stability of the emulsions prepared with the WPM/SA B suspensions during technological treatments.....	145
7.3.1	Design of heat-stable WPM/SA B emulsions .....	145
7.3.2	Freeze/thaw stability of the WPM/SA B emulsions .....	153
7.4	Conclusion.....	154
General Conclusion .....		157
Further Outlook .....		163
7.5	Scientific outlook.....	164
7.6	Technological outlook .....	166
References .....		169

Table of contents

Table of Figures	195
Table of Tables	203

## List of abbreviations

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$V_d$ : Volume of dispersed phase (m<sup>3</sup>)

$m_p$ : Mass of aggregates (kg)

$v_{Stokes}$ : Gravitational separation rate (m/s)

$\rho_1$ : Density of the continuous phase (kg/m<sup>3</sup>)

$\rho_2$ : Density of the dispersed phase respectively (kg/m<sup>3</sup>)

$\rho_p$ : Aggregate density (kg/m<sup>3</sup>)

$\Delta A$ : Total interfacial area (m<sup>2</sup>)

$\Delta G$ : Interfacial free energy (J)

A4F: Asymmetric Flow Field-Flow Fractionation

BSA: Bovine Serum Albumin

C: Surface coverage degree

CasNa: Sodium caseinate

CLSM: Confocal laser scanning microscopy

$d_{3,2}$ : Surface mean diameter

$d_{4,3}$ : Volume mean diameter

GPC: Gel permeation chromatography

HPLC: High performance liquid chromatography

Igs: Immunoglobulins

LF: Lactoferrin

LUT: Look up table

MC: Micellar casein

MM: Molar mass

MW: Molecular weight

pI: Isoelectric point

R<sub>g</sub>: Gyration radius

SA: Strand-like aggregate

SA<sub>4</sub>: Strand-like aggregate prepared with whey protein solution at 4 wt%

SA<sub>9</sub>: Strand-like aggregate prepared with whey protein solution at 9 wt%

SA<sub>Na</sub>: Strand-like aggregate prepared with whey protein solution at 4 wt% and 40 mM of NaCl

SDS: Sodium dodecyl sulfate

TEM: Transmission electron microscopy

## List of abbreviations

WP: Whey protein

WPC: Whey protein concentrate

WPM A: Whey protein microgel prepared with the powders A

WPM AC: Whey protein microgel prepared with the powders A after centrifugation, i.e. without caseins and residual native whey proteins.

WPM: Whey protein microgel

$\alpha$ -La:  $\alpha$ -lactalbumin

$\beta$ -Lg:  $\beta$ -Lactoglobulin

$\Delta P_L$ : Laplace pressure (Pa)

$\eta$ : Continuous phase viscosity (Pa/s)

$D$ : Fat droplet diameter (m)

$a$ : Specific surface area (m<sup>2</sup>)

$g$ : Gravitational acceleration (m/s<sup>2</sup>)

$r$ : Fat droplet radius (m)

$\gamma$ : Interfacial tension (N/m)

## General Introduction

---

In western countries, food supply is abundant and diversified thanks to an innovative food industry that constantly provides new products to consumers in terms of sensory and nutritional properties, and services i.e. food with extended shelf life, ready-to-use foods, foods for on-to-go consumption, *etc.* However, the food industry has to compose with a complex and sometimes contradictory consumer's demand, e.g. fresh foods with extended shelf-life, low processed and ready-to-eat foods, traditional foods with high safety standard, *etc.* Some of these demands can be tackled by the use of food additives for instance to improve food stability during long-term storage. However, more and more consumers have increased apprehension toward the list of additives written on food packaging, e.g. carrageenan, xanthan, modified starch, lecithin. The designation of the additives by the code "E..." increases this apprehension. In addition, for most of the consumers, the way the food products is manufactured in the food industry remains a "black box" and they don't understand the need of using additives (Baines & Seal, 2012). Fueled by mass media coverage of isolated and questionable practices that hit the whole food sector, some consumers believe the food industry is focused on profit with little concerns about their expectations and demands. Recently, with an aim to put consumer's mind at ease, a new trend called "clean-label" has emerged and the food industry looks for the manufacture of more natural, meaning additive-free or 100 % natural (Baines & Seal, 2012; Li & Nie, 2016), and healthy food products (Mollet & Rowland, 2002).

The dairy market is an important share of the French agri-food business in terms of turnover with 1/5 of agri-food turnover, employees (~ 250 000) and with ripple effect on the global French economy (energy, packaging, *etc.*). Five French dairy companies are among the top 25 of the worldwide dairy companies. However, the dairy market is constantly evolving with tough competition at world level. The end of the milk quotas in Europe, the decrease in milk powder imports by China, the competition of proteins of other origin (plant proteins), and many other economic and political decisions have been at the source of the decrease in milk prices over the last two years. At the same time, the global consumption of dairy products will continue increasing over the next few years. Driven by the world population growth and the global incomes growth, a strong demand on developing markets like North Africa, the Middle East and East Asia is predicted. On a tight market with a high level of competition at world level, French dairy manufacturers are looking for a better valorization of milk components. The milk proteins, i.e. caseins and whey proteins, have always been considered as the "white gold of the milk". In fact, the milk proteins have a high nutritional value and numerous functional properties such as gelling, emulsifying and foaming. Therefore, milk proteins are key elements in the incredible diversity

of textures encountered in dairy products, e.g. in cheeses or yogurt, and they have always led to innovations in the dairy sector.

Current scientific knowledge on the structure and interactions of milk proteins allow designing aggregates with different sizes and morphologies such as the fibrils, microgels and fractal aggregates of whey proteins. A defined type of aggregates is formed under strict experimental conditions that could change depending on the characteristic of the protein powder mainly its composition. Up to now, the functional properties of these aggregates in dairy products has been barely studied. In order to evaluate these functional properties with the aim to improve the valorization of milk proteins, a major research project called “PROFIL: for Multi-Functional Protein Assemblies for Innovation in Dairy Industry” was launched (2014 - 2020). This project brings together a large number of French dairy companies, i.e. Bel, CF&R, Lactalis, Isigny Ste Mère, SODIAAL (Entremont, Candia, Eurosérum), Laïta (Even Santé Industrie, Epi Ingrédient), Savencia, and several research institutes i.e. INRA-Agrocampus ouest UMR STLO, INRA UR BIA, ONIRIS, LUBEM Université de Bretagne Occidentale, Université du Maine and Université de Rennes 1. The PROFIL project is financially supported by the Regional councils of Brittany and Pays de la Loire. The west part of France is the most important milk producing region in France and one of the most important in Europe with 10% of the European production. The “milk valley” regroups dairy farmers, dairy companies and researchers from the west part of France to create technical and scientific excellence in the dairy industry. It is conducted by INRA and managed by BBA which is an industrial association. The aim of this project is to use the plasticity and the multifunctional properties of milk proteins:

- to create dairy products without non-dairy additives (“clean label” trend) which allow to satisfy the consumers’ expectations in term of naturalness.
- to create innovative products corresponding to new uses and new consumption habits. For instance, products containing a high level of whey proteins for specific population e.g. elderly persons, sportsmen.

Therefore, PROFIL aims at strengthening the link and the transfer of knowledge between public research institutes and French dairy manufacturers in order to reinforce the positions and competitiveness of these latter.



PROFIL is a 6-year project, funding 11 PhD thesis. It is organized in 4 axes (Fig 1):

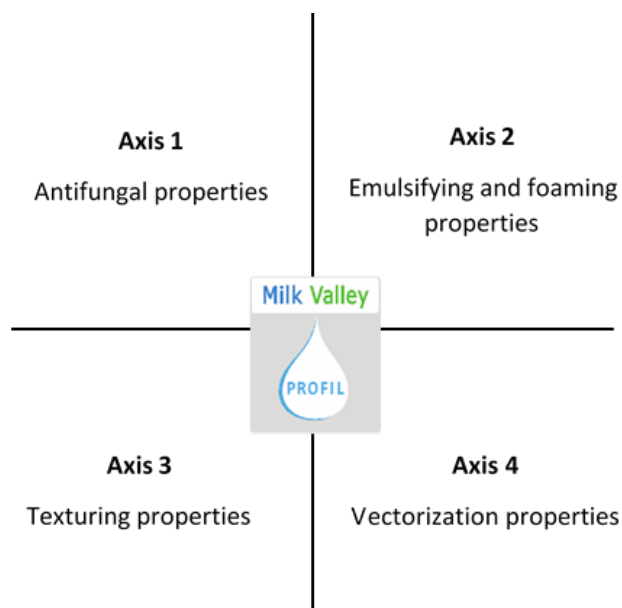


Figure 1: Organization of the PROFIL project

My PhD thesis, starting in March 2014, belongs to the axis “Emulsifying and foaming properties”. It is entitled “Combined effect of the whey protein aggregates and caseins on dairy emulsions stability during technological treatments”. The underlying objective of my PhD thesis was to use selected whey proteins aggregates to stabilize oil-in-water emulsions during technological treatments, i.e. heating and freeze/thaw cycle.

Many food products are emulsions, e.g. sauces, creams, cheeses. They consist of two immiscible phases, an oil phase and an aqueous phase, one dispersed in another and separated by an interface. Most food emulsions are oil-in-water emulsions, in which the aqueous phase is the continuous phase and the oil phase is the dispersed phase. Because of their structure, dairy emulsions are thermodynamically unstable systems. The fat droplets are subjected to creaming, flocculation and coalescence but these evolutions are easily controlled by an appropriate homogenization and the formation of a viscoelastic protein interfacial layer around the oil droplets. However, the appliance of additional stresses (e.g. heating, freeze/thaw cycle) occurring during their manufacture, handling and subsequent use by the consumers are able to modify some compounds in the emulsion and could induce destabilization (for instance gelation). Therefore, to control emulsion stability against stresses, dairy manufacturers add non-dairy additives e.g. carrageenan, xanthan, pectin, lecithin. As mentioned earlier, consumers pay increasing attention to the composition of the products they eat and they are looking for more natural, sustainable and healthy products (David Julian McClements & Gumus, 2016). Consequently, dairy

manufacturers are seeking to reduce or eliminate non-dairy additives from their products according to the "clean-label" approach. At the same time, whey proteins are increasingly used in a large variety of food products due to their high nutritional properties (e.g. in infant milk formulas, beverages for sportsmen and elderly persons). Whey proteins are extremely sensitive to heat treatment. They have excellent gelling properties above the critical concentration of gelation but this constitutes a drawback in food products where a fluid texture is required (e.g. in emulsion beverages). Whey protein aggregates are suggested to be an efficient alternative to native whey proteins to stabilize emulsions during heat treatment at high protein concentrations (Çakır-Fuller, 2015). Pickering-like emulsions manufactured with whey protein aggregates showed promising capacity to stabilize emulsion during storage (Destribats, Rouvet, Gehin-Delval, Schmitt & Binks, 2014). However, information on the stability of whey proteins aggregate emulsions, particularly during technological treatments (heating, freeze/thaw cycle), are scarce and need further scientific studies to be fully understood and controlled. In addition, the presence of a small amount of caseins improved the heat stability of the whey protein emulsions (Dickinson & Parkinson, 2004; Parkinson & Dickinson, 2004). The caseins are the main protein fraction in bovine milk. They are often present simultaneously with the whey proteins in various dairy ingredients and food products. Therefore, understanding the competition between the caseins and the whey protein aggregates for the fat droplet surface seems essential to control emulsion stability during technological treatments.

The present work was divided into seven chapters. The **chapter 1** is dedicated to the current knowledge about the whey protein aggregates, the procedures used for their production, some generalities on the emulsion stability and a specific focus on the stability of the whey protein aggregate emulsions during technological treatments. **Chapter 2** describes the goals and the objectives of this work. **Chapter 3** presents the materials and methods used during the thesis. **Chapters 4 to 7** correspond to the main results of the work. In the first part (**Chapter 4**), the impact of whey proteins powder ageing on the structure of the aggregates was raised. In **Chapter 5**, the aggregates with the most promising ability to stabilize emulsions during the technological treatments were selected. The exact composition and the functional properties of the whey protein aggregate suspension were also determined. In **Chapter 6**, the capacity of the whey protein microgels to stabilize the oil-in-water emulsions during the technological treatments was investigated. The importance of the powder composition and of the influence of traces of caseins on emulsion stability was highlighted. The hypothesis and the mechanism brought to light were tested and checked with strand-like aggregates of whey proteins and with mixture of microgels and strand-like aggregates (**Chapter 7**). Finally, a general conclusion that highlighted the main results and the further outlooks were described.

## Chapter 1: Literature review

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Heat-induced whey protein aggregates are promising supramolecular structures for the formation and stabilization of oil-in-water emulsions. The first part of this literature review describes the characteristics and some properties of the fibrils, fractal aggregates and whey protein microgels. Generalities on emulsions and their physical instabilities during the technological treatments are presented in a second part. The two main approaches for the preparation of stable emulsions are then exposed. The first one consists of the formation of a steric barrier of whey protein aggregates at the surface of the fat droplets based on the principle of Pickering-like emulsions. The second one relates the stabilization of the fat droplet surface by non-aggregated proteins and the use of protein aggregates to modify the properties of the continuous phase (e.g. viscosity, heat stability). The last part of the review focuses on the stability of emulsions prepared with whey protein aggregates in the absence or presence of non-aggregated proteins during technological treatments (heating, freezing). The effect of the caseins presence on the emulsion stability during the technological treatments was also addressed.

**The main aims of the work presented in this chapter were to:**

- Summarize the conditions of formation, and the main structural characteristics and functionalities of the whey protein aggregates
- Remind some generalities on the oil-in-water emulsions and their destabilization mechanisms during technological treatments
- Present the last advances on Pickering-like emulsions prepared with whey protein aggregates and emulsions prepared with a combination of aggregates and non-aggregated proteins.

## Table of contents

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<b>1.1</b>	<b>Whey protein aggregates .....</b>	<b>7</b>
1.1.1	Milk proteins .....	7
1.1.2	Characteristics of the whey proteins aggregates .....	8
1.1.2.1	Fibrils.....	8
1.1.2.2	Strand-like aggregates .....	10
1.1.2.3	Microgels .....	12
1.1.3	Functionalities of the whey proteins aggregates .....	14
1.1.3.1	Physical stability .....	14
1.1.3.2	Interfacial properties .....	15
<b>1.2</b>	<b>Emulsions and whey proteins aggregates .....</b>	<b>16</b>
1.2.1	Emulsion definitions .....	16
1.2.2	Emulsion formation .....	17
1.2.3	Destabilization mechanisms of the emulsions during technological treatments.....	18
1.2.3.1	Flocculation.....	19
1.2.3.2	Creaming.....	20
1.2.3.3	Partial coalescence .....	21
1.2.3.4	Coalescence .....	22
1.2.4	Pickering and Pickering-like emulsions .....	22
1.2.5	Whey protein aggregate emulsions .....	26
1.2.6	Emulsions containing whey proteins <i>versus</i> whey protein aggregates: stability during technological treatments.....	27
<b>1.3</b>	<b>Whey protein and casein emulsions .....</b>	<b>30</b>
1.3.1	Whey proteins and caseins competitive: adsorption for the oil droplet surface .....	30
1.3.2	Emulsions containing caseins <i>versus</i> a mixture of whey proteins and caseins: stability during technological treatments.....	31
<b>1.4</b>	<b>Conclusion.....</b>	<b>32</b>

## 1.1 Whey protein aggregates

### 1.1.1 Milk proteins

The whey protein fraction and the casein fraction are the two major protein fractions in milk (20 and 80% in bovine milk, respectively). In contrast to caseins, the whey proteins fraction is still soluble after acidification of a raw skim milk at pH 4.6 (Farrell Jr. et al., 2004) or after rennet-driven coagulation. The bovine whey protein fraction is composed of globular proteins (Table 1), essentially  $\beta$ -Lactoglobulin ( $\beta$ -Lg, the major bovine whey protein),  $\alpha$ -Lactalbumin ( $\alpha$ -La), Bovine Serum Albumin (BSA), the Immunoglobulins (Igs) and Lactoferrin (LF).

Table 1: Mains proteins in cow milk and their proportions

	Caseins (80%)				Whey proteins (20%)	
Main proteins	$\alpha_{s1}$ -Casein	$\alpha_{s2}$ -Casein	$\beta$ -Casein	$\kappa$ -Casein	$\beta$ -Lactoglobulin	$\alpha$ -Lactalbumin
proportion (%)	40	10	35	12	50-60	20

The casein fraction is composed of four main caseins (Table 1):  $\alpha_{s1}$ -Casein ( $\alpha_{s1}$ -Cas),  $\alpha_{s2}$ -Casein ( $\alpha_{s2}$ -Cas),  $\beta$ -Casein ( $\beta$ -Cas) and  $\kappa$ -Casein ( $\kappa$ -Cas). The organization of the caseins depends on their mineralization. In presence of calcium and phosphate, the caseins are mainly in the form of micellar casein which is the supramolecular structure encountered in milk. One casein micelle is composed of several thousands of caseins and 8 % in of amorphous calcium phosphate nanoclusters (Holt, Carver, Ecroyd & Thorn, 2013)(Fig 2). In the absence of divalent or multivalent ions, caseins exist as an equilibrium of individual monomers and small assemblies ( $\sim 15$  caseins) (Pitkowski, Durand & Nicolai, 2008). In the rest of the manuscript, the term “caseins” refers to the equilibrium of individual monomers and small assemblies and “micellar caseins” refers to the natural supramolecular assembly of caseins and minerals.

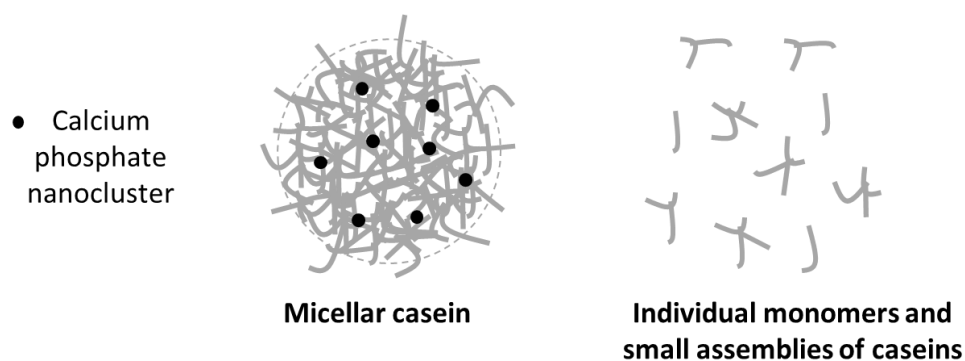


Figure 2: Scheme of the micellar casein and individual monomers and small assemblies of caseins

### 1.1.2 Characteristics of the whey proteins aggregates

Whey proteins (WP) are commonly used in food products owing to their diversified functional properties and exceptional biological value due to richness of essential amino acids, binding properties for vitamins, minerals, fatty acids, etc. (Madureira, Pereira, Gomes, Pintado & Malcata, 2007; Morr & Ha, 1993; Smithers, 2008). The functional properties of the WP, i.e. gelling, foaming and emulsifying properties, have been extensively studied (de Wit, 1998; Ha & Zemel, 2003; Kinsella & Whitehead, 1989). Recently, an increasing interest for the functional properties of WP aggregates raised. Indeed, the modification of the WP into aggregates is a way to bring new functionalities to WP and to extend their uses (Guyomarc'h et al., 2014). By adjusting the conditions for their formation, it is possible to obtain various types of WP aggregates such as microgels, fibrils and strand-like aggregates (Jung, Savin, Pouzot, Schmitt & Mezzenga, 2008; Nicolai, Britten & Schmitt, 2011; Havea, Grant, Jiu Wai Hii & Wiles, 2012). These aggregates have particular features regarding their size, morphology, density, composition, type of bounds between proteins in the aggregates. They are most commonly produced by heating a WP solution below the critical concentration of gelation. Heating allows to form stable suspensions of WP aggregates (Nicolai & Durand, 2013). This literature review focuses on the heat-induced WP aggregates called fibrils, strand-like aggregates and microgels.

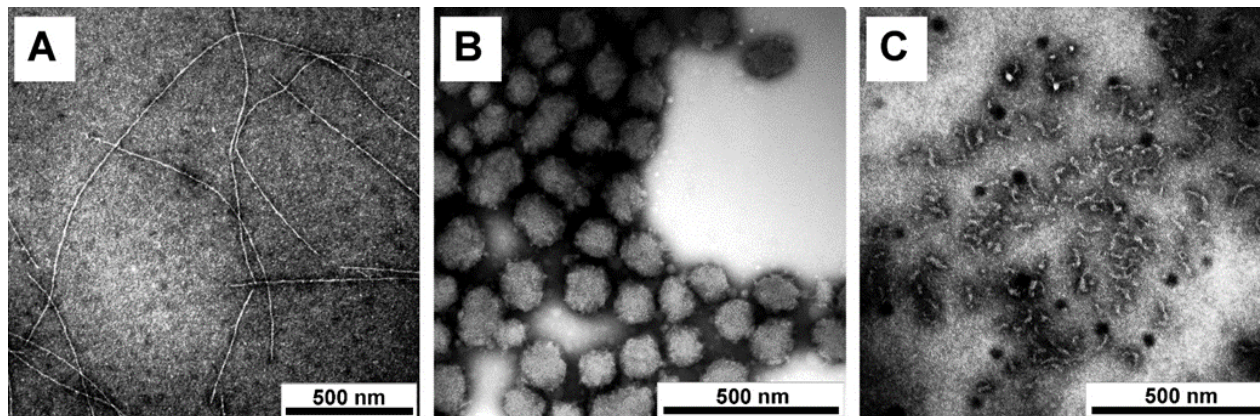


Figure 3: Negative-staining TEM image of  $\beta$ -lactoglobulin aggregates obtained upon heating a 1 wt% protein solution at different pH: (A) pH 2.0; (B) pH 5.8; (C) pH 7.0. Reproduced from Jung et al (2008) with permission of the American Chemical Society.

#### 1.1.2.1 Fibrils

Protein fibrils consist on the association of proteins or peptides into long, insoluble and highly ordered fibrillar structures (Adamcik & Mezzenga, 2012). Protein fibrils can be formed with various globular proteins (WP, ovalbumin, soy proteins...). WP fibrils with a length larger than 1  $\mu\text{m}$  and a width of 3-10 nm (Fig 3A) can be obtained by prolonged heating at high temperature ( $T \geq 80^\circ\text{C}$ ) of a solution at low ionic strength ( $\leq 20 \text{ mM}$ ) and at  $\text{pH} < 2.5$  (Adamcik & Mezzenga, 2012; Kroes-Nijboer, Venema & Linden,

2012). The formation of the WP fibrils includes two steps: first an unfolding and/or an hydrolysis of the WP into peptides and then the association of the unfolded proteins and/or peptides into linear  $\beta$ -sheet-organized aggregates, called fibrils (Akkermans et al., 2008; Kroes-Nijboer, Venema, Bouman & van der Linden, 2011). The general mechanism of the fibril formation is described by a nucleation and elongation model (Adamcik & Mezzenga, 2012). The nucleation consists in a local unfolding and/or the hydrolysis of the WP in order to expose on the protein surface, amino acids prone to establish intermolecular  $\beta$ -sheets. The formation of  $\beta$ -sheet-organized oligomers, or nucleus, is usually the limiting step (lag phase) of the fibril formation. Then, the proteins associate in protofilaments in a cross- $\beta$ -sheet configuration ( $\beta$ -sheets perpendicular to the elongation direction) and these protofilaments are also able to associate sidewise into twisted ribbons to form mature fibrils (Adamcik & Mezzenga, 2012), as indicated on Figure 4.

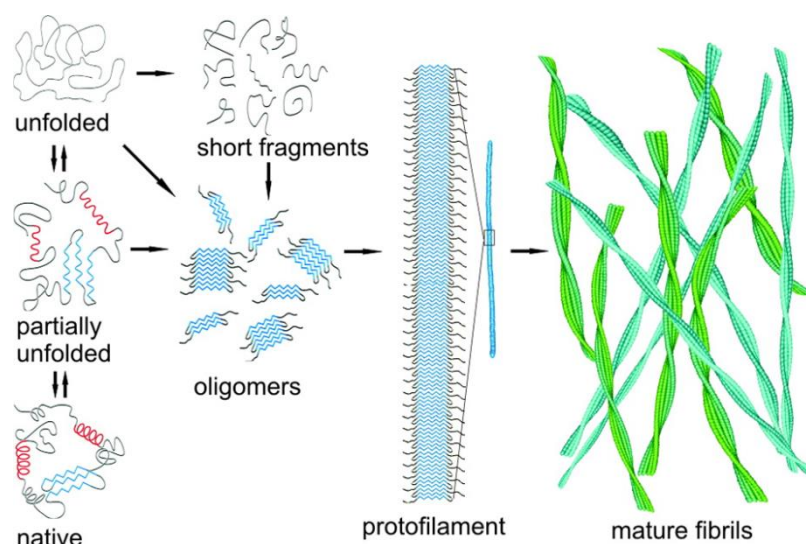


Figure 4: Scheme of the mechanism of amyloid fibrils formation. Reproduced from Adamcik & Mezzenga (2012) with permission of the America Chemical Society.

Akkermans et al. (2008) showed that only the peptides that were the most hydrophobic, with low charge and with a high ability to form  $\beta$ -sheets, were able to form fibrils. This explains that the conversion level of the fibrils was low, below 50 % (Kroes-Nijboer et al., 2011). However, the conversion yield can be increased by increasing the protein hydrolysis rate (Kroes-Nijboer et al., 2011), the heating time, the protein concentration (Bolder, Vasbinder, Sagis & van der Linden, 2007) and the temperature (Loveday, Wang, Rao, Anema & Singh, 2012). An acceleration of the fibril formation could be obtain by microwave heating (Hettiarachchi, Melton, Gerrard & Loveday, 2012). The authors suggested that microwave heating initially accelerated the self-assembly of  $\beta$ -Lg by a non-thermal effects on unfolding, nucleation,

and subsequent stacking of  $\beta$ -sheets, rather than promoting  $\beta$ -Lg partial hydrolysis. The fibril formation are modulated by the addition of  $\beta$ -casein, glycerol or sorbitol to the WP solution (Dave, Loveday, Anema, Jameson & Singh, 2014b; Dave, Loveday, Anema & Singh, 2016). Protein glycation also prevented fibril formation due to the low ability of the glycated peptides to self-assemble into fibrils (Dave, Loveday, Anema, Jameson & Singh, 2014a). The formation of the fibrils takes a long time and in most of the cases the steady state is difficult to reach (Nicolai & Durand, 2013).

#### 1.1.2.2 Strand-like aggregates

Strand-like aggregates are composed of the association of curved strands. For low level of association the term strand-like aggregates is usually used but for high level of association, it is common to talk about fractal aggregates. Strand-like aggregates are formed under conditions of strong electrostatic repulsions, i.e. pH far above the isoelectric point (pI) of the proteins and at low ionic strength ( $\leq 20$  mM). They are also called soluble aggregates. The structure of strand-like aggregates and fractal aggregates prepared with pure  $\beta$ -Lg (Gimel, Durand & Nicolai, 1994; Le Bon, Nicolai & Durand, 1999; Pouzot, Nicolai, Visschers & Weijers, 2005; Renard, Axelos, Boué & Lefebvre, 1996) or with WP solutions at pH7 (Mahmoudi, Mehalebi, Nicolai, Durand & Riaublanc, 2007) was extensively studied (Fig 3C). A two-steps aggregation mechanism based on the nucleation-growth mechanism was proposed by (Aymard, Gimel, Nicolai & Durand, 1996). In the first step, curved strands with 10 nm-diameter and 50 nm-length dimensions were obtained (Donato, Schmitt, Bovetto & Rouvet, 2009; Durand, Gimel & Nicolai, 2002; Jung et al., 2008; Schmitt et al., 2009). They result from the association of denatured monomers and small oligomers (Nicolai et al., 2011). Indeed, the protein structure was modified and became more mobile on heating. Reactive groups, i.e. hydrophobic and thiol groups, initially buried in the inner part of the protein structure, became exposed at the protein surface and led to the association of denatured proteins into small oligomers. The small oligomers are linked by disulfide bonds and when a critical concentration of oligomers is reached, they associate into primary aggregates (Fig 5). At low protein concentration, the curved strands persisted at steady state. At higher concentration and/or by reducing the electrostatic repulsions between the curved strands, a second aggregation can take place conducting to their association into larger fractal aggregates (Nicolai et al., 2011; Nicolai & Durand, 2013; Phan-Xuan et al., 2011).



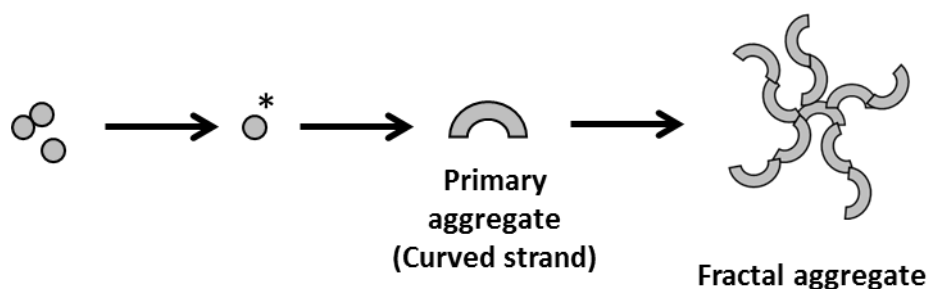


Figure 5: Scheme of the mechanism of formation of curved strand and fractal aggregates. Adapted from Nicolai, Britten & Schmitt (2011).

In the absence of salt, the second aggregation only occurred at protein concentration approaching the gelation concentration and a step aggregate size increase with protein concentration was observed. By adding NaCl, the second aggregation was observed at lower protein concentration and the increase in the aggregate size with protein concentration was gentle (Baussay, Bon, Nicolai, Durand & Busnel, 2004; Mahmoudi et al., 2007; Mehalebi, Nicolai & Durand, 2008; Phan-Xuan et al., 2011, 2013; Schmitt, Bovay, Rouvet, Shojaei-Rami & Kolodziejczyk, 2007). Figure 6 illustrates the evolution of the size and the morphology of WP aggregates according to protein concentration and NaCl content in protein solutions. Protein surface hydrophobicity would be the driven force of the aggregation but disulfide bonds were formed in a second time between the denatured proteins. The critical gelation concentration of WP solutions decreased with the decrease in pH or with the increase in the salt concentration (Mahmoudi et al., 2007; Mehalebi et al., 2008).

The heat-induced WP aggregates have a similar structure than the  $\beta$ -Lg aggregates but a different composition because of the co-aggregation of the different WP (Nicolai et al., 2011). The fractal dimension determined for the WP aggregates at different protein concentrations was  $df = 1.7$  and  $df = 2$  in absence and presence of NaCl, respectively (Mahmoudi et al., 2007). Hence, protein density in the aggregates decreased and its molar mass increased when the radius of the fractal aggregates increased; protein density in the aggregates increased with increasing NaCl concentration in WP solutions (Nicolai & Durand, 2013). For the fractal aggregates, the conversion yield was high and only 5 wt% of the  $\beta$ -Lg molecules were not aggregated at steady state (Mehalebi et al., 2008).

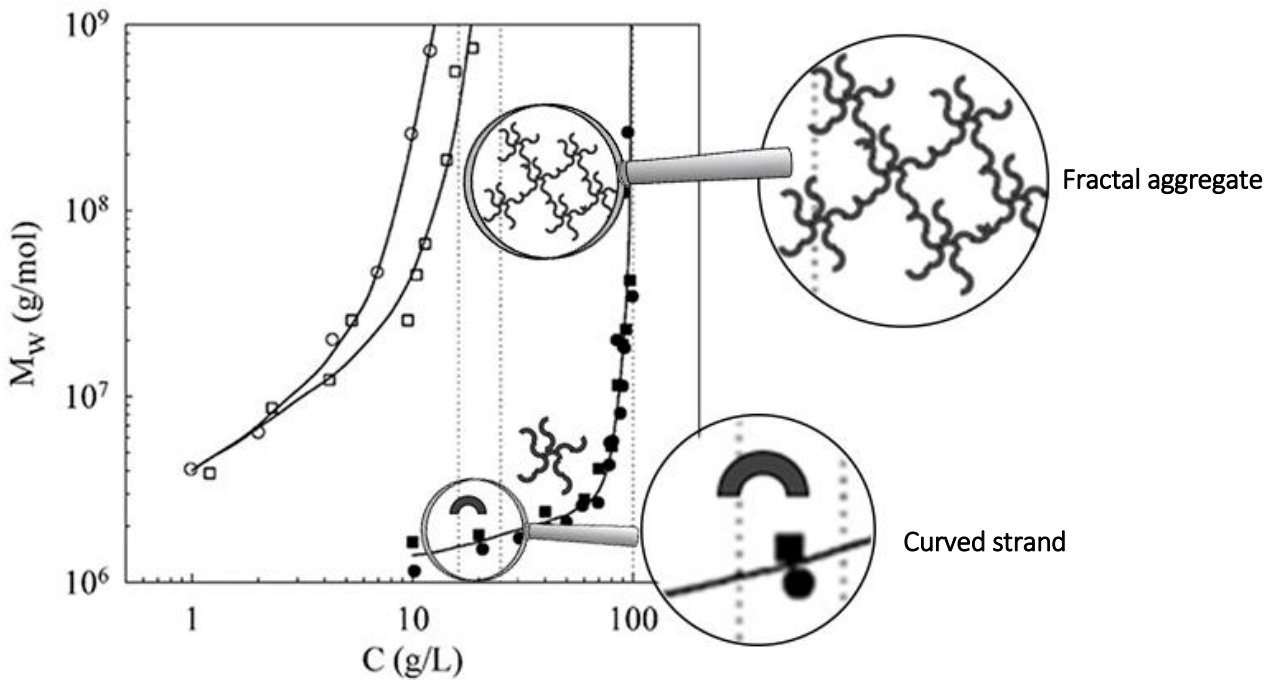


Figure 6: Concentration dependence of the weight-average molar mass of WPI (squares) and  $\beta$ -Lg (circles) aggregates formed in the presence of 0.1 M NaCl (open symbols) or in the absence of added salt (solid symbols). The solid lines are guides to the eye. The dotted lines indicate the critical gelation concentration:  $C_g \approx 100$  g/L for WPI and  $\beta$ -Lg in the absence of added salt,  $C_g \approx 25$  g/L for WPI, and  $C_g \approx 15$  g/L for  $\beta$ -Lg at 0.1 M NaCl. Adapted from Mahmoudi, Mehalebi, Nicolai, Durand and Riaublanc (2007) with permission of the America Chemical Society.

#### 1.1.2.3 Microgels

A microgel suspension consists on a colloidal dispersion of gel-like particles. The microgels have to be dispersed in a solvent, swollen by the solvent and have to exhibit a size between 10-1000 nm (Pelton & Hoarse, 2011). The promising ability to stabilize emulsions by different types of microgels was recently reviewed (Dickinson, 2015).

Whey protein microgels (WPM) are formed under conditions of weak electrostatic repulsions, i.e. in presence of divalent salt ( $\text{Ca}^{2+}$ /protein molar ratio  $\geq 2$ ) or close to the pI of the WP (Fig 3B). The WPM are usually prepared by heating pure  $\beta$ -Lg or WP solutions at pH 5.7-5.9 in the absence of added salt or at pH 7 in the presence of calcium. As for the other mechanisms of aggregation, a nucleation step and growth step was proposed to explain the formation of the WPM (Phan-Xuan et al., 2013). Schmitt et al. (2010) described the formation of the WPM by a heat-induced unfolding of the WP, which exposed hydrophobic residues on the protein surface and then their rapid aggregation into small dense primary aggregates ( $\sim 9$  nm). Then, these primary aggregates associated into larger structures of spherical shape of about 200-300 nm of diameter in which the primary aggregates are piled up like the scales in a closed pinecone. Protein surface hydrophobicity would be the driven force of the aggregation but disulfide bonds were formed in a second time between the proteins in the WPM. The disulfide bonds would

stabilize the WPM after changing the pH of the suspension in a very large pH range, i.e. between pH 2 - 4 and pH 5.5 - 8 (Schmitt et al., 2010). Murphy, Cho, Farkas & Jones (2015) confirmed that the non-covalent interactions played a key role in the formation of the WPM because they were still observed when the solution was heated in the presence of reducing agents. Krebs, Devlin & Donald (2009) have suggested that the microgels consisted on a dense packing of short  $\beta$ -sheet cross-linked fibrils. According to Schmitt et al. (2010) the structure of the microgels is homogeneous up to a scale length of about 10 nm. For some authors, more studies are required to get an unambiguous picture of the internal structure of the WPM (Nicolai, 2016).

During WPM formation, the pH of the solution increased (Moitzi et al., 2011; Phan-Xuan et al., 2011). Phan-Xuan et al. (2011) hypothesized that the pH increase was due to the protein denaturation and/or the partial charge neutralization of the proteins in the WPM leading to a depletion of  $H^+$ . Consequently, the charge density of the WPM increased during heating and stabilized the WPM suspension due to enhanced electrostatic repulsions between the WPM. However, the pH increase concomitantly conducted to side reactions such as the formation of strands instead of WPM. The proportion of WPM, strands and residual native proteins varied with the pH, as well as the temperature and duration of heating (Phan-Xuan et al., 2011). When the steady state was reached, the solution does not contain native proteins anymore (Nicolai, 2016). A microgel conversion rate of 85 % can be obtained either with pure  $\beta$ -Lg or WP (Destribats et al., 2014; Phan-Xuan et al., 2011) with appropriate conditions of heating. The WPM can be purified from a WPM suspensions containing WPM, strands and residual native proteins by centrifugation 26,900 *g* for 15 min (Donato et al., 2009).

The formation of the WPM was affected by different factors such as the temperature of heating, the protein concentration, the origin of the proteins (type of powder used for their formation), the pH of the solution and the presence of salt (i.e.  $CaCl_2$ ). The impact of the pH of the protein solution on the formation of the WPM was extensively studied (Donato et al., 2009; Jung et al., 2008; Kharlamova, Inthavong, Nicolai & Chassenieux, 2016; Mehalebi et al., 2008; Murphy et al., 2015; Phan-Xuan et al., 2011; Schmitt et al., 2009). The control of the pH is more important for the formation of the WPM than of the fractal aggregates (Kharlamova et al., 2016). In the absence of salt, it was found that the appropriate pH and temperature to obtain a high level of WPM was between 5.7 - 5.9 and at 85°C respectively (Donato et al., 2009). Moreover, the WPM formation depends strongly on the nature and the concentration of proteins (Schmitt, Bovay, Vuilliomenet, Rouvet & Bovetto, 2011). In the above mentioned study increasing the  $\alpha$ -La/ $\beta$ -Lg ratio increased the amount of soluble aggregates and decreased the amount of WPM after heating. This was explained by a decrease in the surface hydrophobicity of the primary aggregates containing larger amount of  $\alpha$ -La. Moreover, the WPM conversion rate was reduced from 70 - 85 % to 65 % by demineralization of the WP solution (prepared

with a WP isolate powder) before heating, showing the importance of the mineral content of the WP solution (Schmitt et al., 2011). This could explain the compositional variability of the WPM suspensions obtained after heating different WP solutions prepared using different powder batches. Phan-Xuan et al. (2013) confirmed the impact of the salt composition of the WP solution, especially the presence of divalent cations such as  $\text{Ca}^{2+}$ . These authors showed it was possible to form WPM at pH 7 in the presence of  $\text{CaCl}_2$ . In fact, the net charge of the WP would be the key element to control the formation of the WPM since it had to be between -5 and -3 to obtain a stable suspension of WPM. The protein net charge is a function of the pH of the solution and the calcium/protein molar ratio (R) (Nicolai, 2016). Phan-Xuan et al. (2014) found that this molar ratio has to be between  $1 \leq R < 3$  to form WPM at pH 7. WPM can be formed by controlling the amount of other cations such as  $\text{Fe}^{2+}$  or  $\text{Mg}^{2+}$  in the WP solution. So the WPM can be formed by decreasing the net charge of the proteins by a specific binding of protons or by adding divalent cations but not by the only screening of the protein electrostatic repulsions with NaCl (Nicolai, 2016). Indeed, WPM were not formed at pH7 by adding NaCl, even at a concentration as high as 0.4 M. Another way to form WPM was described by Sarkar et al. (2016). The WPM were prepared from a WP solution at 10 wt% and at pH7 which was heated at 90°C during 30 min to form a gel. Then, this gel was mixed in a phosphate buffer and pre-homogenized with a blender during 10 min to obtain the microgels.

### 1.1.3 Functionalities of the whey proteins aggregates

Some new and promising functionalities of the aggregates described in the previous section have recently been reviewed (Dickinson, 2015; Nicolai, 2016; Nicolai et al., 2011; Nicolai & Durand, 2013). In this section, we will focus on the physical stability and the interfacial properties of the aggregates.

#### 1.1.3.1 Physical stability

When the steady state was reached, stable suspensions of WP aggregates were obtained. If the dispersing phase of the aggregates was unchanged, they remained heat stable (Nicolai & Durand, 2013). Increasing the WP aggregate concentration before a second heat treatment or adding salt and/or changing the pH could lead to the precipitation or the gelation of the aggregates.

A gelation induced by the reduction of the electrostatic repulsions between the aggregates, i.e. by adding salt and/or changing pH, is referred to cold gelation (Nicolai et al., 2011). Cold-set gels usually required a slow increase in the ionic strength and/or a slow pH change of the aggregate suspension to form. WPM gave coarse gels whereas the soluble aggregates (i.e. strand-like aggregates) gave homogeneous fine-stranded networks. Moreover, a larger protein concentration was needed to obtain a cold-set gel of WPM than of soluble aggregates. This seems to be related to the higher density of the WPM compared to soluble aggregates (Nicolai, 2016). Cold-set gels of fibrils was formed by using divalent cations such as  $\text{Ca}^{2+}$  (Veerman, Baptist, Sagis & van der Linden, 2003).

When the protein concentration was too low to form a gel, the pH variation or the addition of salt to the aggregate suspension led to the aggregation and the precipitation of the aggregates. The colloidal stability of the WPM was studied in aqueous phase containing or not NaCl (Destribats et al., 2014). These authors indicated that the WPM were stable between pH 2 and 4 and between pH 6 and 8 in the absence of salt and at pH 2 and between pH 5.2 and 8 in the presence of 150 mM of NaCl. The WPM aggregated and settled down in the pH range  $4 < \text{pH} < 5.5$  and  $3.5 < \text{pH} < 5.5$  without or with salt, respectively. The fibrils were insoluble in the pH range from pH 4 to pH 6.5 (Loveday, Su, Rao, Anema & Singh, 2011). The fractal aggregates were more heat stable than native proteins in the presence of salt and at neutral pH (Ryan et al., 2012). Heating fractal aggregates instead of native proteins allowed decreasing the size, modifying the morphology and increasing the solubility of the particles obtained, which result in a decrease in the turbidity and the viscosity of the heated solution. Fractal aggregates of small size and compact structure with high charge density had a lower propensity to secondary heat-induced interactions in the presence of salt. A pre-aggregation of the WP was shown to improve the heat stability of WP solution (Shen, Fang, Gao & Guo, 2017). However, to the best of our knowledge the stability of these aggregates in a complex aqueous phase, i.e. containing sugar and salts at various concentrations, during either a heat treatment at high temperature (120°C) or a freeze (-18°C)/thaw cycle has not been studied yet.

#### 1.1.3.2 Interfacial properties

The interfacial properties of the fibrils, fractal aggregates and microgels of WP at model interface was studied by different authors (Jung, Gunes & Mezzenga, 2010; Mahmoudi, Axelos & Riaublanc, 2011; Mahmoudi, Gaillard, Boué, Axelos & Riaublanc, 2010; Murphy, Farkas & Jones, 2016). Jung et al. (2010) showed that a suspension of fibrils decreased more rapidly the air-water surface tension than native proteins. This behavior was due to the residual native proteins and peptides as it was not observed after dialysis of the fibrils suspension (after removing residual native proteins and peptides). The fibrils gave more rigid and more fragile interface than the native proteins. Mahmoudi et al. (2010) compared the air-water interfacial behavior of the native WP and the fractal aggregates of WP prepared at 80°C and pH 7. By drop tensiometry, they exhibited the same adsorption behavior at interface and the same ability to form an interfacial viscoelastic film. The authors suggested the adsorption behavior of the fractal aggregates was mainly defined by the residual non-aggregated protein fraction (at least 5 %) which was in sufficient amount to fully cover the interface. Because of their larger size and smaller diffusion coefficient, the aggregates reached the interface after the residual native proteins. The structure of the aggregates affected their spreading at air-water interface (Mahmoudi et al., 2011). Aggregates of spherical shape formed by dynamic heating at 100 - 120°C seemed to deform less at air-water interface than fractal aggregates. However, a partial disruption of both aggregates in smaller

entities at the interface was observed. This observation was in agreement with the study of Murphy et al. (2016). These authors also indicated that the protein concentration and the size of the WPM affected their interfacial adsorption. At low protein concentration, small (200 nm) and large (300 nm) WPM completely disrupted at the air-water interface whereas at high protein concentration only a deformation and a spreading of the WPM were observed. The deformation was higher for the smaller WPM. The WPM adsorption was faster and the interfacial elasticity increased more rapidly with small WPM than with large WPM.

The fractal aggregates and the WPM exhibited more promising properties for a use as natural surfactants in food products than fibrils. Indeed, due to their low conversion yield, the heating time required for their formation and the fact they are not fully soluble between 4 and 6.5 (pH range of most food products), the use of fibrils in food products is compromised (Nicolai & Durand, 2013). Since the present work focused on the emulsion stability during technological treatments, the capacity of the WP aggregates to stabilize emulsions were detailed in the next part.

## 1.2 Emulsions and whey proteins aggregates

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### 1.2.1 Emulsion definitions

Oil-in-water emulsions consist of mixtures of two immiscible liquids, in which the oil phase is dispersed as small spherical droplets in the water phase (McClements, 2005). Most of the food emulsions are oil-in-water emulsions such as milk, cream, dressing, beverages and sauces. They are constituted of three regions, all of which contributing to the physicochemical properties of the emulsions:

- The dispersed oil phase in the form of droplets. The type of oil will define the amount of fat crystals, their size and their polymorphism at defined temperature. The oil volume fraction, the distribution of the droplet size and their interactions are key elements of the stability and rheology of the emulsions.
- The continuous phase surrounding the oil droplets. Numerous factors of the continuous phase impact the properties of the emulsions: the pH, the ionic strength, the nature and the amount of the molecular species present (protein, salt, sugar, *etc.*). The viscosity of the continuous phase is of tremendous importance for its long-term stability.
- The interface or the oil droplet surface covered by amphiphilic molecules. The nature of the emulsifiers plays an important role on the charge and the visco-elastic properties of the interface and on the emulsion properties (McClements, 2005). Biopolymers such as proteins, solid particles, small surfactants are among the water soluble emulsifiers used to stabilize oil-in-water

emulsions. They are dispersed in the water phase before the oil is added while stirring (Vilgis, 2015).

### 1.2.2 Emulsion formation

Milk is a natural oil-in-water emulsion but in most other food emulsions, the dispersion of the oil phase in the aqueous phase required a large amount of energy brought by mechanical operations such as high-speed mixing or high-pressure homogenization (McClement, 2005). The mechanical energy input allows the disruption and the mixing of the oil as small droplets into the water phase (Schubert, Ax & Behrend, 2003; Walstra, 1993; Walstra & Smulders, 1998). The increase in the interfacial area between the two immiscible phases increases the interfacial free energy of the system compared to the situation before mixing or homogenizing. The interfacial free energy ( $\Delta G$  in J) needed to increase the total interfacial area ( $\Delta A$ , m<sup>2</sup>) of the emulsion is defined by the equation:

$$\Delta G = \gamma \Delta A$$

where,  $\gamma$  is the interfacial tension (N/m) between the oil and water phases. The rupture of large droplets into small ones requires overcoming the internal pressure of the oil droplets. This latter is defined by Laplace pressure ( $\Delta P_L$  in Pa):

$$\Delta P_L = \frac{2\gamma}{r}$$

where  $r$  is the droplet radius (m). Therefore, the energy input required to disrupt oil droplets is higher when the oil droplets are smaller at constant interfacial tension (Lee et al., 2013).

The size of the oil droplet dispersion constitutes a key element for the organoleptic properties (e.g. texture, appearance, taste), the nutritional properties and the stability of the food emulsions. Two processes acting in opposite direction are responsible for the mean oil droplet diameter of the emulsion (Karbstein & Schubert, 1995):

- The disruption of the oil droplets into smaller ones during the mechanical treatment (high-speed mixing, high-pressure homogenization).
- The rapid destabilization of the formed oil droplets by re-coalescence (McClement, 2005).

To control the formation of the oil-in-water emulsions, food technologists used amphiphilic molecules or emulsifiers. A simple emulsifier is like a bi-block molecule, composed of a hydrophobic segment and a hydrophilic segment, which exhibits an affinity for the two immiscible phases and by a positioning at the interface reduces the interfacial tension. Proteins are large emulsifiers that contain many

hydrophilic and hydrophobic parts. To be a good emulsifier, it has to adsorb and to orient itself rapidly at the oil droplet interface in order to form a protective layer preventing oil droplet re-coalescence (Karbstein & Schubert, 1995). When the amount of emulsifier is in excess for the full coverage of the interface, the mean oil droplet diameter can be controlled by the energy input (homogenization pressure).

In the food industry and for lab scale studies, oil-in-water emulsions are often prepared using two successive homogenization steps. The primary homogenization which is usually performed with a high-speed blender or a rotor stator, is used to prepare a coarse emulsion. At this stage, the oil and water phases are converted into large droplets of oil dispersed in the water phase. The secondary homogenization conducted on a high-pressure homogenizer is used to reduce the size of the oil droplets.

### 1.2.3 Destabilization mechanisms of the emulsions during technological treatments

Food emulsions are usually classified in the macro-emulsions; their mean oil droplet diameter is between 0.1 and 50  $\mu\text{m}$ . They are thermodynamically unstable and tended to separate unless they are stabilized (Karbstein & Schubert, 1995). Emulsion stability is kinetically controlled (Dickinson, 1994). Traditionally, the emulsion stability is defined as the resistance of the emulsion to change over time. In our case, it is the ability of the emulsions to keep its aspect during technological treatments (i.e. heating, freeze/thaw cycle). These changes could be of physical or chemical origin (McClement, 2005) but in this section and in our work, only the physical instabilities are taking into account (Fig 7).



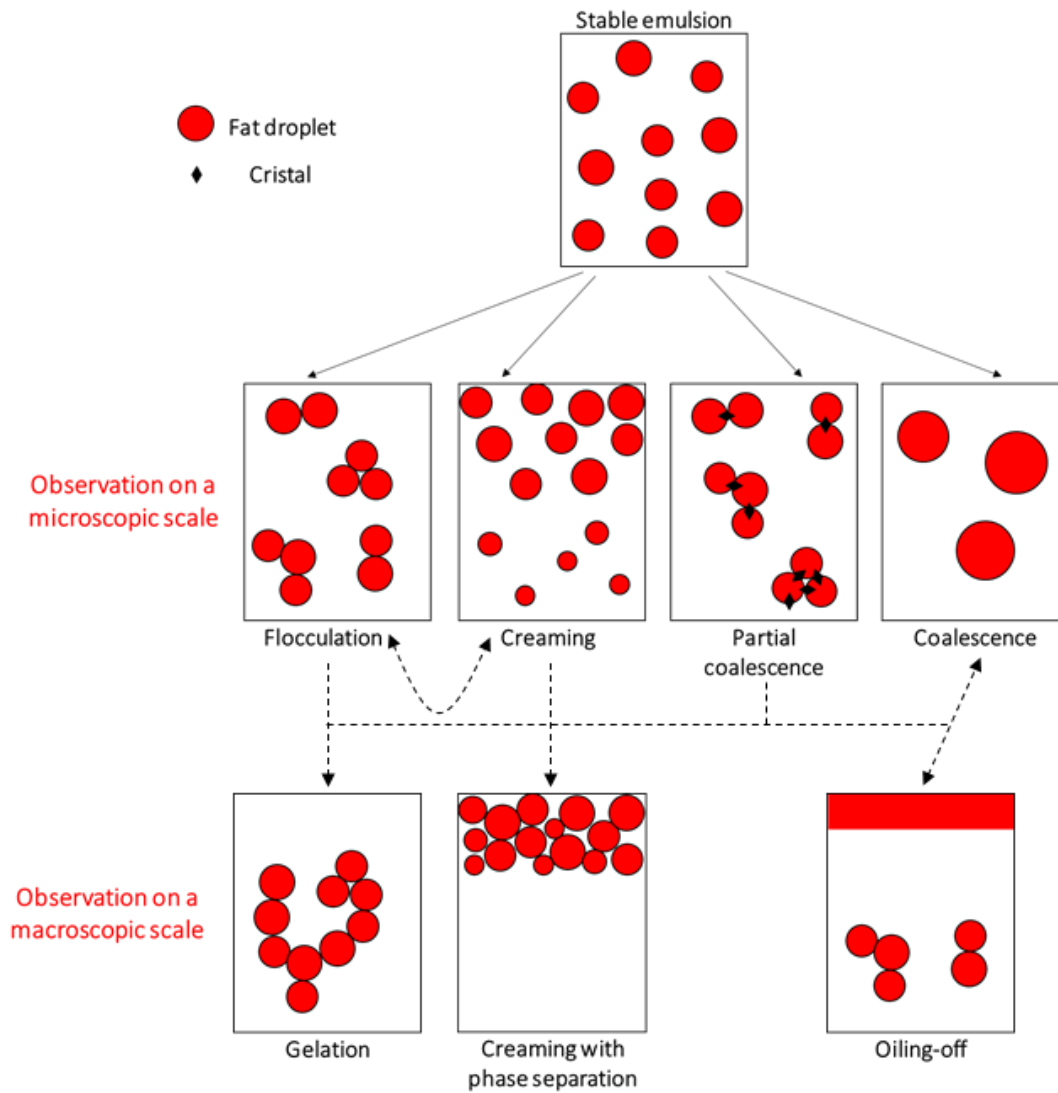


Figure 7: Physical instabilities occurring during technological treatments of oil-in-water emulsions

#### 1.2.3.1 Flocculation

Oil droplet flocculation is the aggregation of two or several oil droplets that keep their individual integrity (McClements, 2005). The flocculation comes from an inability of the emulsifiers to inhibit the close contact of the oil droplets (Dickinson & Stainsby, 1982). A good approximation of the emulsion stability is obtained by the DLVO theory. The sum of the attractive van der Waals interactions and the electrostatic repulsive interactions between the oil droplets allow to obtain the DLVO interaction potential. At each distance between the oil droplets, the sign of the sum of the two forces determines the tendency of the droplets to agglomerate or to separate. If the electrostatic repulsive interactions are superior to the attractive van der Waals interactions, the droplets are separated and conversely. In flocculated emulsions, the oil droplets are in weak interactions, which can be disrupted, at least

temporally, by shaking, stirring or pH variation (Dalgleish, 1997). Flocculation is also reversible because of the non-disruption of the protective stabilizing layer at the fat droplet interface (Dickinson, 1997). The flocculation leads to an increase in the particle size of the emulsions, which can undergo enhanced creaming (Dalgleish, 1997). Oil droplet flocculation can also be responsible for an increase in the emulsion viscosity and in some cases for the formation of a soft gel. The gel consists in an interconnected tridimensional network of oil droplets (Demetriades, Coupland & McClements, 1997b, 1997a; Bijsterbosch, Bos, Dickinson, Opheusden & Walstra, 1995). Depending on the products, the viscosity increase can be desirable or undesirable (McClement, 2005). When the increase in viscosity is desired, it can lead to the decrease in the oil droplet creaming.

Two types of flocculation are distinguishable, the depletion flocculation and the bridging flocculation:

- The depletion flocculation is due to the presence in the continuous phase of the emulsion of non-adsorbing colloidal particles such as protein aggregates or polysaccharides. The exclusion of the non-adsorbing colloidal particles from the narrow region surrounding the oil droplets generates an attractive osmotic force strong enough to overcome the repulsive forces between the oil droplets i.e. steric and electrostatic (McClements, 2005). The interaction between the oil droplets is generally very weak and is reversible by dilution. The depletion flocculation is observed in emulsions with a high droplet packing density and need a minimal amount of non-adsorbed colloidal particles to occur. This minimal particle concentration decreases with the increase in the size droplets and of the effective volume fraction of colloidal particles (McClements, 2000). As the thickness of the depletion zone is approximately equal to the radius of the non-adsorbing particles, the structure of these latter is of primary importance. Extended and stiff particles are particularly effective to induce oil droplet depletion flocculation.
- The bridging flocculation occurs when a molecule or a particle adsorbs to the surface of more than one oil droplets and connect them together (Dalgleish, 1997). It is favored by a lack of emulsifiers, a large interfacial area and a droplet formation time shorter than the adsorption time of the emulsifiers (McClements, 1999). For example, bridging flocculation is observed in casein micelles-stabilized emulsions at protein concentration (McCrae & Lepoetre, 1996).

#### 1.2.3.2 Creaming

Creaming is a gravitational separation leading to a concentrated layer of oil droplets on the top of the emulsion without a change in the droplet size distribution (McClement, 2005). In the oil-in-water emulsion, the creaming is observed because the oil phase has a lower density than the aqueous phase. The creaming is a progressive phenomenon. Initially, a vertical gradient of oil droplets concentration appears in the emulsion. Subsequently, a creamed top layer separates from the serum bottom phase

(Dickinson, 1997). Generally, the creaming is reversible as the oil droplet size distribution is unchanged. During the creaming, the oil droplets are subjected to two opposing forces, the gravitational force that gives the oil droplets an upward movement and the frictional force that limits and slows down this movement. The creaming velocity or the gravitational separation rate ( $v_{Stokes}$  in m/s) is expressed by the Stokes' law equation:

$$v_{Stokes} = - \frac{2gr^2(\rho_2 - \rho_1)}{9\eta}$$

where  $g$  corresponds to the gravitational acceleration ( $m/s^2$ ),  $r$ , the droplet radius (m),  $\rho_1$  and  $\rho_2$  the density of the continuous phase and dispersed phase respectively ( $kg/m^3$ ) and  $\eta$ , the continuous phase viscosity (Pa/s).

Beside the droplet size, the flocculation of the oil droplets is also an important factor affecting the creaming velocity (Dickinson, 1997). The flocculation of the oil droplets, that creates aggregates of oil droplets with a larger size than the individual oil droplets, can increase their creaming. Also, by decreasing the distance between the oil droplets, the creaming can facilitate oil droplet flocculation and coalescence. The creaming can be prevented or limited by decreasing the droplet size, the difference of density between the two phases for instance by using dense emulsifiers or by increasing the viscosity of the continuous phase. Emulsion beverages exposed to high temperatures of storage ( $> 30^\circ C$ ) often exhibit a creamed oil droplet layer on the top of the emulsion due to a decrease in the viscosity of the continuous phase.

### 1.2.3.3 Partial coalescence

Partial coalescence occurs only in emulsions that contain a mixture of liquid and solid fat phase, as it is the case for milk fat. A decrease in the emulsion storage temperature can induce the crystallization of the fat inside the droplets. The partial coalescence appears when solid fat crystals of one droplet breaks up the surface and penetrates inside another droplet (Boode, 1992; Boode & Walstra, 1993; Boode, Walstra & de Groot-Mostert, 1993; Walstra, 1993). The mechanical strength of the fat crystal network inside the droplets prevents their complete merging; the fat droplets keep their initial shapes (Boode, 1992; Coupland, 2002; Dickinson & McClements, 1995; Walstra, 1996; Walstra, 2003). Hence, the fat droplets form clusters with irregular shapes. The partial coalescence occurs either in the continuous phase or at the surface of the air bubble after adsorption. The partial coalescence leads to the increase in the emulsion viscosity (McClement, 2005). Under some conditions (emulsion with low fat droplet packing density), it can also facilitate fat droplet creaming. Unlike flocculation and creaming, the partial coalescence is irreversible. If an emulsion with partially coalesced fat droplets is warmed, a fusion (coalescence) of the fat droplets is observed.

### 1.2.3.4 Coalescence

The process for which two or several oil droplets merge together to form a single larger droplet is called coalescence. This reduces the interfacial free energy of the system due to the decrease in the contact area between oil and water (McClements, 2005). The coalescence occurs after a time of contact of the oil droplets. The interface composition is a key element to control oil droplet coalescence. Large (steric hindrance) and charged (electrostatic repulsion) emulsifiers are the most efficient to prevent oil droplet coalescence. Coalescence is irreversible. An abundant droplet coalescence results in the formation of an oil layer at the top of the emulsion, called oiling off. Sometimes this phenomenon is observed when the emulsions are subjected to alternating positive and negative temperatures. Flocculation, creaming and partial coalescence can lead at the coalescence and consequently to the oiling off.

### 1.2.4 Pickering and Pickering-like emulsions

Pickering emulsions have benefited of increasing interest in the last few years (Berton-Carabin & Schroën, 2015; Dickinson, 2012; Hunter, Pugh, Franks & Jameson, 2008), especially in the field of food science (Fig 8).

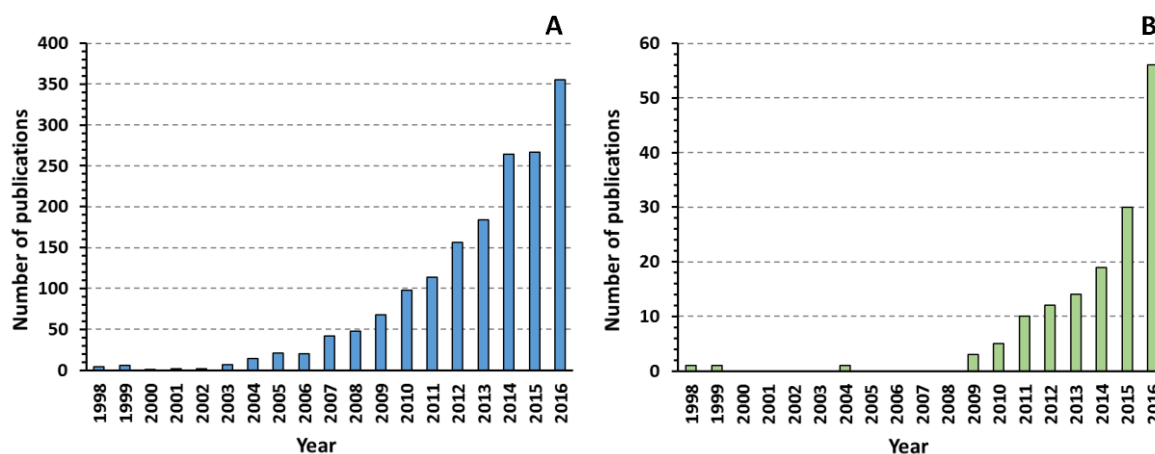


Figure 8: Number of publications related to (A) Pickering emulsions and (B) Pickering emulsions and food by year. Data from Thomson Reuters Web of Science.

Pickering emulsions (Pickering, 1907; Ramsden, 1903) also called solid-stabilised emulsions consist in emulsions stabilized by densely packed solid colloidal particles (Binks, 2002; Leal-Calderon & Schmitt, 2008). The colloidal particles, partly wetted by oil and by water, form a steric barrier at the interface ensuring excellent protection against coalescence (Horozov & Binks, 2006). The particle affinity for both the oil and water phases determines the particle contact angle at interface ( $\theta$ ) and the particle ability to

stabilize oil-in-water emulsions or water-in-oil emulsions (Fig 9). Particles preferentially wetted by water (contact angle  $< 90^\circ$ ) stabilize oil-in-water emulsions and particles preferentially wetted by oil (contact angle  $> 90^\circ$ ) stabilize water-in-oil emulsions (Berton-Carabin & Schroën, 2015). Once at the interface, the particles are usually considered as irreversibly adsorbed because the energy required for their desorption is extremely high compared to the thermal energy (Kaz, McGorty, Mani, Brenner & Manoharan, 2012). In oil-in-water emulsion, this energy depends mainly on the contact surface area of the particle at the oil phase. The particle density at the oil droplet surface (particle coverage rate) and the difficulty to remove the adsorbed particle from the interface (depending on the particle size and its wettability by the oil phase) defined the level of protection given by the particles. To be an efficient emulsifier, the particle size has to be at least an order of magnitude smaller than the oil droplet size (Dickinson, 2012). The high physical stability of the Pickering emulsions during long-term storage constitutes the main advantage of their potential use in food products (Berton-Carabin & Schroën, 2015). In return, one important limitation is that only few solid particles used so far (for instance starch granules, oil crystals or flavonoids nanoparticles) are food-grade ingredients.

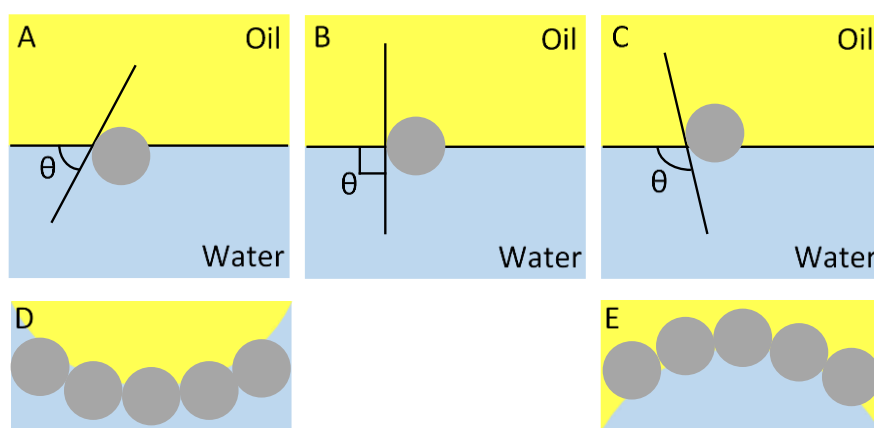


Figure 9: Position of a spherical particle at a planar oil–water interface for a contact angle (measured through the aqueous phase) less than  $90^\circ$  (A), equal to  $90^\circ$  (B) and greater than  $90^\circ$  (C). Putative particles positioning at a curved interface: for  $\theta < 90^\circ$ , stabilization of o/w emulsions (D), for  $\theta > 90^\circ$ , stabilization of w/o emulsions (E). Adapted from Aveyard, Binks & Clint (2003).

Aside solid colloidal particles, soft colloidal particles exhibiting particle-like features in term of dimension but without their non-deformable character were also considered for the stabilization of food emulsions (Berton-Carabin & Schroën, 2015). Soft particles are maintained internally by weak interactions and consequently their structure can change after modifying external parameters such as temperature,

solvent composition, *etc.* They are able to change their morphology and may also change their composition once adsorbed at interface (Schmitt & Ravaine, 2013). The soft particles have not exactly the same mechanism of emulsion stabilization than the solid particles. Indeed, the swelling and the structural changes of the soft particles at the interface have also to be considered (Schmidt et al., 2011). The soft particles are able to spread and to interpenetrate at interface (Destribats et al., 2014; Murphy et al., 2016; Schmidt et al., 2011). By this mechanism, they were proposed as alternative ingredients to stabilize emulsions. The terms “Pickering-like emulsions” or “Mickering emulsions” were proposed to define emulsions stabilized by soft particles (Schmidt et al., 2011).

Zein, soy or milk protein (WPM aggregates or casein micelles) particles were successfully used to stabilize oil-in-water emulsions through a Pickering-like mechanism (Destribats et al., 2014; Folter, Ruijven & Velikov, 2012; Gao et al., 2013; Liu & Tang, 2013, 2014). Heating protein solution is an efficient way to prepare particles with aim to stabilize Pickering-like emulsions. Destribat, Rouvet, Gehin-delval, Schmitt & Binks (2014) used WPM to stabilize coarse emulsions in a large range of pH and ionic strength (between pH 2 - 8 and at ionic strength of 0 and 150 mM NaCl). All the emulsions were extremely stable against coalescence (i.e. at least 18 months). The WPM organized differently at interface as a function of their surface charge that was tunable by changing the pH and adding salts to the emulsions. Emulsions prepared with charged WPM exhibited low surface coverage rate (~ 50 %). Charged WPM limited oil droplet coalescence but they were responsible for the bridging of neighboring oil droplets at the source of a high flocculation rate. Emulsions prepared with neutral WPM appeared less flocculated than emulsions prepared with charged WPM. The neutral WPM displayed a complete coverage of the oil droplet surface. Under this condition, the spreading of the WPM and the formation of a 2D-network at the oil droplet surface was suggested in agreement with Murphy, Farkas & Jones (2016). The formation of a steric barrier around oil droplets was suggested to limit the phenomenon of coalescence. WPM were also used to improve the stability of water-in-water emulsions (Murray & Phisarnchananan, 2016; Nguyen, Nicolai & Benyahia, 2013) and foams (Schmitt, Bovay & Rouvet, 2014). Close to the pH of neutral charge, WPM self-aggregated at air-water interface leading to an arrest of the drainage as well as of the disproportionation of the air bubble (Schmitt et al., 2014). The behavior of the WPM at interface was correlated to the WPM solution properties at the same pH. More recently, a study investigated the stability of Pickering-like emulsions prepared with purified  $\beta$ -Lg fibrils (Gao et al., 2017). As for the WPM emulsions, the fibril-stabilized emulsions exhibited a long-term stability. However, the pH influenced the stability of the fibrils-stabilized emulsions. The fibrils showed an excellent ability to stabilize oil-in-water emulsions far from the pH of neutral charge of the fibrils, pH ~ 5.2, but the fibrils aggregated and were responsible for the emulsion destabilization (with large droplet size) when they were uncharged. The stability of the emulsions was also observed for a narrow fibril concentration

## Chapter 1: Literature review

range. Below the fibril concentration of optimal emulsion stability, the insufficient fat droplet surface coverage induced oil droplet coalescence. Above the concentration of optimal stability, the excess of fibril in the continuous phase induced the flocculation of the fat droplet through a depletion-flocculation mechanism. Similarly, the fibrils of WP were a better foaming agent than native or denatured WP at low protein concentration (1 - 3 wt%). They improved the foaming capacity and the foam stability (Oboroceanu, Wang, Magner & Auty, 2014).

WP aggregates other than the WPM and the fibrils were also used to stabilize emulsions. The stability of the emulsions prepared with heat-induced WP aggregates was enhanced compared to native whey protein-stabilized emulsions (Kim, Cornec & Narsimhan, 2005; Moro, Gatti & Delorenzi, 2001). The foaming properties of soluble aggregates were also improved compared to the foaming properties of native proteins (Moro, Baez, Ballerini, Busti & Delorenzi, 2013; Schmitt et al., 2007). The size, the charge and surface hydrophobicity of the WP aggregates influenced the emulsion forming and stabilizing properties (Lam & Nickerson, 2015). A decrease in the size of the aggregates improved the emulsion forming property due to their fastest migration at the oil droplet surface and the emulsion stability. The ultrasound treatment of a heat-induced WP aggregates suspension increased its emulsifying properties i.e. emulsifying activity and emulsion stability, by a modification of the structure and the size of the aggregates (Shen et al., 2017). The emulsion stability was higher in pH conditions away from the pH of neutral charge of the aggregates. In concentrated emulsions (60 %wt of oil), the use of WP aggregates with a radius in the range from 25 to 40 nm led to an increase in the emulsion viscosity (Knudsen, Øgden & Skibsted, 2008). The authors explained the viscosity increase by the increase in the oil droplet interactions due to the presence of aggregates at the oil droplet surfaces. At lower oil volume fraction (20 wt%), other authors indicated a decrease in the viscosity of the emulsions prepared with WP aggregates (at a concentration of 5 g/L) compared to the emulsions prepared with native proteins due to a decrease in the interaction between the oil droplets (Bernard, Regnault, Gendreau, Charbonneau & Relkin, 2011). In the other hand, Moro et al. (2013) showed a decrease in the emulsifying properties (emulsifying capacity and emulsion stability) of a WP solution at pH 6.8 heated at 85°C for different time (from 1 to 15 min). According to Dybowska (2011), the emulsion properties were enhanced with the increase in the heating temperature applied to form the aggregates. These authors explained the improved emulsion stability by a higher viscosity of the continuous phase containing WP aggregates and/or by the adsorption at the droplet surface of the aggregates in a second protein layer. These different and by some way contradictory results could be explained by the structure of the emulsions resulting from the process of emulsification (different interfacial area) and the composition of the WP aggregate suspensions (different proportion of aggregates and non-aggregated proteins). The

aggregate adsorption at fat droplet surface would be facilitated by increasing the energy input for increasing the interfacial area and the proportion of aggregates in the suspension.

In spite of promising emulsion stability properties, WP aggregates exhibit two main drawbacks for a use in Pickering-like emulsions. WPM-stabilized emulsions are stable *versus* coalescence when the WPM fully covered the droplet surface. However, WPM are large and dense particles, meaning their number is limited in the emulsions. Consequently, at defined protein concentration the WPM-stabilized emulsions are coarser than the one prepared using non-aggregated proteins. The protein concentration has to be increased (more than one decade) to decrease the oil droplet size significantly (Nicolai, 2016). Another possibility is to use smaller WP aggregates, i.e. strand-like aggregates, but the mechanism of fat droplet stabilization could be different even if a detailed comparison was not done until now. As mentioned earlier, the yield of formation of the fibrils is low, meaning the amount of non-aggregated proteins and peptides in the fibril suspension at “steady state” remains high. These latter competed with fibrils for the adsorption at fat droplet interface and modified the emulsion properties (Jung et al., 2010). Hence, their removing could be an essential step before using fibrils for emulsion stabilization.

### 1.2.5 Whey protein aggregate emulsions

In Pickering emulsions, the particles stabilize the oil droplets. However, the diffusion and the adsorption of the particles at interface are slow. Hence, a difficulty encountered in real food systems is that other surface-active entities compete with solid or soft particles for interface (Dickinson, 2012). Small molecular emulsifiers such as milk proteins positively or negatively affected the stability of the emulsions prepared with silica particles (Pichot, Spyropoulos & Norton, 2010) or cellulose particles (Murray, Durga, Yusoff & Stoyanov, 2011). This resulted from either the interaction of the small molecular emulsifiers with the particles or a competition between the entities for the oil droplet surface (Berton-Carabin & Schroën, 2015). Serfert et al. (2014) prepared emulsions with a solution of WP fibrils with a conversion rate lower than 50 %. Therefore, the fibril suspension contained a mixture of fibrils and non-aggregated proteins and/or peptides. Better emulsifying activities were observed for the  $\beta$ -Lg fibrils solution than for the unheated  $\beta$ -Lg solution at pH 2 and 3. Blijdenstein, Veerman & van der Linden (2004) and Peng, Simon, Venema & van der Linden (2016) combined the use of native WP as emulsifiers and WP fibrils as non-adsorbed colloids to stabilize oil-in-water emulsions against creaming. The presence of fibrils in the continuous phase induced an increase in the emulsion viscosity. However, below a critical concentration of fibrils, a depletion-flocculation phenomenon leading to enhanced creaming was observed. Above this critical concentration, the emulsions were kinetically stabilized (absence of creaming) although no fibril gel network and droplet gel network was formed (Peng et al.,



2016). The authors indicated that the long fibrils were more efficient to stabilize the emulsions against creaming than the short ones. However, the emulsification process seems to induce a shortening of the fibrils (Serfert et al., 2014). If the stabilizing properties of the fibrils is mainly due to its presence in the continuous phase, their addition in the emulsion after the dispersion of the oil phase should be considered. Interestingly, the presence of non-aggregated proteins was shown to impact the protein composition at the fat droplet surface of WP microparticles emulsions (Çakır-Fuller, 2015). This suggests a competition between the WP microparticles and the non-aggregated WP for the oil droplet interface. Similarly, a combination of fractal aggregates and native  $\beta$ -Lg synergistically improved the foaming properties of the solution, i.e foam stability and foamability (Rullier, Axelos, Langevin & Novales, 2009, 2010; Rullier, Novales & Axelos, 2008). The presence of native  $\beta$ -Lg improved the mixture foamability because the fractal aggregates, especially the larger ones, were poorly surface active. However, the fractal aggregates reduced the foam drainage. The non-aggregated proteins adsorbed preferentially at the air-water interface and, if their amount was sufficient, played the role of anchors for the fractal aggregates that could lead to stable foam films (Rullier et al., 2009).

These examples show that it is possible to design stable emulsions (and foams) with a mixture of WP aggregates and non-aggregated WP. However, in most of the mentioned studies the repartition of the aggregates and the non-aggregated proteins between the fat droplet surface and the continuous phase of the emulsion was not studied in details although it could help to have a better understanding of the key parameters involved in emulsion stabilization.

### 1.2.6 Emulsions containing whey proteins *versus* whey protein aggregates: stability during technological treatments

Emulsions containing WP are extremely heat sensitive. Heated above 70°C, the WP unfold and then aggregate (Griffin, Griffin, Martin & Price, 1993; Roefs & De Kruif, 1994; Sawyer, 1968; Verheul, Roefs & de Kruif, 1998). The heat denaturation of the WP was responsible for the emulsion instability (Çakır-Fuller, 2015; Dybowska, 2011; Euston, Finnigan & Hirst, 2000; Hunt & Dalgleish, 1995; McClements, 2004; Sarel et al., 2014). The instability of the WP emulsions during heating was due to the proteins adsorbed at the oil droplet surface as well as at the protein in the continuous phase (Euston et al., 2000). At low protein concentration (below 3 wt%), a heat-induced flocculation of the oil droplets occurred (Çakır-Fuller, 2015; Dickinson & Parkinson, 2004). Sliwinski, Roubos, Zoet, van Boekel & Wouters (2003) indicated that the oil droplets irreversibly aggregated on heating. The amount of adsorbed proteins at the oil droplet surface increased on heating (Euston et al., 2000; Sliwinski et al., 2003) leading to a protein interfacial layer thicker than a monolayer (Dickinson, Rolfe & Dalgleish, 1989; Hunt & Dalgleish,

1994). Removing the non-absorbed proteins from the emulsion decreased drastically the aggregation of the oil droplets. It was deduced that both the adsorbed proteins and the non-adsorbed proteins were necessary for the oil droplets aggregation to occur (Euston et al., 2000). Increasing the WP concentration increased the flocculation of the oil droplets and the emulsion viscosity until the critical concentration of gelation was reached. The critical concentration of gelation was estimated at around 3 wt% of non-adsorbed WP (Çakır-Fuller, 2015), confirming that the concentration of the non-adsorbed WP in the continuous phase played a key role on the emulsion heat stability (Çakır-Fuller, 2015; Dybowska, 2011; Euston et al., 2000; Reiffers-Magnani, Cuq & Watzke, 1999; Sliwinski et al., 2003). Above the critical concentration of gelation, the heat-denatured WP in the continuous phase played a “glue” role (Euston et al., 2000) connecting the different oil droplets in a continuous network. Below this concentration, the amount of proteins was not enough to form a continuous network of denatured proteins and oil droplets. The heat stability of the WP emulsions was dependent on the pH and the ionic strength (Demetriades et al., 1997a; Hunt & Dalgleish, 1995). Increasing the ionic strength or adjusting the pH closer to the pI of the WP reduced the emulsion heat stability.

Only few studies deal with the stability of emulsions containing WP aggregates during heating. The heat treatment (90°C, 30 min) of the emulsions containing WPM-stabilized oil droplets increased the surface protein load (Sarkar et al., 2016). After heating, the oil droplet surface was covered by a continuous network of aggregated WPM instead of individual WPM isolated at the oil droplet surface before heating. Moreover, the WPM network connected neighboring oil droplet after heating. The authors concluded that the structure and the reactivity of the WPM were affected by the heat treatment and the interface. The heat stability of the WP emulsions at high protein concentration was enhanced by using microparticulated WP (Çakır-Fuller, 2015). The heat stability of the WP microparticle suspensions was correlated with the heat stability of the emulsions. The presence of non-aggregated proteins in the suspension of WP microparticles (~ 25 %) induced a gelation of the emulsion above a critical concentration of non-aggregated WP of 3 %. For the fibril emulsion, Peng et al. (2016) have shown that the fibril emulsion stability was not affected after 7 days of heating at 45°C.

The freeze/thaw stability of the emulsions containing WP and WP aggregates has received little attention. Most of the studies highlighted the impact of the WP in model emulsions for ice cream applications. The stability of the WP emulsions during a freeze/thaw cycle depended on the freezing temperature. Up to -5°C, only the oil phase partially crystallized and the WP emulsion was stable after thawing (Fig 10). As soon as the water crystallized (at temperature below -5°C), the emulsions exhibited oiling off after thawing. Water crystallization promoted the extensive coalescence of the oil droplets (Thanasukarn, Pongsawatmanit & McClements, 2004b). The emulsion freeze/thaw stability was affected

by the composition of the continuous phase (Thanasukarn, Pongsawatmanit & McClements, 2004a). By the screening of the electrostatic repulsions, which brought the oil droplets closer together, the addition of NaCl decreased the emulsion freeze/thaw stability. In contrast, the addition of sucrose enhanced the emulsion freeze/thaw stability due to its cryo-protector effect.

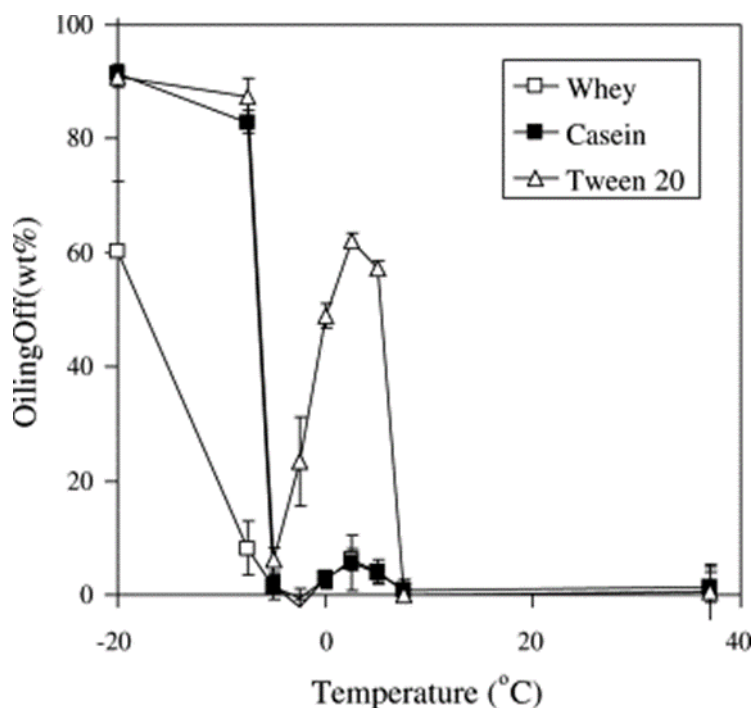


Figure 10: Free oil (wt%) accumulating on the top of 20 wt% hydrogenated palm oil-in-water emulsions after being stored at different temperatures due to oiling off. The emulsions were stabilized by: (a) 2 wt% Tween 20; (b) 1 wt% whey protein isolate; (c) 1 wt% casein. Reproduced Thanasukarn, Pongsawatmanit & McClements (2004b) with permission of Elsevier.

The partial coalescence of the fat droplets at the surface of the gas bubbles is a key step during the ice cream manufacture. The milk proteins, i.e. casein micelles or WP, form a thick and viscoelastic interfacial layer at the surface of the fat droplets limiting their partial coalescence (Goff, 1997a). Therefore, small molecular surfactants (lecithin, mono- and di-glycerides) are added to the mix in order to displace the milk proteins from the interface and facilitate the partial coalescence of the fat droplets, as well as facilitate the adsorption of the partially coalesced fat droplets at the air bubble interface (Goff, 1997b). The caseins are more easily displaced from the fat droplet surface than the WP (Wilde, Mackie, Husband, Gunning & Morris, 2004) that form cohesive interface and hinder fat droplet partial coalescence (Dalgleish, 1996). The ice cream obtained in the presence of small surfactants had a smooth texture and improved thawing stability due to a uniform distribution of small air bubbles (Barford & Krog, 1987; Goff, 1997a). A mixture of WP aggregates and caseins in replacement of native WP promoted a smaller and a more uniform air bubble size distribution in whipped-frozen emulsions in presence of surfactants (Relkin, Sourdet, Smith, Goff & Cuvelier, 2006).

### 1.3 Whey protein and casein emulsions

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As indicated in the previous sections, the solutions of WP aggregates that have been used to prepare oil-in-water emulsions often contained a protein fraction that was not included in the aggregates. This results from either the intentional mixing of different protein fractions prior emulsification or the incomplete incorporation of the proteins in the aggregates (non-aggregated WP, peptides) during their preparation or the inherent presence in the protein powder of proteins that do not participate to the formation of the WP aggregates (caseins, peptides). Indeed, WP powders prepared at the industrial scale can have variable compositions (amount and type of proteins, amounts of sugar and salt) that could affect their physicochemical and functional properties (Carl Holt et al., 1999). Depending on the method used to produce the WP powders, a variable amount of caseins could be present in the powder (Coppola, Molitor, Rankin & Lucey, 2014). For these reasons, in this section we address some results on the emulsion WP and caseins under the form of an equilibrium between individual caseins and small assemblies.

#### 1.3.1 Whey proteins and caseins competitive: adsorption for the oil droplet surface

In oil-in-water emulsions containing protein-coated, the composition and the concentration of the proteins adsorbed at the fat droplet surface depended on the composition and concentration of the proteins in the continuous phase. In emulsions prepared with a mixture of caseins and WP, the proteins were in competition with each other for the oil droplet surface (Dalgleish, 1997; Hunt & Dalgleish, 1994; Ye, 2008). At low protein concentration, the WP absorbed in preference to caseins at the oil droplet surface (Ye, 2008). In contrast, at high protein concentration i.e. above 3 wt%, the caseins adsorbed in preference to the WP (Hunt & Dalgleish, 1994; Ye, 2008). Caseins are widely used as emulsion stabilizing agent in food due to their high flexibility and surface activity (Doxastakis, 1989). They are either dispersed in solution as individual molecules or in the form of small aggregates (HadjSadok, Pitkowski, Nicolai, Benyahia & Moulai-Mostefa, 2008; Panouillé, Nicolai, Benyahia & Durand, 2005). These aggregates contain about 15 caseins (Pitkowski et al., 2008). The differential protein adsorption was attributed to different protein conformation at the oil droplet surface and different protein aggregation state in the continuous phase. The caseins can cover more interfacial area than the WP (Hunt & Dalgleish, 1994). Hence, a smaller amount of caseins was needed to stabilize the same interfacial area compared to whey proteins. The higher adsorption of WP than caseins at low protein concentration was due to their limited spreading at interface (Mackie, Miggins & Dann, 1993). At high protein concentration, the caseins adsorbed in greater proportion because they are mainly aggregated in the continuous phase of the emulsion and adsorbed in the form of casein aggregates at the fat droplet

surface (Magesh Srinivasan, Singh & Munro, 1996). An increase in the protein concentration in whey protein/casein emulsions resulted in an increase in the amount of adsorbed proteins due to the formation of a secondary protein layer at the fat droplet interface (Ye, 2008). However, Hunt & Dalgleish (1994) indicated that the WP were unable to form a secondary protein interfacial layer when the caseins were already adsorbed at the fat droplet surface because the caseins form loops and tails extending in the continuous phase. Presently, no information is available in the scientific literature on the competition between WP aggregates and caseins for the oil droplet surface.

### 1.3.2 Emulsions containing caseins *versus* a mixture of whey proteins and caseins: stability during technological treatments

The emulsions prepared at 2 wt% caseins were heat stable at 90°C or 121°C for 30 min and 15 min, respectively (Hunt & Dalgleish, 1995). Only a slight increase in the surface mean diameter of the droplet was observed for the emulsion heated at 121°C for 15min (Srinivasan, Singh & Munro, 2002). The simultaneous presence of caseins and WP conferred a synergistic stabilization to emulsions against heating (Parkinson & Dickinson, 2007). In the absence of caseins, a thin layer of WP covered the droplet surface. The thickness of the WP interface increased in the presence of caseins due to the long dangling tail ensuring a steric stabilization of the oil droplets; such emulsions had enhanced protection against heat-induced destabilization (Dickinson & Parkinson, 2004; Parkinson & Dickinson, 2004) (Fig 11).

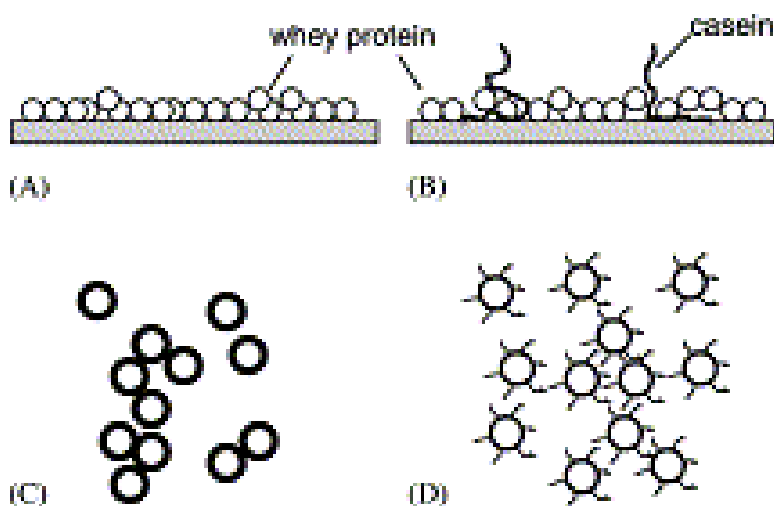


Figure 11: Schematic representation of the effect of a small addition of sodium caseinate on the colloidal structure of a heat-treated WPI-B emulsion: (A) thin layer of globular whey protein molecules adsorbed at the oil–water interface; (B) thicker layer with disordered casein molecules incorporated; (C) reversibly flocculated heat-treated WPI-B emulsion (high viscosity); (D) non-flocculated emulsion (low viscosity) sterically stabilized by low density of dangling casein tails. Reproduced from Dickinson & Parkinson (2004) with the permission of Elsevier.

The replacement of only 5 %wt of the WP content by caseins allowed eliminating the heat-induced viscosity increase in the WP emulsions. Therefore, a very small amount of caseins was sufficient to improve the heat stability of the WP emulsion.

Some authors reported that emulsions containing caseins were sensitive to a freeze/thaw cycle (Cramp, Docking, Ghosh & Coupland, 2004; Palanuwech & Coupland, 2003; Thanasukarn et al., 2004b). Thanasukarn et al. (2004b) showed a better stability during freeze/thaw cycle of the WP emulsions than the casein emulsions. Contradictory results suggested that the caseins form a thicker interfacial layer responsible for the increased emulsion freeze/thaw stability compared to the WP emulsion (Cramp et al., 2004; Palanuwech & Coupland, 2003). In the best of our knowledge, no study highlighted the impact of the replacement of a fraction of WP aggregates by caseins on the emulsion freeze/thaw stability.

## 1.4 Conclusion

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The emulsions prepared with native WP are easily destabilized during heating at high protein concentration due to a gelation mechanism. Results on WP emulsion freeze/thaw stability are rather contradictory. An analysis of the scientific literature highlights that the use of WP aggregates instead of native WP could be a solution to improve the stability of emulsions during technological treatments. WP fibrils, WPM and strand-like aggregates of WP are putative efficient aggregates for this functionality. Because of their low level of conversion, the extensive heating time required for their formation, their low solubility between pH 4 and 6.5 (pH range of most food products) and their disruption during the emulsification process, the use of fibrils for the stabilization of protein-rich emulsions during technological treatments appears complicated. The fractal WP aggregates and the WPM have been reported to form stable emulsions. However, their ability to stabilize WP-rich emulsions during technological treatments received only little attention. One study mentioned that WP microparticles-coated oil droplet flocculated during heating. The stability of WP-rich emulsions was enhanced by replacing native WP by WP partially aggregated (mixture of particles and non-aggregated WP). The concentration of non-aggregated WP limits the emulsion stability on heating but the underlying mechanism is not clearly understood. From these results, it appears that a minimum amount of non-aggregated WP is necessary to limit oil droplet flocculation but above a critical amount, the emulsion gelled. The amount of non-aggregated WP in the suspensions intended to prepare stable emulsions is probably dependent on the characteristics of the emulsions (oil fraction, oil droplet mean diameter, protein concentration). Hence, controlling the fraction of non-aggregated WP in the WP aggregate suspension for a large diversity of emulsions is difficult to imagine in an industrial context. The caseins, which can be naturally present in the WP ingredients produced at the industrial scale, improve the heat

## Chapter 1: Literature review

stability of WP emulsions. The role played by the presence of caseins on the heat stability of WP aggregate emulsions has not yet been studied. The concomitant presence of caseins and WP aggregates was shown to modify the stability of the emulsions during freeze/thaw cycle.





## Chapter 2: Aims and strategy of the PhD

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The production of fluid WP-rich emulsions that are stable during technological treatments (heating, freeze/thaw cycle) in the absence of non-dairy additives is a great challenge for dairy manufacturers. In this context, the aim of my PhD research project was to investigate the combined properties of the WP aggregates and the caseins to control the stability of the emulsions during technological treatments. The strategy has been developed into four main complementary parts including (i) the selection of WP aggregates with promising ability to stabilize oil-in-water emulsions during technological treatments based on the literature review; (ii) the characterization of their technological properties (interfacial and emulsifying properties, heat stability, freeze/thaw stability); (iii) the study of the contribution of the adsorbed proteins at the fat droplet surface and the proteins in the continuous phase on the emulsion stability; and (iv) the proposition of a mechanism of stabilization of the emulsions during technological treatments.

**The main aims of the work presented in this chapter were to:**

- Define the context of the PhD
- Highlight the aims and the research questions
- Explain the strategy of the PhD

Table of contents

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Chapter 2: Aims and strategy of the PhD.....	35
1.1 Context of the PhD .....	37
1.2 Aim, objective and strategy .....	40
1.3 Originality of the research project.....	42

## 2.1 Context of the PhD

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Among the dairy products, many fluid emulsions are oil-in-water emulsions containing WP. They are subjected to technological treatments, i.e. heat treatments and freeze/thaw cycle, in order to reduce/control the microbial load of the emulsions and to inactivate/control some enzymes that would otherwise cause quality loss. Since the emulsions are thermodynamically unstable systems, these treatments may affect their physical stability. The emulsion may lose its fluidity (become highly viscous or gelled), include pieces of gel or precipitates, or exhibit oiling off.

Some of the above mentioned quality defects are assigned to the unfolding and aggregation of the WP on heating. The impact of heating depends on the WP concentration of the emulsions and especially on the non-adsorbed protein fraction. The heat stability of the emulsion is more difficult to control in WP-rich emulsions such as in some nutritive emulsion beverages (liquid infant formula, drinks for sportsmen and the elderly). Preliminary tests on the heat stability of emulsions prepared with WP suspensions at different protein concentrations (Fig 12) confirmed published results. The heat stability of the WP emulsions decreased drastically with protein concentration. A gelation of the emulsions was observed as soon as  $\sim 4$  wt% of proteins (protein concentration in the WP suspension used to prepare the emulsion). This explains that non-dairy additives are usually used to prevent the heat instability of emulsions containing WP.

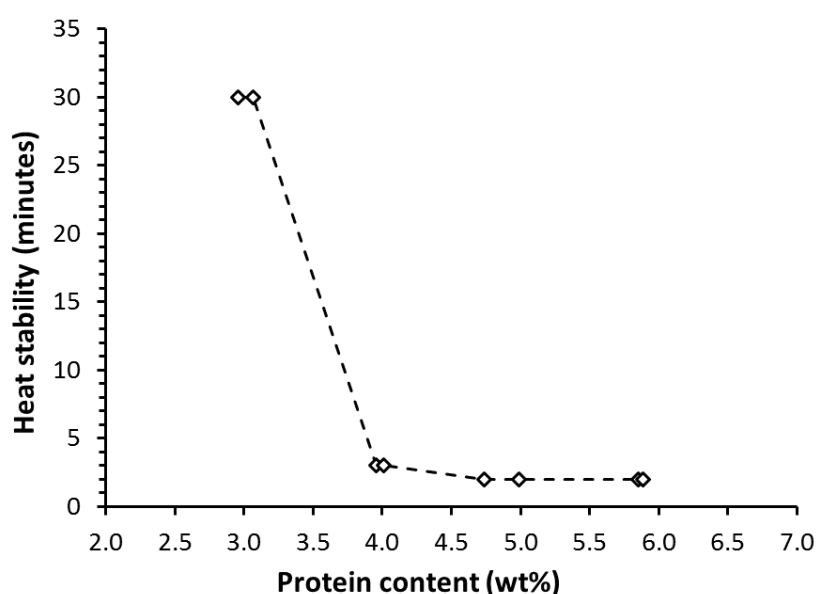


Figure 12: Heat stability at 120°C of emulsions prepared with whey protein suspensions at various protein concentrations. The dotted lines are guides to the eye.

Emulsions prepared with WP dispersed in water were unstable after storage at a temperature below  $-5^{\circ}\text{C}$ . After thawing, a phase separation (oiling off) was observed in the emulsions. The addition of sucrose improved the freeze/thaw stability of the WP emulsions. The freeze/thaw stability of WP emulsions prepared in milk permeate (soluble phase of milk, which composition include minerals and lactose) was evaluated at the beginning of the PhD project in order to check if its freeze/thaw behavior is closer to the one of the WP emulsions in water or in a solution of sucrose. Preliminary results indicated that the WP emulsions in milk permeate were extremely stable to freezing at  $-18^{\circ}\text{C}$  and subsequent thawing compare to WP emulsions in water (Fig 13). This constitutes an advantage for creams or sauces that have to be stable during a freeze/thaw cycle. However, some of these products contain heat-aggregated proteins (used in the product formulation of formed during a heat-treatment prior to the freezing stage).

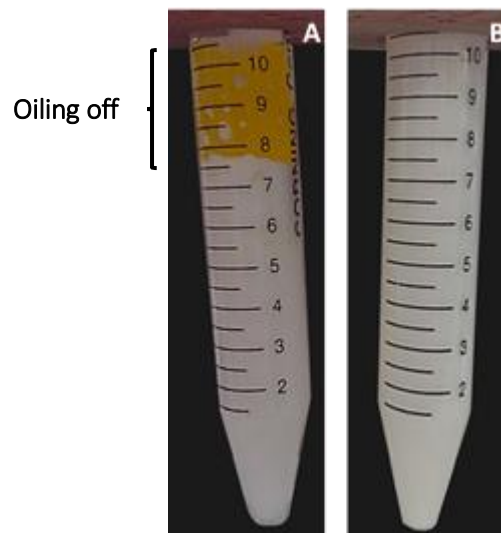


Figure 13: Picture of whey protein emulsion prepared in water (A) or in milk permeate solution (B) after a freeze/thaw cycle

To the best of our knowledge, the stability of such products to freeze/thaw cycle is unknown. For some other applications such as in ice cream manufacture, an excessive stability of the fat droplets is unfavorable. The crystallized fat droplets have to aggregate and coalesce partially when the emulsion is whipped. The WP form a viscoelastic interfacial layer limiting the partial coalescence of the fat droplets. Presently, the stability of the fat droplets is controlled by the use of small surfactants that displace the proteins from the interface at low temperature, but dairy manufacturers are looking for an alternative to satisfy the consumers.

Indeed, modern consumers look for more and more food products offering extra-benefits such as health, wellness, naturalness and pleasure. They are particularly attentive to food products containing

less negatively perceived processed ingredients and reduced amounts of additives. However, such formulations are generally physically and chemically less stable (Palzer, 2009). Proposing alternative formulations that meet the challenge of reducing the amount of additives without affecting the food product stability especially during technological treatments will offer competitive advantages to the dairy companies. Milk proteins and particularly the WP benefit of a positive perception by the consumers for their excellent nutritional properties and naturalness. The functionalities of the WP could be advantageously diversified by controlled aggregation. Based on the literature review, we suggest the WP aggregates can be incorporated as functional ingredients in the formulation of food products that are **rich in whey proteins**. These products have to be resistant to technological treatments (heating, freeze/thaw cycle) in the absence of non-dairy additives ("**clean label**").

WP aggregates exhibit promising emulsion stabilization properties. Pickering-like emulsions prepared with whey protein microgels adsorbed at the fat droplet surface inhibit their coalescence and WP fibrils dispersed in the continuous phase of the emulsion stop fat droplet creaming. Moreover, the stability of WP emulsions during technological treatments depend on the stability of both the proteins adsorbed at the droplet surface and the proteins (native or aggregated) in the continuous phase (Çakır-Fuller, 2015; Euston et al., 2000). Therefore, understanding the mechanism of stabilization of the emulsions during the technological treatments requires an increased knowledge of the contribution of the WP aggregates adsorbed at the fat droplet surface and those in the continuous phase. In addition, various WP aggregates regarding their size and morphologies could have different contributions to the emulsion stability. To clarify this structure/function relationship, the impact of the morphology and the size of the aggregates on the emulsion stability will also be studied.

In dairy emulsions, the proteins are of different nature (WP, caseins) and in different structural states (aggregated, non-aggregated). Milk proteins and silica or cellulose particles exhibit synergistic effect on emulsion stability (Murray et al., 2011; Pichot et al., 2010). Despite interesting surface stabilization properties in unheated emulsions, the presence of the whey protein microgels at the fat droplet surface could induce the flocculation of the fat droplets on heating (Sarkar et al., 2016). One way to get round this obstacle would be the selective replacement of the whey protein microgels at the fat droplet surface by other proteins such as the caseins. In fact, the caseins adsorbed at the droplet surface enhance the stability of the WP emulsions during heating (Dickinson & Parkinson, 2004; Parkinson & Dickinson, 2004), and during a freeze/thaw cycle. The use of caseins instead of non-dairy additives would allow the formulation of 100% dairy emulsions. Moreover, some WP powders naturally produced at the industrial scale contain traces of caseins (Coppola et al., 2014). Caseins preferentially adsorbed at the droplet surface compared to the WP especially at high protein concentration (Hunt & Dalgleish,

1994; Ye, 2008). Therefore, if the WP aggregates adsorbed at the fat droplet surface are responsible for the emulsions destabilization during the technological treatments, we hypothesize the emulsion stability could be enhanced by:

- Covering the fat droplet surface by a minimal amount of caseins
- Dispersing the WP aggregates mainly in the continuous phase.

## 2.2 Aim, objective and strategy

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This work was conducted in collaboration with the main French dairy companies. One recommendation was to develop model emulsions as close as possible to real dairy emulsions. The emulsions were prepared at pH 7 with anhydrous milk fat as dispersed phase and WP suspensions in the soluble phase of milk (milk permeate) as continuous phase. For this reason, we will talk about fat droplets in the rest of the manuscript. The technological objective of my PhD is to propose solutions able to prevent the irreversible emulsion destabilization during technological treatments. The emulsion destabilization was investigated at macroscopic and microscopic scales. Visual changes, such as the gelation, the loss of fluidity of the emulsions or the two immiscible phase separation (oiling off), constitutes the main criteria of the emulsion destabilization at the macroscopic scale. In the rest of the manuscript, **stable emulsions refer to emulsions after technological treatments (heating or freeze/thaw cycle) that do not display the above-mentioned physical destabilization**. It is important to specify that a creaming leading to a vertical fat droplet gradient could be considered as a macroscopic destabilization. In a first attempt, we did not use this criterion to discriminate stable and unstable emulsions since this change is reversible after agitation and was inherent to the structure of our emulsions (see after). The emulsion destabilization at microscopic scale was referred to the flocculation and coalescence of the fat droplets. These changes were deduced from light scattering measurements and confocal laser scanning microscopy (CLSM) observations. For characterizing the emulsions by CLSM, the fat droplet diameter should not be too small. For this reason, the targeted mean fat droplet diameter in the emulsions was fixed at 1.5 - 2  $\mu\text{m}$  and explains that some emulsions creamed especially if the fat droplets were flocculated or coalesced. We decided to prepare model emulsions at 30 % (v/v) fat in order to have a total fat droplet surface area, which is in the range of the interfacial area encountered in commercial dairy emulsions. In the model emulsions, the total fat droplet surface area is around 1000  $\text{m}^2/\text{L}$ , whereas it is generally between around 100  $\text{m}^2$  and 2000  $\text{m}^2$  per liter in commercial dairy emulsions. The average distance between the fat droplets in the model emulsions is close to 1  $\mu\text{m}$ . The mechanism responsible for the emulsion destabilization was also investigated in regard of the composition (by SDS-PAGE) and the structure of the proteins at the fat droplet surface and in the continuous phase (observations by TEM). The final

objective of my PhD thesis is to suggest **new ways for improving the stability of fluid emulsions during technological treatments without using additives**. A special attempt is on **emulsions rich in whey proteins** due to the technological challenges and the economic relevance regarding these products.

In order to achieve the objective, several scientific questions have to be answered:

- What is the protein stability of the aggregates in the continuous phase of the emulsions during the technological treatments?
- Do the whey protein aggregates adsorbed at the fat droplet surface influence the emulsion stability during the technological treatments?
- Does the morphology of the whey protein aggregates affect the stability during the technological treatments of dairy emulsions?
- What is the combined effect of the whey protein aggregates and caseins on the stability of dairy emulsions?

The strategy adopted to tackle these issues was separated in three parts (Fig 14):

- 1) Aggregates with different morphologies and sizes were studied and those with the most promising abilities to stabilize oil-in-water emulsions were selected. The selected aggregate suspensions were characterized. The functional properties of the aggregate suspensions were evaluated (heat stability, freeze/thaw stability, interfacial and emulsifying properties). The heat stability and the freeze/thaw stability of the aggregate suspensions will help to understand the heat stability and freeze/thaw stability of the continuous phase of the emulsions (**Chapter 5**).
- 2) To determine the impact of the adsorbed WP aggregates at the fat droplet surface and the importance of the composition of the WP aggregate suspension, the stability during technological treatments of the emulsions prepared with either the WP aggregates or a mixture of WP aggregates and caseins were compared. The comparison was performed at different scales (from the characterization of the fat droplet surface to the visual aspect of the emulsion) (**Chapter 6**).
- 3) In order to propose a general mechanism for the stabilization of the emulsions during technological treatments, a second set of experiments was conducted with emulsions prepared with a second kind of aggregate and with a mixture of aggregates (**Chapter 7**).

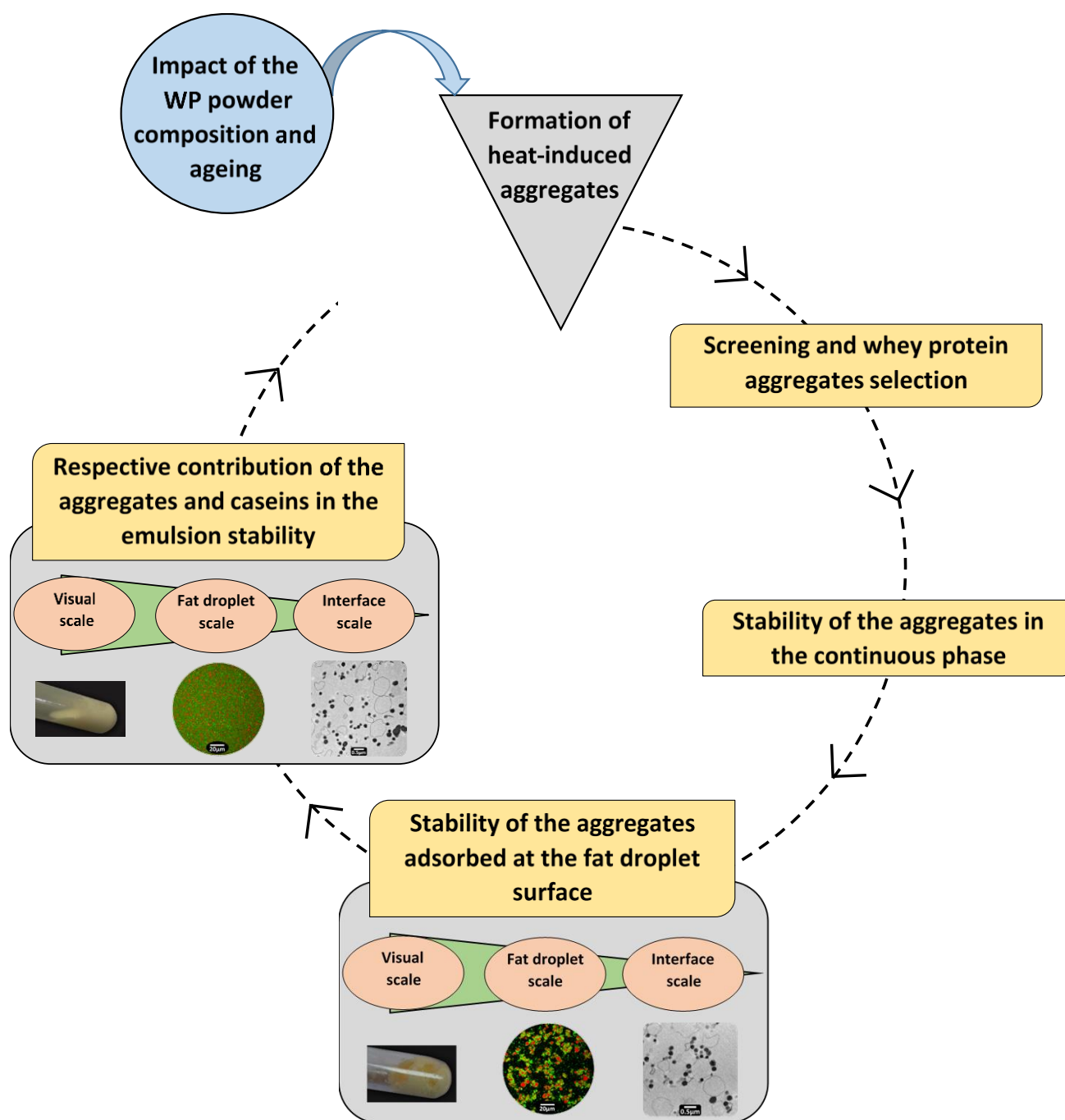


Figure 14: Scheme representing the thesis strategy

## 2.3 Originality of the research project

Previous research studies were conducted on the stability of the WP emulsions and sodium caseinate emulsions during technological treatments. In contrast, only few studies have been conducted on the emulsifying properties of the WP aggregates and on their ability to stabilize emulsions over time.



Moreover, valorizing the combined effects of the WP aggregates and a small amount of caseins constitutes a new approach for the stabilization of the WP-rich emulsions during technological treatments.

In order to obtain results easily transposable to the industry, this work involved the use of industrial raw materials (WP powder, sodium caseinate powder, milk ultrafiltrate permeate, anhydrous milk fat). Such ingredients are much more complex than pure protein, oil or salt, adding a level of complexity and an originality to this study.



## CHAPTER 3: Materials and methods

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## Table of contents

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<b>CHAPTER 3: Materials and methods .....</b>	<b>45</b>
<b>3.1 Materials .....</b>	<b>47</b>
<b>3.2 Protein suspensions and emulsion preparation .....</b>	<b>48</b>
3.2.1 Whey protein, sodium caseinate and micellar casein suspensions .....	48
3.2.2 Strand-like aggregate suspensions.....	48
3.2.3 Whey protein microgel suspensions.....	49
3.2.4 Preparation of protein-coated emulsions .....	50
<b>3.3 Stability of protein-coated emulsions and protein suspensions during technological treatments.....</b>	<b>50</b>
3.3.1 Heat stability .....	50
3.3.2 Freeze/thaw stability .....	50
<b>3.4 Protein particle characterization .....</b>	<b>51</b>
3.4.1 Size distribution of the protein particles.....	51
3.4.2 Gyration radius and molar mass of protein particles.....	51
3.4.3 $\xi$ -potential of the protein particles.....	52
3.4.4 Morphology of the protein particles.....	52
3.4.5 Composition of the strand-like aggregate suspensions and the whey protein microgel suspensions.....	52
3.4.6 Interfacial properties of the protein particles.....	54
<b>3.5 Emulsion characterization .....</b>	<b>54</b>
3.5.1 Fat droplet size distribution .....	54
3.5.2 Microstructure .....	54
3.5.3 Quantification and composition of the proteins adsorbed at fat droplet surface .....	57

### 3.1 Materials

Whey protein (WP) powders, sodium caseinate (CasNa) powder, micellar casein (MC) powder and milk permeate powder were provided by dairy companies (confidential origin). Anhydrous milk fat (melting point 32°C) was from Corman (Corman, Limbourg, Belgium).

Two different WP powders (A and B) were used. The detail of their compositions is shown in Table 2. The total protein content, casein content and mineral composition were determined by Kjeldahl method, by SDS-PAGE under reducing conditions (see below, section 3.4.5.2) and by atomic absorption spectrometry (Varian 220FS spectrometer, France), respectively. The WP powder A contained mainly  $\alpha_s$ -caseins and  $\kappa$ -casein (identified by mass spectrometry). CasNa powders contained 87.7 wt% of proteins.

Table 2: Powders composition

	<i>Whey protein powder A</i>	<i>Whey protein powder B</i>	<i>Sodium caseinate powder</i>	<i>Micellar casein powder</i>	<i>Milk permeate powder</i>
<i>Protein (w/w %)</i>	88.8	88.8	87.6	77.8	-
<i>Casein/protein (w/w %)</i>	18.41	6.49	99.2	92.9	-
<i>Ca<sup>2+</sup> (w/w %)</i>	0.31	0.17	-	-	0.31
<i>Mg<sup>2+</sup> (w/w %)</i>	0.02	0.02	-	-	0.12
<i>Na<sup>+</sup> (w/w %)</i>	0.09	0.15	-	-	0.63
<i>K<sup>+</sup> (w/w %)</i>	0.16	0.11	-	-	2.74

Due to different casein contents in WP powder A and WP powder B, the mean extinction coefficient of the proteins at 280 nm ( $\epsilon_{280\text{nm}}$ ) was determined for each powder by the slope of a calibration curve. A  $\epsilon_{280\text{nm}}$  of 1.14 L/g/cm and 1.13 L/g/cm was obtained for the proteins in the WP powder A and the WP powder B, respectively.

Table 2 also provides the detailed mineral composition (determined by atomic absorption spectrometry) of the WP powders and of the milk permeate powder.

## 3.2 Protein suspensions and emulsion preparation

### 3.2.1 Whey protein, sodium caseinate and micellar casein suspensions

WP suspensions, CasNa suspensions and MC suspensions were prepared by dispersing WP powder A or B, CasNa powder and MC powder at different concentrations (wt%) in a milk permeate solution prepared at 5.6 wt% in ultra-pure water. This dispersing phase had a composition similar to that of the dispersing phase of milk. The pH of the WP, CasNa and MC suspensions was adjusted to 7 by adding NaOH at 1 M. Sodium azide (0.05 % w/v; Sigma-Aldrich, USA) was then added to the protein suspensions to prevent bacterial growth. The suspensions were called WP A suspension (for powder A), WPB suspension (for powder B), CasNa suspension and MC suspension.

### 3.2.2 Strand-like aggregate suspensions

Strand-like aggregates (SA) were prepared according to a protocol adapted from (Mahmoudi et al., 2010). A suspension was prepared by dispersing the WP powder A at 4 wt% protein in ultra-pure water. The mixture was gently stirred at room temperature for at least 4 h. Then, the pH of the suspension was set at 7 by adding NaOH at 1 M. The protein suspension was preheated at 40°C in a water bath and then heated to 80°C in less than 1 min with a home-made tubular exchanger immersed in a water bath set at 95°C. Subsequently the suspension was maintained at this resting temperature for 15 h in order to form the aggregates (SA). The SA suspension was concentrated twice by ultrafiltration using a 10kDa spiral organic membrane (Helicon, Millipore, France) connected to a Millipore filtration system (PROLAB Millipore MSP 006239, Millipore, France). The concentrated SA suspension was diluted at various protein concentrations between 2.6 and 6.6 wt% using milk permeate solutions, which concentration was chosen to obtain after dilution protein suspensions in the soluble phase of milk. The pH of the SA suspensions was adjusted to 7 by adding NaOH at 1 M. Sodium azide (0.05 % w/v) was then added to protein suspensions to prevent bacterial growth. This suspension was called SA<sub>4</sub> suspension.

Two types of large SA aggregates (fractal aggregates) were prepared with whey protein powder A. WP powder A was dissolved at 9 wt% proteins in ultra-pure water for the first aggregate and at 4 wt% in ultra-pure water containing 40 mM of NaCl for the second one. The pH of the suspensions was adjusted to 7. Exactly the same protocol for heat treatment and dilution was applied as for the SA<sub>4</sub> suspension. The obtained suspensions were called SA<sub>9</sub> and SA<sub>Na</sub> suspensions, respectively.

When necessary, concentrated suspensions of CasNa were added to SA suspensions in order to adjust the casein/whey protein weight ratio.

### 3.2.3 Whey protein microgel suspensions

Whey protein microgels (WPM) were produced according to Bovetto, Schmitt, Beaulieu, Carlier & Unterhaslberger (2007). WP powder A was dissolved at 4 wt% proteins in ultra-pure water. The suspension was adjusted to pH 5.8 by adding HCl at 1 M and was gently stirred at room temperature for at least 4 h. The protein suspension was preheated at 40°C and then heated up to 80°C in less than 1 min with a home-made tubular exchanger immersed in a water bath set at 95°C. Then the suspension was maintained at 80°C for 1 h.

The WPM suspension was concentrated twice by ultrafiltration with a Millipore filtration system (PROLAB Millipore MSP 006239, Millipore, France) using a 10 kDa spiral organic membrane (Helicon, Millipore, France). A range of WPM suspensions was prepared by diluting the concentrated protein suspension with concentrated milk permeate solutions. The concentration of the milk permeate solutions was chosen to obtain WPM suspensions after dilution with a soluble phase similar to the soluble phase of milk. The protein concentration in the diluted WPM suspensions was between 2.7 and 6.5 wt%. The pH of the WPM suspensions was adjusted to 7 by adding NaOH at 1 M. Sodium azide (0.05 % w/v) was then added to protein suspensions to avoid bacterial growth. The obtained suspensions were called WPM A suspensions.

After heating, the WPM can be recovered by centrifugation at 27 000 *g* for 15 min at 20°C using a Avanti J-26S XP centrifuge (Bekman Coulter, USA) and the pellet was redispersed with ultra-pure water and centrifuged twice (Donato et al., 2009). Then, the washed pellet was dispersed at around 3 or 6 wt% in a permeate solution at 5.6 wt% and the suspension was adjusted at pH 7 by adding NaOH at 1 M. These suspensions were called WPM AC suspensions. Sodium azide (0.05 % w/v) was added to the WPM suspensions to avoid bacterial growth.

A second type of WPM was prepared with WP powder B. WP powder B was dissolved at 4 wt% proteins in ultra-pure water and was adjusted to pH 6.6. Exactly the same protocol for heat treatment and concentration was applied as for the WPM A suspensions. The obtained suspensions were called WPM/SA B suspensions.

In some preparations, concentrated suspensions of CasNa were added to WPM A, WPM AC and WPM/SA B suspensions in order to vary the casein/whey protein weight ratio.

### 3.2.4 Preparation of protein-coated emulsions

Oil-in-water emulsions were prepared with 30 % (v/v) anhydrous milk fat and 70 % (v/v) of aqueous phase containing various amounts of proteins. To avoid the formation of fat crystals, the anhydrous milk fat and protein suspensions were heated at 60°C and then mixed together. The mixture was pre-emulsified with a rotor stator homogenizer (Heidolph Silent Crusher M, Schwabach, Germany) set at 18000 rpm for 5 min. Subsequently, the coarse emulsion was passed through a homogenizer (Stansted, Harlow, Essex, UK) at a pressure of 5 MPa (5 passes). In the result section, the protein concentrations indicated in the text and in the figures referred to the protein concentration of the suspensions used to prepare the emulsions (protein concentration in the continuous phase of the emulsions).

## 3.3 Stability of protein-coated emulsions and protein suspensions during technological treatments

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### 3.3.1 Heat stability

In order to measure the heat stability of the emulsions, a series of 3 glass tubes per emulsion was filled with 2 mL of emulsion. The first tube was used for particle size measurements. In the second tube, 20 µL of Nile Red (0.125 % in propylene glycol) were added to the emulsion to clearly visualize fat release (oiling off). Volumes of 20 µL of Nile Red and 10 µL of Fast Green (1 % in water) were added into the third tube that was used for confocal laser scanning microscopy (CLSM) analysis. The glass tubes were immersed in an oil bath set at 120°C (Huber, Germany) and they were respectively heated for 1, 2, 3, 5, 10, 20 and 30 min. Immediately after heating, the tubes were cooled and a visual analysis was performed to detect any traces of heat-induced modifications (thickening, gelation, fat release).

Exactly the same protocol was performed for measuring the heat stability of the protein suspensions. However only 2 glass tubes were prepared per suspension. The first glass tube was filled with the protein suspension only and 10 µL of Fast Green (1 % in water) was added to the second tube for CLSM analysis.

### 3.3.2 Freeze/thaw stability

The freeze/thaw stability of the emulsions and protein suspensions was also analysed. For that purpose, 10 mL of each emulsion and 10 mL of each protein suspension were placed in a tube, which was kept at -18°C for at least 1 week. Samples were thawed in an oven set at 50°C for 2 h and were analyzed visually to detect any trace of freeze/thaw-induced modifications (thickening, gelation, fat release).



### 3.4 Protein particle characterization

#### 3.4.1 Size distribution of the protein particles

A Zetasizer Nano ZS (Malvern Instrument, Worcestershire, UK) was used to determine the size (hydrodynamic diameter) and the size distribution of the particles in protein suspensions before and after technological treatments by using dynamic light scattering (DLS). To avoid multiple particle effects, protein suspensions were diluted 10 times for WP A, WPB and SA suspensions and 100 times for WPM suspension with milk permeate solution at 5.6 wt%.

The diluted protein suspensions were placed in a plastic cell and analyzed at 25°C in a backscattering configuration at 173° for 120 s. Each sample was analyzed in triplicate. The general purpose model (non-negative least-square model) was used to analyze experimental data and the results corresponded to the mean intensity of 10 runs. The diffusion coefficient ( $D_t$ ) was extracted from the fit of the correlation curve and was used to calculate the Z-average hydrodynamic diameter of the particles ( $D_h$ ) with the Stokes- Einstein equation:

$$D_h = \frac{K_B T}{3\pi\eta D_t}$$

where  $K_B$  is the Boltzmann's constant,  $T$  is the temperature and  $\eta$  is the solvent viscosity.

A refractive index of 1.45 was used for protein particles (Zhang, Arrighi, Campbell, Lonchamp & Euston, 2016) and 1.34 for the solvent (milk ultrafiltration permeate) in order to calculate the volume-size representation.

#### 3.4.2 Gyration radius and molar mass of protein particles

For each suspension, the protein aggregates were separated on an Asymmetric Flow Field-Flow Fractionation (A4F) module connected to a short channel Eclipse Dualtec (Wyatt Technology Corporation, Santa Barbara, USA) with a trapezoidal geometry and a tip-to-tip length of 145 mm. To introduce the sample into the channel, an autosampler Infinity HP 1260 Bio-Inert with a cooling module (Agilent technologies, Santa Clara, USA) was used. The channel was coupled to a pump, Infinity HP 1260 Bio-Inert (Agilent technologies, Santa Clara, USA), controlled by software OpenLab CDS (Agilent technologies, Santa Clara, USA). A regenerated cellulose membrane with a cut-off of 10 000 Da (Wyatt Technology Corporation Santa Barbara, USA) and a 350  $\mu$ m polyester spacer were used. The three online detectors were coupled to the channel, a MALLS instrument with 18 angles Dawn® HELEOS™ (Wyatt, Santa Barbara, USA), a refractometer 1260 RID (Agilent technologies, Santa Clara, USA) and a multiple

wavelength detector MWD RL (Agilent technologies, Santa Clara, USA). The ASTRA software (Wyatt Technology Corporation, Santa Barbara, USA) were used for the data acquisition and processing. The Berry extrapolation was used with a second order polynomial fit and a refractive index increment ( $dn/dc$ ) of 0.1851 mL/g. These extrapolations allowed to calculate the molar mass (MM) and the gyration radius ( $R_g$ ) of the particle directed towards the detectors. Ultra-pure water with 0.02 w/v% sodium azide, 45 mM NaCl at pH 7 and filtered through a 0.1  $\mu$ m Durapore membrane (Millipore, France) was used as a liquid carrier.

The samples (40 g/L) were diluted 100 times with the liquid carrier. The samples were introduced into the channel and were fractionated according to this method in 102 min. A crossflow rate of 2 mL/min was applied for the focusing step, followed by the introduction of the sample (62.5  $\mu$ L) at 0.20 mL/min and the elution at 0.5 mL/min. Then, the cross flow was gradually reduced from 2 to 0 mL/min.

### 3.4.3 $\xi$ -potential of the protein particles

The  $\xi$ -potential of the WP suspensions, SA suspensions and WPM suspensions diluted 10 times in a milk permeate solution at 5.6 wt% was measured in triplicate using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). After 2 min of equilibration, measurements were performed at an applied voltage of 50 V. The  $\xi$ -potential was calculated using the Smoluchowski model.

### 3.4.4 Morphology of the protein particles

The morphology of the protein particles (SA<sub>4</sub> and WPM) was observed by transmission electron microscopy (TEM). The sample was diluted 100 times and a volume of 15  $\mu$ L was deposited on a carbon grid. The sample was then stained using a 2 % uranyl acetate solution during 2 min. After two milli-Q water rinsing, the sample was dried for 20 min at 50°C. The sample was observed using a transmission electron microscope JEOL JEM-1230 (JEOL, Japon) operating at 80 kV.

### 3.4.5 Composition of the strand-like aggregate suspensions and the whey protein microgel suspensions

The SA suspension contained SA, caseins and residual native WP. The residual native WP were quantified by gel permeation chromatography (GPC) after precipitation of the caseins and the non-native WP at pH 4.6 (see below). The amount of caseins was determined by SDS-PAGE under non-reducing conditions (see below).

According to Donato et al. (2009), WPM suspensions included WPM, SA, caseins and residual native WP. The proportion of WPM in the WPM suspensions is defined as the difference between the total protein content and the protein content in the supernatant after centrifugation at 27 000 *g* for 15 min at 20°C (Schmitt et al., 2011). The centrifugation was performed using a Avanti J-26S XP centrifuge (Bekman Coulter, USA). For protein quantification, WPM suspensions and supernatants were diluted 100 times and at 20 times, respectively, with denaturing buffer (8 M urea, 0.05 M trisodium citrate, 5 g/L SDS, 0.2 M Tris, 10 mM DiThioTreitol). The mixture was left at 37°C for 1 h. Proteins were quantified by UV Adsorption (UVmc<sup>2</sup>, Safas, France) at 280nm. Residual native WP were quantified by gel permeation chromatography (GPC) after precipitation of the caseins and the non-native WP at pH 4.6 (see below). The amount of caseins was determined by a SDS-PAGE under non-reducing conditions (see below). The proportion of SA in the WPM suspensions was deduced from the total protein content and the amount of WPM, caseins and residual native WP.

#### 3.4.5.1 Gel filtration

A Yarra SEC 3000 column (Phenomenex, Le Pecq, France) connected to high performance liquid chromatography (HPLC) was used. This latter contained a Water 2695 separation system, a Waters 2489 double wavelength detector and Empower (Milford, USA) acquisition as well as some data processing software. The elution flow was 0.8 mL/min using a 0.05 M phosphate buffer with 0.1 M NaCl at pH 7. Proteins were detected at 214 nm.

#### 3.4.5.2 SDS-PAGE

Protein suspensions diluted at 1 g/L with the denaturing buffer were analysed under non-reducing and reducing conditions (*For non-reducing condition*: 0.5 M Tris-HCl pH 6.8, 20 % glycerol, 2 % SDS, containing 0.5% bromophenol blue; *For reducing condition*: 0.5 M Tris-HCl pH 6.8, 20 % glycerol, 2 % SDS, containing 0.5% bromophenol blue, 6 M urea, 0.15 M DiThioTreitol). After 1 h of incubation at 37°C, 10 µL of each diluted protein suspension was loaded in the sample well (equal amounts of proteins in each well, 10 µg) of a precast 4 - 20 % polyacrylamide gel electrophoresis (mini-protein TGX precast gel, Bio-Rad Laboratories, Dublin, Ireland). Electrophoresis was carried out at a constant current (200 V) for 45 min. Gels were rinsed with distilled water and were stained with Bio-safe Coomassie G250 (Bio-Rad Laboratories, Dublin, Ireland) for at least 60 min. A MW marker (kit Precision Plus Protein Standards 10 – 250 kD, Bio-Rad Laboratories, Dublin, Ireland) was employed for molecular weight (MW) calibration. The gels were scanned by Image Scan II (Amersham, Bioscience). The amount of caseins in each sample was quantified using a casein titration calibration curve using the densitometry software (Image Quant TL 1D, Amersham, Bioscience).

### 3.4.6 Interfacial properties of the protein particles

The compression isotherms (surface pressure *versus* area) were determined for the WP (WP) and for the aggregates (SA, WPM) using a two-symmetrical barrier Langmuir trough (Micro Through XL Kibron, Helsinki, Finland) with a surface area of 22007 mm<sup>2</sup>. The barriers were composed of Teflon and the trough of stainless steel with PTFE coating. The trough was filled with approximately 60 mL of ultra-pure water at pH 7. A small amount of proteins was spread at the air–water interface in the shape of small droplets thanks to a microsyringe. The amount of proteins spread for WP, SA and WPM was 10 µg, 7 µg and 40 µg, respectively. The equilibrium was reached after 30 min, and then, the sample was compressed at a constant rate of 10 mm/min until a maximum pressure of 25 mN was reached. The experiment was conducted at 20°C.

## 3.5 Emulsion characterization

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### 3.5.1 Fat droplet size distribution

The size (volume mean diameter  $d_{4,3}$ ) and the size distribution of the fat droplets in visually stable emulsions (before and after technological processing) was measured by laser light scattering (LLS) using a Mastersizer 2000 (Malvern Instruments, Worcestershire, UK) with two laser sources. Emulsions were dispersed in the machine cell containing 100 mL ultra-pure water with the aim of reaching 8 % obscuration. If necessary, the samples were diluted in 1 % sodium dodecyl sulfate (SDS) solution in order to separate flocculated fat droplets. For fat droplets, the refractive indexes used were 1.458 and 1.460 at 633 and 466 nm, respectively and for the dispersing phase it was set at 1.33. All analyses were performed in triplicate and the fat droplet size was determined by the volume mean diameter ( $d_{4,3} = \sum n_i d_i^4 / \sum n_i d_i^3$ , where  $n_i$  is the number of particles with diameter  $d_i$ ).

### 3.5.2 Microstructure

#### 3.5.2.1 Confocal laser scanning microscopy

A NIKON Eclipse-TE2000-C1si (NIKON, Champigny sur Marne, France) confocal laser scanning microscope (CLSM) was used to characterize emulsion microstructure. For unheated and freeze/thaw emulsions, 10 µL of Nile Red and 5 µL of Fast Green were added to 1 mL of emulsion to stain the fat globules and the proteins respectively, and the samples were mixed gently. For heated emulsions, only the samples containing both fluorescent probes were analyzed (see section 3.3.1). The emulsions were covered with aluminum foil in order to protect the fluorescent probes until sample analysis. They were kept at  $19 \pm 1^\circ\text{C}$  for at least 15 min before analysis, and then 50 µL of each emulsion were placed on a glass slide. Observations were conducted at excitation wavelengths of 633 nm for Fast Green dye and

543 nm for Nile Red dye. Observations were performed at  $19 \pm 1^\circ\text{C}$  using a  $\times 100$  oil immersion objective. Two dimensional images were acquired for each sample in  $512 \times 512$  pixel slices.

The images obtained in CLSM were analyzed according to a procedure modified from Silva, Legland, Cauty, Kolotuev & Floury (2015). A white top-hat filter was applied to improve the fat droplet contrast before smoothing the image in order to remove acquisition noise. Then, images were processed with an Otsu threshold method to get a binary image and LUT (look up table) inversion was made to convert fat droplets as white (255) and the aqueous phase as black (0). The inter-fat droplet distances (distance between the surface of the fat droplets and its closest neighbor) were obtained from the reconstructed image. This analyse allows to observe the repartition of the fat droplets in the emulsions. Indeed, if the fat droplets are distributed homogeneously in the emulsion, the distribution of the inter-fat droplet distance will be narrow. On the contrary, a larger distribution will indicate an inhomogeneous repartition of the fat droplets in the emulsion (Fig 15A and 15B). This method is complementary to the granulometry. Indeed, to use the laser light scattering method, the emulsion is necessarily diluted in water and stirred. These steps constitute limitations of the method because they can disrupt the weak bonds between fat droplets. Fiji software was used to perform the image analysis (Schindelin et al., 2012). Specific plugins as MorphoLibj (Arganda-Carreras et al, 2016) and Granulometry (Legland, Devaux, Bouchet, Guillon & Lahaye, 2012) were required to quantify the morphological features. These plugins were downloaded at [http://www.pfl-cepia.inra.fr/index.php?page=morpholibj\\_en](http://www.pfl-cepia.inra.fr/index.php?page=morpholibj_en), [http://www.pfl-cepia.inra.fr/index.php?page=ijGranulometry\\_en](http://www.pfl-cepia.inra.fr/index.php?page=ijGranulometry_en), respectively.

The data acquired were processed with the free software R (R Core Team, 2016). The emulsion microstructure before and after heat treatment (after a heating time of 30 min for heat-stable emulsions or the heating time just greater than that required to destabilize emulsions in other cases) was monitored by calculating the point by point difference of the area under the curve (AUC) of the inter-particle distance distribution between the non-heated and heated emulsions. The curve obtained (Dif AUC) is an indication of the modification of the microstructure of the heated emulsion; a negative value at a specific distance between two fat droplets indicates a decrease in the proportion of fat droplets having this specific inter-fat droplet distance after heat treatment and inversely a positive value refers to an increase in the proportion of this inter-fat droplets distance in the heated emulsion (Fig 15C).

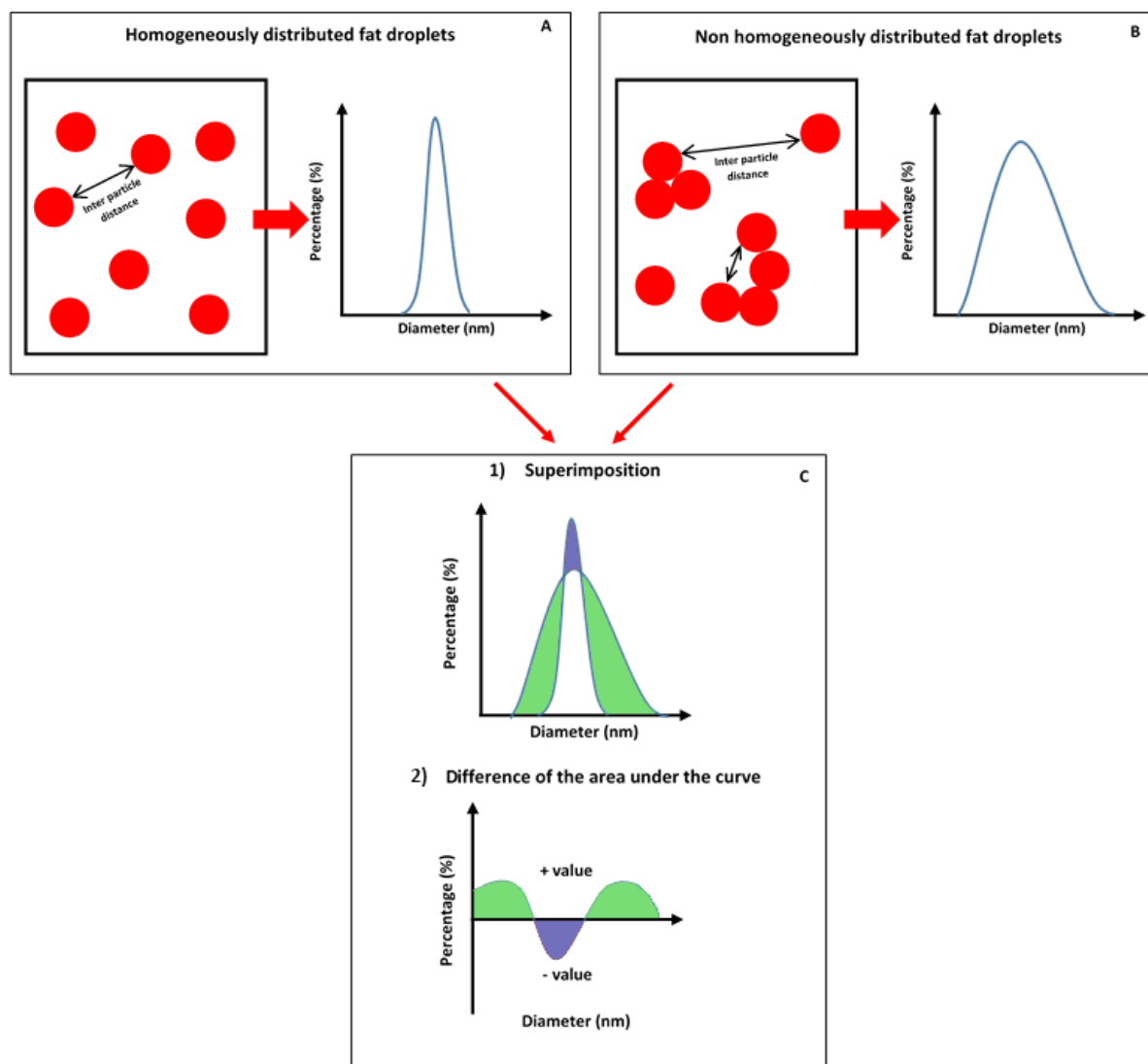


Figure 15: Schema of the image analysis

### 3.5.2.2 Transmission electron microscopy

The microstructure of the emulsions was also observed by transmission electron microscopy (TEM). A small fraction of the emulsion was placed into a liquid solution of agar 1.5 % (w/v) at 40°C and the sample was cooled for agar gelation. Then, a part of the agar gel trapping the emulsion was cut and placed for fixation into 1.5 mL of 3 % glutaraldehyde in 0.1 M phosphate buffer at pH 7.2 for 4 h at 4°C. The sample was rinsed in one bath of phosphate buffer at pH 7.2 and then with distilled water. Then, it was dehydrated with successive baths containing increasing concentrations of ethanol (30, 50, 70, 85, 95 and 100 % v/v). The impregnation was first made in propylene oxide (1 bath 50 % ethanol/50 % propylene oxide and 1 bath 100 % propylene oxide) and secondly in epon resin (1 bath 50 % propylene oxide/50 % epon, and 3 baths 100 % epon). Finally, the sample was included in epon and the resin was

left 3 days at 55°C for polymerization. An ultramicrotome Leica EM UC7 (Leica Microsystems, Germany) equipped with a diamond knife was used to prepare ultrathin sections (80 nm) of the resin containing the sample inclusion. These sections placed on a TEM grid, were negatively stained with 4 % (w/v) uranyl acetate and were observed using a JEOL JEM 1400 transmission electron microscope (JEOL, Japon) operating at 120 kV. Images were recorded on camera Gatan Orius SC 1000 (Digital Micrograph software) at Microscopy Rennes Imaging Center platform (MRic), situated at University Rennes 1, France.

### 3.5.3 Quantification and composition of the proteins adsorbed at fat droplet surface

#### 3.5.3.2 Protein quantification

Proteins were recovered from the fat droplet surface according to the method of Patton & Huston (1986) modified by Surel et al. (2014). Briefly, a mixture of 2 mL of emulsion and 2 mL of 50 % (w/v) sucrose solution was placed using a syringe at the bottom of a 15 mL centrifuge tube containing 10 mL of 5 % (w/v) sucrose solution. Tubes were centrifuged at 3 000 *g* for 2 h, 10°C, in order to separate the fat droplets (and adsorbed proteins) on the top, the sucrose separating solution in the middle and the continuous phase (with unabsorbed proteins) at the bottom of the centrifuge tube. Immediately after centrifugation, tubes were frozen at -18°C and then meticulously sawed at the limits of the upper and middle phase, and the middle and bottom phase in order to recover proteins in the three fractions. Cream phase (with fat droplet) was washed by SDS and was centrifuged at 3 000 *g* for 1 h, 10°C, in order to separate the fat in the upper phase and adsorbed proteins in the lower phase.

The lower phase (containing proteins adsorbed at the fat droplet surface only) were diluted at least 5 times with denaturing buffer (8 M urea, 0.05 M trisodium citrate, 5 g/L SDS, 0.2 M Tris, 10 mM DiThioTreitol), and the mixture was left at 37°C for 1 h. Proteins were quantified by UV Adsorption (UVmc<sup>2</sup>, Safas, France) at 280 nm.

The surface protein load (mg/m<sup>2</sup>) was determined from the amounts of protein adsorbed at the interface of fat droplets and diameter of the fat droplets. The diameter of the fat droplets was determined in the cream phase separated by centrifugation before protein recovery by laser light scattering (section 3.5.1). Conventionally, the surface mean diameter ( $d_{3,2}$ ) is used to calculate the surface protein load. However, for some emulsions a small peak assumed to correspond to WP aggregates was observed in addition to the fat droplet peak. It shifted the  $d_{3,2}$  to lower fat droplet diameter and give aberrant results. In the emulsions without this small peak, the mode and the  $d_{3,2}$  of the fat droplet distribution were very close. Therefore, for all emulsions the mode of the largest peak

was used to calculate the surface protein load. Surface protein load was quantified for emulsions that were visually stable (heat stability on macroscopic scale).

### 3.5.3.3 Protein composition

The proteins adsorbed onto the fat droplet surface of the emulsions were identified and quantified by SDS-PAGE (see above, part 3.4.5.2). The amounts of caseins in each sample were quantified by SDS-PAGE using a casein titration calibration curve with the densitometry software (Image Quant TL 1D, Amersham, Bioscience). The surface WP load was deduced from the results obtained for the caseins and the total amounts of proteins in each well.

Figure 16 displays an overview of the different analysis performed on the emulsions and protein suspensions.

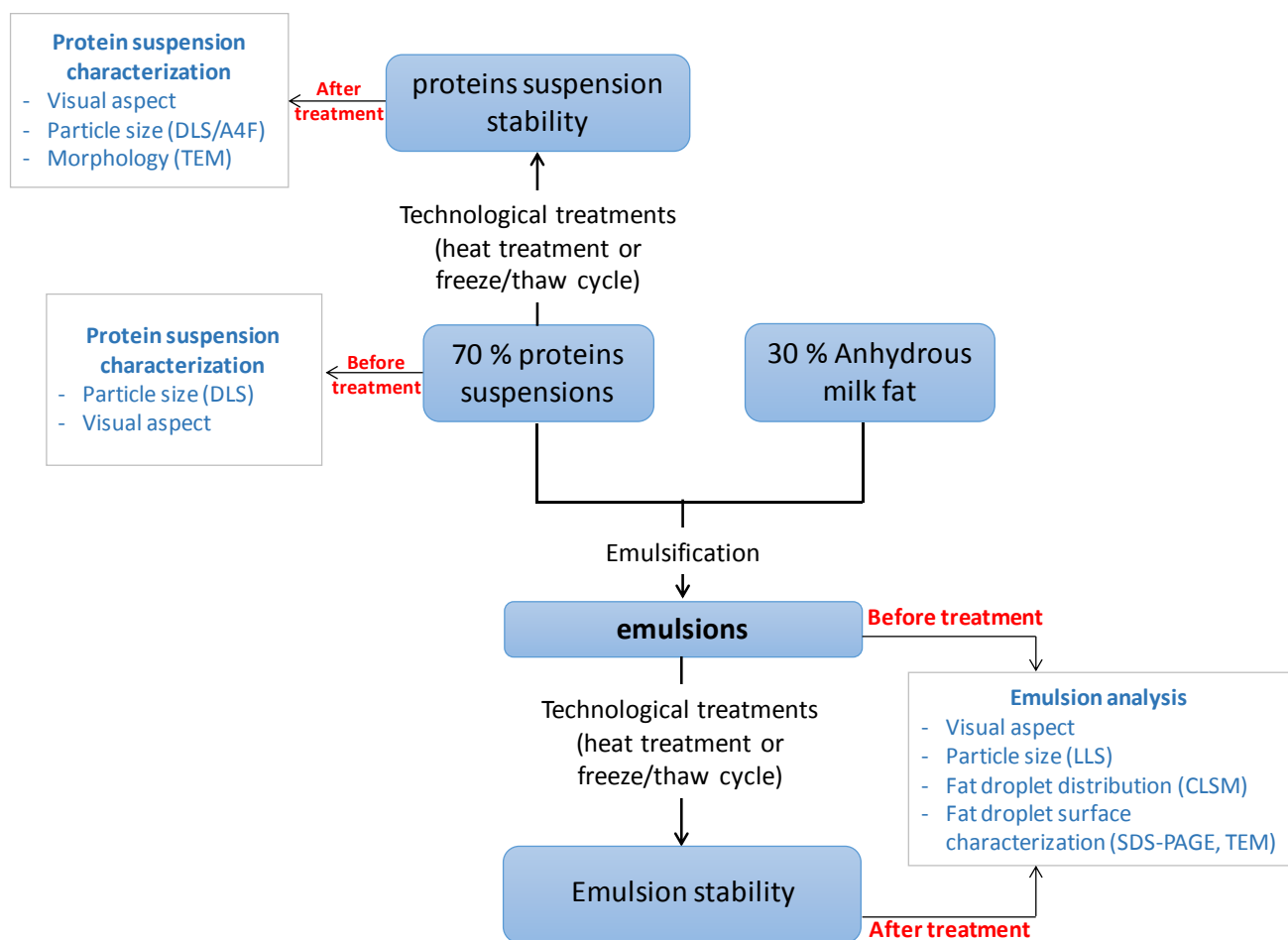


Figure 16: Diagram summarizing the different steps of preparation and analysis of the protein suspensions and emulsions



## CHAPTER 4:

### Impact of whey proteins powder ageing on the structure of heat-induced aggregates.

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This chapter aims at evaluating the impact of WP powder ageing on the structure of the aggregates formed on heating under controlled conditions. Indeed, the experiments of the PhD thesis were realized over a 3 years' period and the question of the reproducibility of the heat-induced aggregates as a function of the powder storage time was raised. The structural modifications occurring during the storage of the WP powders were evaluated. This study demonstrated that the level of protein lactosylation and the amount of aggregated proteins in dry state increased during the storage of the powder at high temperature (i.e. 40°C). The structural changes (size, morphology) of the WP aggregates formed by heating a WP solutions prepared with the aged powders (from 0 to 15 months) were characterised. Powder ageing at 40°C showed an evolution of the structure of the heat-induced aggregates and a simultaneous decrease in the native protein content and the formation of advanced glycation end products in the powder. For the heat-induced aggregates produced using solutions prepared with WP powders stored at 20°C, no structural difference was observed between fresh or aged powders, even if the level of protein lactosylation increased during storage. A part of these results has been published in the following paper: Norwood, E.-A., Chevallier, M., Floch-Fouéré, C. L., Schuck, P., Jeantet, R., & Croguennec, T. (2016). Heat-Induced Aggregation Properties of Whey Proteins as Affected by Storage Conditions of Whey Protein Isolate Powders. *Food and Bioprocess Technology*, 9(6), 993–1001.

**The main aims of the work presented in this chapter were to:**

- Identify the structural changes in stored powders, which can impact the heat aggregation functional properties of the whey proteins.
- Understand the role of the composition and the ageing of the powder in the aggregates formation
- Define appropriate storage conditions for the whey protein powder used during my PhD

## Table of contents

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<b>CHAPTER 4: Impact of whey proteins powder ageing on the structure of heat-induced aggregates.....</b>	<b>59</b>
<b>4.1 Introduction .....</b>	<b>61</b>
<b>4.2 Materials and methods.....</b>	<b>62</b>
4.2.1 Whey protein powder .....	62
4.2.2 Whey protein powder storage conditions .....	62
4.2.3 Powder composition .....	62
4.2.4 Browning Index .....	63
4.2.5 Level of $\beta$ -Lg lactosylation.....	63
4.2.6 Heat treatment of whey protein suspensions.....	63
4.2.7 Analysis of the heat-induced aggregates .....	63
<b>4.3 Powder state after storage .....</b>	<b>64</b>
<b>4.4 Heat-induced aggregation .....</b>	<b>68</b>
<b>4.5 Conclusion.....</b>	<b>72</b>

## 4.1 Introduction

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As soon as the beginning of my PhD thesis, the question of the reproducibility of the structure of the heat-induced aggregates over a 3 years' period was raised. For this reason, we studied the impact of the powder ageing on the ability to form the WP aggregates.

The powder ageing was known to influence the structural properties of protein-rich powders. The kinetics of structural changes in the dry state were dependent on the powder composition and the storage conditions (Haque et al., 2012). It has been observed that the composition of the powder grain surface evolved during storage, leading to changes in the properties of the micellar casein powder. In fact, the porosity and surface hydrophobicity of the particle surface increased, likely due to fat migration (Fyfe et al., 2011; Gaiani et al., 2009). Moreover, an evolution of protein lactosylation (Anema, Pinder, Hunter & Hemar, 2006; Guyomarc'h, Warin, Donald Muir & Leaver, 2000; Stapelfeldt, Nielsen & Skibsted, 1997), the formation of Maillard reaction products (e.g. furosine, hydroxymethylfurfural, etc.) and extensive protein cross-linking leading to large aggregates (Le, Bhandari & Deeth, 2011; Le, Bhandari, Holland & Deeth, 2011) were observed during the storage of whole milk and skimmed milk powders.

These structural changes were responsible for the modification of some techno-functional properties of the powder components, e.g. solubility (Haque, Bhandari, Gidley, Deeth & Whittaker, 2011; Havea, 2006; Mimouni, Deeth, Whittaker, Gidley & Bhandari, 2010; Schokker et al., 2011) and interfacial properties (foaming, emulsifying) of the milk proteins (Thomas, Scher, Desobry-Banon & Desobry, 2004). The rehydration kinetics and the protein solubility decreased with the storage time at temperature greater than room temperature (Anema et al., 2006; Fyfe et al., 2011; Haque et al., 2010). The Maillard reaction which occurs during the powders storage could have undesirable consequences on the emulsifying activity of the sodium caseinate/lactose or glucose mixture (Hiller & Lorenzen, 2010). Moreover, as shown by Norwood et al. (2016), foaming properties of the WP powders can also be influenced by the storage conditions (storage temperature, powder water activity) and the storage duration. However, the influence of the powder ageing on the ability of the WP to form heat-induced aggregates was not studied yet.

In this study, a WP powder at water activity ( $a_w$ ) close to industrial storage conditions ( $a_w = 0.23$ ) was produced from fresh liquid WP concentrate. The powder was then stored at representative storage temperature conditions from 4°C to 40°C. The structural modifications of the proteins in the powder state for periods of up to 15 months and the structure of the aggregates obtained after heating WP suspensions were characterised as a function of the time of storage of the powder.

**Note:** This part of my PhD thesis was performed in collaboration with another PhD student: Eve-Anne Norwood. These results were published in *Food and Bioprocess Technology*, 2016, 9, 993-1001. The trials were mainly conducted by Eve-Anne Norwood. I mainly participated in the second part focused on “heat-induced aggregation”. Due to the collaboration and because we wanted to control the powder ageing from the date of its manufacture, the powders and the protocols used in this part were not the same as in the rest of my PhD thesis manuscript. For only this part, a different materials and methods section was described.

## 4.2 Materials and methods

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### 4.2.1 Whey protein powder

The WP powder was obtained by spray drying a WP concentrate originating from ultrafiltration and diafiltration of the skimmed milk microfiltrate. Provided by a dairy company based in Brittany (France), the concentrate had a dry matter of 27.8 wt% and contained 95 wt% milk proteins. Spray drying was carried out to produce fine powders ( $\sim 60 \mu\text{m}$ ), using a three-stage spray drying pilot, Bionov (GEA, Niro Atomizer, Saint-Quentin-en-Yvelines, France). The flow was set at around 100 L/h and the inlet and outlet temperatures were fixed at 169°C and 66°C respectively. After drying, the WP powder with  $a_w = 0.23$  had a water content of 7.5 wt%. Immediately after drying, the powder was packed under air in airtight tins of 375 g capacity.

### 4.2.2 Whey protein powder storage conditions

The WP powder tins were stored in chambers set at 4, 20 and 40°C for periods of up to 15 months. After 0.5, 1, 3, 6, 9, 12 and 15 months of storage, 1 tin per temperature condition was opened in order to analyse the structural and functional properties of the powders.

### 4.2.3 Powder composition

Native proteins were quantified during powder storage according to Gulzar, Bouhallab, Jardin, Briard-Bion & Croguennec (2013) by gel permeation chromatography (GPC) using a Yarra SEC 3000 column (Phenomenex, Le Pecq, France) connected to a high performance liquid chromatography (HPLC) apparatus comprising a Waters 2695 separation system, a Waters 2489 double wavelength detector and Empower software (Milford, USA) for acquisition and data processing. The protein elution was performed with a flow of 0.8 mL/min using a 0.05 M phosphate buffer at pH 7 containing 0.1 M NaCl. Proteins were quantified by absorbance at 214 nm.

## CHAPTER 4: Impact of whey proteins powder ageing on the structure of heat-induced aggregates.

### 4.2.4 Browning Index

The browning index of powders was determined by measuring  $L^*a^*b^*$  parameters with a colorimeter (Konica Minolta Photo Imaging France SAS, Roissy, France) using the formula:

$$\text{Browning Index} = \frac{[100(x - 0.31)]}{0.17}$$

with  $x = \frac{(a+1.75L)}{(5.645L+a-3.012b)}$  (Maskan, 2001)

### 4.2.5 Level of $\beta$ -Lg lactosylation

The  $\beta$ -Lg percentage of lactosylation was determined by mass spectrometry after protein separation on reverse phase HPLC with a C4 VYDAC column (214TP5215, 150 × 2.1 mm) (GRACE, Columbia, USA). The analysis was carried out with 15  $\mu$ L of a 0.5 wt% solution reconstituted in milliQ water diluted 2/3 with a buffer solution of trifluoroacetic acid (TFA) 0.212 %. The flow applied was 0.25 mL/min with an mixture of buffer A (water + 0.106 % TFA) and buffer B (water + 80 %  $\text{CH}_3\text{CN}$  + 0.1% TFA). The column was equilibrated with a mixture containing 37 % buffer B and linear gradients of 37 to 60 % buffer B from over 40 min, then 60 to 90 % over 2 min, and 90 to 37 % over 4 min. At the end of the column, a fraction of the eluate entered a QSTAR XL mass spectrometer (MDS SCIEX, Ontario, Canada) at a flow of 75  $\mu$ L/min. Proteins were ionized with an ion sprayer source before determining their mass with a time of flight scanner previously calibrated with  $\beta$ -CN peptide from casein  $\beta$  (193-209). Mass acquisition was carried out in the mass range of 500 to 3000 m/z and a tension of 5000 V.

### 4.2.6 Heat treatment of whey protein suspensions

The protein suspensions obtained after dissolving in milli-Q water (4 wt%) the non-aged WP powder (stored at 4°C – Ref) and the WP powders stored for 3, 6, 12 and 15 months were adjusted to pH 5.8, 6.2 and 6.6. The samples were heated in a water bath at 80°C for 1 h and then quenched in cold water. After heat treatment, 0.02 % sodium azide was added to each sample. The samples which were analysed the same day (i.e. GPC and dynamic light scattering (DLS) experiments) were kept at room temperature and those that were not analysed the same day (i.e. TEM observations) were stored at 4°C.

### 4.2.7 Analysis of the heat-induced aggregates

Particle size distribution of the heat-treated dispersions was determined by DLS using a Zetasizer Nano ZS (Malvern Instrument, Worcestershire, UK). Measurements were performed in triplicate at 20°C measuring the backscattered light at 173° over 120 s. Prior to measurement, samples were diluted 100 times with milli-Q water. Average size diameters (Z-average) were reported. TEM was performed using negative staining. Samples were diluted to 160 mg/L protein and stained using a 2 % uranyl acetate

solution (Ayache, Beaunier, Boumendil, Ehret & Laub, 2007). Samples were observed using a JEOL JEM 1400 transmission electron microscope (Croissy-sur-Seine, France) operating at 120 kV. Images were recorded with a GATAN Orius SC 1000 camera (Digital Micrograph software).

### 4.3 Powder state after storage

Immediately after packaging ( $t_0$ ), WP powder contained 4.7 wt% proteins that precipitated at pH 4.6 i.e. caseins and denatured WP. These were considered to be proteins that had denatured during spray drying or prior processing steps. Moreover, no structural or functional modification was detected during 15-months' storage at 4°C. The powder stored at 4°C was therefore taken as a reference (Ref) and any difference from Ref properties was taken to be a structural and/or functional evolution.

Browning is an essential criterion for powder acceptability since the colour is non-compliant for use in many applications. At  $t_0$ , the browning index of WP powder was 15, typical for a non-aged powder as shown in Fig 17. During storage at 20°C, changes in the browning index were very slight or nul over 15 months, whereas powders stored at 40°C browned during storage, starting from as early as 15 days of storage. These results are in agreement with those of Stapelfeldt et al. (1997) and Burin, Jouppila, Roos, Kansikas & Buera (2000) who showed that milk powder turned brown as a function of time and temperature during storage at  $T \geq 25^\circ\text{C}$ . Li-Chan (1983) reported that under accelerated storage conditions (37°C for 42 days and  $a_w$  of 0.75), the browning of the WP powders was correlated with a decrease in the residual lactose as well as a decrease in lysine availability. Other authors have also shown that powder browning in the presence of sugar was correlated with a loss of amino acids (Martinez-Alvarenga et al., 2014).

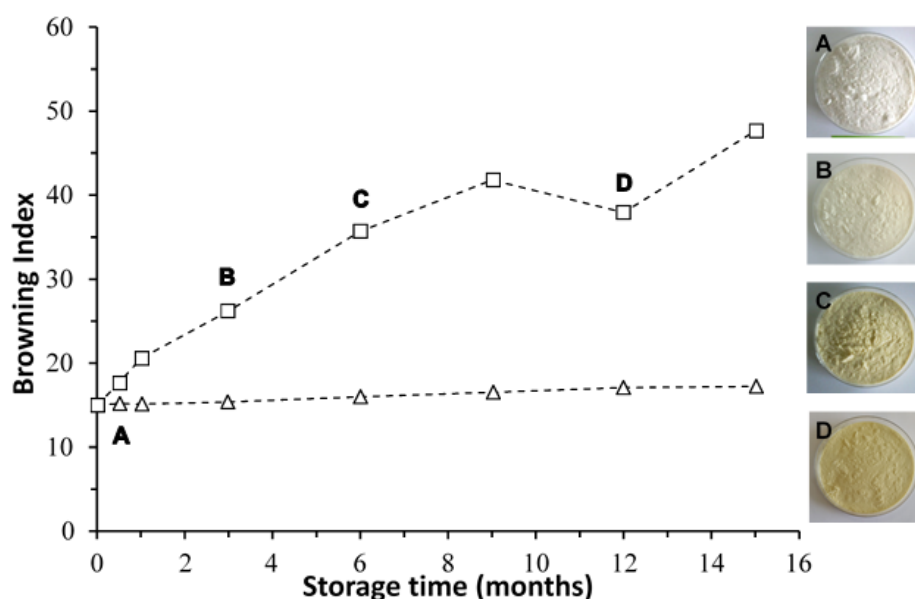


Figure 17: Evolution of Browning Index for powders of  $a_w$  0.23 stored at 20°C (triangles) and 40°C (squares) as a function of storage time, with relative error < 0.7 %. (A-B) Images of powders after 1 month of storage at 4°C and 3, 6 and 12 months of storage at 40°C, respectively.

#### CHAPTER 4: Impact of whey proteins powder ageing on the structure of heat-induced aggregates.

Protein solubility at pH 4.6 is a current criterion to determine the amount of WP in the native state. On this basis it is possible to obtain the fraction of native and denatured/aggregated proteins in powders during storage. Powders stored at 20°C were thus stable until 15 months (Fig 18). Similar behaviour was observed for powders stored at 40°C less than 3 months; from this date, the fraction of native proteins started to decrease, with a concomitant increase in denatured/aggregated proteins. After 9-months' storage at 40°C, powders presented ~ 32 wt% denatured/aggregated protein. Morr & Ha (1993) reported that storage of WPC powder resulted in partial denaturation and progressive polymerization. The amount of native proteins therefore decreased from 60 to 33 wt% during 7 days of storage at 80°C. Furthermore, storage at 25°C for a year caused the polymerization of 18 % of  $\beta$ -Lg monomers. Havea (2006) reported that these proteins tended to unfold during storage at 40°C, making them more available to the formation of aggregates linked by disulphide bridges. Protein-protein interactions have been observed by other researchers (Anema et al., 2006; Mimouni et al., 2010; Zhou & Labuza, 2007). This has also been reported for WP dry heated in a powder state (Gulzar, Bouhallab, Jeantet, Schuck & Croguennec, 2011).

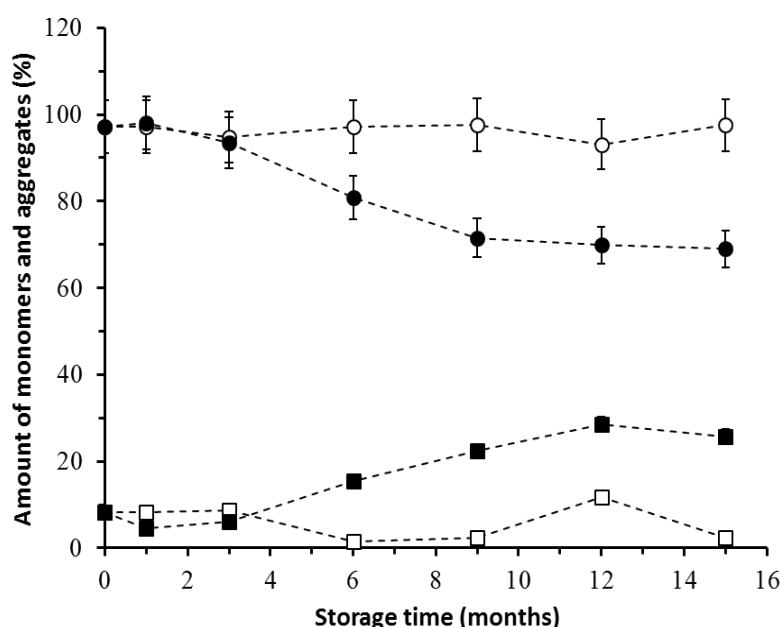


Figure 18: Amount of native (circles) and denatured/aggregated (squares) proteins for powders of aw 0.23 stored at 20°C (empty symbols) and 40°C (full symbols) as a function of storage time.

Among the non-aggregated proteins that were detected on reverse phase chromatography at the characteristic retention time of  $\beta$ -Lg and  $\alpha$ -La, some were lactosylated. The fraction of lactosylated  $\beta$ -Lg (Fig 19), which could carry one or more lactose molecules per protein, was determined by mass spectrometry. Only the results for  $\beta$ -Lg are presented due to the difficulty of detecting lactosylation on  $\alpha$ -La with storage time.

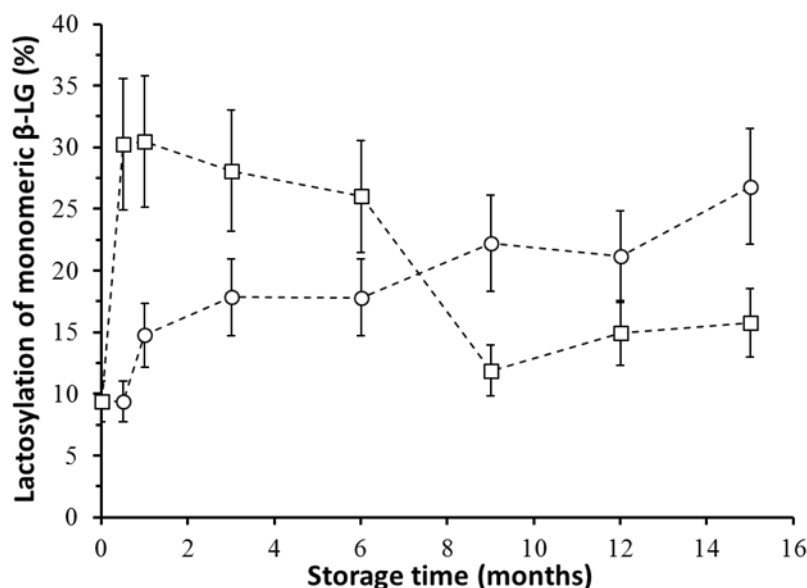


Figure 19: Evolution of lactosylation of  $\beta$ -Lg stored at 20°C (circles) and 40°C (squares) as a function of storage time.

The results of protein lactosylation have to be treated with caution. Indeed, these results are only estimated since ionization efficiency could happen differently during mass spectrometry analysis according to the level of lactosylation of the proteins. However, as the number of lactose molecules bound per protein was low (mainly one or two lactose molecules), the ionization efficiency between proteins should be close. At  $t_0$ , 10 % of the WP were lactosylated. The percentage increased regularly, reaching 27 % after 15 months of storage at 20°C. In powders stored at 40°C, lactosylated proteins evolved faster, reaching 30 % after 15 days of storage, then remained stable up to 3 months of storage before decreasing. The decrease after 3 months' storage matched a concomitant increase in protein aggregation. This suggested participation of Maillard degradation products in aggregation reactions (Guyomarc'h et al., 2000; Morgan et al., 1999). As shown in Fig 20, powders with  $a_w$  0.23 stored at 40°C for up to 12 months first presented a sharp increase in protein lactosylation (Fig 20A), which then decreased towards protein aggregation (Fig 20B). SDS PAGE showed that aggregates comprised disulphide bridges and also other covalent bonds (data not shown). The latter are thought to be linked to the presence of lactose degradation products in the medium forming covalent protein crosslinks



#### CHAPTER 4: Impact of whey proteins powder ageing on the structure of heat-induced aggregates.

(Enomoto et al., 2007; Guyomarc'h et al., 2014; Le, Deeth, Bhandari, Alewood & Holland, 2013; Li et al., 2009).

During storage at 20°C, changes in the browning index were very slight or null over 15 months, in accordance with the relatively small changes in protein lactosylation. It is interesting to note that the browning index at 40°C steadily increased while the level of protein lactosylation reached a maximum between 15 days and 3 months of storage (see Fig 17 and Fig 19).

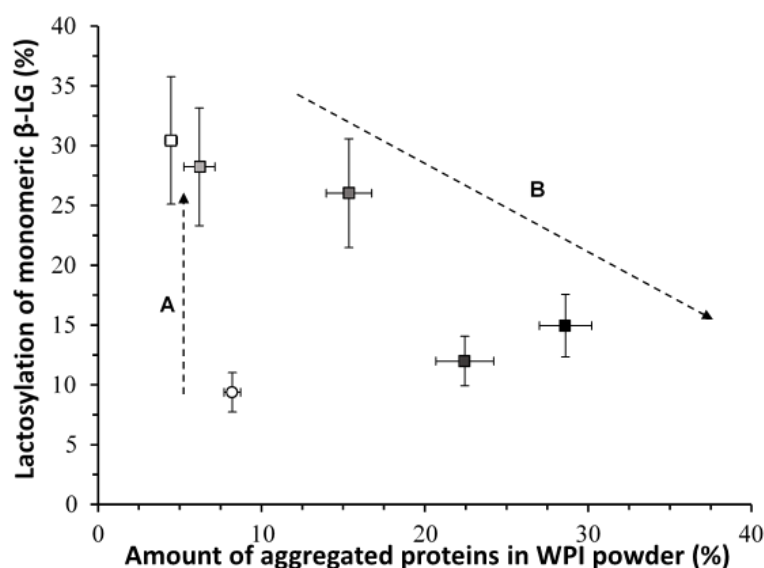


Figure 20: Correlation between lactosylation and aggregation for Ref (empty circle) and powders with aw 0.23 stored at 40°C (empty square=1 month; light grey square = 3 months; grey square=6 months; dark grey square=9 months; black square = 12 months) as a function of storage time.

Browning is the consequence of the extent of the Maillard reaction between amino groups and lactose molecules generating coloured compounds and aromatic molecules. In fact, although it had been thoroughly diafiltered before spray drying, a very small amount of lactose may still have remained in the WP powder produced (~ 1.2 wt%) and that would not limit development of the Maillard reaction. Moreover, it has been reported that temperature affects the extent of lactosylation during storage (Guyomarc'h et al., 2000). As powder browning mainly reflects deterioration of lactose molecules interacting with proteins, it is likely that free lactose molecules were in the process of binding to protein while others that were already bound (fewer) were degraded into aggregates and tended to colour the powder from the first month of storage. For longer storage times (> 6 months), degradation of lactose molecules overtook the binding of new lactose molecules (possibly because there were no longer any free lactose molecules in the powder), resulting in increasing the browning index and aggregation while decreasing the fraction of lactosylated proteins. These results are in agreement with previous studies showing that storage at high temperature is followed by an increase in the amount of Maillard reaction products (Ipsen and Hansen 1988; Kieseker and Clarke 1984). However, aggregation might also be due

to heat-induced denaturation of proteins that unfold and thus aggregate. Norwood et al. (2016) confirmed the role of lactose during powder storage.

#### 4.4 Heat-induced aggregation

The stored WP powders were analysed for changes in their heat-induced aggregation properties. WP are widely used for their ability to form stable aggregates under controlled conditions (Donato et al., 2009), generally associated with the formation of intra- and inter-molecular interactions that occur during heating (Roefs & De Kruif, 1994). It is known that heat-induced aggregation is very dependent on pH (Hoffmann & van Mil, 1999).

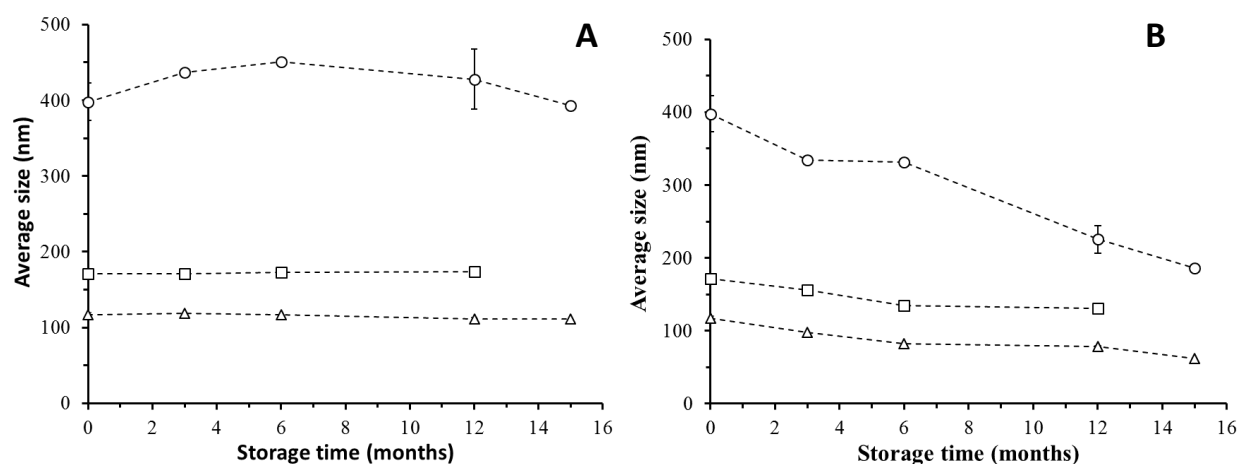


Figure 21: Average aggregate sizes as a function of storage time for powders with aw 0.23 stored at 20°C (A) and 40°C (B) after heat treatment at pH 5.8 (circles), pH 6.2 (squares) and pH 6.6 (triangles).

Heat-induced aggregation of reconstituted WP powders was studied at pH 5.8, 6.2 and 6.6 (Fig 21). Several studies have shown that heating generates microgels at pH 5.8, strands at pH 6.6 and a mixture of both at pH 6.2 (Nicolai et al., 2011; Nicolai & Durand, 2013; Phan-Xuan et al., 2011). Figure 21 shows the evolution of heat-induced aggregate size as a function of powder storage time and temperature after 1 h of static heating of WP suspensions (4 wt%) adjusted to a given pH. The aggregate size of Ref was around  $400 \pm 25$  nm,  $170 \pm 1$  nm and  $120 \pm 3$  nm at pH 5.8, 6.2 and 6.6, respectively. As expected, the WP powders stored at 20°C showed relative stability with regard to heat-induced aggregate size after 15 months of storage, whatever the pH. In fact, no significant variation was registered in DLS after repetition. However, storage at 40°C provided very different results. Heat-induced aggregate size decreased as a function of storage time regardless of pH. This decrease was significant from 3 months. At pH 5.8, 6.2 and 6.6, size decreased to  $225 \pm 20$  nm,  $130 \pm 2$  nm and  $78 \pm 2$  nm after 12 months, respectively.

The heat-induced aggregates from WP powders stored for 12 months at 40°C were observed by TEM (Fig 22). In the Ref samples, heat-induced aggregates consisted of microgels (spherical units) at pH 5.8

#### CHAPTER 4: Impact of whey proteins powder ageing on the structure of heat-induced aggregates.

(Fig 22A) and a mixture of smaller individual particles, mostly spherical and strand-like aggregates, at pH 6.2 (Fig 22C) and 6.6 (Fig 22E), respectively. Images of heat-induced aggregates using 12-months stored powders still showed spherical units although smaller than in Ref at pH 5.8 (Fig 22B), mostly strands at pH 6.2 (Fig 22D), and only strands at pH 6.6 (Fig 22F). The morphological features of Ref were not in agreement with the results reported by Phan-Xuan et al. (2011) for samples heat treated at pH 6.2 and 6.6. According to the latter authors, only strands could be formed at pH 6.6 and mostly strands at pH 6.2. The difference between our results and those of Phan-Xuan et al. (2011) might be related to the age of the powder as the shapes of the aggregates changed with storage time. It is also possible that differences in powder composition may affect the morphology of the aggregates. Phan-Xuan et al. (2013) indicated that small variations in amount of calcium could shift microgels to fine strands and *vice-versa*.

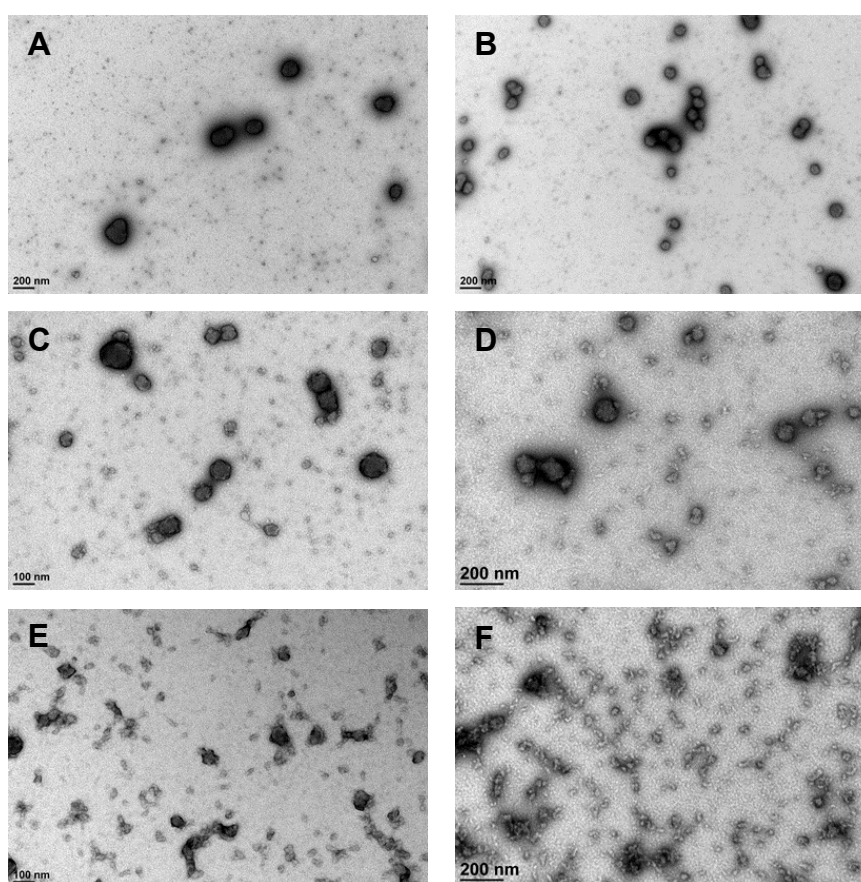


Figure 22: TEM images of negatively stained whey protein Ref (A, C, E) and powder stored for 12 months at 40°C (B, D, F) when heat treated at pH 5.8 (A and B), pH 6.2 (C and D) and pH 6.6 (E and F).

Upon heating, WP move to a reactive state. The protein structure is modified, free to unfold, exposing their hydrophobic core and free thiol groups so that they are able to interact with nearby molecules. First, covalent oligomers are formed linked by disulphide bridges (Croguennec, O’Kennedy & Mehra, 2004; Surroca, Haverkamp & Heck, 2002). Their structure is flexible and when the number of oligomers

#### CHAPTER 4: Impact of whey proteins powder ageing on the structure of heat-induced aggregates.

exceeds a critical level, they form in relatively monodisperse structured aggregates (Nicolai et al., 2011; Schmitt et al., 2009). Their presence seems to be a condition for the formation of microgels (Da Silva Pinto et al., 2012; Schmitt et al., 2009), but according to Schmitt et al. (2010) small aggregates resulting from an association of oligomers are only a stage in the formation of final heat-induced aggregates. In fact, these small aggregates appear to be the source of new large aggregates, first linked by hydrogen and hydrophobic bonds, and then stabilized by disulphide bridges. Other authors have hypothesized that the heat-induced aggregation originates from growth and nucleation processes (Aymard et al., 1996; Bromley, Krebs & Donald, 2006; Phan-Xuan et al., 2011), the nuclei being the oligomers formed in the early stages of aggregation on which the denatured proteins aggregate (Phan-Xuan et al., 2013). Environmental conditions (pH, ionic strength, nature of salts) govern heat-induced aggregation as they determine the ability of proteins to unfold, change the net charge and thus the interaction between proteins which finally affect oligomers formation.

It was possible to see on TEM images that not all aggregates were identical, but depended on the pH suspension when heat treated. At pH 5.8, microgel types were visible while at pH 6.2 and 6.6, mixtures of microgels and strands were observed. Phan-Xuan et al. (2014) highlighted the importance of the net charge density of native protein in the type of heat-induced aggregate formed, and therefore the key role of the pH suspension during heat treatment (Hoffmann & van Mil, 1999). Around pI, aggregates form a spherical structure (microgels) which is reinforced by new covalent bonds (Giroux, Houde & Britten, 2010), whereas when the pH increases, aggregate size decreases and strands are observed (Phan-Xuan et al., 2011). This can be explained by the repulsion generated by surface charges of denatured proteins, thereby limiting further aggregation. In our samples at  $\text{pH} \geq 6.2$ , a mixture of strands and microgels was present, in agreement with Hoffmann & van Mil (1999) and Phan-Xuan et al. (2011). In fact, heat-induced aggregation favours the formation of microgels before strands (Nicolai, 2015) as the pH increases upon aggregation, leading to an increase in the protein net charge and then a shift from microgels to strands.

Structural findings confirmed that heat-induced aggregation was not significantly different from the Ref when the powders were stored at 20°C, although lactosylation was observed following 15 months of storage. When powders were stored at 40°C, the amount of native protein decreased in favour of aggregation in the dry state. According to Phan-Xuan et al. (2013), as oligomers are formed from native proteins, heat-induced aggregates are smaller because of the reduction in amount of native protein. A steady state is reached when the suspension no longer contains native monomers. Our results were consistent with those of Jones, Adamcik, Handschin, Bolisetty & Mezzenga (2010) that explained that heat-induced aggregates are smaller probably due to a reduction in available protein monomers. The literature also indicates that the reduction in aggregate size is due to the presence of sugar which modifies native proteins into advanced glycation end products (Da Silva Pinto et al., 2012). Proteins are

#### CHAPTER 4: Impact of whey proteins powder ageing on the structure of heat-induced aggregates.

found to be less flexible and thus lose some of their ability to form aggregates and then inhibit protein aggregation (Lee, Park, Paik & Choi, 2009). Indeed, in our study, proteins were partly lactosylated, without subsequently aggregation in a dry state during storage at 20°C, and no changes in heat-induced aggregation could be observed.

Moreover, heat-induced aggregates decreased in size at pH 6.2 and 6.6 but their shape moved from microgels to strands. If strands are precursors of microgels, the decrease in the amount of native protein after powder storage at 40°C could explain the change in aggregate morphology (Lee et al., 2009). The extensive binding of lactose molecule and Maillard reaction products on WP (mainly on amino groups) stored at 40°C could also increase their net charge. Consequently, clustering of oligomers upon heating might rapidly reach a critical charge density, thus limiting aggregation, and strands would then form.

To summarize, a mechanism for heat-induced aggregation of stored WP powders under the experimental conditions tested is proposed in Fig 23.

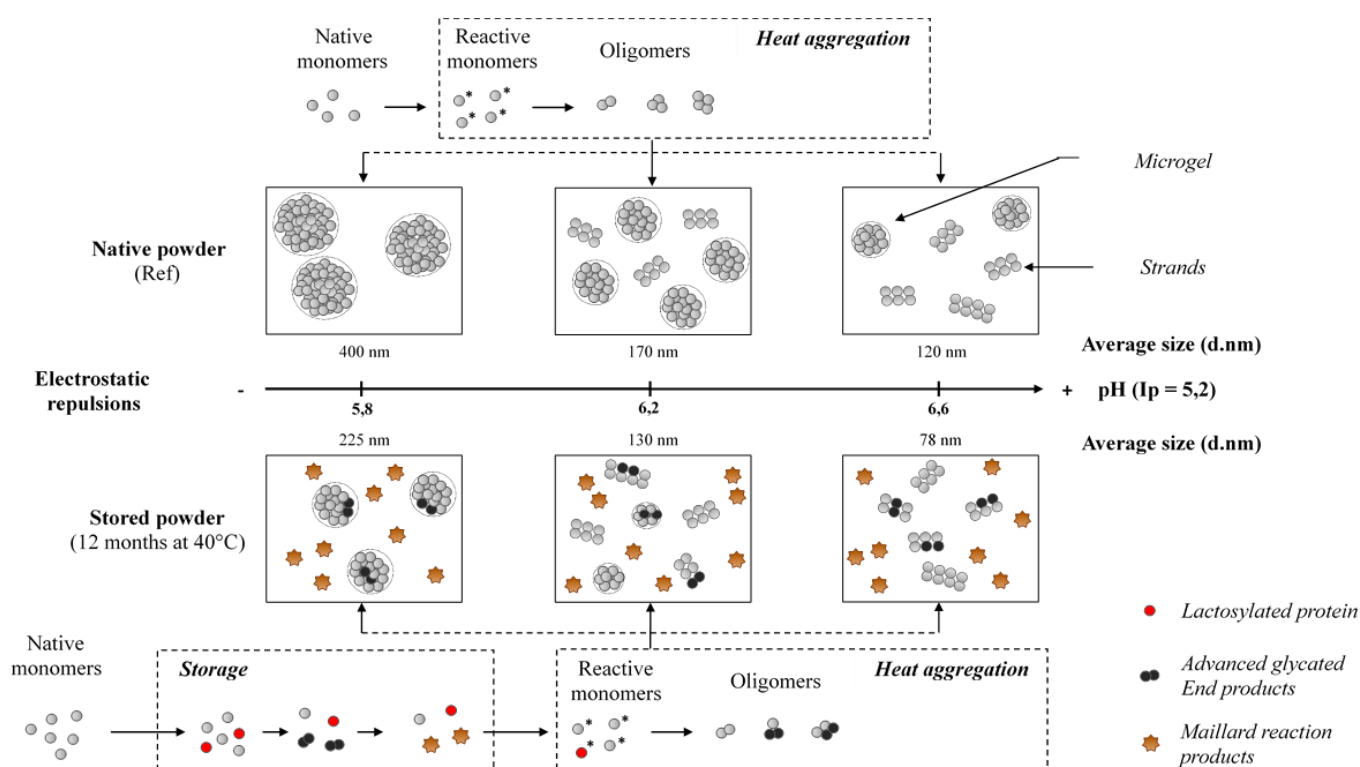


Figure 23: Proposed scheme for formation of heat-induced aggregates of native and stored powders as a function of pH. From Norwood et al. (2016)

## 4.5 Conclusion

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In this study, the consequences of storage conditions representative of the current modes of delivery of WP powders were investigated. These storage conditions resulted in structural changes that could explain the variability of functional properties of WP powders. Interestingly, the results also highlighted that the production of the heat-induced aggregates are reasonably reproducible over a 15 months' period when the WP powder is stored at temperature below 20°C. The results obtained in this study allowed us to define appropriate storage conditions for the WP powder used during my PhD thesis. The WP powders were stored at temperature below 20°C, in order to control the formation of the WP aggregates during the 3 years' period of my PhD thesis. This storage temperature was reported to delay structural changes in the powder that could impact the behavior of the WP suspension on heating.

## CHAPTER 5: Selection, composition and characterization of the structure and functional properties of whey protein aggregates.

The aim of this chapter is to select and characterize the suspensions of WP aggregates likely to present a good capacity to stabilize WP-rich emulsions during technological treatments, i.e. heating (120°C) and freezing/thawing (-18°C). The production procedure of the WP aggregates was based on bibliography. Their ability to stabilize oil-in-water emulsions during technological treatments was investigated during a first screening. The term “stable emulsion” after applying technological treatments (heating, freeze/thaw cycle) referred to an emulsion with an absence of oiling off, gelation and change in flowability. This study demonstrated that the morphology and size of the aggregates impacted the heat and freeze/thaw stability of the emulsions. Suspensions containing fractal aggregates (diameter up to 1000 nm) were not selected during a first screening because of their low ability to stabilize the emulsions during heating. Microgels suspensions and strand-like aggregates (diameter of ~ 80 nm) suspensions were selected for their good ability to stabilize emulsions. For the selected aggregates suspensions, the size distribution and morphology of the aggregates and the precise composition of the suspension were determined. A significant influence of the WP powder composition due to industrial production batch variation on the structure of the protein aggregates was observed. The heat stability and freeze/thaw stability of the WP aggregate suspensions at varying concentrations as well as their interfacial and emulsifying properties were evaluated. A part of these results has been published in the following paper: Chevallier, M., Riaublanc, A., Lopez, C., Hamon, P., Rousseau, F., & Croguennec, T. (2016). Aggregated whey proteins and trace of caseins synergistically improve the heat stability of whey protein-rich emulsions. *Food Hydrocolloids*, 61, 487–495 and Chevallier, M., Riaublanc, A., Lopez, C., Hamon, P., Rousseau, F., Thevenot, J., & Croguennec, T. Increasing the heat stability of whey protein-rich emulsions by combining the functional role of WPM and caseins. *Food Hydrocolloids*, in press.

### The main aims of the work presented in this chapter were to:

- Characterize the composition of the whey protein aggregate suspensions
- Select whey protein aggregates with a good ability to stabilize whey protein-rich emulsions during technological treatments, i.e. heating and freezing/thawing
- Investigate the functional properties (heat stability and the freeze/thaw stability and emulsifying ability) of the selected aggregates suspensions
- Understand the composition role of the powder on the structure of the aggregates formed

## Table of contents

<b>CHAPTER 5: Selection, composition and characterization of the structure and functional properties of whey protein aggregates .....</b>	<b>73</b>
<b>5.1 Introduction .....</b>	<b>75</b>
<b>5.2 Selection and characterization of the whey protein aggregates .....</b>	<b>75</b>
5.2.1 Selection of the whey protein aggregates: potentialities of the strand-like aggregates and the microgels .....	75
5.2.2 Aggregate screening based on their ability to stabilize emulsions during technological treatments .....	77
5.2.3 Composition and characterization of the selected aggregate suspensions .....	78
5.2.3.1 Composition of the aggregate suspensions .....	78
5.2.3.2 Structural characterization of the aggregates.....	82
<b>5.3 Functional properties of the aggregate suspensions.....</b>	<b>85</b>
5.3.1 Heat stability and freeze/thaw stability of the aggregate suspensions.....	85
5.3.1.1 Heat stability of the aggregate suspensions .....	86
5.3.1.2 Freeze/thaw stability of the proteins suspensions.....	88
5.3.2 Emulsifying capacity of the aggregate suspensions .....	88
5.3.2.1 Interfacial properties of the protein suspensions at the air-water interface .....	89
5.3.2.2 Emulsifying capacity of the protein suspensions .....	90
<b>5.4 Impact of the whey proteins powder composition on the structure of the aggregates .....</b>	<b>91</b>
5.4.1 Casein effect .....	92
5.4.2 Calcium effect .....	93
<b>5.5 Conclusion.....</b>	<b>94</b>



## 5.1 Introduction

Nicolai and Durand (2013) suggested that the heat induced aggregation of the WP give rise to new entities providing new functionalities. WP aggregation can be controlled by adjusting the chemical characteristics of the aqueous phases, to produce various types of WP aggregates. For instance, strand-like aggregates (curved strands and fractal aggregates) are formed at pH 7 (Mahmoudi et al., 2010) whereas particulate aggregates (whey protein microgels, WPM) are formed in a narrow range of pH (5.8 - 6.0) and at low ionic strength (Schmitt et al., 2010). Fibrils are obtained by heating at 80°C for 22 h a WP suspension at pH 2 and at very low salt concentration (Jung et al., 2008; Nicolai et al., 2011). In addition, slight variations in the protein (Croguennec et al., 2014; Guyomarc'h, Nono, Nicolai & Durand, 2009) and salt (Phan-Xuan et al., 2013) compositions of the WP suspensions was shown to affect the structure of heat-induced WP aggregates.

WP aggregates exhibit different functionalities than the native WP. Heat-induced aggregates in a stable suspension at steady state (after prolonged heating) are automatically heat stable (Nicolai & Durand, 2013). For this reason, these aggregates were hypothesized to increase the thermal stability of beverages (Ryan, Zhong & Foegeding, 2013). Schmitt et al. (2014) showed that WPM had efficient foam stability property especially at pH close to their pI i.e. pH 4.8. The emulsion stability was reported to be improved by WPM (Destribats et al., 2014) and fibrils of WP (Peng et al., 2016).

In this chapter, different types of WP aggregates were produced according to already published procedures in order to investigate the impact of their morphology and their size on the heat stability and freeze/thaw stability of the emulsions. To our knowledge, such comparison has not been done yet. The aggregate suspensions with the best ability to stabilize WP emulsions for technological treatments (heating, freezing/thawing) were characterized on a structural and functional point of view. For the functional properties, the emulsifying properties and stability of the aggregates during heat treatment and freeze/thaw cycle were determined. Finally, the impact of the powder composition, which is inherent to industrial batch-to-batch variation, on the aggregates formation was also evaluated.

## 5.2 Selection and characterization of the whey protein aggregates

### 5.2.1 Selection of the whey protein aggregates: potentialities of the strand-like aggregates and the microgels

To investigate the impact of the morphology of the aggregates, two types of aggregates were produced: strand-like aggregates (SA) and whey protein microgels (WPM). The procedures for their production are well described in the bibliography. In addition, SA of different sizes (from individual curved strand to

large fractal aggregates) were obtained by varying the protein concentration and the ionic strength of the WP suspensions. It is probable that the structure of the SA changed slightly in the absence and presence of salt but, except their average hydrodynamic diameter, this particular aspect was not investigated.

As illustrated on figure 24, the size of the SA increased with WP concentration in the suspension (Mahmoudi et al., 2007). On this basis, SA were produced by heating the WP suspension at two different protein concentrations: SA were produced at a protein concentration of 4 wt% ( $SA_4$ ) and fractal aggregates ( $SA_9$ ) were produced at a protein concentration of 9 wt% which was just below the critical gelation concentration of the WP suspension (Fig 24).

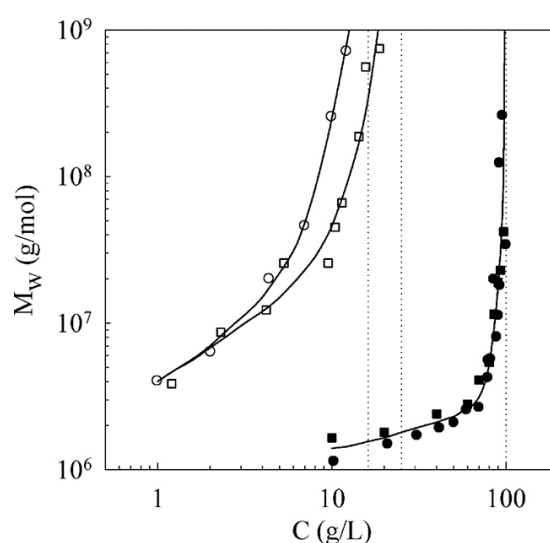


Figure 24: Concentration dependence of the weight-average molar mass of WPI (squares) and  $\beta$ -Lg (circles) aggregates formed in the presence of 0.1 M NaCl (open symbols) or in the absence of added salt (solid symbols). The solid lines are guides to the eye. The dotted lines indicate the critical gelation concentration:  $C_g \approx 100$  g/L for WPI and  $\beta$ -Lg in the absence of added salt,  $C_g \approx 25$  g/L for WPI, and  $C_g \approx 15$  g/L for  $\beta$ -Lg at 0.1 M NaCl. (Mahmoudi, Mehalebi, Nicolai, Durand & Riaublanc, 2007)

Other fractal aggregates were produced by adding NaCl in the WP suspension before heating. The NaCl concentration has to be chosen carefully regarding the protein concentration in the suspension as the critical concentration of gelation decreases with increasing NaCl concentration (Mahmoudi et al., 2007). Some preliminary tests showed that the largest aggregate ( $SA_{Na}$ ) produced at a protein concentration of 4 wt% were obtained after adding 40 mM of NaCl in the suspension. In contrast, the size of the WPM changed only slightly with protein concentration. In consequence, only a single protein concentration (4 wt%) was selected to produce the WPM.

These four types of aggregates were produced with the WP powder A. They will be hereinafter referred to as WPM A for whey protein microgels, and  $SA_4$ ,  $SA_9$ ,  $SA_{Na}$  for strand-like aggregates produced with either 4 wt% of protein or 9 wt% of protein and at 4 wt% of protein with 40 mM NaCl, respectively.

### 5.2.2 Aggregate screening based on their ability to stabilize emulsions during technological treatments

Emulsions at 3 wt% of proteins (dispersed in milk ultrafiltration permeate) and 30 % (v/v) of milk fat were prepared with the four types of aggregates (i.e. WPM A, SA<sub>4</sub>, SA<sub>9</sub>, SA<sub>Na</sub>). All the emulsions appeared fluid just after homogenization at 40 MPa. The microstructure of the WPM A, SA<sub>4</sub> and SA<sub>9</sub> emulsions was similar in terms of fat droplet size and spatial distribution of the fat droplets in the emulsions. The size (volume mean diameter  $d_{4,3}$ ) of the fat droplets was at  $1.4 \pm 0.1 \mu\text{m}$  both in absence and in presence of SDS. Only the SA<sub>Na</sub> emulsion was flocculated. The size of the fat droplets of the SA<sub>Na</sub> emulsion was  $5.1 \mu\text{m}$  in the absence of SDS and  $1.5 \mu\text{m}$  in the presence of SDS.

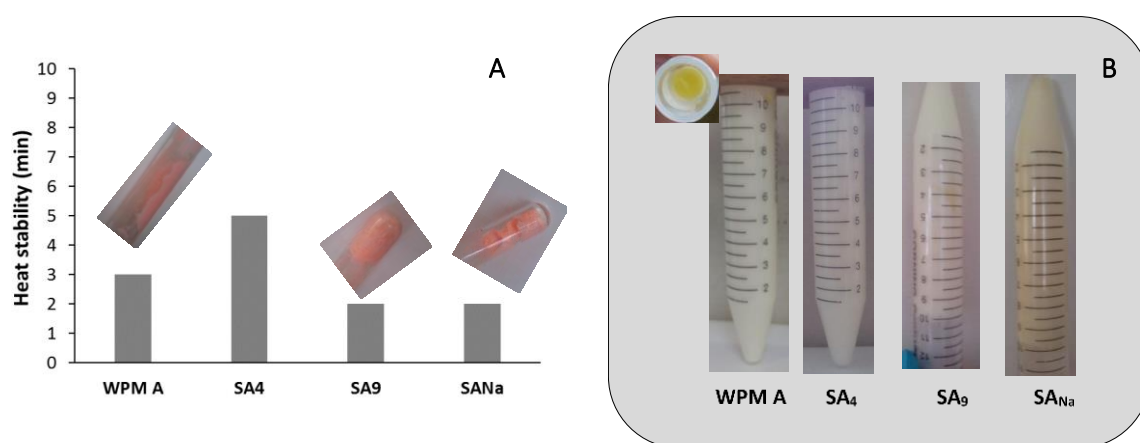


Figure 25: Heat stability of the emulsions (A) and pictures of emulsion after the freeze/thaw cycle (B).

The heat stability of the four emulsions was evaluated after 1, 2, 3, 5, 10, 20 and 30 min of heating at 120°C. The heat stability time indicated on figure 25A represents the time just below than that required to destabilize the emulsions, meaning without change in the macroscopic aspect of the emulsion such as loss of flowability, trace of aggregation or gelation. Emulsions appeared gelled at 5 min of heating for WPM A emulsion and at 3 min of heating for SA<sub>9</sub> and SA<sub>Na</sub> emulsions. The SA<sub>4</sub> emulsion was visually destabilized at 10 min of heating. This latter did not gel but a change of its flowability and small fat spot at the top of the emulsion were observed at macroscopic scale. At the microscopic scale, the particle size determined by dynamic light scattering increased from  $1.7 \mu\text{m}$  to around  $10 \mu\text{m}$  in SA<sub>4</sub> emulsion due to the flocculation of the fat droplets.

After a freezing at -18°C and thawing, only the SA<sub>4</sub> emulsion was visually stable (Fig 25B). The emulsions reconstituted with WPM A exhibited a phase separation, i.e. a fat layer on the top of the emulsion appeared after a freeze/thaw cycle. The SA<sub>9</sub> and SA<sub>Na</sub> emulsions were gelled. CLSM observations of the SA<sub>9</sub> and SA<sub>Na</sub> emulsions immediately after thawing showed an inhomogeneous distribution of the fat

droplets in the emulsion confirming the macroscopic observation (Fig 26A and B). The distribution of the proteins appeared also inhomogeneous after the freeze/thaw cycle; they were more concentrated around the fat droplets suggesting that SA<sub>9</sub> and SA<sub>Na</sub> formed a network entrapping the fat droplets.

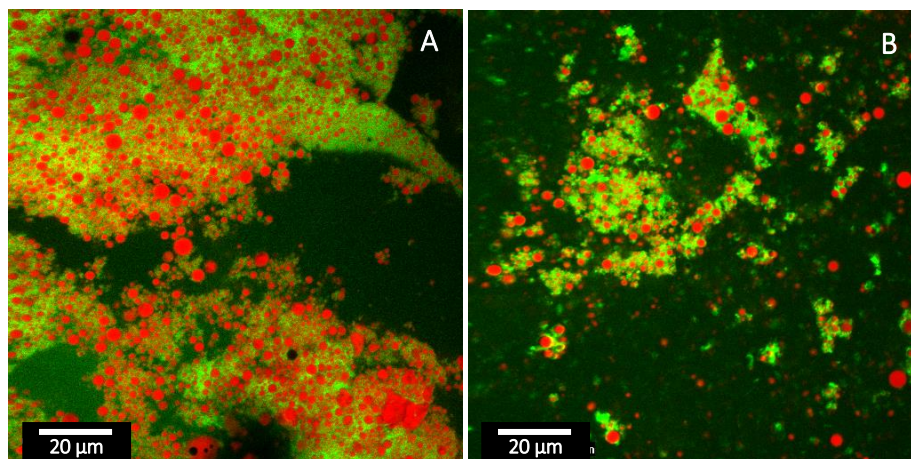


Figure 26: Confocal Laser Scanning Microscopic (CLSM) images of SA<sub>9</sub> emulsion (A) and SA<sub>Na</sub> emulsion (B) after a freeze/thaw cycle. Proteins are in green (labelled with fast green) and fat is in red (labelled with Nile red).

After this rapid screening, SA<sub>9</sub> and SA<sub>Na</sub> were not retained for further studies because they exhibited low ability to stabilize emulsions during heating and they gelled during the freeze/thaw cycle. In contrast, the WPM A and SA<sub>4</sub> were selected for the following studies. Emulsions prepared with WPM A and SA<sub>4</sub> were more heat stable than the other emulsions and they kept a fluid aspect after a freeze/thaw cycle, even if trace of oiling off was observed for WPM A emulsion.

### 5.2.3 Composition and characterization of the selected aggregate suspensions

#### 5.2.3.1 Composition of the aggregate suspensions

The mechanism of formation of the WP aggregates is usually not complete (presence of residual native proteins) and side aggregation reactions leading to aggregates other than targeted aggregates may also occur. Hence, the suspensions of WP aggregates may contain a mixture of molecular and supramolecular entities. In the cases of SA<sub>4</sub> suspension, the presence of residual native WP in addition to strand-like aggregates was reported by Mehalebi, Nicolai & Durand (2008). SA and residual native WP were reported in the WPM suspension (Donato et al., 2009). The WP powder used in this study also contained some caseins (around 18 % on a protein weight basis), that could be incorporated in the aggregates or leaved as “free” caseins (noted caseins in the following) in the SA suspension and WPM A suspension.

A quantification of each entity in the aggregates suspensions prepared with the WP powder A was performed and is addressed in Figure 27.

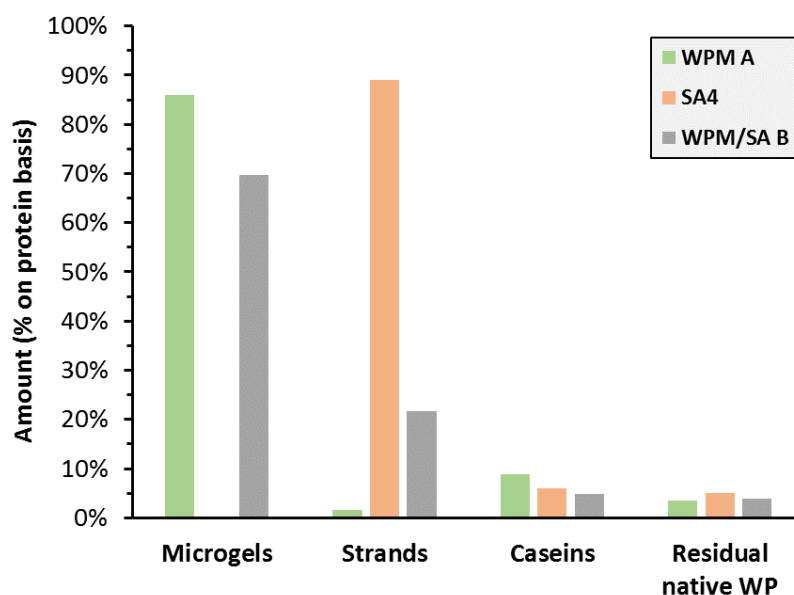


Figure 27: Proportion of the entities in the selected aggregates suspensions: WPM A suspension (green), SA<sub>4</sub> suspension (orange) and WPM/SA B suspension (grey).

The SA<sub>4</sub> suspension was composed of 89 % (on weight protein basis) of strand-like aggregates, 6 % of caseins and 5 % of residual native WP. The WPM A suspension was composed of 86 % WPM, residual native WP (3.6 %) and caseins (8.8 %) (Fig 27). It contained also some strand-like aggregates (1.6 %). The presence of strand-like aggregates in WPM suspension was suggested to be due to the pH increase occurring during the WPM formation (Donato et al., 2009). The obtained WPM conversion rate was in line with other published studies on WPM formation (Destribats et al., 2014; Phan-Xuan et al., 2011). The WPM A suspensions contained more caseins that was not incorporated in the aggregates but less residual native WP than the SA<sub>4</sub> suspensions. Note that the proportion of caseins was lower than in the WP powder due to the presence of some caseins in the WP aggregates.

One limitation of the method of quantification of the different protein fractions in the suspensions is that the mean protein extinction coefficient used to determine the protein concentration in the WPM A suspension and in its supernatant after removing the WPM by centrifugation was considered the same; it was based on the protein composition of the powder. However, the proteins composition in the whole suspension and in its supernatant of centrifugation was different; the proportion of caseins was higher in the supernatant. The extinction coefficient is lower for the caseins (0.81 L/g/cm; Pitkowski, Durand & Nicolai, 2008) than for the WP. Therefore, the protein concentration in the supernatant was slightly

overestimated. Hence, the amount of WPM which was determined by difference between the total protein concentration and the concentration of the proteins in the supernatant was slightly underestimated in favor of strand-like aggregates (residual native WP and caseins were determined independently using calibration curves). However, SDS-PAGE analysis of the supernatant of the WPM A suspension after centrifugation confirmed the presence of a significant amount of strand-like aggregates (one band in the stacking gel and a smear at the top of the gel under non-reducing condition indicated the presence of aggregates). The strand-like aggregates were dissociated mainly as  $\beta$ -Lg and  $\alpha$ -La monomers but also caseins under reducing conditions (Fig 28).

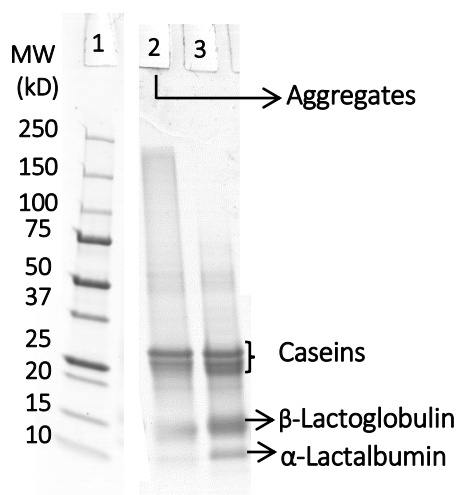


Figure 28: SDS-PAGE of the proteins in the supernatant of WPM A suspension under non-reducing conditions (2) or under reducing conditions (3). Molecular weight marker (1).

In order to test the reproducibility of the procedure of formation of the WPM and the strand-like aggregates, another whey protein powder (WP powder B) was used as starting material. The technological scheme used to obtain the WP powder B was different than the one used to obtain the WP powder A but the two powders had the same protein content ( $\sim 90$  wt%) and the same manufacturer reference. For confidential reasons we did not have access to both technological schemes. The amounts of caseins and  $\text{Ca}^{2+}$  were lower in WP powder B compared to WP powder A (see section 3.1). Exactly the same protocol was applied to prepare WPM suspensions and  $\text{SA}_4$  suspensions with WP powder B. Surprisingly, it was not possible to obtain the same aggregates with the same protocols using WP powder B although protein powder content was almost similar in the two powders. In fact, after 1 h of heat treatment, the WP suspension at 4 wt% protein (pH 5.8) gelled (Fig 29). According to Bovetto et al. (2007), the pH of formation of the WPM could evolved with the composition of the WP powder. Phan-Xuan et al. 2011 indicated that in the presence of  $\text{Ca}^{2+}$ , the pH of formation of

## CHAPTER 5: Selection, composition and characterization of the structure and functional properties of whey protein aggregates.

the WPM was shifted toward 7. To determine the optimal pH of the WPM formation with WP powder B, the heat treatment was applied at different pH from 5.8 to 7 (Fig 29).

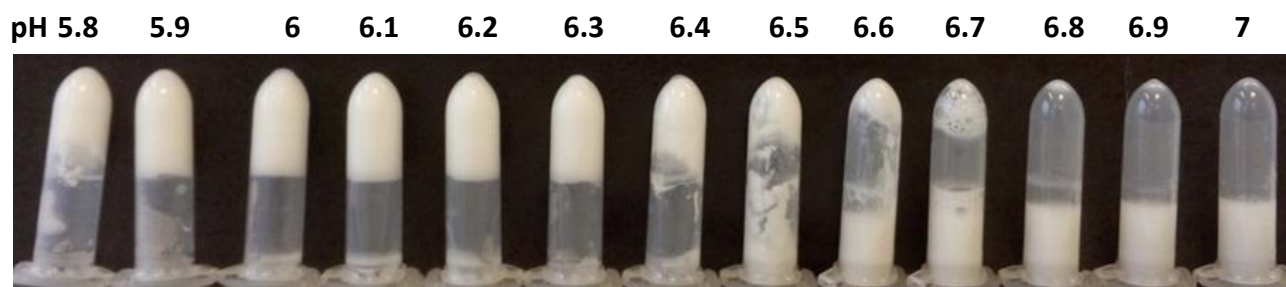


Figure 29: Picture of the whey protein B suspension at different pH after heat treatment.

Below pH 6.5, the WP suspensions gelled during the heat treatment. At pH 6.5 and 6.6, the suspensions were opaque after heating which is one of the characteristics of the WPM suspensions. However, at pH 6.5 some particles settled out on ageing, suggesting their aggregation due to too low electrostatic repulsions between them. At pH 6.7 and above, the heated WP suspensions were more turbid than opaque, suggesting a mixture of different aggregates. In fact, as shown by Phan-Xuan et al. (2011), the pH had a crucial importance in the formation of WPM. Above a critical pH, the heated suspension could contain a mixture of strand-like aggregates and WPM. Moreover, an analysis of the particle size distribution showed that only the suspension at pH 6.6 had a mono-modal size distribution. So, it seems possible to produce a WPM suspension with WP powder B at pH 6.6. A suspension of strand-like aggregates could not be obtained with WP powder B in the pH range tested. No translucent suspension which is characteristic of SA suspensions was observed at any pH.

An analysis of the WPM B suspension indicated it was composed of 69.8 % of WPM, 21.6 % of aggregates other than WPM (assumed strand-like), 4.8 % of caseins and 3.8 % of residual native WP (Fig 27). This suspension was considered as a mixture of WPM and SA, so it was called WPM/SA B suspension in the following. The high proportion of SA was confirmed by a SDS-PAGE analysis of the supernatant of WPM/SA B suspension (Fig 30). The observation of one band in the stacking gel and a smear at the top of the separating gel under non-reducing condition indicated the presence of aggregates. The increase in the bands intensity corresponding to the monomers of  $\beta$ -Lg et  $\alpha$ -La under reducing condition showed that they were composed of WP. Interestingly these aggregates seemed to be mainly composed of  $\alpha$ -La.



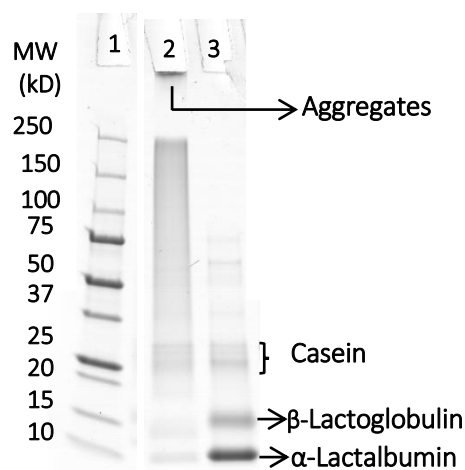


Figure 30: SDS-PAGE of the protein in the supernatant WPM/SA B suspension under non-reducing conditions (2) or under reducing conditions (3). Molecular weight marker (1).

Based on the above results, four protein suspensions were selected for studying their ability to stabilize emulsion during technological treatments i.e. heating, freezing/thawing:

- WPM A suspension and SA<sub>4</sub> suspension which contained mainly one type of aggregates and a small amount of residual native WP and caseins;
- WPM/SA B suspension that was composed of a mixture of WPM and strand-like aggregates and a small amount of residual native WP and caseins;
- WPM AC suspension, which was a purified fraction of WPM by centrifugation of the WPM A suspension

As far as we know, a purified fraction of SA was not attainable at pilot scale as easily as for WPM purified fraction, and for this reason was not selected for emulsion formation.

#### 5.2.3.2 Structural characterization of the aggregates

The aggregate suspensions were characterized in terms of size distribution and surface charge by dynamic light scattering and by  $\xi$ -potential measurements, respectively. The results are shown in Table 3 and Figure 31.

Table 3: Z-average, size distribution,  $\xi$ - potential of aggregates suspensions

	WPM A	WPM AC	SA <sub>4</sub>	WPM/SA B
<b>Z-Average (nm)</b>	300 ± 5	330 ± 30	80 ± 2	235 ± 25
<b>Distribution (nm)</b>	120 - 950	100 - 1200	20 - 340	100 - 1000
<b><math>\xi</math>- potential (mV)</b>	-17.1 ± 0.6	-	-14.4 ± 1.2	-



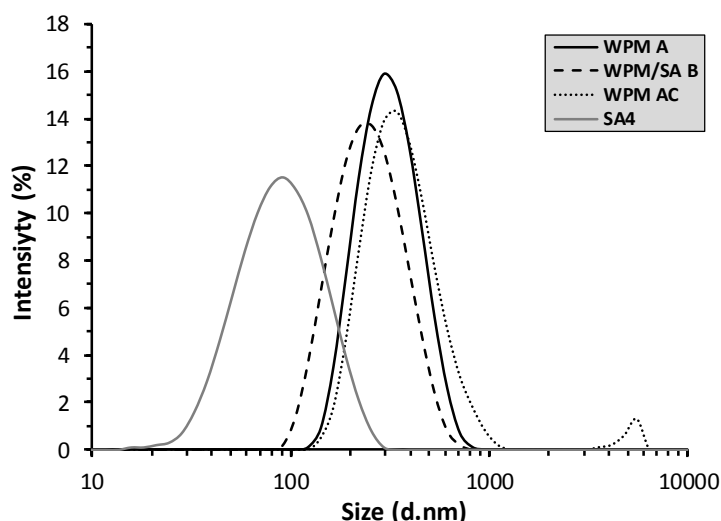


Figure 31: Particle size distribution of the WPM A suspensions at 3% (black line), of the WPM AC suspensions at 6.8 wt% (dotted black line), of the SA<sub>4</sub> suspensions at 3 wt% (grey line) and of the WPM/SA B suspensions at 3 wt% (dashed black line).

The Z-average diameter of the WPM A (300 nm) was in agreement with previous published results on WPM (Destribats et al., 2014; Sarkar et al., 2016; Schmitt et al., 2010). The Z-average diameter of the WPM after centrifugation of the WPM A suspension called (i.e. after removing the caseins and the WP not included in the WPM) was slightly larger (330 nm) than in the WPM A suspension probably due to the procedure used for their recovery that could select the largest entities. In addition, a small intensity peak was observed for particles with large hydrodynamic radius. It probably came from insufficient dispersion of the WPM after centrifugation (see below). The Z-average diameter of SA<sub>4</sub> was around 80 nm and was in accordance with Mahmoudi et al. (2007). The Z-average diameter of the particles in the WPM/SA B suspension was intermediate between WPM suspension and SA<sub>4</sub> suspension. As indicated by Phan-Xuan, Durand & Nicolai (2011), the increase in the pH of WPM formation could decrease the WPM size. It is also probable that the presence of strand-like aggregates that are smaller in size than the WPM in the WPM/SA B suspension shifted the Z-average of the particles to smaller value. As dynamic light scattering was not the most appropriate method to determine the size distribution of a mixture of aggregates, the analysis of the WPM/SA B suspension was performed by A4F-MALLS and compared to other aggregate suspensions (Fig 32). The WPM/SA B suspension was analyzed before and after centrifugation, in order to observe with more precision the soluble aggregates. The fraction analyzed after centrifugation was called WPM/SA B sup. The separation profiles of the WPM A and WPM/SA B suspensions showed that the WPM contained in the two suspensions seemed eluted between 35 and 70 min. Moreover, the molar mass (MM) was in the same range (Table 4) suggesting that WPM observed in the two suspensions had very similar structure. The separation profile obtained for the WPM/SA B supernatant showed one peak eluted just after ~ 20 min

corresponding of the elution of strand-like aggregates. These results confirmed that two types of aggregates were present in the WPM/SA B suspension. They also suggested that the aggregates in the supernatant of WPM/SA B suspension had a structure comparable to strand-like aggregates in SA<sub>4</sub> suspension but with a slightly smaller MM. To confirm the analysis, it would be interesting to recover each eluted fraction for an observation by TEM.

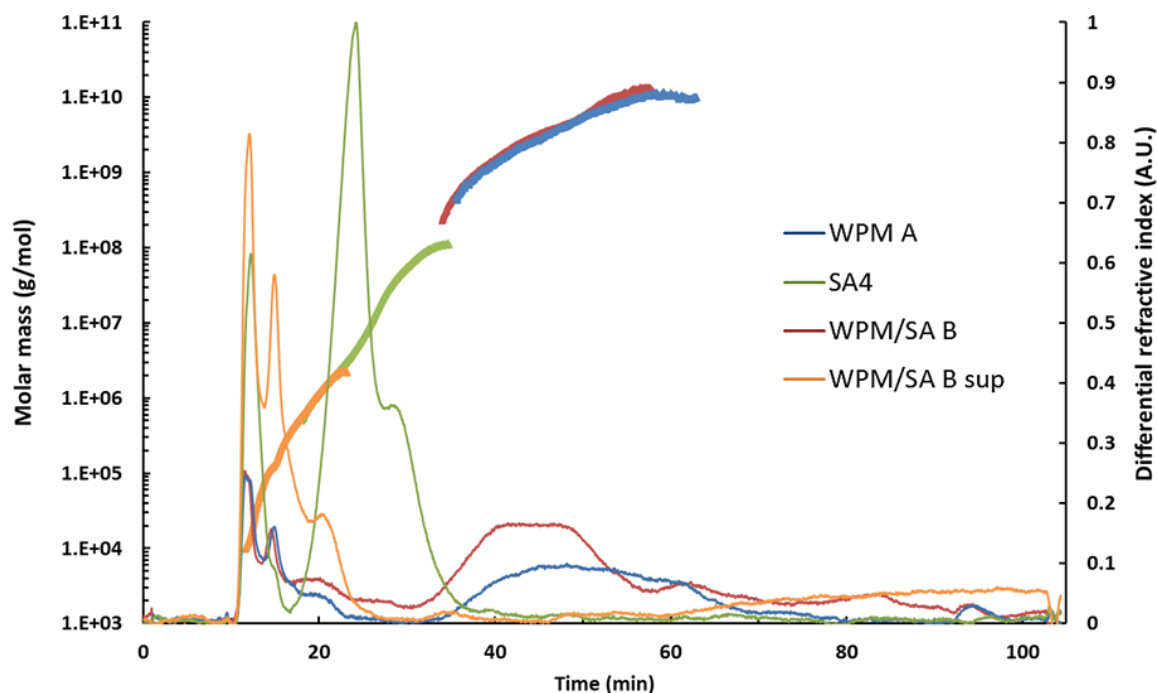


Figure 32: Separation profiles of the whey protein aggregates suspensions using asymmetrical flow field-flow fractionation coupled with multiangle laser light scattering (AF4-MALLS). Thin line, differential refractive index measurements; Thick line, online calculated molecular mass

Overall, the aggregate gyration radius determined by A4F-MALLS was in accordance with the results obtained in DLS (Table 4). The gyration radius of the WPM in the WPM/SA<sub>4</sub> was larger than the one observed by DLS. The strand-like aggregates in the WPM/SA B suspension could have a higher influence on the size determined by DLS.

Table 4: Molar mass and gyration radius calculated for the aggregates suspension in A4F.

	WPM A	SA <sub>4</sub>	WPM/SA <sub>4</sub>
MM (g/mol)	5.9E+09	1.9E+07	3.7E+09
% Standard deviation	1.3	4.8	15.7
Rg (nm)	193.3	31.1	172.7
% Standard deviation	2.3	4.4	2.2

The  $\xi$ -potential values of the particles in WPM A and SA<sub>4</sub> suspensions were in the same range (Table 3). The value is mainly defined by the dispersed phase of the aggregates, that was milk ultrafiltration permeate in both cases.

The morphologies of the aggregates (Fig 33) confirmed the presence of mainly WPM (Fig 33A) and strand-like aggregates with a very low level of branching (Fig 33B), respectively. Their size was in agreement with the Z-average diameter determined by dynamic light scattering and with the results reported in the bibliography (Jung et al., 2008).

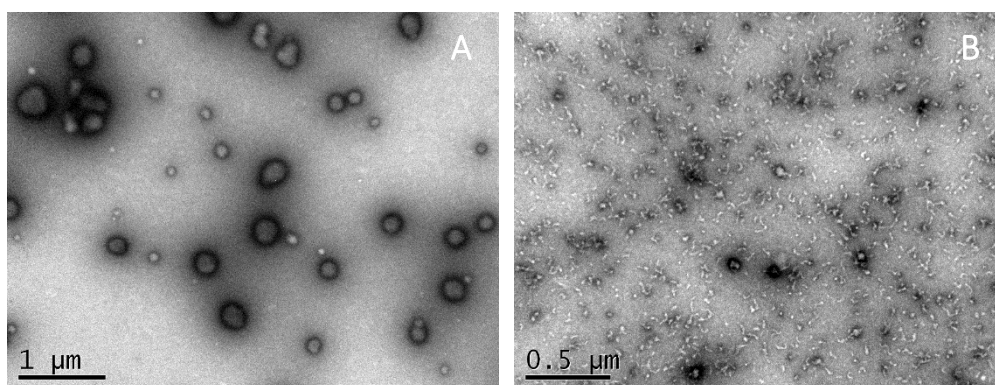


Figure 33: Negative-staining Transmission Electronic Microscopy (TEM) image of (A) WPM A suspension and (B) SA<sub>4</sub> suspension.

### 5.3 Functional properties of the aggregate suspensions

#### 5.3.1 Heat stability and freeze/thaw stability of the aggregate suspensions

The stability during technological treatments of emulsions containing protein coated-fat droplets is mainly dependent on the protection brought by the protein adsorbed at the fat droplet surface but also on the stability of the proteins in the dispersed phase. For example, it has been shown that the heat stability of whey protein-stabilized fat droplets is dependent on the concentration of native WP in the dispersed phase (Çakır-Fuller, 2015; Dybowska, 2011; Euston et al., 2000). These authors showed that above a critical concentration, the WP gelled on heating (Çakır-Fuller, 2015; Dybowska, 2011; Euston et al., 2000; Hunt & Dalgleish, 1995; David Julian McClements, 2004; Sural et al., 2014). During the freezing stage, the ice crystal growth increases the ionic strength and concentrates the aggregates in the remaining liquid phase. These changes may destabilize the aggregates that form large particles after thawing (Hashizume, Kakiuchi, Koyama & Watanabe, 1971). Then, the properties (stability to heating and to freezing/thawing) of the aggregates suspensions in the milk ultrafiltration permeate were investigated as a putative source of emulsion instability. By this approach, the objective was to acquire knowledge on the stability of protein aggregates in order to discriminate if emulsion instability

originated from the adsorbed aggregates at fat droplets surface and/or the aggregates in the continuous phase.

### 5.3.1.1 Heat stability of the aggregate suspensions

The heat stability of the aggregate suspensions was evaluated in a large range of protein concentrations (from 2 wt% to 7.4 wt%) corresponding to the protein concentration range in the continuous phase of the emulsions (chapters 5 and 6).

First, the macroscopic destabilisation of the aggregate suspensions was evaluated visually and was related to a change of flowability, a gelation or the appearance of traces of aggregation. The greatest heating time allowing the obtaining of stable protein suspensions with regard to the above criteria was plotted as a function of the protein concentration in the suspensions (Fig 34). A heat stability of 30 min meant that the emulsion was visually stable after the longer heating time at 120°C investigated (30 min). A suspension of unheated whey proteins (WP A) was treated under the same conditions to highlight the beneficial of the structuration of the proteins into aggregates.

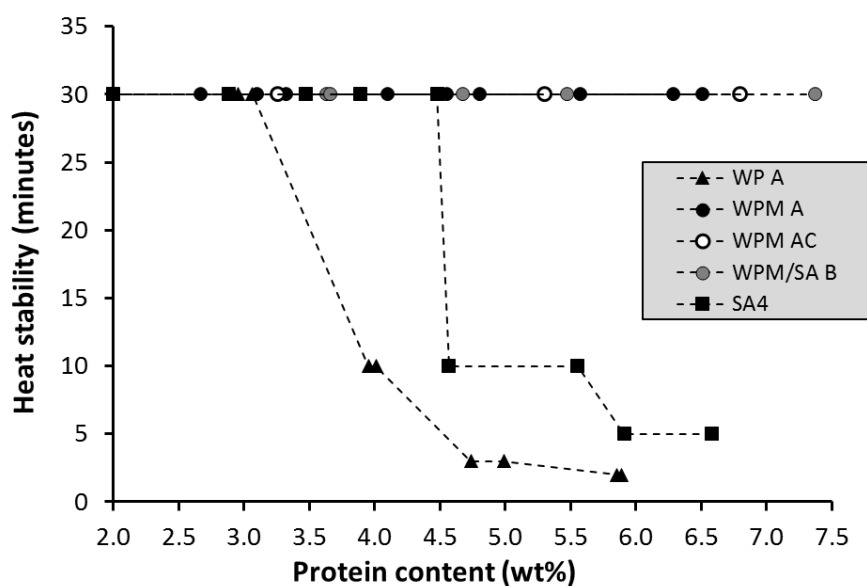


Figure 34: Heat stability of the protein suspensions: WP A suspension (black triangle), SA<sub>4</sub> suspension (black square), WPM A suspension (black circle), WPM AC suspension (open circle) and WPM/SA B suspension (grey circle).

At 4 wt% protein concentration and above, the WP A suspensions gelled. Below the critical concentration of gelation (between 3 and 4 wt%), the DLS analysis showed that some aggregates were formed during heating: the Z-average diameter of the particles in a WP A suspension at 3 wt% in the milk ultrafiltration permeate was  $275 \pm 2$  nm after 30 min of heating at 120°C.

# CHAPTER 5: Selection, composition and characterization of the structure and functional properties of whey protein aggregates.

A similar behavior was observed for heated SA<sub>4</sub> suspensions, but the gelation concentration range was shifted toward higher protein concentration (> 4 wt%). SA<sub>4</sub> suspensions at low protein concentrations ( $\leq 4.5$  wt%) were visually stable for more than 30 min at 120°C (Fig 34) but an increase in the Z-average diameter of the aggregates was observed by DLS: the Z-average diameter of the aggregates in the SA<sub>4</sub> suspension at 4.5 wt% proteins was  $152 \pm 1$  nm after 30 min of heating at 120°C instead of  $80 \pm 2$  nm before heating.

In contrast, the WPM in WPM A, WPM AC, WPM/SA B suspensions were visually heat stable up to 30 min and corroborated the results of Donato et al (2007). However, the Z-average diameter of the WPM in the WPM AC suspension exhibited an increasing trend during heating. This trend is not observed in the WPM A (Fig 35) and WPM/SA B suspension. A previous study (Liu et al., 2016) indicated that WPM heated in the dispersed phase of milk in the presence of caseins and native WP are able to form large aggregates with a size between 1 and 100  $\mu\text{m}$ . Such heat-induced changes were contradictory to our observations suggesting that the amounts of caseins and native WP in WPM A and WPM/SA B suspensions were probably too low. In the opposite, WPM aggregation was only observed after the removal of the caseins and the residual native proteins from the WPM A suspension by centrifugation.

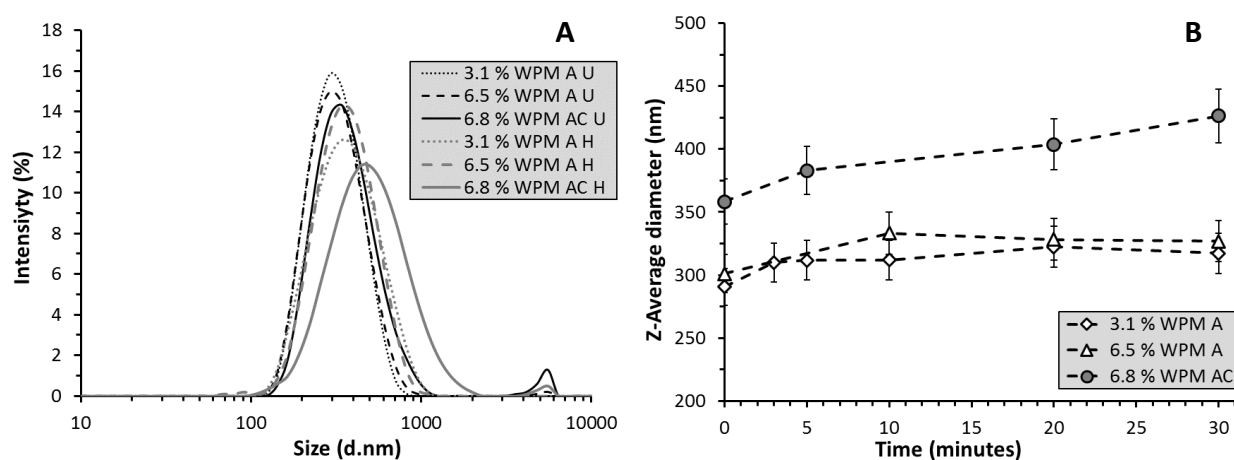


Figure 35: (A) Particle size distribution in the WPM suspensions before heat treatment (black line) and after heat treatment (grey line) at various protein concentrations (3.1 wt%, dotted line; 6.5 wt%, dashed line) and in the WPM AC suspension (6.8 wt%, continuous line). (B) The evolution of the Z-average of the particles in the WPM A suspensions as at various proteins concentrations (3.1 wt%, open diamond; 6.5 wt%, open triangle) and in WPM AC suspension (6.8 wt%, grey circle) a function of heating time at 120°C. The dotted lines are guides to the eye.

No sign of aggregation was identified in the WPM/SA B suspension although it contained a fraction of SA (~ 22 %) in addition to WPM. However, the amount of SA in the WPM/SA B suspension only reached

1.6 % for the highest protein concentration tested (7.4 wt%). Therefore, the amount of SA was probably too low to create a visible aggregation in WPM/SA B suspensions.

Compared to unheated proteins, a pre-aggregation of the proteins shifted the heat stability of protein suspensions toward higher concentration. The heat stability of the protein suspensions depended on the structure of the aggregates. Only WPM suspensions remained fluid in the whole range of protein concentration tested after heating.

### 5.3.1.2 Freeze/thaw stability of the proteins suspensions

The freeze/thaw stability of the aggregates suspensions adjusted at different concentrations was tested after a storage at  $-18^{\circ}\text{C}$  ( $\geq 1$  week). All the aggregate suspensions appeared fluid and no sign of aggregation of the particles was detected by DLS after thawing (Fig 36).

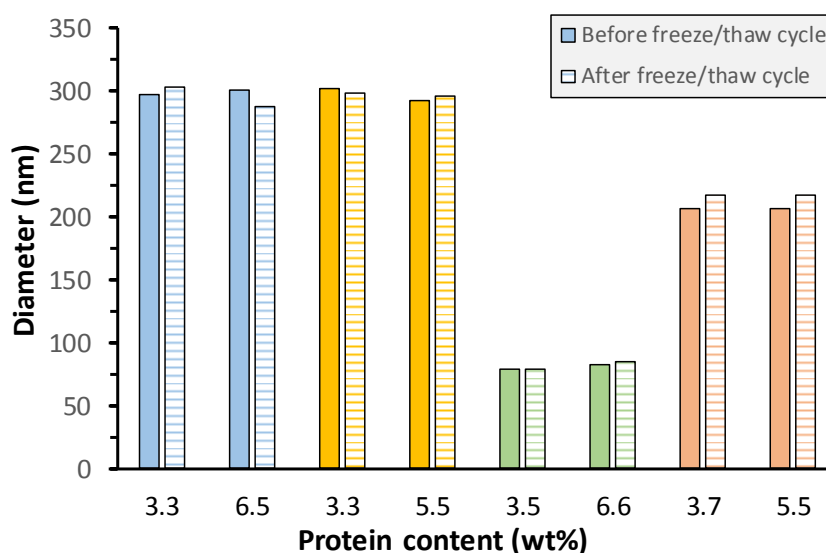


Figure 36: Size of the particles before (full rectangle) and after freezing/thawing cycle (striped rectangle) in WPM A suspensions (blue), WPM AC suspension (yellow), SA<sub>4</sub> suspension (green) and WPM/SA B suspension (orange).

### 5.3.2 Emulsifying capacity of the aggregate suspensions

The emulsifying capacity of the aggregate suspensions determines the size of the fat/oil droplets obtained. At a given concentration, the more the interfacial area created during homogenization, meaning the smaller the size of the droplets generated, the greater is the protein emulsifying ability (Ye, 2011). The protein emulsifying ability is mainly governed by the structure and the flexibility of the protein (Euston & Hirst, 1999; Kato & Yutani, 1988, 1988). Euston & Hirst (1999) showed the impact of the structure of the casein micelle on its emulsifying capacity. It was demonstrated that the

demineralization of the casein micelle improved its emulsifying capacity probably by enhancing its spreading at the interface (Lazzaro et al., 2017). The preheating of  $\beta$ -Lg or WP suspensions leading to the formation of aggregates before emulsification decreased the emulsion capacity of the proteins (Dybowska, 2011; Moro et al., 2013). Similarly, the interfacial area occupied by the aggregates may change depending on their structure and their ability to spread at interface. We measured this property by using a model interface (air-water interface). In parallel, the emulsifying capacity of the aggregates was evaluated from the size of the fat droplets generated by using a standardized process for forming the emulsions.

### 5.3.2.1 Interfacial properties of the protein suspensions at the air-water interface

The interfacial properties of the aggregates were determined by measuring the surface pressure ( $\Pi$ )–surface area (A) isotherm at the air–water interface on a Langmuir balance (Fig 37A). Different amounts of proteins were spread at the air–water interface for being in the range of measurement of the equipment. For a sake of comparison, the surface areas were normalized by the amount of proteins spread at the air–water interface. The results obtained for the aggregates in the WPM A suspension and the SA<sub>4</sub> suspension were compared to the one obtained for the unheated proteins (WP A suspension).

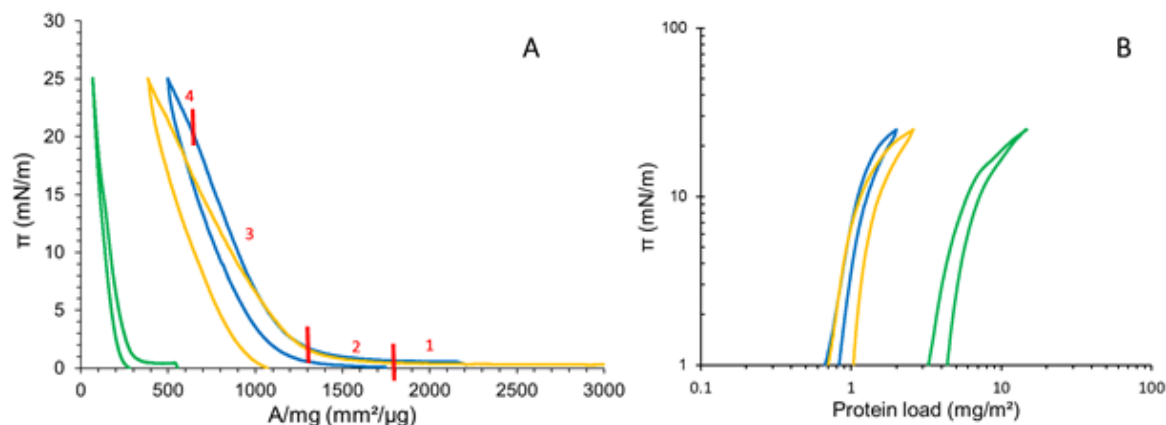


Figure 37:  $\pi/A$  isotherm (A) and  $\pi$ /Protein load isotherm (B) of WP A suspension (blue line), for SA<sub>4</sub> suspension (yellow line) and for WPM A suspension (green line) at air–water interface

Typical protein isotherms characterized by a gaseous phase, a liquid phase, a compressed phase and a saturated phase from high to low surface area (phases 1, 2, 3, and 4 on the compression isotherm, respectively) were observed. These phases are identified on the surface pressure ( $\Pi$ )–surface area (A) isotherm of unheated WP (Fig 37A). The isotherms for unheated proteins and for stand-like aggregates (SA<sub>4</sub>) was almost equivalent (Fig 37A) at least in the phases 1 and 2 during the compression of the

interface. The same protein surface coverage is required to induce an increase in the surface pressure. In other words, the amount of strand-like aggregates and unheated proteins at the air-water interface for observing an increase in the surface pressure was similar (Fig 37B). This suggests that the strand-like aggregates did not adsorb randomly at the air-water interface but spread as starfishes on the interface rather than having some strands extended into the aqueous solution. The slope of the phase 3 of the compression isotherm and the hysteresis (gap between the compression curve and the decompression curve) differed between SA<sub>4</sub> and unheated proteins. An interface composed of SA<sub>4</sub> was more compressible than of unheated proteins and the hysteresis was also more pronounced. The hysteresis reflects the presence of attractive interactions within the interfacial film. These interactions originate from the presence of attractive groups on the surface of the proteins or aggregates, and exposed by the conformational changes occurring during their adsorption at the interface. The results indicated that the interactions are stronger or more numerous in an interface made of strand-like aggregates than of unheated WP.

The phase transitions on the compression isotherm of the WPM A suspension were shifted to lower surface area. The protein density in the WPM maybe an explanation for this particular behavior. For the same amount of proteins located at the interface, the amount of surface covered by the WPM is lower than for unheated WP and SA<sub>4</sub> (Fig 37A). This indicated that the WPM did not spread completely at the air-water interface otherwise a similar behavior than for the other suspensions would be obtained.

In addition, the slope of the compression isotherm was more than 3 times steeper than for the unheated WP. The slope of the isotherm compression and the weak hysteresis suggested that the WPM deformability and the attractive interactions between the WPM at the interface were low. These results were not in line with other published studies on WPM properties at fluid interface (air-water) (Murphy et al., 2016). These authors showed that the WPM isotherm tended to be similar to the native WP isotherm suggesting the ability of the WPM to disrupt at the interface under low surface load condition (gaseous phase). However, these authors indicated that smaller WPM (with an average hydrodynamic diameter around 200 nm) rearranged more rapidly than larger ones (with an average hydrodynamic diameter around 300 nm). In the WPM A suspension, WPM had the characteristics of large WPM regarding their average hydrodynamic diameter ( $300 \pm 5$  nm).

#### 5.3.2.2 Emulsifying capacity of the protein suspensions

Emulsions with 30 % (v/v) of milk fat and 70 % (v/v) of aggregate suspensions (WPM A suspension, WPM AC suspension, SA<sub>4</sub> suspension and WPM/SA B suspension) at different protein concentrations were prepared. Emulsions prepared in the same conditions with WP A suspensions were used as control. The



procedure of emulsion formation consisted of a pre-emulsification of the milk fat and the proteins suspensions followed by a homogenization of the mixture at 5 MPa (5 passes). The emulsifying capacity of the protein suspensions were characterized regarding the average volume diameter of the fat droplets in the presence of SDS (Fig 38).

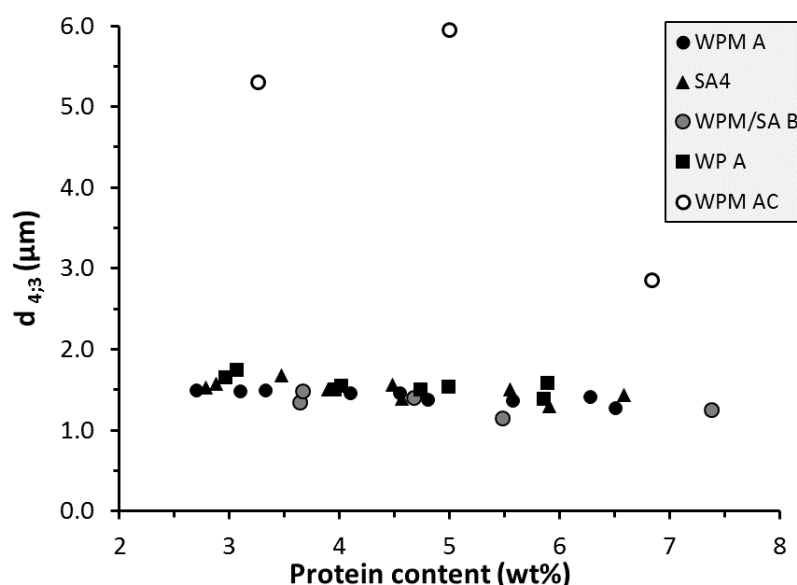


Figure 38: Fat droplet size ( $d_{4,3}$ ) of WP A emulsions (black square), SA4 emulsions (black triangle), WPM A emulsions (black circle), WPM AC emulsions (empty circle) and WPM/SA B emulsions (grey circle) at various protein concentrations in the dispersing phase

The average volume diameter ( $d_{4,3}$ ) of the fat droplets in the presence of SDS was reported in Figure 38. The average volume diameter of the fat droplets at constant protein concentration was rather constant for all emulsions except those made with the WPM AC suspensions. Increasing the protein concentration in the emulsions induced a slight decrease in the  $d_{4,3}$ . Consequently, the emulsifying capacity of the aggregates suspensions was similar between the aggregate suspensions except for the WPM AC suspension. The comparison of the fat droplets  $d_{4,3}$  of the WPM A suspensions and the WPM AC suspensions suggested that the caseins and the residual native proteins played a key role in the emulsifying capacity of the aggregate suspensions. They were responsible for the major fat droplet surface created during the homogenization stage.

#### 5.4 Impact of the whey proteins powder composition on the structure of the aggregates

Two similar WP powders in term of protein purity but manufactured using two different technological schemes and stored under different conditions (duration, temperature) led to two different behaviors

when WP powders were dispersed in water and subsequently heated. The conditions of heating leading mainly to WPM in the WP A suspension (prepared with WP A powder) and induced the gelation of the WP B suspension (prepared with WP B powder). A compositional analysis of the two powders indicated slight differences, especially regarding the amount of calcium and caseins (Table 2, chapter 3).

To have a better understanding of the compositional elements of the WP powders that affect the structural properties of the heat-induced aggregates, complementary investigations were conducted. The impact of the slight difference of composition (casein, calcium) between the two WP powders was studied in order to know if they were at the source of the different behavior on heating (part 5.2.3).

#### 5.4.1 Casein effect

Caseins were more abundant in the WP A powder (18.4 %) than in the WP B powder (6.5 %). We first checked if the amount of caseins was a key parameter for the WPM formation at pH 5.8.

The influence of the presence of caseins in WP suspensions during heating has already been shown (Guyomarc'h et al., 2009). The caseins prevented the growth of the WP aggregates by a chaperone-like mechanism (Guyomarc'h et al., 2009; Morgan, Treweek, Lindner, Price & Carver, 2005; O'Kennedy & Mounsey, 2006; X. Zhang et al., 2005). Moreover, O'Kennedy & Mounsey (2006) indicated that micellar caseins promoted WP denaturation but controlled their aggregation. In both cases (mixture of individual caseins and small casein assemblies, and micellar caseins), increasing the casein amount in WP suspension would decrease the size of the heat-induced aggregates of WP.

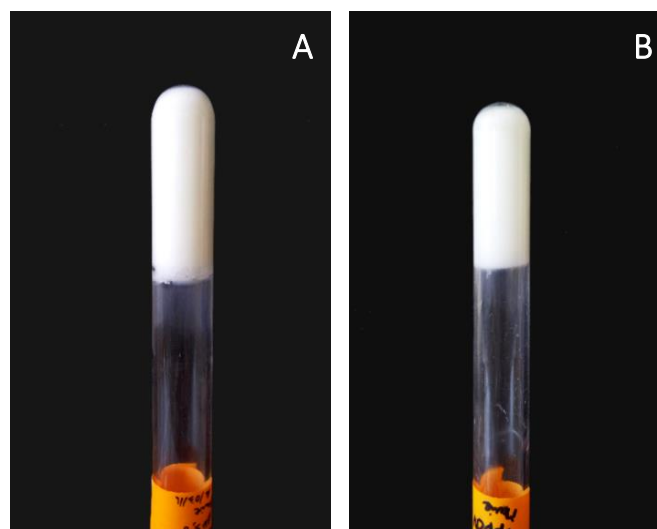


Figure 39: Pictures of 4 wt% of whey protein suspension B with an addition of CasNa (A) or an addition of MC (B)

## CHAPTER 5: Selection, composition and characterization of the structure and functional properties of whey protein aggregates.

The structure of the caseins (mixture of individual caseins and small assemblies of caseins or micellar caseins) in the WP powder A and WP powder B was unknown. However the presence of micellar caseins would be surprising as the SDS PAGE analysis of the WP powders mainly indicated the presence of casein  $\alpha_{s1}$ , casein  $\alpha_{s2}$ , casein  $\kappa$  but not of casein  $\beta$ . In order to test the influence of the amount of caseins on the structure of the aggregates obtained after heating WP suspensions at pH 5.8, the amount of caseins in WP B suspension (4 wt%) was increased to equal the amount of caseins in WP A suspension by adding either sodium caseinate (mixture of individual caseins and small assemblies of caseins) or micellar caseins. The WP B suspensions enriched in caseins were heat treated and after 1 h of heating at 80°C a gel was obtained in both cases (Fig 39). This indicated that the amount of caseins and the association state of the casein in the WP powders was not responsible for the different behavior of WP A and WP B suspensions on heating.

### 5.4.2 Calcium effect

The two powders also differed by their calcium content. Phan-Xuan et al. (2013) reported that the presence of three  $\text{Ca}^{2+}$  per protein in a 4 wt%  $\beta$ -Lg suspension at pH 7 allowed to obtain a particulate gel resulting from the association of  $\beta$ -Lg microgels. In fact, one of the main parameters for the formation of the WPM or  $\beta$ -Lg microgels is the net charge of the native proteins. Phan-Xuan et al. (2014) showed that  $\beta$ -Lg microgels were not formed when the net charge of the protein was highly negative, i.e. higher than -5. Under this condition, mainly strand-like aggregates were formed. On the other hand, if the amount of free  $\text{Ca}^{2+}$  was too high, the net charge of the protein is decreased and a gelation of the protein suspension could be observed. The pH and the amount of  $\text{Ca}^{2+}$  are the two main ways to control the net charge of the native proteins. However, in our study the pH of formation of the WPM was the same for the two WP suspensions (pH 5.8) and the amount of  $\text{Ca}^{2+}$  was lower in WP B powder than in WP A powder (see part 3.1 Table 2). Hence, the net charge of the proteins in the WP B suspension would be higher than in the WP A suspension which is in favor of the formation of strand-like aggregates but not a gel. However, the potential measurement gives the same value for the proteins in the two powders. The amount of  $\text{Ca}^{2+}$  in the WP powder does not seem to explain the observed behavior of WP B suspension on heating.

The heat-induced aggregation of WP suspensions depends on numerous factors; the impact of the composition and the storage conditions (chapter 4) of the powder were tested here. These studies were not able to explain the gelation of the WP B suspension at pH 5.8 whereas WP A suspension heated under the same condition lead to WPM. However, they are additional paths to explore. A recent

publication showed that the ratio between caseins,  $\text{Ca}^{2+}$  and WP constituted the key parameter to control WP aggregation (Nguyen et al., 2016).

## 5.5 Conclusion

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The main conclusions of chapter 5 are the following:

- Among the aggregates suspensions obtained from the WP powder A, the SA<sub>4</sub> suspension and WPM A suspension showed promising capacity to stabilize WP-rich emulsions during technological treatments, i.e. heating and freezing/thawing. They were selected for further studies. WPM AC suspension was also selected in order to investigate the influence of the other proteins naturally present in industrial WP powders on the emulsion stability.
- Another WP powder (WP powder B) containing different casein and calcium contents compared to the WP powder A allowed to obtain a mixture WPM and strand-like aggregates: the WPM/SA B suspensions. This mixture of aggregates was also tested for its capacity to stabilize WP emulsions.
- Fractal aggregates (SA<sub>9</sub> and SA<sub>Na</sub>) rather connected the fat droplets during emulsification giving viscous emulsions that gelled during the heat treatment and a freeze/thaw cycle. As one of the objectives of the project was to obtain fluid emulsions that were resistant to heat-treatment and/or exhibited a controlled stability during a freeze/thaw cycle, they were not selected for further studies.
- The selected aggregate suspensions showed the same emulsifying capacity except for the WPM AC suspension. At equal protein concentration, WPM covered less amount of interface than SA<sub>4</sub> and unheated proteins. This could explain the WPM AC suspension gave the coarser fat droplet dispersion. The interactions were stronger within the interfacial film of strand-like aggregates than of WPM.
- The properties of the aggregates suspensions indicated that the heat stability is dependent on the structure of the aggregates and the protein concentration. Aggregates suspensions containing WPM were extremely heat stable at all concentrations tested. Conversely, SA<sub>4</sub> suspensions gelled on heating when the concentration of proteins increased. On the other hand, WP aggregation did not impact the freeze/thaw stability of the suspensions. These results could be useful to understand the mechanism of emulsion stabilization and particularly the influence of the aggregates in the dispersed phase.

## CHAPTER 6: Stability of emulsions containing whey protein microgels revealing the beneficial impact of caseins

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The stability of emulsions containing whey protein microgels (WPM) was investigated during technological treatments i.e. heat treatment (120°C) and freeze/thaw cycle. It is known that WPM exhibit excellent ability to stabilize emulsions against coalescence. However, information relating to their capacity of protection during technological treatments is still lacking. The heat stability of the emulsions prepared with the whole WPM A suspensions at various protein concentrations and the WPM A suspension after removing the caseins and the WP not included in the WPM by centrifugation (WPM AC suspension) were compared. The emulsion prepared with the WPM AC suspension was less heat stable than the emulsions prepared with the whole WPM A suspensions. The heat stability of the emulsions prepared with the whole WPM A suspensions was dependent on the protein concentration of the emulsion. Emulsions were more heat stable at high protein concentrations (6 wt%) than at low protein concentrations (3 wt%). From an analysis of the protein composition at fat droplet surface and emulsion observations using electronic microscopy, we suggested that WPM at the fat droplet surface were responsible for a gelation of the emulsion. Interestingly, by taking advantage of the competition between proteins for interface, WPM adsorbed at fat droplet surface can be replaced by caseins and emulsion heat stability was improved. Therefore, these results showed the crucial role of a small amount of caseins in the heat stability of the WPM emulsions. The presence of caseins also contributed to the freeze/thaw stability of the WPM emulsions. A part of these results has been published in the following paper: Chevallier, M., Riaublanc, A., Lopez, C., Hamon, P., Rousseau, F., Thevenot, J., & Croguennec, T. Increasing the heat stability of whey protein-rich emulsions by combining the functional role of WPM and caseins. *Food Hydrocolloids*, in press.

### The main aims of the work presented in this chapter were to:

- Quantify the stability of emulsions prepared with the whey protein microgels during heating and a freeze/thaw cycle
- Demonstrate the competitive adsorption of the proteins at fat droplet surface
- Evaluate the contribution of the proteins adsorbed at the fat droplet surface and the proteins in the continuous phase to emulsion stability
- Offer suggestions to control the stability of emulsions containing whey protein microgels

## Table of contents

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<b>CHAPTER 6: Stability of emulsions containing whey protein microgels revealing the beneficial impact of caseins.....</b>	<b>95</b>
<b>6.1 Introduction .....</b>	<b>97</b>
<b>6.2 Heat stability of the emulsions .....</b>	<b>98</b>
6.2.1 Characteristics of the unheated emulsions prepared with WPM A and WPM AC suspensions.....	98
6.2.2 Characteristics of the heated emulsions prepared with WPM A and WPM AC suspensions.....	101
6.2.3 Influence of fat droplet surface composition.....	104
6.2.4 Mechanism of emulsion stabilization.....	106
6.2.4.1 Influence of the adsorbed proteins on the heat stability of the emulsions .....	106
6.2.4.2 Capacity of the WPM to bridge fat droplet surface .....	112
6.2.5 Design of heat-stable emulsions containing WPM .....	115
<b>6.3 Freeze/thaw stability of the emulsions .....</b>	<b>117</b>
<b>6.4 Conclusion.....</b>	<b>120</b>

## 6.1 Introduction

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Consumers demands in terms of food constantly evolve at the pace of scientific knowledge with recent concerns for more natural (Li & Nie, 2016) and healthy products (Mollet & Rowland, 2002). Due to their functional properties and exceptional biological value, e.g. richness in essential amino acids, binding properties for vitamins, minerals and fatty acids (Morr & Ha, 1993; Smithers, 2008), there is a trend to increase the use of WP in a large range of food products such as emulsion beverages (Çakır-Fuller, 2015). These products often undergo one or several technological treatments as freezing (-18 °C) or heating (72 or 120 °C) to extend their shelf life and to ensure their microbial stability.

WP-rich emulsions are heat sensitive due to the rapid denaturation/aggregation of WP at temperatures above 70°C (Griffin et al., 1993; Roefs & De Kruif, 1994; Sawyer, 1968; Verheul et al., 1998). The heat-induced modifications could have detrimental consequences on the final appearance of the products. In liquid dairy products containing fat droplets, i.e. dairy emulsions, heat-treatments can cause the aggregation of the fat droplets and even the gelation of the emulsions if WP concentration and/or the fat droplet volume fraction are too high (Çakır-Fuller, 2015; Demetriades et al., 1997b; Euston et al., 2000; Hunt & Dalgleish, 1995; Jost, Baechler & Masson, 1986; Yamauchi, Shimizu & Kamiya, 1980; Yost & Kinsella, 1992). It has been shown that the heat stability of WP emulsions is dependent both on the heat stability of the proteins adsorbed at the fat droplet surface and on the concentration and heat stability of the proteins (native or aggregated) in the continuous phase (Çakır-Fuller, 2015; Dybowska, 2011; Euston et al., 2000). The presence of WP aggregates instead of native WP in the continuous phase of the emulsion improves emulsion heat stability at high protein concentrations (Çakır-Fuller, 2015). The ability of adsorbed WP aggregates to stabilize fat droplets on heating was less studied and requires further investigations to be fully understood.

Emulsions prepared with WP dispersed in water were stable up to the freezing temperature (i.e. -5°C) but they were destabilized by ice crystal growth when the temperature decreased further (Thanasukarn et al., 2004b). The addition of sucrose to WP emulsion resulted in an improving of its stability during a freeze/thaw cycle (Thanasukarn et al., 2004a). This was confirmed by the preliminary results obtained in this work which revealed that the WP A emulsions were stable to freezing at -18°C and subsequent thawing (see chapter 2). WP form a thick and viscoelastic interface (Dickinson, 2001) that stabilizes the fat droplets against various stresses. This constitutes an advantage for emulsions that have to resist freeze/thaw cycles but a drawback for instance in the process of making ice cream, in which a controlled destabilization of the fat droplets is required. To the best of our knowledge, information on the ability of the WP aggregates to stabilize emulsion during freeze/thaw cycle is still lacking.

WPM stabilize emulsion droplets with exceptional resistance to coalescence (Destribats et al., 2014). Under certain conditions, the WPM spread at the fat droplet interface (Destribats et al., 2014), and their structure can be disintegrated into small aggregates (Murphy et al., 2016). These rearrangements or the preferential orientations of the WPM at the fat droplet surface could modify their behavior during technological treatments. Emulsions stabilized by soft WPM particles but produced differently than in the previous studies (Destribats et al., 2014; Murphy et al., 2016), exhibited a heat induced flocculation of neighboring fat droplets by a mechanism of fusion of adsorbed particles (Sarkar et al., 2016).

The objective of the work was to study the heat stability and freeze/thaw stability of oil-in-water emulsions prepared with heat-stable WPM in a large range of WP concentrations. A specific emphasis was placed on the contributions of the WPM adsorbed at the fat droplet surface and the WPM dispersed in the continuous phase of the emulsion. The role played by residual caseins in the stability of the emulsion was also investigated.

## 6.2 Heat stability of the emulsions

### 6.2.1 Characteristics of the unheated emulsions prepared with WPM A and WPM AC suspensions

Various emulsions were prepared with the whole WPM A suspensions at different protein concentrations ranging from 2.7 wt% to 6.5 wt%. By increasing the protein concentration of the emulsions, the amount of caseins and WP not included in the WPM increased proportionally to the WPM A suspension concentration. Two additional emulsions were prepared with the WPM AC suspension (i.e. WPM A suspension after centrifugation in order to remove the caseins and WP not included in the WPM), one at 3.2 wt% and the second at 6.8 wt% proteins. The emulsions were fluid, except the emulsions prepared with the WPM AC suspensions that were highly viscous and creamed slowly. The small volume of dispersed phase released during the creaming had a very low turbidity (Fig 40) compared to the turbidity of the WPM AC suspensions indicating that the WPM were almost completely in interaction with the creamed phase.



Figure 40: Picture of the emulsion prepared with the WPM AC suspension at 3.2 wt% proteins. A creamed layer appeared at the top rapidly (less than one hour).



CHAPTER 6: Stability of emulsions containing whey protein microgels  
revealing the beneficial impact of caseins

The size (volume mean diameter  $d_{4,3}$ ) of the fat droplets in the emulsions prepared with the WPM A and WPM AC suspensions before heat treatment is presented in Figure 41. When protein concentration in the emulsions prepared with the whole WPM A suspensions increased, the size of the fat droplets determined by laser light scattering in the presence of SDS slightly decreased from 1.5 to 1.3  $\mu\text{m}$ . The size of the fat droplets is significantly higher in the emulsions prepared with the WPM AC suspensions, in which the volume mean diameter in the presence of SDS were 5.3  $\mu\text{m}$  and 2.9  $\mu\text{m}$  for emulsions at 3.2 wt% and 6.8 wt% of proteins, respectively. The emulsion containing 3.2 wt% of proteins was not plotted in the graph. Decreasing the protein concentration or removing the caseins and WP not included in the WPM probably reduced the amount of surface active entities available for covering the fat droplet interface during homogenization (Dickinson, 2010). This was confirmed by analyzing the emulsions prepared with the WPM AC suspension by laser light scattering in the absence of SDS and by CLSM. In the absence of SDS, the average size of the particles in the emulsions increased to 57  $\mu\text{m}$  and 44  $\mu\text{m}$  indicating the flocculation of fat droplets for emulsions at 3.2 wt% and 6.8 wt% of proteins, respectively.

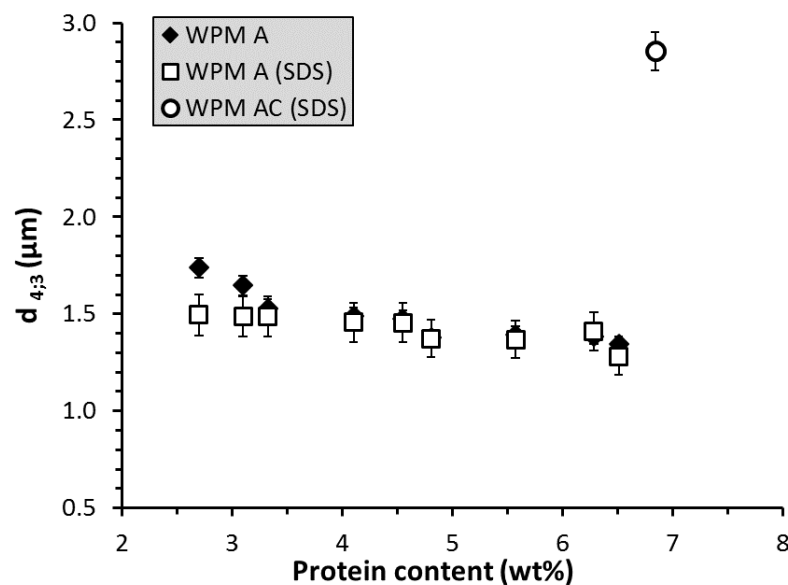


Figure 41: Volume mean diameter ( $d_{4,3}$ ) of the particles in the emulsions before heat treatment determined by laser light scattering: for the emulsions prepared with the whole WPM A suspensions at various protein concentrations with (empty square) or without SDS (black diamond) and for the emulsion prepared with the WPM AC suspension at 6.8 wt% proteins with SDS (empty circle).

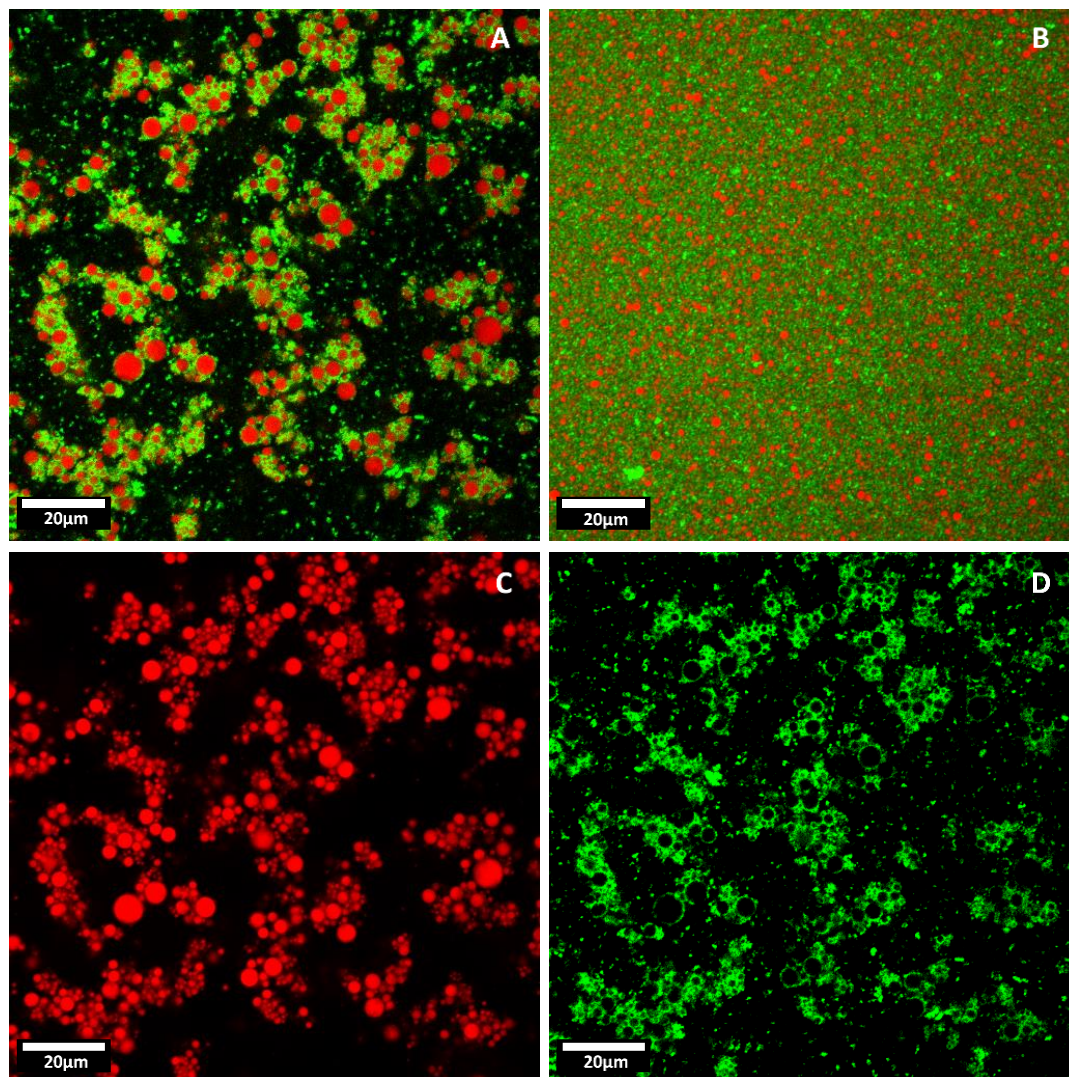


Figure 42: Confocal Laser Scanning Microscopic (CLSM) images of emulsions before heat treatment: (A) emulsion at 6.8 wt% proteins prepared with the WPM AC suspension (to remove the caseins and whey proteins not included in WPM); (B) emulsion at 6.5 wt% proteins prepared with the whole WPM A suspension. Co-localisation of fat (in red) and proteins (in green). Individual images showing the fat droplets repartition (C) and on the protein repartition (D) in the emulsion at 6.8 wt% proteins prepared with the WPM AC suspension; overlay of these 2 images corresponds to (A).

The CLSM images showed that WPM formed bridges between neighboring fat droplets (Fig 42A, 42C & 42D). This occurs when insufficient surface active entities are available during homogenization to fully saturate the fat droplet surface (Dickinson, 2010a, 2010b). WPM are large, dense particles with an internal protein concentration of  $\sim 150 \text{ g.L}^{-1}$  (Phan-Xuan et al., 2011). At the protein concentration investigated, the amount of WPM particles was probably insufficient and facilitated the bridging flocculation of the fat droplets during homogenization (Fig 42D). The heterogeneous repartition of the fat droplets in this emulsion is highlighted by the wide distribution of the inter-fat droplet distances calculated by CLSM image analysis (Fig 43).

## CHAPTER 6: Stability of emulsions containing whey protein microgels revealing the beneficial impact of caseins

In all other emulsions, the repartition of fat droplets appeared to be homogenous on the CLSM image (Fig 42B). The size of the fat droplets seemed slightly higher in the absence of SDS than in the presence of SDS but only at the lower protein concentrations (below 3.5 wt%) (Fig 41). However, this did not affect the microstructure of the emulsion. CLSM image analysis indicated that the inter-fat droplet distance distribution was independent of the protein concentration in the emulsions (Fig 43). The different microstructure at CLSM scale between the emulsion prepared with the whole suspension of WPM and the emulsion prepared with the suspension of WPM after centrifugation (Fig 42A and 42B) may explain the differences of texture observed.

### 6.2.2 Characteristics of the heated emulsions prepared with WPM A and WPM AC suspensions

The heat stability of the emulsions was evaluated at 120°C. After each heating time of up to 30 min, emulsions were visually observed to detect any signs of macroscopic destabilization, such as a change in flowability, gelation or the appearance of traces of oiling off. The heating time just before that required to destabilize the emulsion was represented according to the protein concentration of the emulsion. A heat stability of 30 min indicated the emulsion was not visually destabilized during the course of the heat treatment.

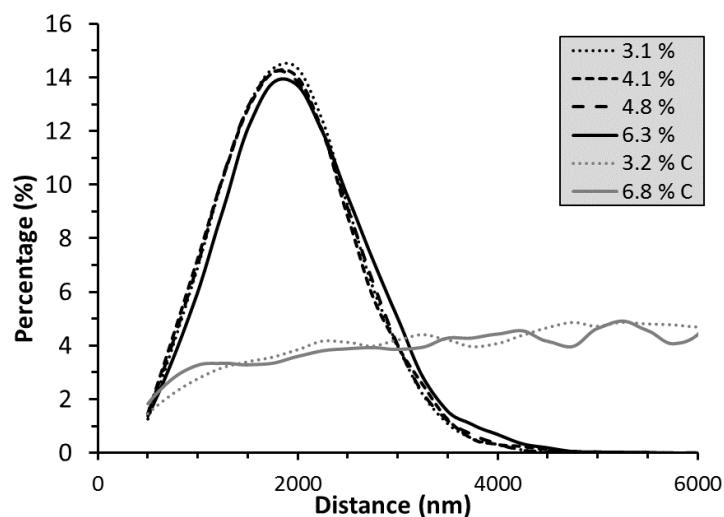


Figure 43: Distribution of the inter-fat droplet distances of emulsions before heat treatment: for the emulsions prepared with the whole WPM A suspensions at 3.1 wt% (black dotted line), 4.1 wt% (black small dashed line), 4.8 wt% (black large dashed line), 6.3 wt% proteins (black continuous line), and for the emulsions prepared with the WPM AC suspensions at 3.2 wt% (grey dotted line) and 6.8 wt% proteins (grey continuous line).

CHAPTER 6: Stability of emulsions containing whey protein microgels  
revealing the beneficial impact of caseins

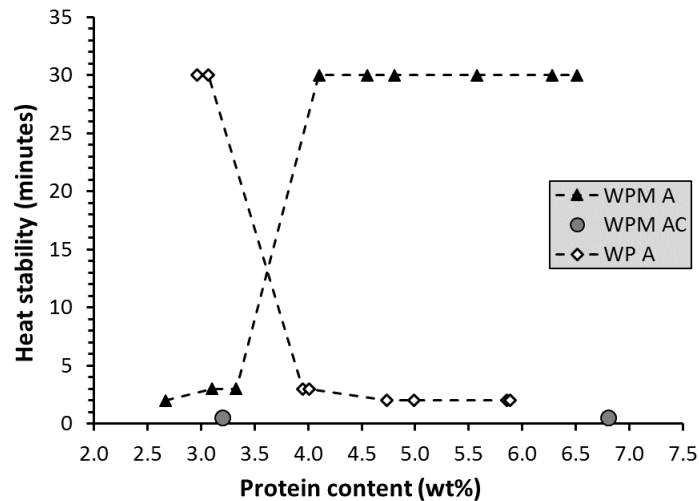


Figure 44: Heat stability of emulsions prepared with the whole WPM A suspensions (black triangle), with the WPM AC suspensions (grey circle) and of WP A emulsions (grey diamond) as a function of protein concentration. The dotted lines are guides.

The heat stability of emulsions prepared with the whole WPM A suspensions exhibited two very different behaviors at low and high protein concentrations with an abrupt transition at around 3.5 - 4 wt% proteins in the continuous phase (Fig 44). The emulsions were more heat stable at high protein concentrations (at least 30 min at 120°C). The fat droplets in the emulsions prepared with more than 4.5 wt% proteins showed only slight signs of flocculation as determined by particle size measurements (Fig 45). The emulsions showed the same microstructure before and after 30 min of heat treatment (Fig 46A and 46B); only a slight difference in the distribution of the inter-fat droplet distances was observed by CLSM image analysis (Fig 46E) confirming light scattering measurements in the absence and presence of a SDS.

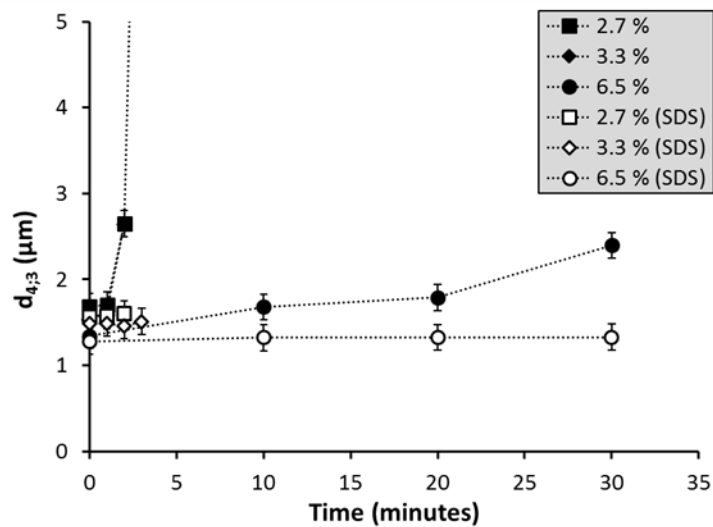


Figure 45: Volume mean diameter ( $d_{4,3}$  in  $\mu\text{m}$ ) of the particles in heated emulsions prepared with the whole WPM A suspensions at various protein concentrations as a function of heating time at 120°C: 2.7 wt% (square); 3.3 wt% (diamond); 6.5 wt% (circle). Fat droplet size measurements were performed by laser light scattering in the absence of SDS (black symbols) and in presence of SDS (empty symbols). The dotted lines are guides to the eye.



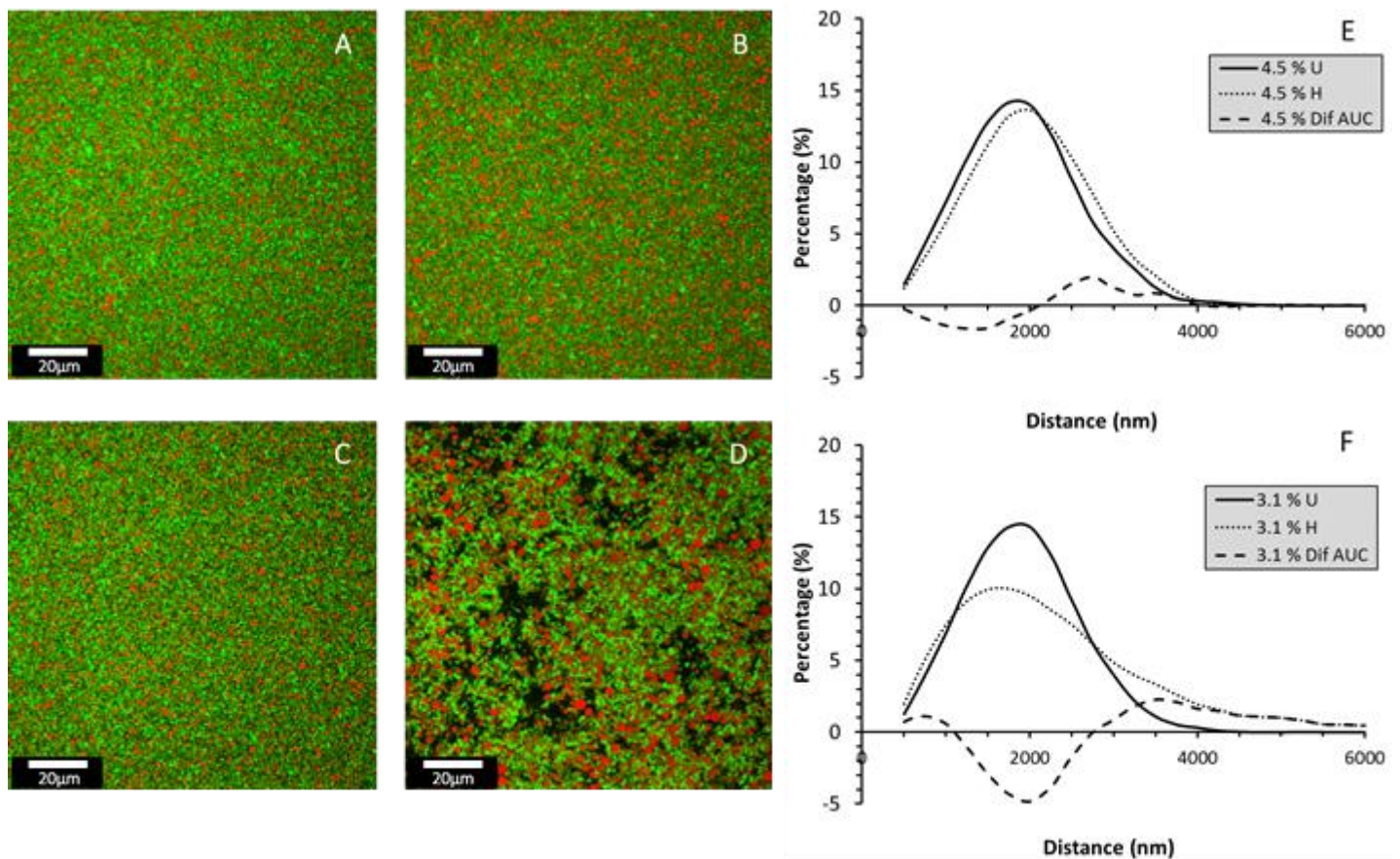


Figure 46: Microstructure analysis of emulsions reconstituted with the whole WPM A suspensions. (A to D) Confocal Laser Scanning Microscopy (CLSM) images of the emulsions reconstituted with the whole WPM A suspensions before (A, C) and after (B, D) heating. Unheated (A) and 5 min heated (B) emulsions at 4.5 wt% proteins; Unheated (C) and 30 min heated (D) emulsions at 3.1 wt% proteins. Fat droplets were stained with Nile Red (red color on the images) and the proteins with Fast green (green color on the images). (E and F) Distribution of the inter-fat droplet distances (distance existing between the fat droplets and its closest neighbor) for emulsions at (E) 4.5 wt% and (F) 3.1 wt% proteins before (continuous line) and after (small dotted line) heating as well as the Dif AUC (point by point difference of the area under the curve of the inter-fat droplet distances between the unheated and heated emulsions (large dotted line)).

In contrast, emulsions at protein concentrations below  $\sim 3.5$  wt% exhibited the first signs of destabilization upon heating at  $120^{\circ}\text{C}$  as early as 3 min (2.7 wt% protein) or 5 min (3.1 and 3.3 wt% proteins). A weak gel was formed and could easily be disrupted by agitation of the emulsions. Before the full heating time of gelation, a flocculation of the fat droplets was observed. Indeed, an increase in the particle size distribution of the emulsions was observed but only without SDS (Fig 45). The repartition of the fat droplets in heated emulsions became inhomogeneous as observed in the CLSM image (Fig 46D) and quantified by the Dif AUC (Fig 46F). This reflects the interaction of many fat droplets, probably at the source of the gelation of the system during prolonged heating. The emulsions were

unstable with regard to heat treatment in the protein concentration range where the fat droplet size before heating changed after adding SDS (Fig 41).

Emulsions prepared with WP A suspensions were represented in Figure 44 in order to compare their heat stability to the heat stability of WPM emulsions. At low protein concentrations, WP A emulsions were more stable than WPM A emulsions but at protein concentrations higher than 3.5 - 4 wt%, the opposite trend was observed; WPM A emulsions were much more heat stable than the WP A emulsions. The emulsions prepared with the WPM AC suspensions were rapidly destabilized on heating at 120°C. After only 1 min of heating, a gel was formed, which progressively contracted and released serum after 3 min. This result seems in line with the result of Sarkar et al., (2016), who showed that the interfacial WPM are in interaction and bridge neighboring fat droplets after heating WPM stabilized emulsions. At about 6.8 wt% protein concentration, the emulsion prepared with the whole WPM A suspension was heat stable suggesting a major contribution of the caseins to the stabilization of the WPM A emulsions.

### 6.2.3 Influence of fat droplet surface composition

In order to understand the difference of heat stability between emulsions containing high and low protein concentrations, and the emulsions prepared with the WPM AC suspensions, the surface protein load was quantified. It corresponds to the amount of proteins adsorbed at the fat droplet surface (expressed in mg/m<sup>2</sup>) and was represented according to the protein concentration in the continuous phase of the emulsions (Fig 47A).

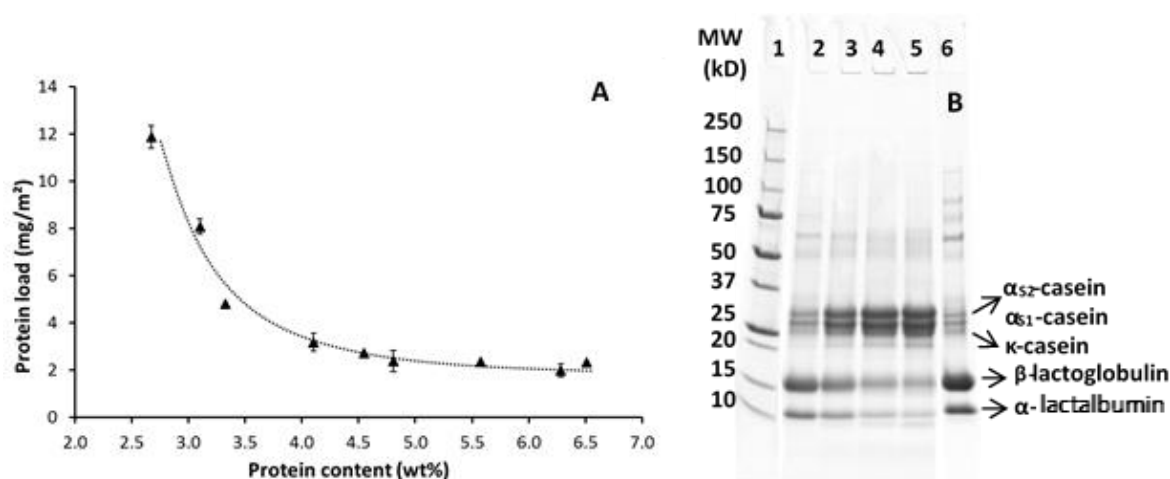


Figure 47: (A) Fat droplet surface protein load in emulsions prepared with the whole WPM A suspensions as a function of protein concentration. The dotted line is guide to the eye. (B) SDS-PAGE of the protein fraction adsorbed onto fat droplet surface in emulsions at different protein concentration; Molecular weight marker (1); emulsions prepared with WPM A suspensions at the following protein concentrations 3.1 wt% (2), 4.1 wt% (3), 4.8 wt% (4), 6.3 wt% (5) and whey protein powder (6). 10 µg of protein was placed in each sample well.

CHAPTER 6: Stability of emulsions containing whey protein microgels  
revealing the beneficial impact of caseins

The increase in the protein concentration of the emulsion led to a decrease in the surface protein load. In fact, the amount of proteins adsorbed at the fat droplet surface significantly decreased from  $11.9 \pm 0.5$  to  $2.4 \pm 0.5$  mg/m<sup>2</sup> when the protein concentration in the WPM A suspensions used to prepare the emulsions increased from 2.7 to 4.8 wt%. Between 4.8 wt% and 6.5 wt% of protein content, the surface protein load remained constant at  $2.3 \pm 0.1$  mg/m<sup>2</sup>, a value consistent with an interfacial layer of protein (Euston & Hirst, 1999; Hunt & Dalgleish, 1994). Note that the surface protein load of the emulsions prepared with the WPM AC suspensions were at  $90 \pm 2$  mg/m<sup>2</sup> and  $99 \pm 2$  mg/m<sup>2</sup> for emulsions at 3.2 wt% and 6.8 wt% of proteins, respectively (not represented on Fig 47).

A qualitative analysis of the type of proteins adsorbed at the fat droplet surface indicated a modification of the protein composition with the increase in the protein concentration of the emulsions in agreement with published works (Hunt & Dalgleish, 1994; Ye, 2008). The proportion of WP, i.e.  $\beta$ -Lg and  $\alpha$ -La, decreased significantly at the expense of caseins with an increase in the protein content of the emulsions (Fig 47B). Although the casein fraction was a minor portion of the WPM A suspensions (8.8 %), caseins almost completely replaced WPM at the surface of the fat droplets at high protein concentrations (more than 4 wt%). Caseins are small flexible proteins with well-defined hydrophobic and hydrophilic regions giving them excellent surface-active properties (Dickinson, 1999). Because of their structure, caseins preferentially adsorb at the fat droplet surface compared to WP (Hunt & Dalgleish, 1994; Ye, 2008). This seems to be exacerbated when WP are aggregated into WPM. When the concentration of caseins was sufficient (at high protein concentrations in the present study), they covered the fat droplet surface and hindered the adsorption of WPM. The stabilizing role of the adsorbed caseins was responsible for the higher flocculation resistance of fat droplets during heat treatment (Dickinson & Parkinson, 2004; Parkinson & Dickinson, 2004). At the same time, the WPM were released in the continuous phase where they are heat-stable (Fig 48). At low protein concentrations (3 wt%), the amount of caseins in the emulsion is too much limited for a complete coverage of the fat droplet surface (Fig 48). This could allow the adsorption of WPM during the homogenization stage, explaining the larger surface protein load and the slight change in the fat droplet diameter in the presence and absence of SDS (Fig 41). The presence of WPM at the surface of the fat droplets would be responsible for the lower heat stability of the emulsions. They promoted the flocculation of the fat droplets and gelation of the emulsions at low protein concentrations (3 wt%) and in the emulsions prepared with the WPM AC suspensions. As the suspensions of WPM (i.e. in the absence of fat) at the same protein concentration as in the aqueous phase of the emulsions were stable on heating, the presence of WPM at the surface of the fat droplets was considered as the main factor responsible for the emulsion instability, as already suggested by other authors (Sarkar et al., 2016) It is

a possibility that WPM adsorption at the fat droplet surface resulted in some structural rearrangements, as already indicated by a number of authors (Destribats et al., 2011; Destribats et al., 2014; Murphy et al., 2016) and made them more reactive on heating, facilitating the interactions between neighboring fat droplets.

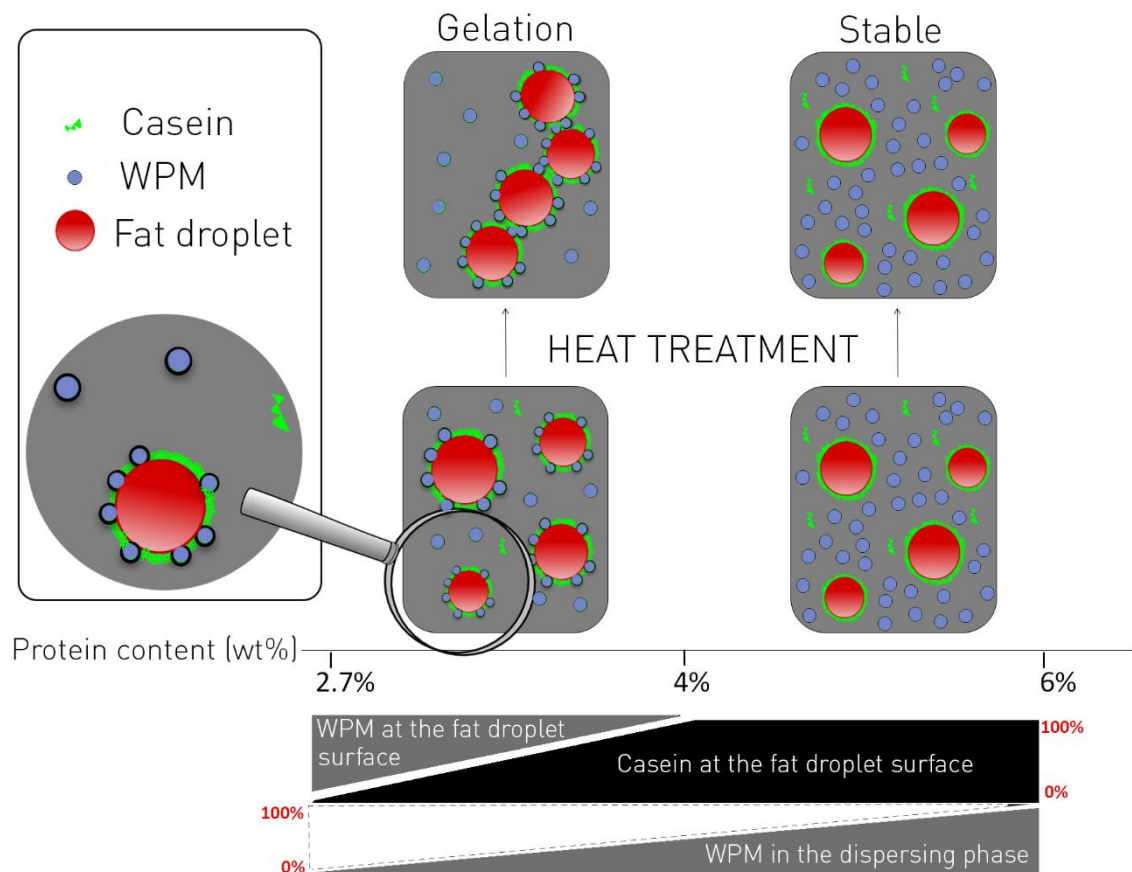


Figure 48: Scheme of the structure of the emulsions prepared with the whole WPM A suspensions at low (below 3.5 wt% proteins) and high (above 4 wt% proteins) protein concentration before and after heat treatment.

## 6.2.4 Mechanism of emulsion stabilization

### 6.2.4.1 Influence of the adsorbed proteins on the heat stability of the emulsions

To demonstrate the role of caseins on the heat stability of the WPM A emulsions, three emulsions were prepared with the WPM A suspension at ~ 2.7 wt% to which an increasing amount of caseins (0.07 wt%, 0.15 wt% and 0.23 wt%) was added. Sodium caseinate was selected because the casein composition of the WP A powder was mainly  $\alpha_{s1}$  and  $\kappa$ -caseins. A WPM A emulsion at 2.7 wt% protein without sodium caseinate addition was also prepared. At this protein concentration the emulsion was unstable during heating. The amounts of sodium caseinate were added to the casein content already present in the 2.7 wt% WPM A suspension (Table 5).



CHAPTER 6: Stability of emulsions containing whey protein microgels  
revealing the beneficial impact of caseins

Table 5: Amount of caseins added and total casein content in WPM A suspension

<b>Added caseins (wt%)</b>	0.00	0.07	0.15	0.23
<b>Total casein content (wt%)</b>	0.24	0.31	0.39	0.47

The addition of a small amount of caseins in the emulsions at 2.7 wt% proteins strongly increased its heat stability. No gelation was observed after 30 min of heating at 120°C for emulsion with 0.47 % of caseins while a gel was formed after only 3 min for the three other emulsions. Even if a slight flocculation of the fat droplets was detected by laser light scattering after 30 min at 120°C, they still appeared distributed homogeneously by CLSM.

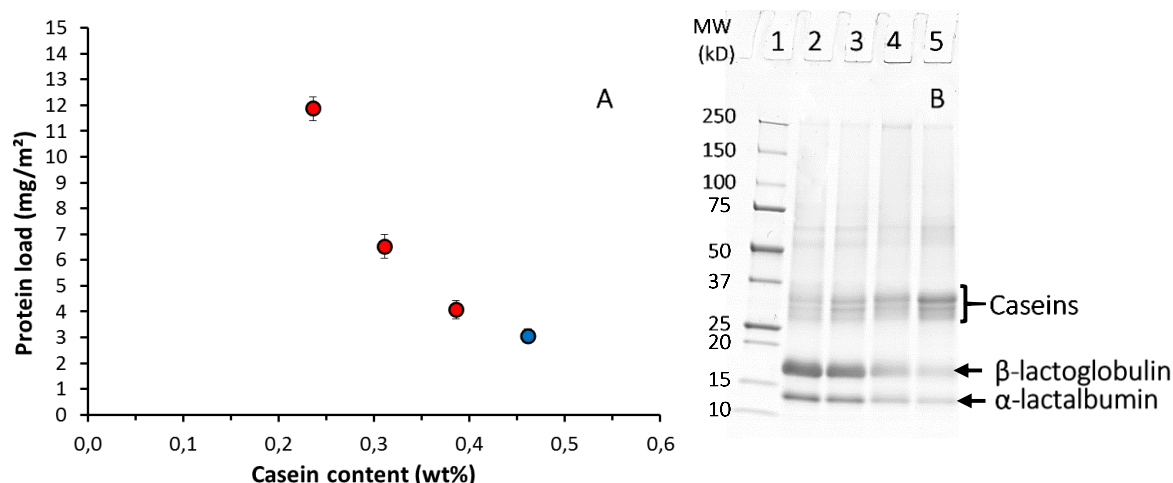


Figure 49: (A) Fat droplet surface protein load in emulsions prepared with WPM A suspensions at ~ 2.7 wt% proteins and various amount of caseins. The heat stability of the emulsion was indicated by colored symbols: gelled emulsions (red circle) and fluid emulsion (blue circle). (B) SDS-PAGE of the protein fraction adsorbed onto fat droplet surface in emulsions prepared with WPM A suspensions at ~ 2.7 wt% various amount of caseins: Molecular weight marker (1); emulsions prepared with WPM A suspensions at 2.7 wt% proteins and 0.24 wt% (2), and 0.31 wt% (3), 0.39 wt% (4), 0.47 wt% (5) of caseins.

The surface protein load in the emulsion at 2.7 wt% proteins was reduced from  $11.9 \pm 0.5$  mg/m<sup>2</sup> in the absence of added casein to  $3.0 \pm 0.3$  mg/m<sup>2</sup> at the higher concentration of caseins (Fig 49A). These results as well as the analysis of the fat droplet surface composition (Fig 49B) confirmed the competitive absorption between caseins and WPM, and the replacement of almost all WPM at the fat droplet surface by caseins. The release of WPM in the continuous phase of the emulsion by adding caseins for covering the fat droplet surface was responsible for the improved emulsion heat stability.

CHAPTER 6: Stability of emulsions containing whey protein microgels  
revealing the beneficial impact of caseins

To confirm the combined effects of the caseins and WPM to stabilize emulsion, new emulsions were designed starting with purified WPM (obtained by centrifugation of the WPM A suspension) and sodium caseinate. The protein concentration in the designed emulsions was chosen to be higher than 4 wt%, protein concentration for which emulsions prepared with the whole WPM A suspension were highly stable on heating (at least 30 min). Three emulsions were prepared with WPM AC suspension at 5.3 wt% protein concentration in which increasing amount of sodium caseinate was added in order to reach casein concentration of 0.2 wt%, 0.5 wt% and 0.7 wt%. A control emulsion was prepared with the WPM AC suspension (free of caseins and WP not included in the WPM) at the same protein concentration (5.3 wt%).

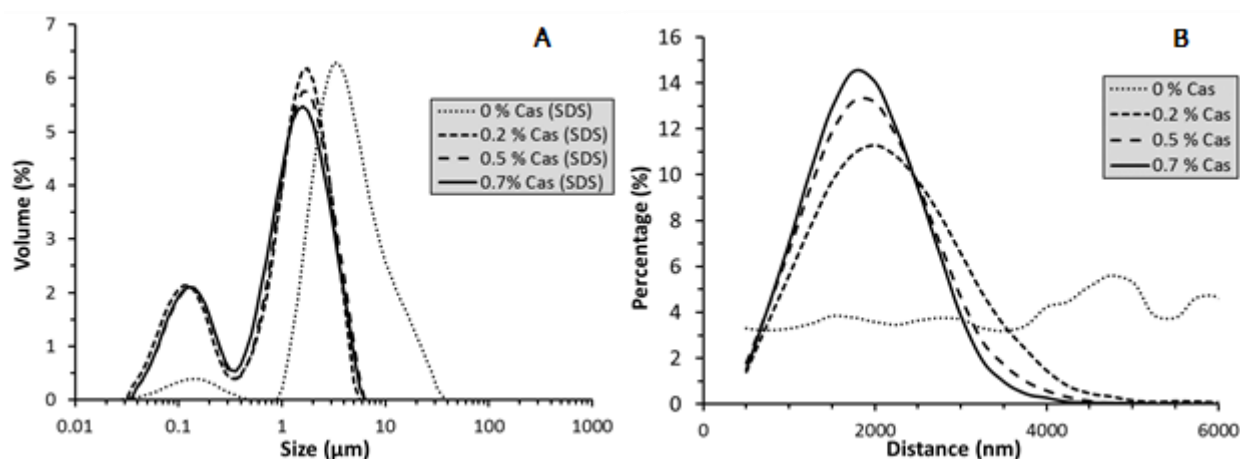


Figure 50: (A) particle size distribution of the emulsions and (B) distribution of the inter-fat droplet distances for emulsions prepared with WPM AC suspension at 5.3 wt% proteins as a function of casein concentration in the continuous phase: dotted line, 0 wt%; small dashed line, 0.2 wt%; large dashed line, 0.5 wt%; continuous line, 0.7 wt% casein.

Just after homogenization, the emulsions were observed viscous in the absence of casein and at low concentration of caseins (0.2 wt% of caseins) and were fluid at higher casein concentrations (with 0.5 wt% and 0.7 wt% of added caseins). At a microscopic scale, the size distribution of the fat droplets of the emulsion in the absence of caseins was shifted toward the larger sizes (Fig 50A).

CHAPTER 6: Stability of emulsions containing whey protein microgels  
revealing the beneficial impact of caseins

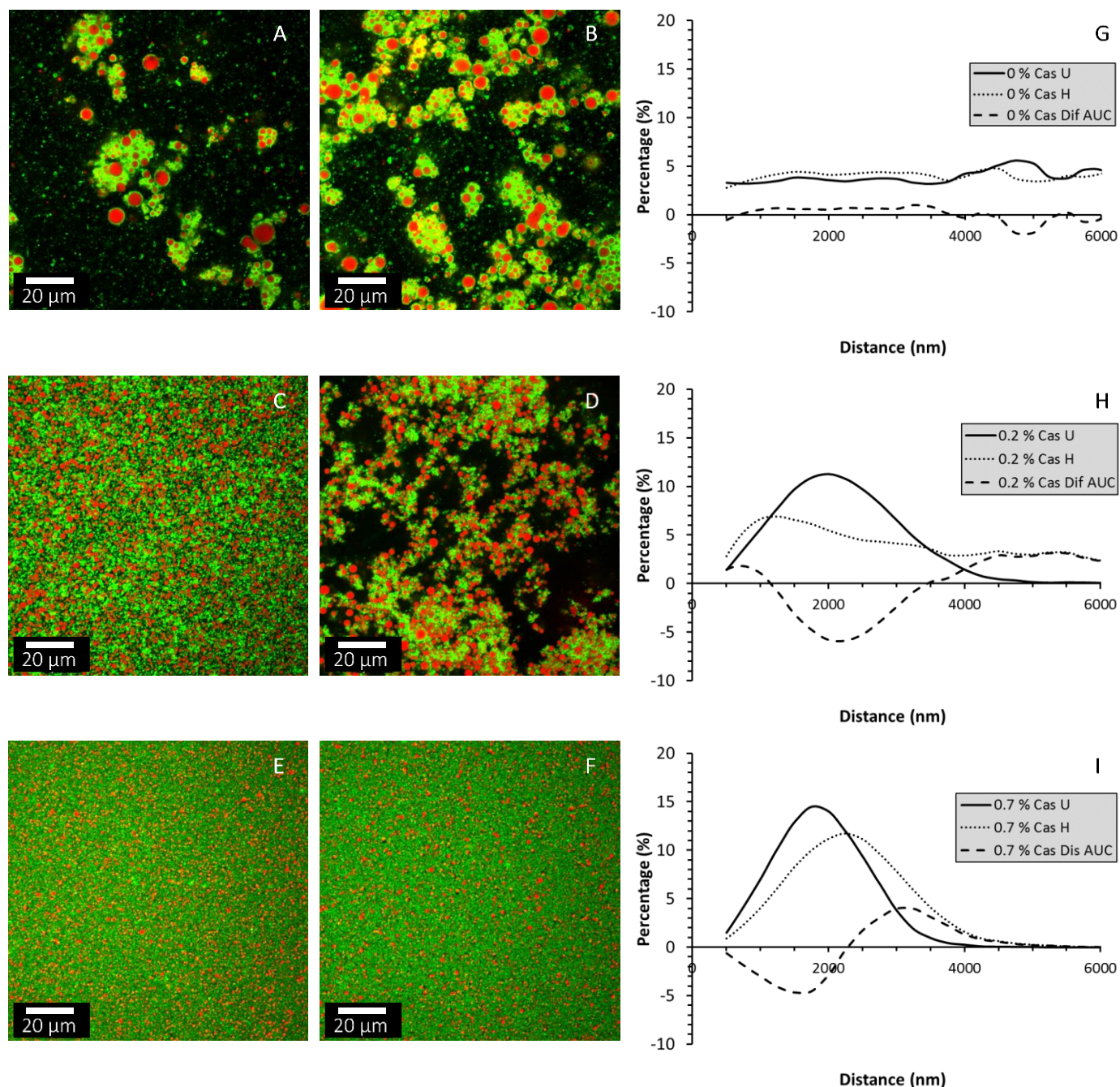


Figure 51: (A to F) Confocal Laser Scanning Microscopy (CLSM) images of the heated emulsions prepared with WPM AC suspensions at 5.3 wt% and (A, B) 0 wt% of caseins (A: unheated and, B: heated 1 min at 120°C); (C, D) 0.2 wt% of caseins (C: unheated and D: after 2 min of heating); (E, F) 0.7 wt% of caseins (E: unheated and F: after 30 min of heating). Fat droplets were stained with Nile Red (red color on the images) and the proteins with Fast green (green color on the images). (G to I) Inter-fat droplet distance distribution for emulsions prepared with the WPM AC suspension at 5.3 wt% and 0 wt% (G), 0.2 wt% (H) and 0.7 wt% (I) of caseins before (continuous line) and after (small dotted line) heating as well as the Dif AUC (point by point difference of the area under the curve of the inter-particle distances between the unheated and heated emulsions; large dotted line).

Moreover, the distribution of the distances between the fat droplets showed a highly inhomogeneous repartition of the fat droplets in the emulsion (Fig 50B). Indeed, some fat droplets were very close while

others were very far from one another. This repartition is characteristic of a high level of flocculation that was confirmed by CLSM observations (Fig 51A). In the emulsion prepared without casein, the volume mean diameters of the particles were 69  $\mu\text{m}$  and 5.9  $\mu\text{m}$  in the absence and presence of SDS, respectively. Fat droplet flocculation indicated the lack of surface active entities available during homogenization to fully saturate the fat droplet surface. Flocculated fat droplets were certainly at the source of the viscosity of the emulsion in the absence of caseins.

In the presence of caseins, the emulsions exhibited almost the same fat droplet distribution (Fig 50A); the volume mean diameter of the fat droplets in the presence of SDS decreased slightly from 1.7  $\mu\text{m}$  to 1.5  $\mu\text{m}$  with the increase in the casein content in the emulsion. However, the distribution of the inter-fat droplet distances was larger for the emulsion of WPM containing 0.2 wt% caseins than for those containing 0.5 wt% and 0.7 wt% of caseins (Fig 50B), which confirmed CLSM observations (Fig 51C and 51E). This suggested that the amount of surface active entities in the emulsion (WPM + caseins) was sufficient to reduce the size of the fat droplets but some fat droplets in the emulsion containing 0.2 wt% caseins kept in close contact after the homogenization stage. This was confirmed by laser light scattering measurements. The volume mean diameters of the fat droplets in the emulsion prepared with WPM and 0.2 wt% of caseins were 9.8  $\mu\text{m}$  and 1.7  $\mu\text{m}$  in the absence and presence of SDS, respectively, showing some flocculation between fat droplets (Fig 52). For the emulsions at 0.5 wt% and 0.7 wt% of caseins the volume mean diameter of the fat droplets stayed the same with or without SDS, showing the absence of fat droplet flocculation. WPM emulsions prepared without casein and with 0.2 wt% of caseins gelled on heating at 120°C after less than 1 min and 2 min, respectively. The emulsions containing 0.5 wt% and 0.7 wt% of caseins were visually stable during the full heating time at 120°C. The repartition of the fat droplets in the WPM emulsion with 0.2 wt% caseins became highly inhomogeneous on heating (Fig 51D). The CLSM images showed an increase in the amount of fat droplets flocculation probably at the source of the gelation of the emulsions. The dif AUC calculation confirmed large changes of the microstructure of the emulsion containing 0.2 wt% of caseins after heating (Fig 51H). These changes originated from the flocculation of the fat droplets only even if fat droplets appeared larger on the CLSM image after heating. The intensification of the flocculation was not observed by the calculation of the dif AUC of the emulsion without casein because the repartition of the fat droplets was already inhomogeneous before heating (Fig 51G).

Fat droplet flocculation was also observed but to a lesser extent by light scattering measurement and the calculation of the dif AUC of the emulsions containing higher amount of caseins (0.5 wt% and 0.7 wt%) (Fig 51I). The volume mean diameters of the fat droplets determined in the absence of SDS in the heated emulsions containing 0.5 wt% and 0.7 wt% caseins were 7.2  $\mu\text{m}$  and 3.0  $\mu\text{m}$ , respectively.

CHAPTER 6: Stability of emulsions containing whey protein microgels  
revealing the beneficial impact of caseins

This result is in line with the observation made with the emulsion prepared with the whole WPM A suspension (containing casein and WP not included in the WPM) at similar protein concentration.

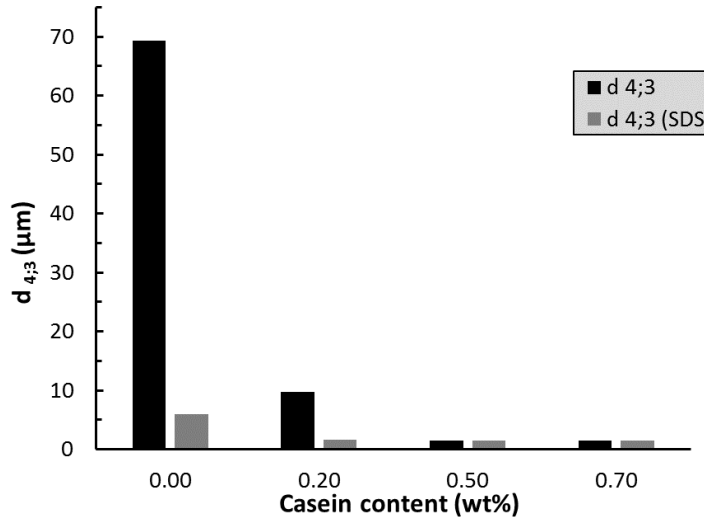


Figure 52: Volume mean diameter ( $d_{4,3}$ ) of the particles in the unheated emulsions prepared with WPM AC suspension at 5.3 wt% and various concentrations of caseins determined by laser light scattering with (grey) or without SDS (black) .

The fat droplet surface protein load was plotted according to the casein content in the emulsions (Fig 53A). On this Figure, emulsion heat stability is represented by colored symbols. A red symbol indicates the presence of visible signs of destabilization of the emulsion during the heat stability test and a blue one represents an absence of visible sign of destabilization of up to 30 min of heating. Increasing the casein concentration reduced the amount of adsorbed proteins. The surface protein load was at  $77 \pm 4.5$  mg/m<sup>2</sup> in the emulsion in the absence of casein and it was reduced to  $1.46 \pm 0.05$  mg/m<sup>2</sup> and  $1.35 \pm 0.02$  mg/m<sup>2</sup> for the WPM emulsions prepared with 0.5 wt% and 0.7 wt% of caseins. The analysis of the adsorbed proteins by SDS PAGE revealed only bands of caseins for the WPM emulsions prepared with 0.5 wt% and 0.7 wt% of caseins. WP were the main adsorbed proteins in the other WPM emulsions (in the absence of casein and with 0.2 wt% of casein). The surface protein load and the composition of the adsorbed proteins strongly suggest that WPM were progressively replaced by caseins at fat droplet surface when the casein content in the emulsions increased (Fig 53B). The surface protein load in emulsions with higher casein content was in line with that determined for emulsions prepared with sodium caseinate (Euston & Hirst, 1999; Hunt & Dalgleish, 1994). The replacement of WPM by caseins at the fat droplet surface was correlated with the increased heat stability of the emulsions. Emulsions were heat stable solely when the WPM were absent from the fat droplet surface.



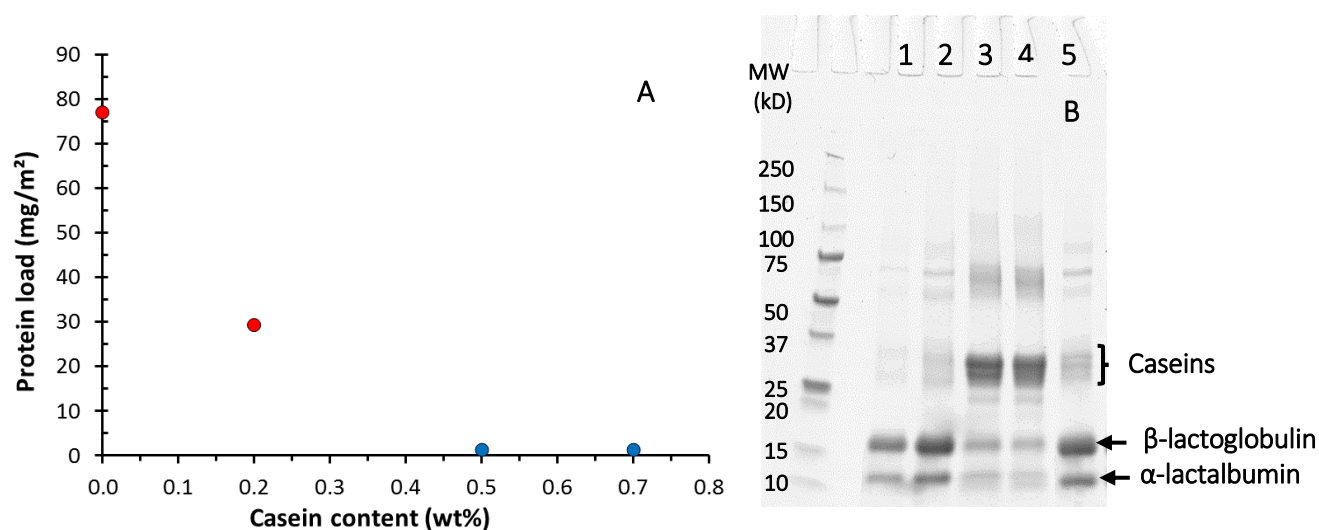


Figure 53: (A) Surface protein load *versus* casein content in the emulsions prepared with WPM AC suspension at 5.3 wt% and increasing amount of caseins. Red circles were for gelled emulsions during the course of heating and blue circles were for heat-stable emulsions during the full heating time. (B) Protein composition of the fat droplet surface of the emulsions prepared with WPM AC suspension at 5.3 wt% in the absence (1) and in the presence of 0.2 wt% (2), 0.5 wt% (3) and 0.7 wt% (4) of caseins determined by SDS-PAGE under reducing conditions. (5) whey protein powder; Mw, molecular weight marker.

#### 6.2.4.2 Capacity of the WPM to bridge fat droplet surface

In order to clarify the mechanism of heat-induced destabilization of the emulsions containing adsorbed WP at the surface of the fat droplets, emulsions prepared with 2.7 wt% and 5.9 wt% of WPM A suspensions and with 2.8 wt% of WPM AC suspension were observed before and after heat treatment by TEM. To remember, WPM emulsion containing 5.9 wt% proteins was heat stable and WPM emulsions at 2.7 wt% and 2.8 wt% proteins were destabilized during heating.

Figure 54 shows the TEM images of the three emulsions before heat treatment. The fat droplet surface of the emulsion prepared with WPM A suspensions after centrifugation was fully recovered by the WPM (Fig 54A) which was in agreement with a limited coalescence stabilization mechanism (Arditty, Whitby, Binks, Schmitt & Leal-Calderon, 2003). In Pickering-like emulsion, when the total amount of particles was not sufficient to fully cover the fat droplet surface, these one coalesce in order to progressively reduced the interfacial area. Due to the irreversible adsorption of the WPM, an increase in the surface coverage degree was observed until coalescence was arrested (Arditty et al., 2003). The particle arrangement at the interface determine the fat droplet size ( $D$ ) (Destribats, Ravaine, Heroguez, Leal-Calderon & Schmitt, 2010):

CHAPTER 6: Stability of emulsions containing whey protein microgels  
revealing the beneficial impact of caseins

$$D = \frac{4C\rho_p d_p V_d}{m_p}$$

Where C was the surface coverage degree,  $\rho_p$  was the WPM density ( $\text{kg/m}^3$ ),  $d_p$  was the WPM diameter (m),  $V_d$  was the volume of dispersed phase ( $\text{m}^3$ ) and  $m_p$  was the mass of WPM (kg). In the present case,  $d_p=3\times 10^{-7}$  m,  $V_d=1.5\times 10^{-4}$   $\text{m}^3$ ,  $\rho_p=150$   $\text{kg/m}^3$ . This equation suggests that all the WPM were adsorbed at the fat droplet surface and that the WPM distribution was monodisperse. A theoretical fat droplet size could be calculated by using this equation assuming a spherical-closed packed particles (78 %), hexagonally-closed packed particles (90 %) and a total fat droplet surface coverage (100 %). For 3.2 wt% ( $m_p=0.0048$  kg) or 6.8 wt% ( $m_p=0.0102$  kg) of WPM the theoretical fat droplet size obtained for different surface coverage degree were indicated in Table 6:

Table 6: Theoretical Fat droplet size at different degree of surface coverage

Degree of surface coverage (%)	78	90	100
<b>D (<math>\mu\text{m}</math>) for 3.2 wt% of WPM</b>	4.4	5.1	5.6
<b>D (<math>\mu\text{m}</math>) for 6.8 wt% of WPM</b>	2.1	2.4	2.6

The results obtained with the theoretical calculation were consistent with the fat droplet mean diameter determined for the emulsions prepared with WPM AC suspension at 3.2 wt% and 6.8 wt%, 5.3  $\mu\text{m}$  and 2.9  $\mu\text{m}$  respectively. For having WPM AC emulsions with a fat droplet mean diameter of 1.7  $\mu\text{m}$  (similar to the emulsions prepared with WPM A suspensions before centrifugation), the same calculation indicated that the WPM AC suspension concentration should be around 10 % (Table 7).

Table 7: Theoretical concentration of WPM needed to obtain a droplet size of 1.7  $\mu\text{m}$ .

Degree of surface coverage (%)	78	90	100
<b><math>m_p</math> (g/L)</b>	83	95	106

Figure 54A showed also that the WPM were located next to each other at the fat droplet surface, which was in agreement with the results reported by Destribats et al (2014). WPM seemed to keep their initial shape on adsorption and no sign of spreading or fusion of neighboring adsorbed WPM were evidenced. Figure 54A also confirmed the capacity of the WPM to bridge two neighboring fat droplets. The bridging flocculation of neighboring fat droplets during the homogenization stage resulted from the lack of surface active entities. This was confirmed by the quasi-absence of isolated WPM in the continuous phase of the emulsion.

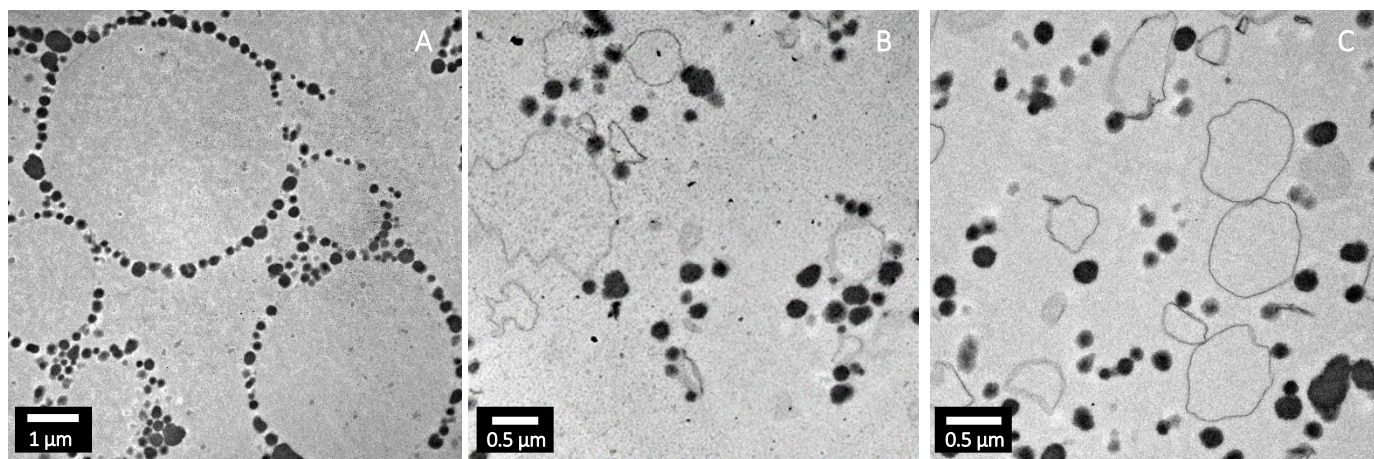


Figure 54: Transmission Electron Microscopy (TEM) images of the unheated emulsions prepared with WPM A suspension after centrifugation at 2.8 wt% (A) and the unheated emulsions prepared with WPM A suspensions at 2.7 wt% (B) and 5.9 wt% (C).

For the emulsion prepared with the WPM A suspension at 5.9 wt% proteins, the fat droplet interface was thin and the WPM were exclusively observed in the continuous phase (Fig 54C). The structure of the fat droplet interface was similar to that observed for a fat droplet interface covered with sodium caseinate (Dalgleish, 1996; Dalgleish, West & Hallett, 1997). This observation was in agreement with the protein composition analysis by SDS PAGE (Fig 47B) and confirmed that the amount of caseins in the emulsion was sufficient to cover the fat droplet surface. This also confirmed that the caseins in the WP powder A were not in a micellar structure since casein micelles form thick and inhomogeneous fat droplet interface (Buchheim & Dejmek, 1997; McClements, 2005; Goff, Verespej & Smith, 1999; Sharma, Singh & Taylor, 1996).

WPM were observed both at the fat droplet surface and in the continuous phase of the emulsion prepared with the WPM A suspension at 2.7 wt% proteins (Fig 54B). The fat droplet surface covered by WPM was very low compared to that covered by the caseins. Some adsorbed WPM appeared shared between neighboring fat droplets confirming the flocculation observed by light scattering measurements. It is probable that the amount of caseins was insufficient to fully cover the fat droplet surface, allowing some WPM to adsorb at the fat droplet surface. These observations proved that the WPM adsorption at the fat droplet surface is modulated by the amount of caseins.

After heating the emulsions, which had adsorbed WPM at the fat droplet surface, a gel was formed. The gel was composed of a network of fat droplets bridged by the WPM (Fig 55A and 55B). This result was in agreement with Sarkar et al., (2016), who showed that the interfacial WPM of neighboring fat droplets interacted after heating. These results confirmed that the main element responsible for the heat-gelation of the WPM emulsions was the WPM adsorbed at the fat droplet surface. However, the WPM



## CHAPTER 6: Stability of emulsions containing whey protein microgels revealing the beneficial impact of caseins

reactivity at the fat droplet surface remains at the moment unexplained. As already mentioned in part 6.2.1, some authors indicated that the WPM were able to rearrange during adsorption at fat droplet surface (Destribats et al., 2014; Destribats et al., 2011; Murphy et al., 2016) and this rearrangement could be at the source of fat droplet bridging on heating. However, no sign of spreading or rearrangements of the interfacial WPM were observed on the TEM images.

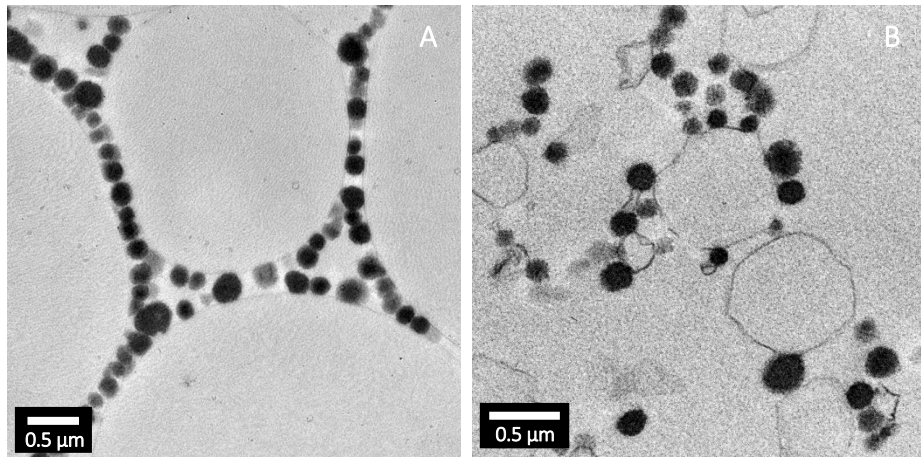


Figure 55: Transmission Electron Microscopy (TEM) images of the heated emulsions prepared with WPM A suspension after centrifugation at 2.8 wt% (A) and the heated emulsion prepared with WPM A suspension at 2.7 wt% (B).

### 6.2.5 Design of heat-stable emulsions containing WPM

The fat droplet surface protein load and the macroscopic heat stability of the emulsions prepared in this chapter 6 of the PhD thesis was represented according to the WP and casein concentration in the emulsions (Fig 56). Heat-stable emulsions were represented with a blue symbol and the unstable emulsions with a red symbol. The emulsions with a protein load above 12 mg/m<sup>2</sup> (i.e. emulsions prepared with the WPM AC suspensions at 3.2 wt%, 5.3 wt% and 6.8 wt% proteins and the emulsion prepared with 5.3 wt% of the WPM AC suspensions, in which 0.2 wt% of sodium caseinate was added) were not plotted in the graph. For the three emulsions prepared without casein the surface protein load was between 75 and 100 mg/m<sup>2</sup> and it was 30 mg/m<sup>2</sup> with 0.2 wt% of added caseins. The four emulsions gelled rapidly on heating (< 2 min).

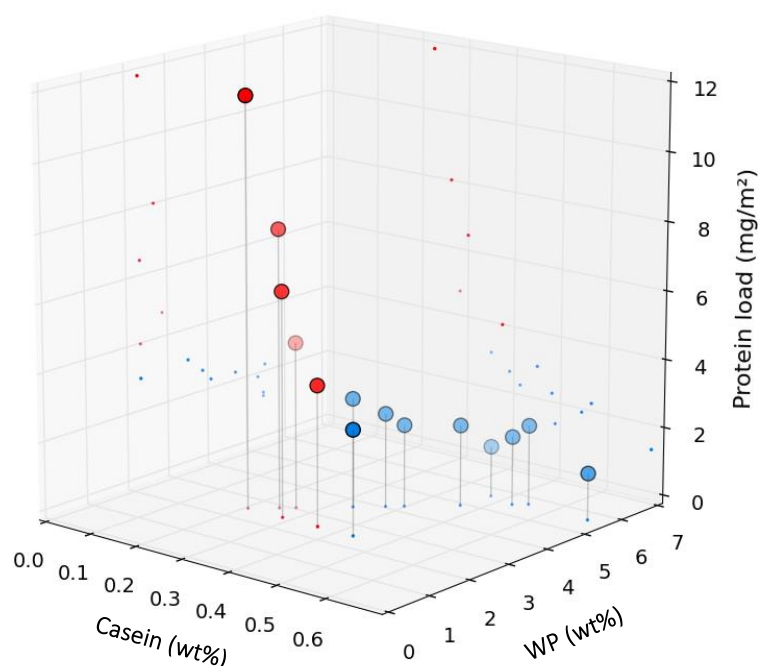


Figure 56: Phase diagram of WPM emulsions with or without added caseins: gelled emulsion (red circle) and stable emulsion (blue circle).

The decrease in the surface protein load was correlated to the increase in the casein content in the emulsions and was associated to a change of the composition of the adsorbed proteins. The whey proteins microgels (WPM) were replaced by caseins, which are much more surface active than the WPM, in emulsions containing high casein content. At high casein content, the surface protein load was constant (1.5 - 3 mg/m<sup>2</sup>) and this value was consistent with a monolayer of caseins (Hunt & Dalgleish, 1994).

A simple calculation allowed determining surface protein load to obtain heat-stable emulsion assuming all caseins are adsorbed at fat droplet surface. The Figure 56 showed that 0.4 % (i.e. 4000 mg/kg) of caseins were necessary to have stable emulsions. The fat droplet diameter of the emulsion ( $D$ ) containing 0.4% caseins was 1.7  $\mu\text{m}$ . If all the caseins were adsorbed at the fat droplet surface, the surface casein load was 3.8 mg/m<sup>2</sup>. Indeed, for 1L of emulsion with  $V_d = 30\%$  (v/v) of fat:

The specific surface area ( $a$ ) was :

$$a = \frac{6}{D} \times V_d$$

In our case :

$$a = \frac{6}{1.7 \times 10^{-6}} \times 300 \times 10^{-6} = 1060 \text{ m}^2$$

So:

$$\text{Protein load} = \frac{4000}{1060} = 3.8 \text{ mg/m}^2$$

For the first heat-stable emulsion, the protein load was 3.2 mg/m<sup>2</sup>. This result was consistent with the theoretical calculation.

The phase diagram also showed a correlation between the emulsion heat stability and the surface protein load. From this phase diagram it is suggested that the heat stability of the emulsions prepared with WPM suspensions followed the all-or-nothing principle:

- with an insufficient amount of caseins in the emulsion, some WPM adsorbed at fat droplet surface during the homogenization stage and the emulsion gelled on heating
- with a sufficient amount of caseins in the emulsion, caseins covered the whole fat droplet surface; the WPM were only in the continuous phase and the emulsion was heat stable.

From these results, it is possible to design heat-stable WP-rich emulsions in the absence of non-dairy additives using a reasoned approach consisting in a control of the repartition of the proteins between the fat droplet surface and the continuous phase. To do this, the emulsion has to contain heat-stable WP aggregates (WPM) and a small amount of caseins because of its combined action on the WPM emulsion heat stability.

- The amount of caseins has to be chosen in function of the fat droplet surface in the emulsion (fat volume fraction and fat droplet size distribution).
- The WPM have to be exclusively in the continuous phase.

### 6.3 Freeze/thaw stability of the emulsions

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All the WPM emulsions prepared in the previous part (6.2 heat stability of the emulsions) were also analyzed for the freeze/thaw stability.

CHAPTER 6: Stability of emulsions containing whey protein microgels  
revealing the beneficial impact of caseins

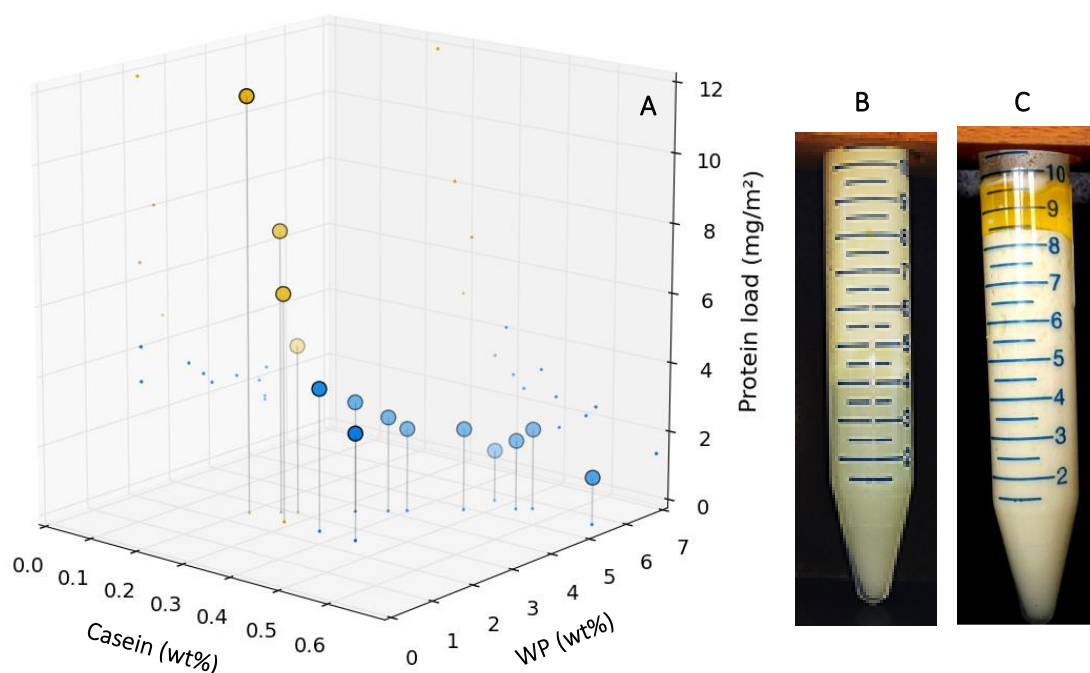


Figure 57: (A) Freeze/thaw stability of WPM emulsions prepared with WPM A or WPM AC suspensions with or without added caseins; Stable emulsions (blue circle) and emulsions exhibiting trace of oiling off (yellow circle). (B) Picture of an emulsion with a creamed layer of fat droplet at the top. (C) Picture of an emulsion with trace of fat release (oiling off).

The Freeze/thaw stability was represented according to the WP concentration and the casein concentration in the emulsion, and the fat droplet surface protein load (Fig 57A). The emulsions with fat droplets having a surface protein load above 12 mg/m<sup>2</sup> (emulsions prepared with the WPM AC suspensions at 3.2 wt%, 5.3 wt% and 6.8 wt% proteins and the emulsion prepared with the WPM AC suspension at 5.3 wt% proteins and 0.2 wt% of added sodium caseinate) were not plotted in the graph. The emulsions prepared with the WPM AC suspensions at 3.2 wt%, 5.3 wt% and 6.8 wt% proteins had a creamed layer of fat droplets at the top of the tube after a freeze/thaw cycle (Fig 57B). The emulsions represented with a yellow symbol and the emulsion prepared with WPM AC suspensions at 5.3 wt% and 0.2 wt% of added sodium caseinate showed sign of oiling off (Fig 57C). The emulsions represented with a blue symbol were stable after a freeze/thaw cycle.

The emulsion behavior during a freeze/thaw cycle had to be put in regard of the protein composition of the fat droplet surface (Fig 58). Only emulsions with the fat droplets completely covered by caseins were stable after a freeze/thaw cycle (Fig 58C).

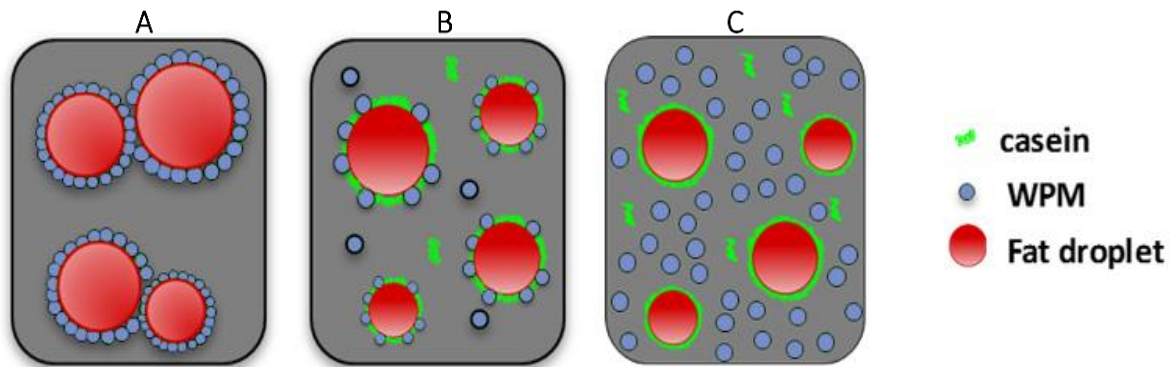


Figure 58: Scheme of the emulsion structure before a freeze/thaw cycle. (A) Emulsion prepared with WPM AC suspension. (B) Emulsion prepared with WPM A suspension containing less than 0.3 wt% of caseins. (C) Emulsion prepared with WPM A suspension containing more than 0.35 wt% of caseins.

The presence of adsorbed WPM at the fat droplet surface led to two different emulsion behaviors during the freeze/thaw cycle. Emulsions with a high density of adsorbed WPM (prepared with WPM AC suspension) were constituted of large and flocculated fat droplets (Fig 58A). They showed a creamed layer of fat droplets after a freeze/thaw cycle which was probably inherent to the initial size distribution of the particles in the emulsions. Even if, no sign of fat release was observed for these emulsions, the coalescence of the fat droplets after one freeze/thaw cycle was revealed by the fat droplet volume mean diameter (Fig 59). Fat droplets in these emulsions seemed to be more resistant against the mechanisms of destabilization induced by the freeze/thaw cycle than emulsions with a low density of adsorbed WPM (see below). The dense packing of the adsorbed WPM could form a thick shell around the fat droplets (steric barrier) limiting the damage of the fat droplet interface by the growth of the fat crystals and the water crystals during the freezing stage (Marefati, Rayner, Timgren, Dejmek & Sjö, 2013). Only a small coalescence of the fat droplet was observed after thawing. The strong binding energy of WPM at the fat droplet interface could prevent its spontaneous desorption (irreversibly adsorption)(Aveyard, Binks & Clint, 2003) and protect the fat droplets against coalescence as postulated for Pickering-like emulsions. The high stability of the fat droplet covered by soft particle (starch granules) during freeze/thaw cycle at  $-18^{\circ}\text{C}$  was shown by Marefati, Rayner, Timgren, Dejmek & Sjö (2013).

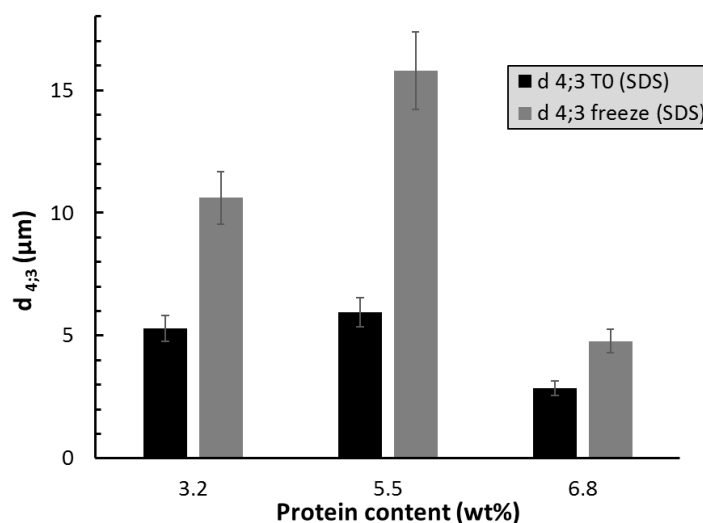


Figure 59: Volume mean diameter ( $d_{4,3}$ ) of the particles in the presence of SDS determined by laser light scattering for the emulsions prepared with the WPM AC suspensions at various protein concentrations before (black) or after freeze/thaw cycle (black).

The emulsions with a low density of adsorbed WPM (Fig 58B) exhibited a fat layer (oiling off) at the top of the tube after a freeze/thaw cycle (Fig 57C). The surface of the fat droplets was constituted of a mixture of caseins and WPM. Taken individually caseins and WPM formed emulsions that resisted to the release of fat after a freeze/thaw cycle. Consequently, the simultaneous presence of caseins and WPM at fat droplet surface would be at the source of the fat release and emulsion instability during a freeze/thaw cycle. Presently, the mechanism leading to the release of liquid fat (oiling off) in such emulsions after a freeze/thaw cycle is still to be discover.

By comparison, emulsions prepared at similar protein concentrations but using unheated WP instead of WPM were stable during the freeze/thaw cycle. These results indicated that it is possible to control the stability of the emulsions by a reasoned formulation of the protein suspensions (by varying the amount of caseins and WPM or by using unheated WP) before preparing the emulsion.

## 6.4 Conclusion

WPM are highly heat stable in solution, but they are responsible for the heat gelation of the emulsions when they are adsorbed at the fat droplet surface. A gel is formed by the interaction of neighboring WPM-covered fat droplets. Similarly, WPM adsorbed at the fat droplet surface were at the source of the emulsion destabilization after a freeze/thaw cycle but the mechanism of destabilization is not understood yet.

CHAPTER 6: Stability of emulsions containing whey protein microgels  
revealing the beneficial impact of caseins

Due to their small size and strong surface-active properties, caseins compete with WPM for adsorption at the fat droplet surface. Hence, by increasing the casein content in the emulsions (amount equivalent to 0.4 wt% of caseins for emulsions having a specific surface area of  $\sim 1000 \text{ m}^2$ ), it is possible to replace WPM by caseins at the fat droplet surface and to improve the emulsion stability during the technological treatments. In fact, caseins protected the fat droplet surface during heating and during a freeze/thaw cycle. Casein concentration can be increased by increasing the total protein concentration or by the adding of sodium caseinate in the WPM suspension.

By a reasoned approach, it is possible to overcome the low heat stability and the high freeze/thaw stability of WP-rich emulsions prepared with unheated WP. In fact, by combining the functional role of WPM and caseins, the heat stability and the freeze/thaw stability of WP-rich emulsions can be controlled.





## CHAPTER 7: Impact of the whey protein aggregate morphology and the presence of caseins on the emulsion stability

The purpose of this chapter was to check if the results obtained on the WPM A emulsions were specific to the emulsions prepared with WPM suspensions or were generic to emulsions prepared with WP aggregates of various morphology (i.e. different size and shape). Emulsions prepared with suspensions of strand-like aggregates (SA<sub>4</sub>) were tested for their heat stability and freeze/thaw stability. The contribution of the aggregates adsorbed at fat droplet surface and those dispersed in the continuous phase to the mechanism of stabilization of the emulsions was analyzed. Results indicated that the protein aggregates adsorbed at fat droplet surface had a key contribution to the destabilization of the emulsions during heating and during freeze/thaw cycle. When SA<sub>4</sub> were mainly adsorbed at fat droplet surface, these latter coalesced and released fat (oiling off) was observed on the top of the emulsions. This phenomenon was prevented in the presence of trace of caseins. By competition for the interface, caseins reduced the amount of SA<sub>4</sub> adsorbed at fat droplet surface and promoted emulsion stability during technological treatments. However, the emulsion heat stability was limited at high protein concentrations (i.e. above 5.6 wt%) by the critical gelation concentration of the SA<sub>4</sub>. Emulsions prepared with a blend of WPM and stand-like aggregates were analyzed under the same conditions. The behavior of the emulsions during technological treatments differed from the theoretical sum of the behaviors of the WPM emulsions and SA<sub>4</sub> emulsions. A part of these results has been published in the following paper: Chevallier, M., Riaublanc, A., Lopez, C., Hamon, P., Rousseau, F., & Croguennec, T. (2016). Aggregated whey proteins and trace of caseins synergistically improve the heat stability of whey protein-rich emulsions. *Food Hydrocolloids*, 61, 487–495.

### The main aims of the work presented in this chapter were to:

- Investigate the emulsion stability prepared with strand-like aggregates
- Discriminate the contribution of adsorbed aggregates and the aggregates in the continuous phase on the emulsion stability
- Confirm the control of emulsion stability by combining the functional role of whey proteins aggregates and caseins
- Approach the stability of the mixed system i.e. with different kind of protein aggregates adsorbed at the fat droplet surface

## Table of Contents

<b>CHAPTER 7: Impact of the whey protein aggregate morphology and the presence of caseins on the emulsion stability .....</b>	<b>123</b>
<b>7.1 Introduction .....</b>	<b>125</b>
<b>7.2 Stability of the SA<sub>4</sub> emulsions during technological treatments.....</b>	<b>126</b>
7.2.1 Design of heat-stable SA <sub>4</sub> emulsions.....	126
7.2.2 Heat stability of SA <sub>4</sub> emulsions at various protein concentrations but at constant casein/SA <sub>4</sub> ratio .....	127
7.2.2.1 Emulsion characteristics before heat treatment .....	128
7.2.2.1.2 Fat droplet surface composition .....	129
7.2.2.2 Emulsion stability to heat treatment .....	132
7.2.2.3 Heat stability of emulsions <i>versus</i> proteins in suspension .....	136
7.2.2.4 Discussion .....	137
7.2.3 Heat stability of the SA <sub>4</sub> emulsions at various casein/SA <sub>4</sub> ratio .....	141
7.2.4 Freeze/thaw stability of the SA <sub>4</sub> emulsions and casein/SA <sub>4</sub> emulsions.....	143
<b>7.3 Stability of the emulsions prepared with the WPM/SA B suspensions during technological treatments.....</b>	<b>145</b>
7.3.1 Design of heat-stable WPM/SA B emulsions.....	145
7.3.2 Freeze/thaw stability of the WPM/SA B emulsions.....	153
<b>7.4 Conclusion.....</b>	<b>154</b>

## 7.1 Introduction

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The heating of a WP solution below the protein gelation concentration results in the formation of soluble aggregates. On prolonged heating, the aggregate solution contains no more reactive monomer and the solution reaches a steady state (Nicolai et al., 2011). These soluble aggregates have different properties than the native proteins used for their production. Many studies reviewed in Nicolai et al. (2011) described improved functional properties of the soluble aggregates in gels, foams, emulsions, etc. (Alting, Hamer, de Kruif, Paques & Visschers, 2003; Dissanayake & Vasiljevic, 2009; Kim et al., 2005; Moro et al., 2001). The structure and the chemical characteristics of the aggregates are modulated by changing the physical-chemical conditions of the protein solution (pH, ionic strength, amount of calcium, protein concentration, etc.) (Nicolai & Durand, 2013). The structure of the aggregates at steady state depends on the repulsions between proteins. By increasing the repulsive strength between the proteins (i.e. near neutral pH and at low ionic strength), the aggregates have strand-like shape with a low level of branching. WP form dense and spherical structures (i.e. WPM) in conditions where the repulsions between proteins are reduced, i.e. at pH 5.8 (Bovetto et al., 2007) or at pH 7 in the presence of calcium (Nicolai & Durand, 2013; Phan-Xuan et al., 2013, 2014). The properties of the aggregates change depending on their structure and chemical characteristics (Nicolai et al., 2011).

In chapter 5 part 5.3, we observed that SA<sub>4</sub> and WPM have different thermal stability at high protein concentration and different interfacial and emulsifying properties. Moreover, a first screening presented Chapter 5 part 5.2.2 revealed that the SA<sub>4</sub> suspensions allowed to prepare emulsions at 3 wt% proteins with a better stability during heating and during freeze/thaw cycle than the emulsions prepared with the WPM A suspension.

To the best of our knowledge, no study reports on the ability of aggregates with different structure to stabilize emulsion against technological treatments. In chapter 6, we observed that WPM adsorbed at the fat droplet surface destabilized the emulsions during heating and during freeze/thaw cycle. So, in order to know if this observation is specific to WPM or if it is a general trend for all types of aggregates at steady state, the heat stability and the freeze/thaw stability of the emulsions prepared with SA<sub>4</sub> suspensions were characterized.

In this chapter, different kinds of WP aggregates suspensions were used to prepare emulsions. Firstly, the ability of SA<sub>4</sub> suspensions (protein concentrations ranging from 2.9 to 6.6 wt%) to stabilize emulsions during the technological treatments was evaluated. In conditions where the emulsions were destabilized during the technological treatments, we investigated the possibility to control emulsion stability by adding an increasing quantity of caseins. In a second time, suspensions containing two kinds of aggregates, i.e. WPM and SA<sub>4</sub>, were used to prepare emulsions. The stability to technological

treatments of designed WPM/SA B emulsions at varying concentrations in proteins with or without added caseins was evaluated.

## 7.2 Stability of the SA<sub>4</sub> emulsions during technological treatments

The ability of SA<sub>4</sub> to stabilize WP emulsions during technological treatments (heat, freeze/thaw cycle) was investigated. Emulsions were prepared with SA<sub>4</sub> suspensions at different protein concentrations (from 2.9 wt% to 6.6 wt%) and without or with added caseins. Note that before adding casein, the SA<sub>4</sub> suspensions used to prepared the emulsions already contained a the emulsions was tested.

### 7.2.1 Design of heat-stable SA<sub>4</sub> emulsions

Figure 60 shows the heat stability of the emulsions according to the WP and the casein concentrations in the emulsion and the protein surface load. A yellow symbol indicates the emulsions that exhibited trace of oiling off during the test of heat stability. In addition, the flowability of the emulsions was affected since the emulsions after heating were visually more viscous than before heating. Stable emulsions are represented by a blue symbol. A red symbol indicates the emulsions that gelled on heating. From Figure 60 it is possible to point out common characteristics explaining emulsion behaviors during the heat treatment:

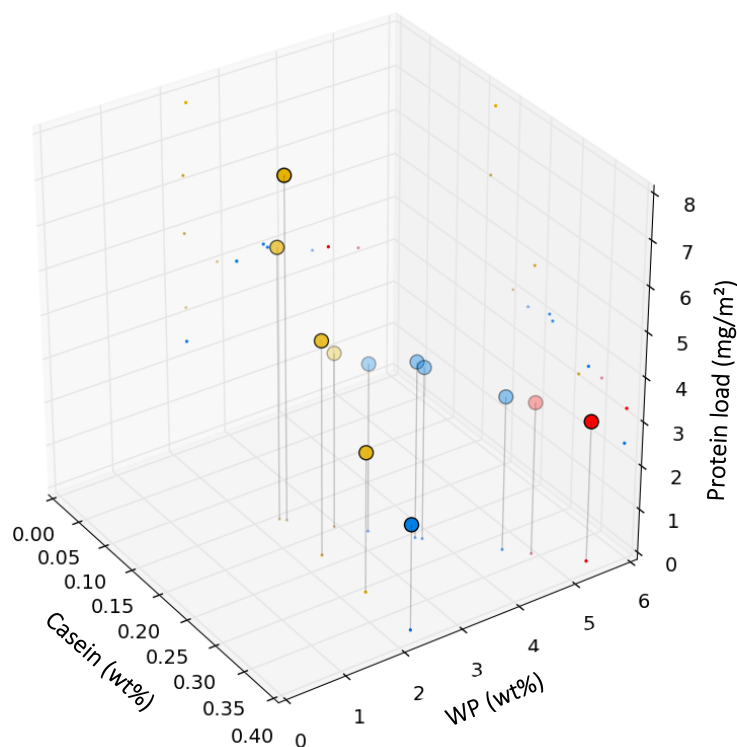


Figure 60: Heat stability of the emulsion prepared with the SA<sub>4</sub> suspension and with or without the added caseins: stable emulsions (blue circle), gelled emulsions (red circle) and emulsions showing oiling off (yellow circle).

## CHAPTER 7: Impact of the whey protein aggregate morphology and the presence of caseins on the emulsion stability

- The emulsions that gelled on heating had in common a high content of SA<sub>4</sub> and a low amount of proteins adsorbed at the surface of the fat droplets (surface protein load around 3 mg/m<sup>2</sup>).
- The emulsions exhibiting fat release after the test of heat stability were prepared with protein suspensions having low concentration of SA<sub>4</sub>. The only emulsion at low SA<sub>4</sub> concentration that was heat-stable, had a high casein content (0.33 wt%). The surface protein load of these emulsions was higher (> 4mg/m<sup>2</sup>) than in the other emulsions, except for the emulsion containing 0.33 wt% caseins. This suggests a competition between the SA<sub>4</sub> and the caseins for the fat droplet surface. The decrease in the surface protein load in the presence of caseins could be attributable to the reduction of the SA<sub>4</sub> adsorption.
- Heat-stable emulsions had a low surface protein load (3 - 4 mg/m<sup>2</sup>), a SA<sub>4</sub> content and a casein content intermediate between the emulsions exhibiting fat release and the gelled emulsions after heating.

In addition, the surface protein load seemed to be dependent on the casein content in the emulsions prepared with SA<sub>4</sub> as already observed for WPM emulsions containing various amount of caseins. To go deeper in the understanding of the mechanism of stabilization during the heating of emulsions prepared with SA<sub>4</sub>, emulsions at various protein concentrations but at constant SA<sub>4</sub>/casein ratio and emulsions at various SA<sub>4</sub>/casein ratio were analyzed at macroscopic and microscopic scales. The contribution of the adsorbed proteins at fat droplet surface and the contribution of the proteins in the continuous phase to the emulsion heat stability were investigated.

### 7.2.2 Heat stability of SA<sub>4</sub> emulsions at various protein concentrations but at constant casein/SA<sub>4</sub> ratio

In this part of the study, the heat stability of emulsions prepared with SA<sub>4</sub> suspensions at protein concentrations ranging from 2.9 wt% to 6.6 wt% (casein content of 6 wt% on protein basis indicating a constant casein/SA<sub>4</sub> ratio in all SA<sub>4</sub> suspensions) was investigated on macroscopic and microscopic scales with a special focus on the protein composition of the fat droplet surface. The heat stability of the emulsions was compared to the heat stability of the proteins in the continuous phase in order to discriminate the origin of the emulsion instability, the fat droplets or the continuous phase. For comparison, emulsions prepared with WP A suspensions (unheated WP) at various concentrations were analyzed under the same conditions.

### 7.2.2.1 Emulsion characteristics before heat treatment

#### 7.2.2.1.1 Emulsion microstructure

Fat droplet size distribution of the emulsions prepared with SA<sub>4</sub> suspensions was determined by light scattering measurements and the results were expressed as the volume mean diameter  $d_{4,3}$ . The same fat droplet size distribution was obtained for all emulsions in the absence or presence of SDS, meaning that fat droplets were not aggregated. The volume mean diameter of fat droplets was  $1.6 \pm 0.1 \mu\text{m}$ . Fat droplets were distributed homogeneously in all emulsions according to CLSM observations (Fig 61A and 61B).

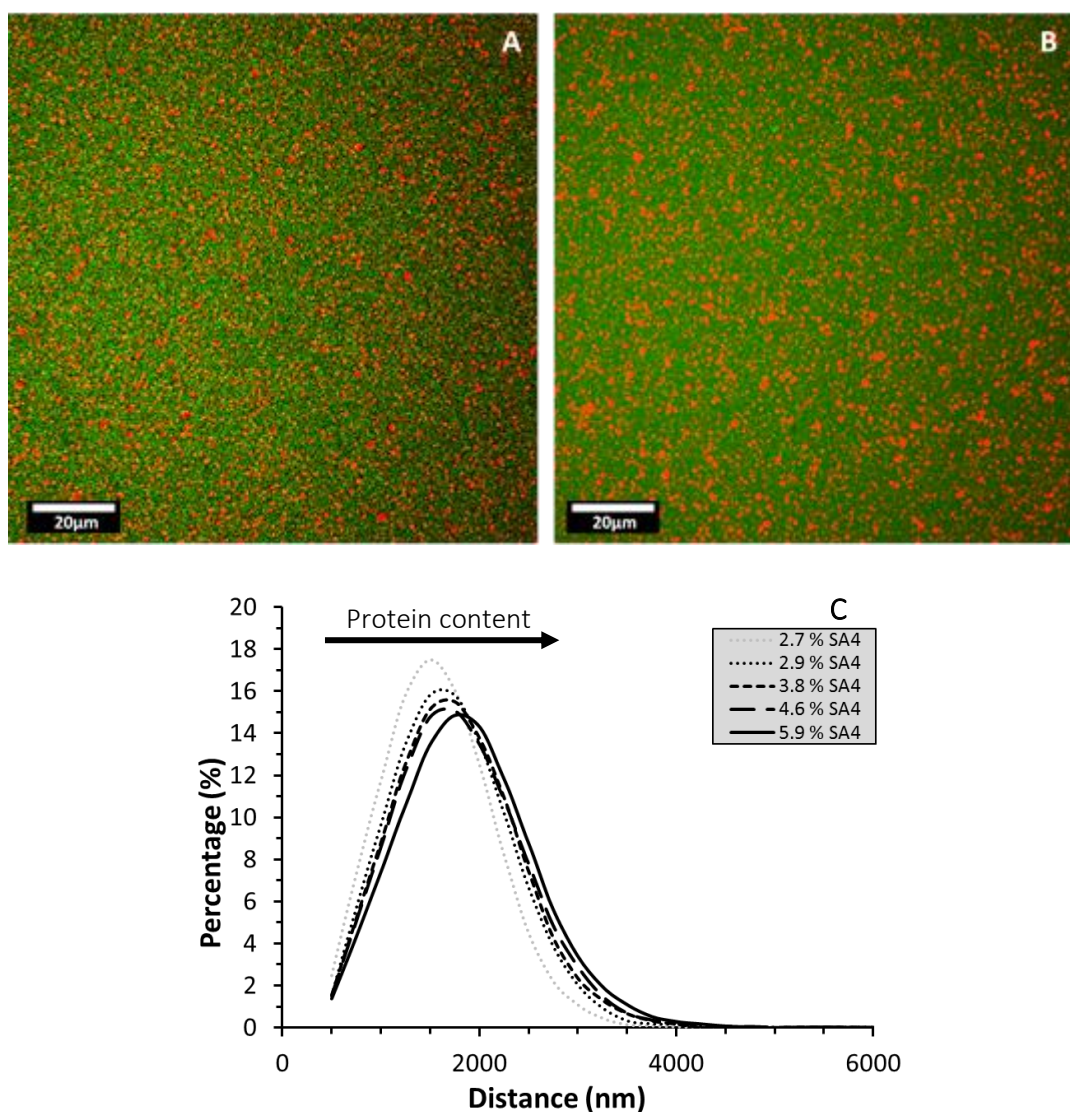


Figure 61: (A and B) Confocal Laser Scanning Microscopy (CLSM) images of SA<sub>4</sub> emulsions at 2.7 wt% (A) and 5.9 wt% (B) before heat treatment. Distribution of the distance between fat droplet surface for SA<sub>4</sub> emulsions as a function of protein concentrations in the continuous phase (A): small grey dotted line, 2.7 wt%; small black dotted line, 2.9 wt%; dashed line, 3.8 wt%; long dashed line, 4.6 wt%; continuous line, 5.9 wt%.

## CHAPTER 7: Impact of the whey protein aggregate morphology and the presence of caseins on the emulsion stability

However, the mathematical analysis of the CLSM images showed that the distribution of the inter-fat droplet distances was wider and the average distance between the fat droplets increased ( $\sim 500$  nm of difference) with the increase in the protein concentration in the SA<sub>4</sub> emulsions. This difference was neither due to a difference of the fat droplet surface area nor a creaming of the fat droplets during the CLSM analysis. This indicated that the fat droplets had a more homogeneous repartition in the SA<sub>4</sub> emulsion at low protein concentration than at high protein concentration probably due to the flocculation of some fat droplets (Fig 61C). The interactions between the fat droplets were weak since no sign of coalescence or flocculation was detected by light scattering measurements. They were probably disrupted by the sample preparation for the light scattering measurement (dilution in water and shearing). This phenomenon is often observed as a result of a depletion-flocculation of the fat droplets in emulsion at high fat volume fraction by non-interacting, non-adsorbed polysaccharides or protein assemblies (Cao, Dickinson & Wedlock, 1990; Dickinson & Golding, 1997; McClements, 2004). This phenomenon was observed in protein-rich emulsions containing casein assemblies (Dickinson & Golding, 1997; Euston & Hirst, 1999). In the present study the SA<sub>4</sub>, which z-average diameter is 80 nm, could be responsible for the observed depletion-flocculation of the fat droplets. By a phenomenon of exclusion from the narrow region surrounding the fat droplets, the SA<sub>4</sub> could create an attractive osmotic force strong enough to overcome the repulsive forces between fat droplets that are consequently driven closer to each other. The depletion-flocculation of the fat droplets is more easily observed by microscopy and image analysis than by light scattering measurement (Dickinson, 2010a).

### 7.2.2.1.2 Fat droplet surface composition

The total amount of proteins adsorbed at the surface of fat droplets was quantified for each emulsion and the results were expressed as surface protein load ( $\text{mg}/\text{m}^2$ ). The surface protein load of SA<sub>4</sub> emulsions depended markedly on the protein concentration in the continuous phase. It was noticeable that the surface protein load decreased from  $7.8 \pm 0.7$  to  $3.9 \pm 0.5$   $\text{mg}/\text{m}^2$  when the protein concentration in the continuous phase increased from 2.9 wt% to 3.5 - 4.0 wt%. Above 3.5 - 4.0 wt% protein in the continuous phase, the surface protein load tended to level off (Fig 62). The surface protein load of the fat droplets of SA<sub>4</sub> emulsions at protein concentration above 3.5 - 4.0 wt% is higher than what expected for a monolayer of adsorbed proteins. For similar total protein contents, the amounts of protein adsorbed at the fat droplet interface were always higher for SA<sub>4</sub> emulsions than for WP A emulsions. Indeed, the surface protein load of WP A emulsions decreased slightly from  $2.4 \pm 0.2$  to  $1.9 \pm 0.5$   $\text{mg}/\text{m}^2$  when the protein concentration in the continuous phase increased (Fig 62).

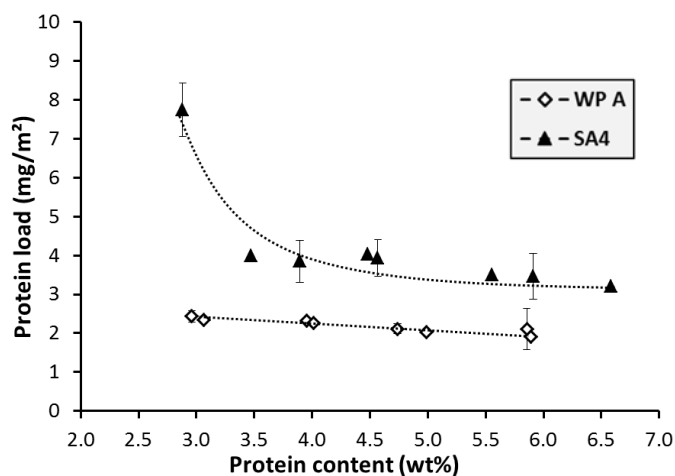


Figure 62: Surface protein load of SA<sub>4</sub> emulsions (black triangle) and WP A emulsions (grey diamond) as a function of the protein concentration in the continuous phase. The dotted lines are guides for the eyes.

As WP ingredients contain different proteins, i.e. globular proteins (mainly  $\beta$ -Lg and  $\alpha$ -La) and caseins, protein composition at the fat droplet surface can be complex and different from the continuous phase. The proteins adsorbed onto the fat droplet surface were quantified after separation by SDS-PAGE (Fig 63A). Based on total protein quantification, equal amounts of proteins were deposited in each well (10  $\mu$ g). SDS-PAGE showed that the composition of the proteins adsorbed at the fat droplet interface differed significantly from the protein composition of the WP ingredient. Caseins were concentrated at the fat droplet interface at the expense of globular proteins, though the casein fraction was only a minor protein fraction in the WP ingredient used in this study (18 % caseins on protein basis in the WP A powder). SDS-PAGE showed that the proportion of caseins adsorbed at the surface of the fat droplets were lower in SA<sub>4</sub> emulsions than in WP A emulsions. The surface casein load was quantified according to the intensity of the casein bands for each electrophoretic track. The surface globular protein load was obtained from the difference between the total surface protein load and the surface casein load. Surface casein and globular protein loads for WP A emulsions and SA<sub>4</sub> emulsions are presented in Fig 63B and 63C, respectively.



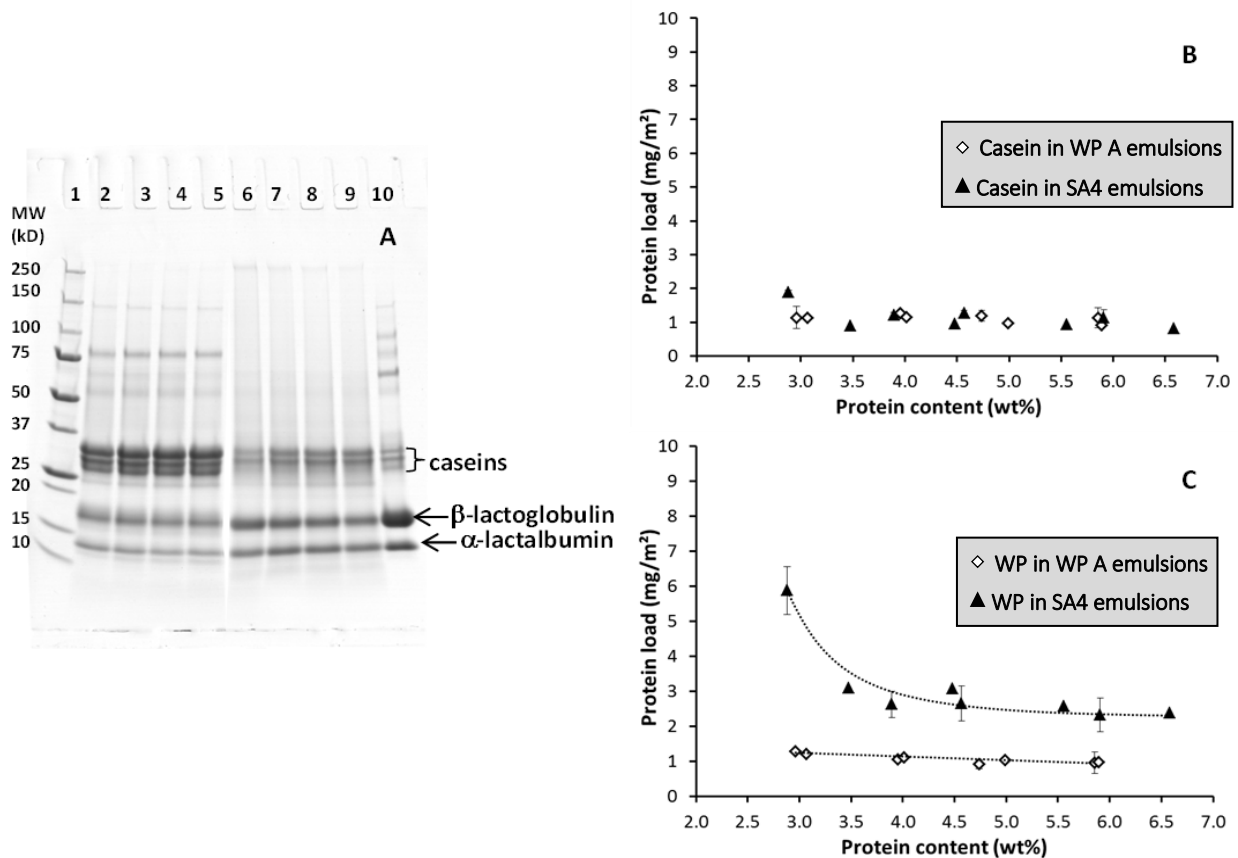


Figure 63: (A) SDS-PAGE of the protein fraction adsorbed onto the fat droplets of WP A emulsions and SA<sub>4</sub> emulsions at different protein concentrations in the continuous phase; Molecular weight marker (1); WP A emulsions at 2.9 wt% (2), 3.9 wt% (3), 4.7 wt% (4), and 5.8 wt% (5) protein content; SA<sub>4</sub> emulsions at 2.8 wt% (6), 3.8 wt% (7), 4.5 wt% (8) and 5.9 wt% (9) protein content; WP A powder (10). (B) Casein and (C) globular whey protein loads for WP A emulsions (grey diamond) and SA<sub>4</sub> emulsions (black triangle) as a function of protein concentration in the continuous phase. The dotted lines are guides for the eyes.

Whatever the emulsion (prepared with SA<sub>4</sub> or WP A suspensions) and regardless of the protein concentration in the continuous phase, the surface casein load was constant ( $1.1 \pm 0.2$  mg/m<sup>2</sup>). The surface globular protein load decreased substantially for SA<sub>4</sub> emulsions when the protein concentration in the continuous phase increased. The quantity of globular proteins at the fat droplet interface decreased from 5.9 to 2.6 mg/m<sup>2</sup> when the protein concentration in the continuous phase increased from 2.9 to 3.9 wt%, respectively, and remained relatively constant above this concentration. In contrast, the surface globular protein load was constant for all protein concentrations in the continuous phase of the WP A emulsions (around  $1.1 \pm 0.1$  mg/m<sup>2</sup>). As the surface protein load and the protein composition of the fat droplets of SA<sub>4</sub> emulsions were stable at protein concentration above 4.5 wt%, an equilibrium between the caseins and SA<sub>4</sub> of the SA<sub>4</sub> suspensions seemed to exist for fat droplet surface.

## 7.2.2.2 Emulsion stability to heat treatment

## 7.2.2.2.1 Heat stability of the emulsions at macroscopic scale

Both SA<sub>4</sub> and WP A emulsions were heated to 120°C from 1 to 30 min in order to detect the first signs of macroscopic destabilisation. Macroscopic destabilisation of the emulsions was evaluated visually and was related to a change of flowability, gelation or the appearance of traces of oiling off (i.e. fat not dispersed as droplets but present instead as a continuous oil phase) on the top of the emulsion samples. The heating time just below that required to observe a destabilization of the emulsion with regard to the above criteria was plotted as a function of the protein concentration in the continuous phase (Fig 64). Macroscopic heat stability of 30 min meant that the emulsion was not visually destabilized after the full heating time at 120°C (30 min).

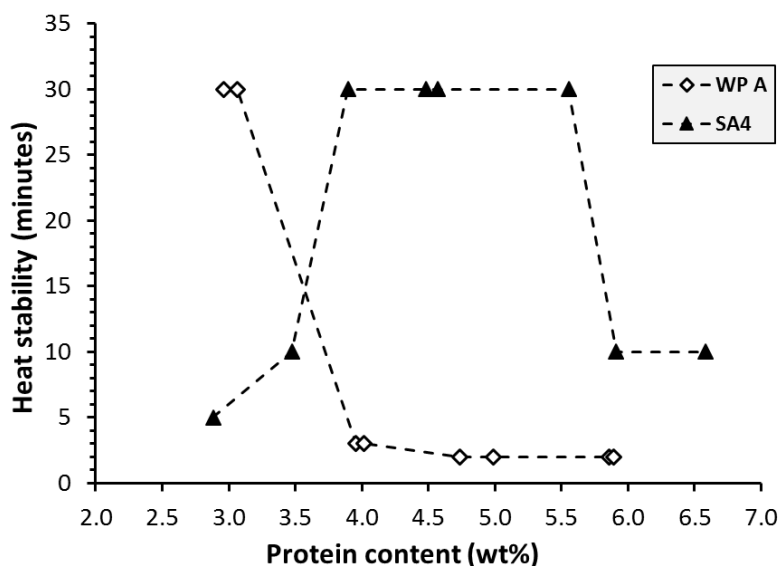


Figure 64: Heat stability of WP A emulsions (grey diamond) and SA<sub>4</sub> emulsions (black triangle) as a function of protein concentration in the aqueous phase. The dotted lines are guides for the eyes.

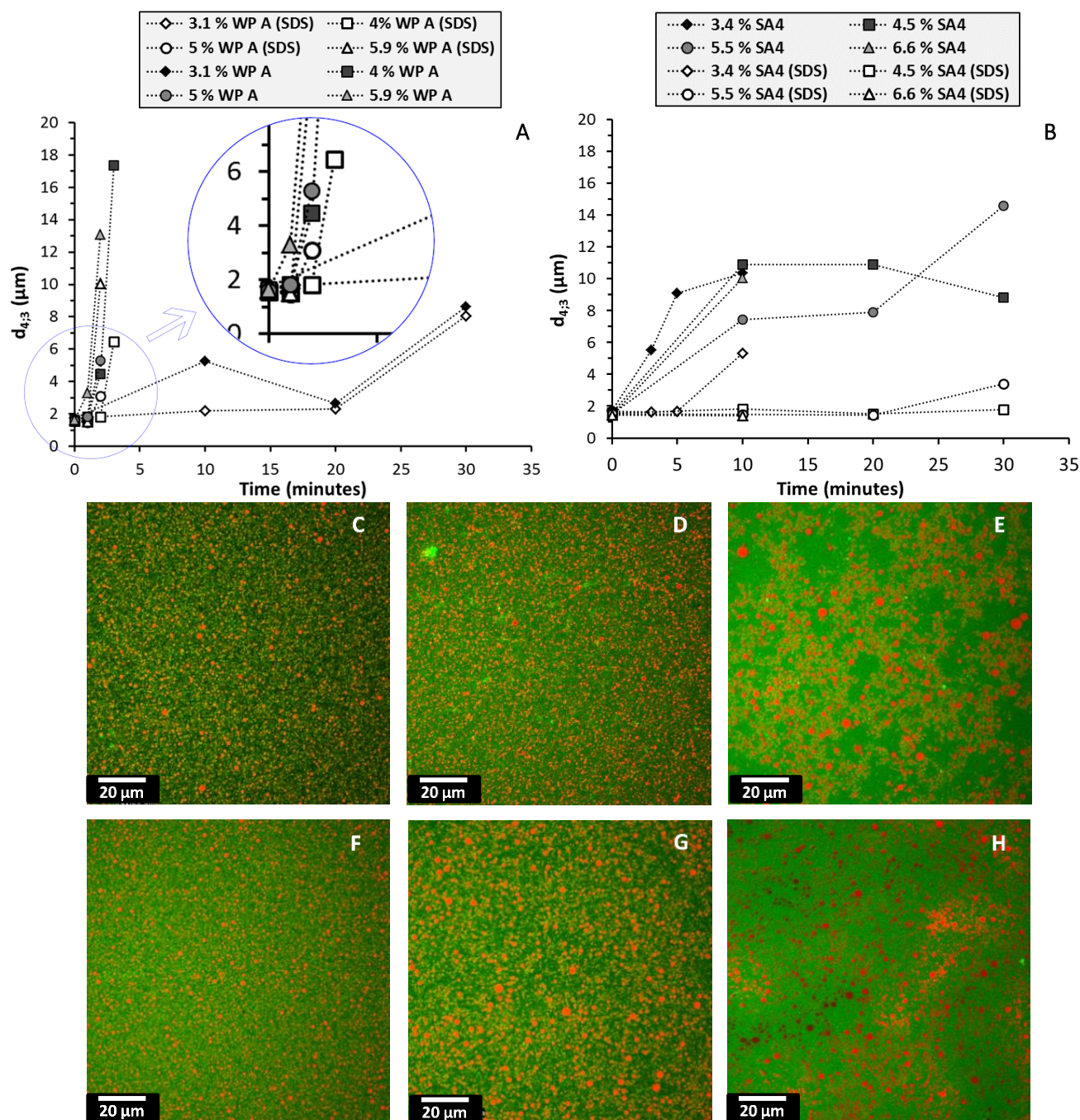
Emulsions prepared with 2.9 wt% and 3.1 wt% WP A suspensions were visually stable to heat treatment (at least 30 min at 120°C), but the macroscopic stability of the emulsion decreased drastically at higher protein concentrations, with gelation after less than 5 min of heating at 120°C. In contrast, SA<sub>4</sub> emulsions were more sensitive to heat treatment at low protein concentrations (i.e. below 3.5 wt%). Emulsions prepared with 2.9 wt% and 3.5 wt% SA<sub>4</sub> suspensions were only stable macroscopically up to 5 and 10 min at 120°C, respectively. Beyond this heating time, these emulsions were more viscous and patches of oiling off were observed. Above 3.5 wt% proteins in the continuous phase, SA<sub>4</sub> emulsions were more stable than WP A emulsions. For intermediate protein concentrations (i.e. between 3.9 wt% and

## CHAPTER 7: Impact of the whey protein aggregate morphology and the presence of caseins on the emulsion stability

5.5 wt%), SA<sub>4</sub> emulsions were the most stable and no sign of destabilization was observed after the full heating time (30 min). At higher protein concentrations in the continuous phase (i.e. above 5.5 wt%), macroscopic stability of SA<sub>4</sub> emulsions decreased, and gelation of the emulsions was observed for heating times longer than 10 min.

### 7.2.2.2.2 Heat stability of the emulsions at microscopic scale

In the visually heat-stable emulsions, particle size was monitored after heat treatment in the absence and presence of SDS (Fig 65A and B). The emulsion prepared with 3.1 wt% WP A suspension was fairly resistant to heat treatment at microscopic scale. There was almost no change in the fat droplet size distribution before or after heating at 120°C. The slight change in the volume mean diameter of the fat droplet observed during heating was due only to some fat droplet flocculation, as the fat droplet distribution of the unheated emulsion was recovered after adding SDS to the heated emulsion (Fig 65A). This result was confirmed by CLSM observations (Fig 65C and 65D). In contrast, WP A emulsions with higher protein concentrations exhibited larger particles at short heating times, as evidenced by laser light scattering in the absence and presence of SDS and then gelation on prolonged heating (Fig 65A). CLSM observations of the WP A emulsions immediately after gelation showed inhomogeneous distribution of the fat droplets in the volume of the emulsion (Fig 65E). Moreover, the fat droplets were visually larger after heating than before heating, suggesting fat droplet coalescence. The continuous phase was also less homogenous after heat treatment; proteins appeared more concentrated around the fat droplets.



## CHAPTER 7: Impact of the whey protein aggregate morphology and the presence of caseins on the emulsion stability

In the emulsions prepared with SA<sub>4</sub> suspension at 2.9 wt%, the particle size increased from 1.7  $\mu\text{m}$  to around 10  $\mu\text{m}$  during heating (Fig 65B) leading to a change in the flowability of the emulsion. This increase was due both to flocculation and/or to coalescence as the initial volume mean diameter of the fat droplets of unheated emulsions was not recovered after adding SDS to heated emulsions (Fig 65B). This was confirmed by the CLSM observations (Fig 65F and 65G). For SA<sub>4</sub> emulsions prepared at 3.9 wt% and 5.5 wt% protein concentration, the volume mean diameter of the fat droplets also increased (Fig 65B) but this was only due to flocculation as the addition of SDS allowed the recovery of the volume mean fat droplet diameter of the unheated emulsions (Fig 65B). The size and the spatial distribution of the fat droplets in the emulsion seemed unaffected by the heat treatment according to the CLSM observations (data not shown), confirming that the level of fat droplet flocculation was probably low. Emulsions prepared with more than 5.5 wt% SA<sub>4</sub> suspension exhibited fat droplet flocculation and even traces of coalescence for longer heating times. The spatial distribution of the fat droplets in these emulsions after heating was fairly uniform (Fig 65H). However, some clusters of fat droplets appeared more intense in the micrographs, suggesting the existence of pieces of gel in the emulsion.

### 7.2.2.2.3 Surface protein load after heat treatment

The surface protein load of fat droplets of heated emulsions was quantified for the greatest heating time before a macroscopic destabilization was noticeable (Fig 66). For all the emulsions, the surface protein load was much higher after heating than before heating. The amount of proteins adsorbed onto fat droplet surface was higher than 6  $\text{mg}/\text{m}^2$  for the emulsions prepared with the WP A suspensions and it tended to increase with the protein concentrations. The surface protein load of the fat droplets of emulsions prepared with the SA<sub>4</sub> suspensions was higher at low protein concentrations and decreased from around 13  $\text{mg}/\text{m}^2$  to 8.4  $\text{mg}/\text{m}^2$  when the protein concentration increased from 3.5 to 6.6 wt%.

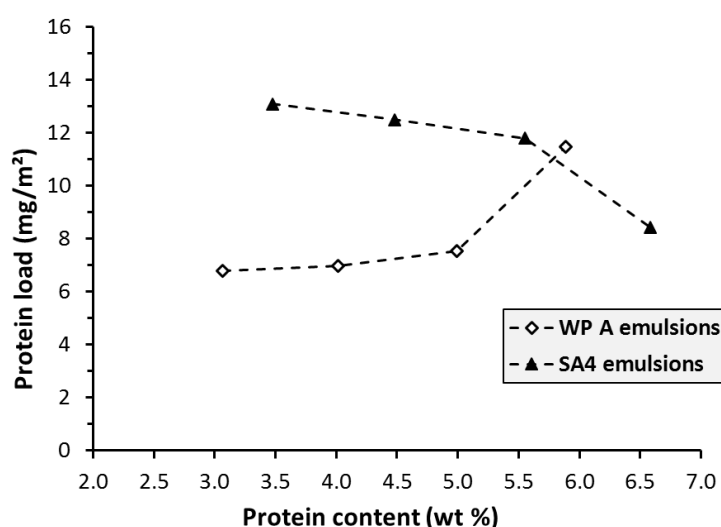


Figure 66: Surface protein load of the WP A emulsions (empty diamond) and SA<sub>4</sub> emulsions (black triangle) at heating time just before a visual destabilization occurred according to the protein concentration in the aqueous phase. The dotted lines are guides for the eyes.

7.2.2.3 Heat stability of emulsions *versus* proteins in suspension

In order to distinguish the effects of the proteins in the continuous phase (i.e. non-adsorbed proteins) from the effect of adsorbed proteins at fat droplet surface on emulsion heat stability, the emulsions and protein suspensions covering the range of protein concentrations of the emulsions were heated at 120°C for up to 30 min. The non-adsorbed protein concentration was determined as the total protein concentration minus the amount of protein adsorbed at the surface of fat droplets. Fig 65 represents the heat stability of WP A and SA<sub>4</sub> emulsions as a function of non-absorbed protein concentrations in the continuous phase and the heat stability of WP A and SA<sub>4</sub> suspensions at equivalent protein concentrations (Fig 67A and 67B).

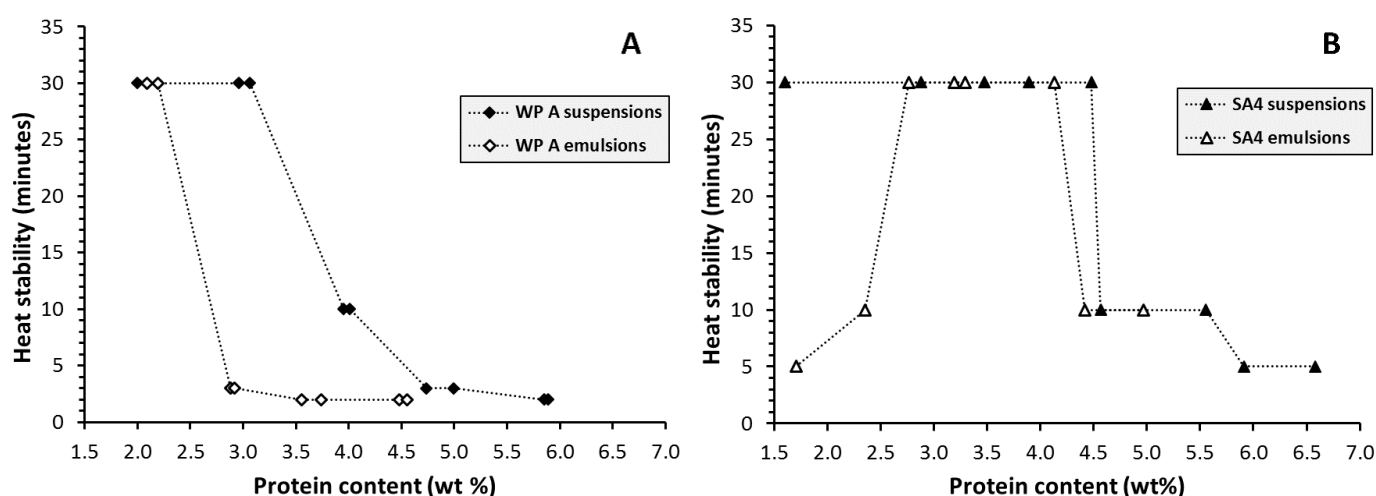


Figure 67: (A) Heat stability of WP A emulsions (grey diamond) as a function of non-adsorbed protein concentration in the continuous phase and WP A suspensions (black diamond) in the same range of protein concentration. (B) Heat stability of SA<sub>4</sub> emulsions (grey triangle) as a function of non-adsorbed protein concentration in the continuous phase and SA<sub>4</sub> suspensions (black triangle) in the same range of protein concentration.

The WP A solutions and WP A emulsions for the lower protein concentrations investigated remained macroscopically stable on heating. However, increasing the protein concentration resulted in gelation of both WP A suspensions and WP A emulsions. Below the critical concentration of gelation of WP A suspensions, dynamic light scattering measurements showed that aggregates were formed during heating: the Z-average diameter of the aggregates in a WP A suspension at 2.9 wt% was ~ 275 nm after 30 min of heating at 120°C. The critical protein concentration of gelation shifted toward higher values when comparing WP A suspensions to WP A emulsions. A WP A suspension at 3 wt% protein was visually stable after 30 min of heating at 120°C (absence of gelation) whereas the emulsion containing the same concentration of non-adsorbed proteins in the continuous phase gelled after 3 min of heating (Fig 67A).

## CHAPTER 7: Impact of the whey protein aggregate morphology and the presence of caseins on the emulsion stability

SA<sub>4</sub> suspensions at low protein concentrations (i.e. below 4.5 wt%) were visually stable for more than 30 min at 120°C (Fig 67B). However, an increase in the Z-average diameter of the aggregates in SA<sub>4</sub> suspensions was observed by dynamic light scattering: the Z-average diameter of the aggregates in the SA<sub>4</sub> suspension at 4.5 wt% protein was 152 nm after 30 min of heating at 120°C instead of 80 nm before heating. Below 3 wt% protein concentration, SA<sub>4</sub> suspensions were visually more stable than SA<sub>4</sub> emulsions containing the same concentrations of non-adsorbed proteins in the continuous phase. Above a protein concentration of 4.5 wt% in the continuous phase, the heat stability of SA<sub>4</sub> emulsions and SA<sub>4</sub> solutions were close. The SA<sub>4</sub> emulsion and SA<sub>4</sub> suspension at 4.4 wt% and 4.6 wt% protein, respectively, were stable for up to 10 min at 120°C.

### 7.2.2.4 Discussion

Emulsions prepared with WP A and SA<sub>4</sub> suspensions at various protein concentrations showed very different heat stability. The decrease in heat stability of WP A emulsions with increasing protein concentrations was observed while the protein load and protein composition at the surface of the fat droplets was almost the same. The range of surface protein load determined in the present study for WP A emulsions (from 2.4 to 1.9 mg/m<sup>2</sup>) was in the range of values reported for WP emulsions by other authors (Euston & Hirst, 1999; Hunt & Dalgleish, 1994; Sliwinski et al., 2003) when the amounts of protein in the continuous phase were not limiting. Under these conditions, proteins in the continuous phase are in competition with each other for adsorption at the fat droplet interface (Dalgleish, 1997; Ye, 2008). The type and amount of proteins that are adsorbed at the fat droplet surface depend on the composition and concentration of the proteins in the continuous phase (Dalgleish, 1997; David Julian McClements, 2004; Ye, 2008). The composition of the fat droplet interface of WP A emulsions was enriched in caseins compared to the composition of the WP A suspensions. Indeed, for protein concentrations above 3 wt% flexible caseins adsorb preferentially over globular proteins at the fat droplet interface due to their high surface activity (Hunt & Dalgleish, 1994; Ye, 2008). For WP A emulsions at the lowest protein concentration, about 30 wt% of the caseins of the emulsion were adsorbed at the fat droplet surface compared to less than 10 wt% of globular WP. The slightly decreasing trend in interfacial protein concentration when the protein concentration in the emulsion increased might have resulted from the increased adsorption of caseins at the interface in place of globular proteins (M. Srinivasan, Singh & Munro, 2002). Indeed, caseins can cover more interfacial area than WP (Hunt & Dalgleish, 1994). However, the change in interfacial protein composition was too limited to be evaluated in view of the precision of the quantification by SDS-PAGE.

Since the fat droplet surface composition barely changed with increasing protein concentrations in the emulsion, it can be assumed that the protein concentration in the continuous phase play a major role

in explaining the gelation of WP A emulsions at high protein concentration (Çakır-Fuller, 2015; Dybowska, 2011; Euston et al., 2000; Reiffers-Magnani et al., 1999). In the present study, WP A emulsions gelled when the non-adsorbed protein concentration in the continuous phase increased above 3 wt%, in agreement with Çakır- Fuller (2015). Below this critical concentration of gelation, only slight flocculation of the fat droplets was observed. The presence of caseins at the interface might protect WP A emulsions against heat destabilization, as indicated by other authors (Dickinson & Parkinson, 2004; Parkinson & Dickinson, 2004). Caseins, especially  $\beta$ -casein, increase the thickness of the thin adsorbed globular protein layer and enhance steric stabilization (Dickinson & Parkinson, 2004). Non-adsorbed proteins formed aggregates on heating but they were too diluted and/or too small to form a gel. It is interesting to note that at higher protein concentrations WP A emulsions gelled at lower concentrations of non-adsorbed proteins in the continuous phase than WP A solutions. This difference suggests that proteins at the interface of fat droplets interact with proteins in the continuous phase on heating and contribute to the gelation of the emulsion. This was confirmed by the increasing surface protein load after heating WP A emulsions. Proteins in the emulsions unfold on heating and interact with other unfolded proteins either in the continuous phase or at the surface of fat droplets (Euston et al., 2000). The interface is then thicker than a monolayer of proteins (Dickinson et al., 1989; Hunt & Dalgleish, 1994). Below the critical concentration of gelation, the protein concentration is not enough to form a continuous network of denatured proteins including fat droplets (Fig 68). On the other hand, denatured WP in the continuous phase have a “glue” role (Euston et al., 2000) connecting the different fat droplets in a network of denatured proteins above the critical concentration of gelation (Fig 68).

In SA<sub>4</sub> emulsions, the surface protein load of fat droplets decreased and the interfacial composition changed with increasing protein concentration in the emulsion. As for WP A emulsions, the proportion of caseins at the fat droplet surface of SA<sub>4</sub> emulsions was higher than in the continuous phase. About the same amounts of casein were quantified in the emulsions but the amounts of globular proteins were always higher in SA<sub>4</sub> emulsions than in WP A emulsions. This can be explained by the adsorption of SA<sub>4</sub> carrying a large number of globular proteins at the same time at the fat droplet surface. Interestingly, increasing the protein concentration in the SA<sub>4</sub> emulsions reduced the surface protein load and especially the surface WP load. This can be explained by the replacement of SA<sub>4</sub> at the fat droplet surface by caseins and non-aggregated WP, these latter being in higher amounts in the continuous phase of the emulsion when the protein concentration increased. However, the sensitivity of quantification by SDS-PAGE identified only a decrease in the globular WP content (aggregates) and was too low to identify an increase in the casein content at the fat droplet interface. This confirms that small surface active molecules present in the continuous phase of the emulsion have a key role in the composition of the interfacial layer through rapid coverage of the interface (Dalgleish, 2006). This result supports the



## CHAPTER 7: Impact of the whey protein aggregate morphology and the presence of caseins on the emulsion stability

observation on the fat droplet compositional change of WPM emulsions when protein concentration increased. However, even for the SA<sub>4</sub> emulsions at high protein concentration, WP remained the more abundant proteins at fat droplet surface. In contrast, they were almost completely excluded from the fat droplet surface of the WPM emulsions at high protein concentration (Chap 6, Fig 47B).

SA<sub>4</sub> emulsions were unstable to heat treatment at low protein concentrations and at high protein concentrations but for different reasons (Fig 68). At low protein concentrations the SA<sub>4</sub> emulsions were characterized by the flocculation and coalescence of fat droplets, resulting in fat patches at the top of emulsions, while at high protein concentration the emulsions gelled. SA<sub>4</sub> emulsions were stable at intermediate protein concentrations. These behaviors can be explained by the protein composition of the fat droplet surface and the protein behavior in the continuous phase. It was shown that the aggregates in SA<sub>4</sub> suspensions were able to interact on heating at 120°C, forming clusters of aggregates or a gel at low and high protein concentrations, respectively. At low protein concentrations (i.e. below 3.9 wt%), the number of strand-like aggregates in the continuous phase of the SA<sub>4</sub> emulsions was low and the fat droplets had the highest levels of protein aggregates on its surface. The amount of caseins adsorbed at fat droplet surface was also low. Under these conditions, the interactions of fat droplets with each other *via* interfacial aggregates was enhanced, resulting in fat droplet flocculation (Fig 68). The close contact of fat droplets resulted in some coalescence and the appearance of patches of oiling off over the heating time. Fat droplet flocculation and coalescence could also explain the changes in emulsion flowability that have been observed. SA<sub>4</sub> emulsions thus had lower heat stability compared to SA<sub>4</sub> suspensions because the instability was mainly governed by the fat droplet surface. Increasing protein concentration in the SA<sub>4</sub> emulsions (3.9 wt% - 5.5 wt%) resulted in an increase in the quantity of SA<sub>4</sub> in the continuous phase and a reduction in SA<sub>4</sub> adsorbed at the surface of fat droplets. The probability of fat droplet interactions *via* interfacial aggregates was reduced in favor of interactions between SA<sub>4</sub> in the continuous phase with SA<sub>4</sub> at the surface of the fat droplets and interactions between SA<sub>4</sub> in the continuous phase. Under these conditions, it is probable that some SA<sub>4</sub> initially present in the continuous phase cross-linked some fat droplets resulting in their flocculation, but no coalescence was observed. At the same time, the concentration of SA<sub>4</sub> in the continuous phase was too low to form a continuous network and the emulsions showed no sign of macroscopic destabilisation over the heating time (Fig 68). At the higher protein concentrations investigated, the interaction of SA<sub>4</sub> with each other in the continuous phase led to the formation of a continuous network (Fig 68). The gelling behavior of the SA<sub>4</sub> suspensions and SA<sub>4</sub> emulsions was almost the same if only the non-adsorbed protein concentration in the continuous phase of the emulsions was taken into account, suggesting that the fat droplets made only a very small contribution to the formation of the continuous aggregate network. This also indicated that caseins cannot be displaced by heat-induced aggregates during

heating. The quantity of caseins at the surface of the fat droplets is enough to ensure the stability of the fat droplet during the heating (Hunt & Dalgleish, 1995; M. Srinivasan et al., 2002).

This study established that the adsorbed SA<sub>4</sub> at fat droplet surface could destabilize the fat droplet surface during heating as already observed for adsorbed WPM. If casein amount in the emulsion is sufficient, the amount of SA<sub>4</sub> at fat droplet surface was reduced. Compared to the WPM emulsions, the continuous phase had a strong influence on the heat stability of the SA<sub>4</sub> emulsions at high protein concentration. Hence, the heat-gelation properties of the SA<sub>4</sub> suspension must be taken into account.

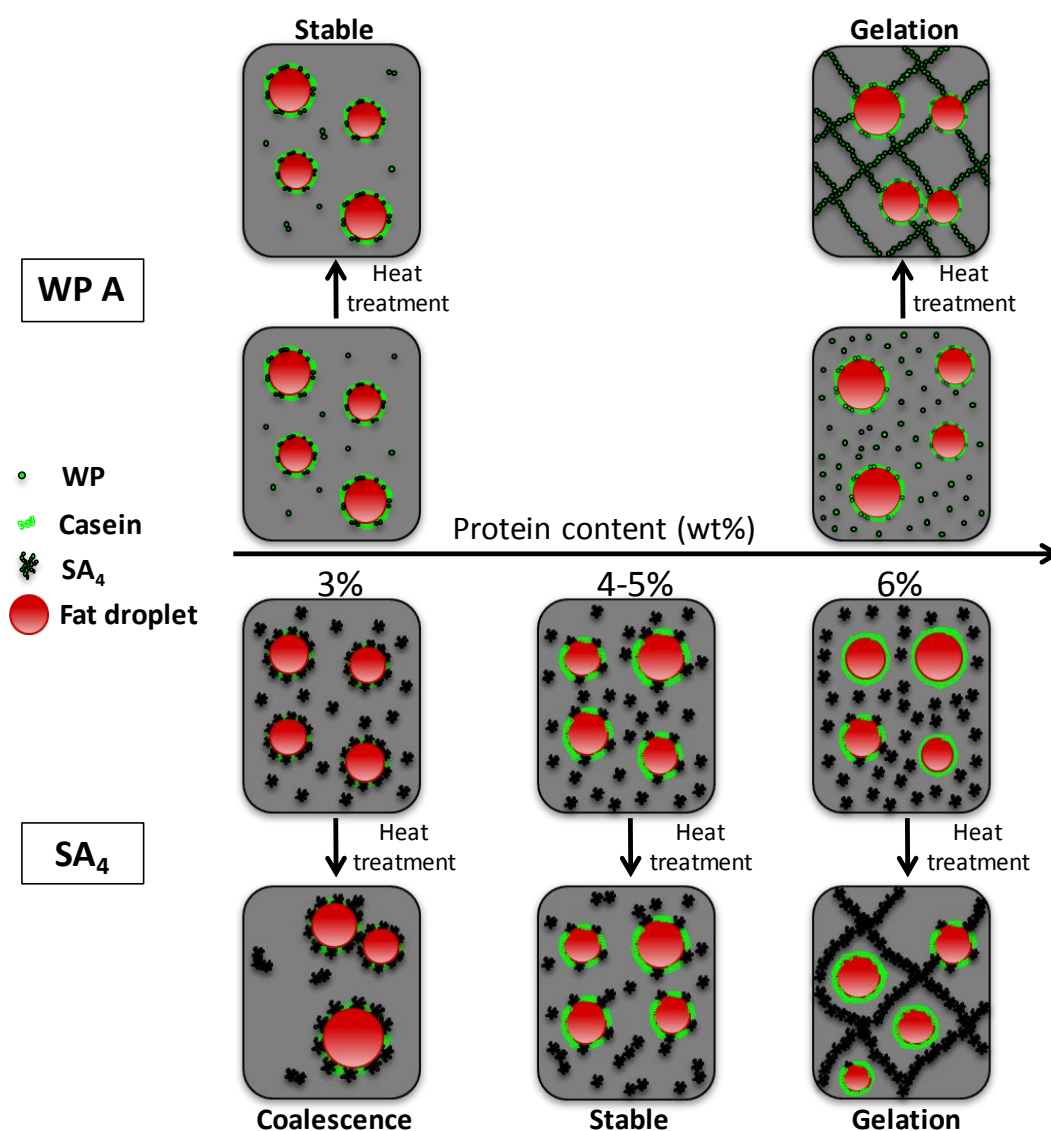


Figure 68: Summary of the structure of the WP A emulsions (top) and SA<sub>4</sub> emulsions (bottom) before and after heat treatment as a function of protein concentration.

### 7.2.3 Heat stability of the SA<sub>4</sub> emulsions at various casein/SA<sub>4</sub> ratio

The objective of this part of the work was to investigate the respective contribution of SA<sub>4</sub> and caseins adsorbed at fat droplet surface on the destabilization of SA<sub>4</sub> emulsions observed at low protein concentration. The strategy consisted in preparing a SA<sub>4</sub> suspension at 2.8 wt% to which increasing amounts of caseins (0.07 wt%, 0.15 wt% and 0.23 wt%) were added. As for the WPM emulsion, the sodium caseinate was selected as casein source because the casein composition of the WP A powder was mainly  $\alpha_{s1}$  and  $\kappa$ -caseins. Note that the amount of caseins added in the protein suspension was cumulated to the casein amount already present in the 2.8 wt% of SA<sub>4</sub> suspension (Table 8). Emulsions were prepared with various casein/SA<sub>4</sub> ratios and their heat stability was characterized.

Table 8: Amount of caseins added and total casein content in SA<sub>4</sub> suspension

<b>Added caseins (wt%)</b>	0.00	0.07	0.15	0.23
<b>Total casein content (wt%)</b>	0.17	0.24	0.32	0.40

Casein addition did not modify the droplet size distribution, with a volume mean droplet diameter for all emulsions of  $1.6 \pm 0.1 \mu\text{m}$  with or without SDS. Fat droplets distribution appeared to be homogeneous for the four emulsions by CLSM observations. A slight shift of the inter-fat droplet distance distribution toward the larger distance was observed with the increase in casein concentration (Fig 69). The shift was much more limited than by changing the protein concentration in the emulsion (Fig 61). As indicated previously it could be explained by the depletion-flocculation of some fat droplets induced by the sodium caseinate assemblies and/or the SA<sub>4</sub> from the continuous phase (see below).

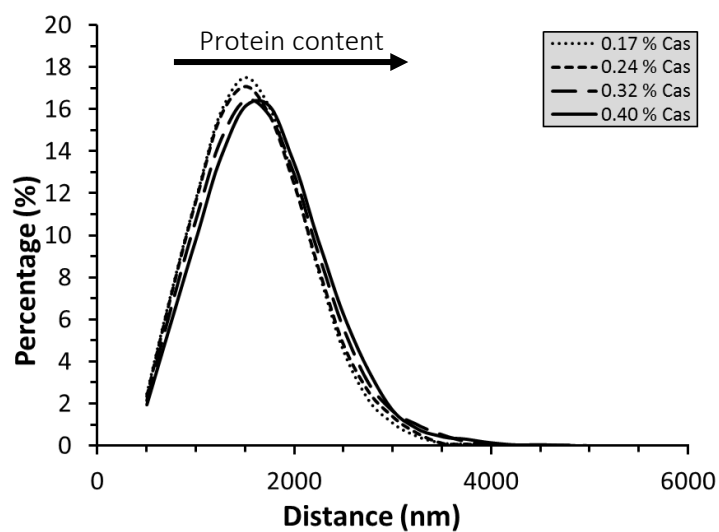


Figure 69: Distribution of the distances between fat droplet surface for the SA<sub>4</sub> emulsions at 2.8 wt% as a function of casein concentrations: small dotted line, 0.17 wt% ; dashed line, 0.24 wt%; large dashed line, 0.32 wt%; continuous line, 0.40 wt%.

# CHAPTER 7: Impact of the whey protein aggregate morphology and the presence of caseins on the emulsion stability

The amount of adsorbed proteins and the composition of adsorbed proteins onto the surface of the fat droplets of the emulsion before heating were analyzed (Fig 70). In the absence of added caseins, the surface protein load of the fat droplets was 6.2 mg/m<sup>2</sup>. It decreased continuously up to 2.4 mg/m<sup>2</sup> with the increase in added caseins up to 0.23 wt% in the emulsions. This value was close to the surface protein load expected for a monolayer of protein (Hunt & Dalgleish, 1994). The surface protein load in casein/SA<sub>4</sub> emulsion with 0.23 wt% of added caseins was lower than that observed in the SA<sub>4</sub> emulsions containing similar amount of caseins but a higher amount of SA<sub>4</sub> (emulsion prepared with a SA<sub>4</sub> suspension at 6.6 wt%). The adsorption of caseins at fat droplet surface was favored by the higher casein/SA<sub>4</sub> ratio in the former emulsion.

SDS-PAGE analysis indicated a decrease in the amount of WP adsorbed at the surface of the fat droplets when the casein/SA<sub>4</sub> ratio increased in the emulsions. The amount of adsorbed caseins remained almost constant but its proportion relative to the total amount of protein adsorbed at the fat droplet surface increased. The protein competition for the fat droplet surface resulted in a replacement of the adsorbed SA<sub>4</sub> by caseins with the increase in casein concentration in the emulsions. Interestingly the  $\alpha$ -La band decreased less drastically than the  $\beta$ -Lg band on SDS-PAGE suggesting that the protein proportion in the non-aggregated WP fraction was different than in the SA<sub>4</sub> in agreement with other studies (Çakır-Fuller, 2015). This author indicated that the  $\beta$ -Lg was more prone to heat aggregation than  $\alpha$ -La. The addition of caseins in the emulsions replaced in preference the SA<sub>4</sub> compared to non-aggregated WP.

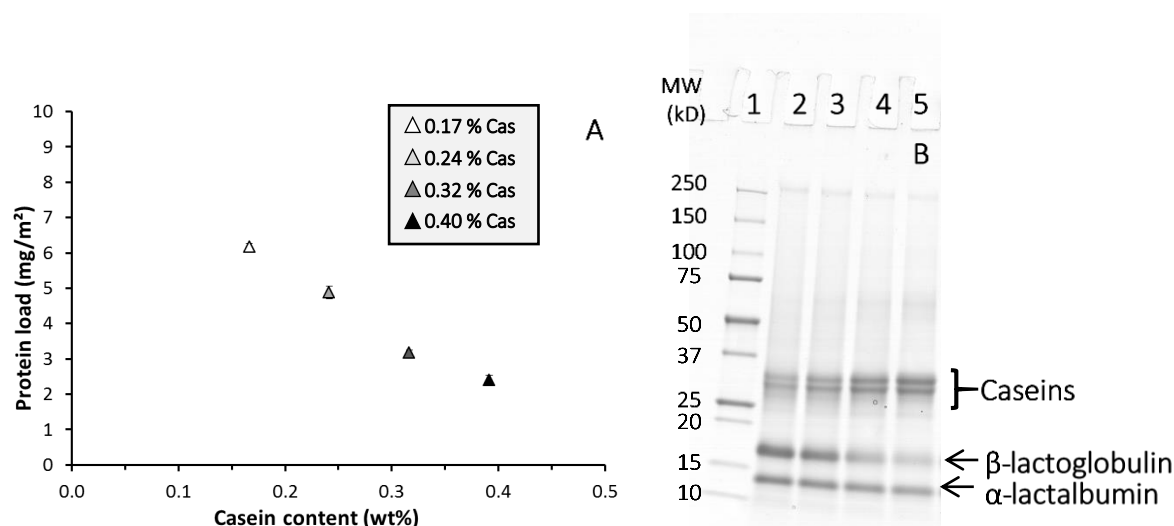


Figure 70: (A) Protein surface load of the fat droplets of SA<sub>4</sub> emulsions (2.8 wt% of SA<sub>4</sub>) containing various amount of sodium caseinate: 0.17 wt% (empty triangle), 0.24 wt% (light grey triangle), 0.32 wt% (dark grey triangle) and 0.40 wt% (black triangle). (B) SDS-PAGE of the adsorbed protein fraction onto fat droplet surface of emulsions prepared with 2.8% of SA<sub>4</sub> suspension and various amount of sodium caseinate: 0.17 wt% (2), 0.24 wt% (3), 0.32 wt% (4) 0.40 wt% (5).; Molecular weight marker (1).

## CHAPTER 7: Impact of the whey protein aggregate morphology and the presence of caseins on the emulsion stability

The heat stability of the emulsions containing various casein/SA<sub>4</sub> ratios was also checked. Unlike the emulsions prepared with the WPM A suspensions (where the heat stability of the emulsions followed the all-or-nothing principle; Chap 6, part 6.2.4.1), the heat stability of the casein/SA<sub>4</sub> emulsions increased progressively with the increase in the casein concentration (Fig 71). The improved heat stability by increasing the casein/SA<sub>4</sub> content in the emulsions was correlated with a reduced surface protein load of the fat droplets. In addition, the more the caseins were absorbed at the fat droplet surface, the more the emulsions were heat stable. From these observations it is suggested that the probability of the fat droplets to interact via interfacial aggregates decreased.

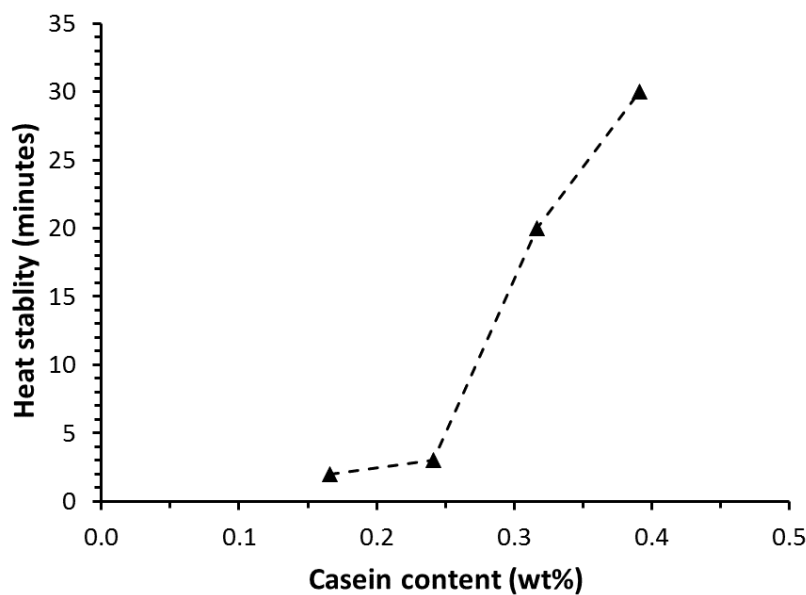


Figure 71: Heat stability of 2.8 wt% SA<sub>4</sub> emulsions (black triangle) as a function of casein concentration. The dotted lines are guides for the eyes.

### 7.2.4 Freeze/thaw stability of the SA<sub>4</sub> emulsions and casein/SA<sub>4</sub> emulsions

The SA<sub>4</sub> emulsions and casein/SA<sub>4</sub> emulsions prepared in the previous part were also analyzed for the freeze/thaw stability.

The stability of the emulsions after one freeze/thaw cycle was represented according to the protein composition of the suspensions used to prepare the emulsions and the surface protein load (Figure 72). The emulsions were visually stable after thawing except two emulsions prepared at low protein concentration (i.e. emulsion at 2.8 wt% and 2.8 wt% with 0.07% of caseins). These latter showed a release of fat (i.e. oiling off) at the top of the tube. They exhibited the highest surface protein load and a high proportion of adsorbed WP at the fat droplet surface (Fig 70). Consequently, the protein composition of the fat droplet surface appeared to play a significant role in the freeze/thaw stability of

## CHAPTER 7: Impact of the whey protein aggregate morphology and the presence of caseins on the emulsion stability

the emulsions. As for the WPM A emulsions, the adsorbed SA<sub>4</sub> onto the fat droplet surface seemed to be responsible for the destabilization of the emulsion.

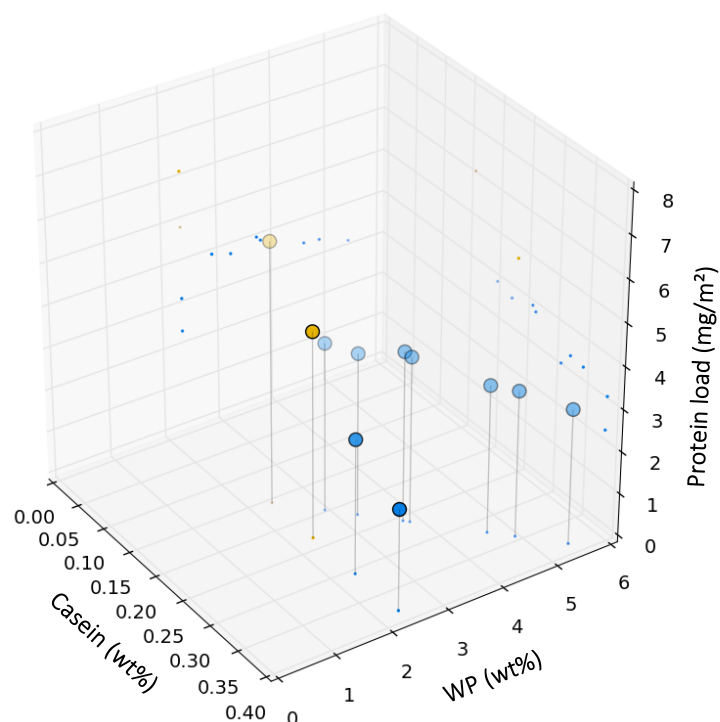


Figure 72: Freeze/thaw stability of the emulsion prepared with the SA<sub>4</sub> suspension and with or without added caseins: stable emulsions (blue circle) and emulsions showing oiling off (yellow circle).

At a microscopic scale, laser light scattering measurements in the absence and presence of SDS indicated that some fat droplets flocculated and others coalesced during a freeze/thaw cycle although the emulsions were visually stable (Fig 73). The changes of SA<sub>4</sub> emulsions microstructure were reduced at high protein concentration probably due to a decreased amount of SA<sub>4</sub> and an increased amount of caseins adsorbed at fat droplet surface.

Therefore, only the protein composition (surface protein load) of the fat droplet seemed to impact the freeze/thaw stability of SA<sub>4</sub> emulsions and casein/SA<sub>4</sub> emulsions. Unlike emulsion heat stability, the protein concentration of the continuous phase did not play a key role.

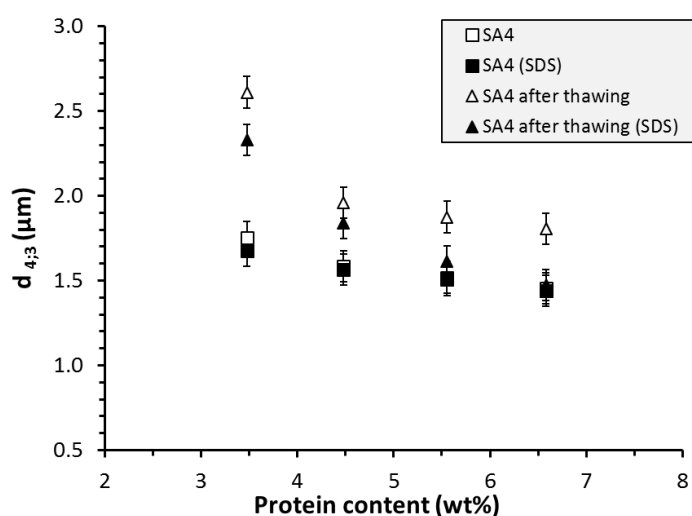


Figure 73: Volume mean diameter ( $d_{4,3}$ ) determined by light scattering measurements with (full black symbol) or without SDS (open symbol) of the particles in the emulsions prepared with the SA<sub>4</sub> suspensions at various protein concentrations before (square) and after one freeze/thaw cycle (triangle) .

### 7.3 Stability of the emulsions prepared with the WPM/SA B suspensions during technological treatments

As shown in chapter 5, the conditions of formation of the WPM were strict and dependent on the composition of the WP powders. Starting from a second powder (WP B powder) and by adjusting the heating conditions of the WP B suspension, a mixture of both whey protein microgels and strand-like aggregates (WPM/SA B suspension) was obtained. More precisely, the WPM/SA B suspension contained 70 % of WPM, 21.5 % of strand-like aggregates, 4.75 % of caseins and 3.75 % of residual native WP. Emulsions prepared with WPM/SA B suspensions at a protein concentration ranging from 3.6 wt% to 7.4 wt% were tested for their stability during technological treatments. Two additional WPM/SA B emulsions in which sodium caseinate was added, were also prepared and tested under the same conditions. The first emulsion was prepared with a WPM/SA B suspension at 3.6 wt% and 0.37 wt% of sodium caseinate; the second emulsion was prepared with a WPM/SA B suspension at 3.7 wt% and 0.14 wt% of sodium caseinate.

#### 7.3.1 Design of heat-stable WPM/SA B emulsions

The macroscopic emulsion heat stability was represented according to the casein concentration and the WP concentration of the WPM/SA B suspension used to prepare the emulsions, and the surface protein load of the fat droplets (Fig 74). A blue symbol indicates that the emulsion was stable during the full heating time whereas a red symbol indicates that the emulsion gelled during the time course of the heat stability test.

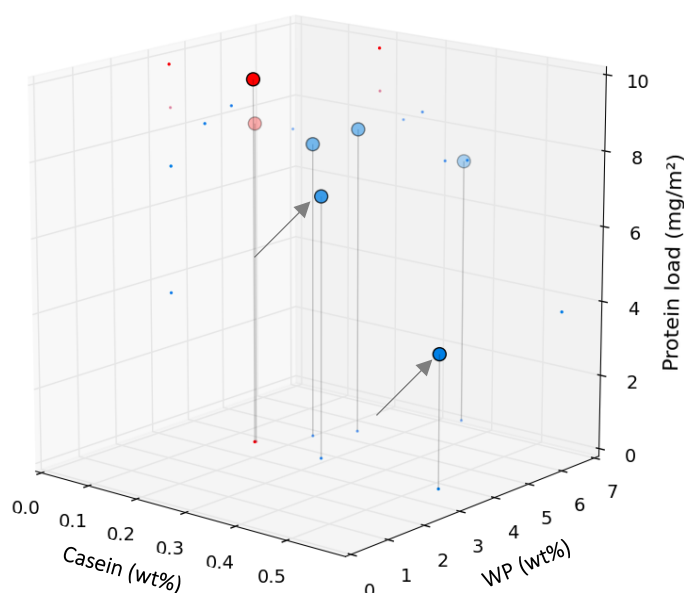


Figure 74: Heat stability of the emulsions prepared with the WPM/SA B suspensions and with or without addition of sodium caseinate: stable emulsions (blue circle), and gelled emulsions (red circle). The grey arrows show the emulsions with added caseins.

As for the WPM A emulsions and SA<sub>4</sub> emulsions, the emulsion heat stability evolved with the protein content of the emulsion. The WPM/SA B emulsions at low protein concentration ( $\leq 3.7$  wt%) gelled after 3 min on heating alike the WPM A emulsions. By increasing the protein concentration up to 4.7 wt%, the emulsion heat stability was improved. The WPM/SA B emulsions that gelled on heating had the lower casein content and the fat droplets with the higher surface protein load. However, compared to heat-stable WPM A emulsions and heat-stable SA<sub>4</sub> emulsions, heat-stable WPM/SA B emulsions had much higher surface protein load. The surface protein load of WPM/SA B emulsions slightly decreased when protein concentration increased. In the absence of added caseins, the casein content of WPM/SA B emulsions was below 0.35 wt%. Based on the calculation of specific surface area of the emulsions ( $\approx 1080 \text{ m}^2/\text{L}$  of emulsion) and the results obtained with WPM A emulsions (when mainly caseins covered the fat droplet surface), it is suggested that the amount of caseins in the WPM/SA B emulsion was not sufficient to cover all the fat droplet surface. Hence, a part of the fat droplet surface could be covered by some WP aggregates explaining the surface protein load observed. However, WPM/SA B emulsions were heat stable for a surface protein load of the fat droplets leading to a destabilization for the other systems studied (WPM A emulsions and SA<sub>4</sub> emulsions) indicating that the surface protein load alone cannot explain emulsion heat stability. In order to explain the heat stability of WPM/SA B emulsions having high surface protein load, a study of the microstructure and of the interface composition before and after heat treatment was performed.



CHAPTER 7: Impact of the whey protein aggregate morphology and the presence of caseins on the emulsion stability

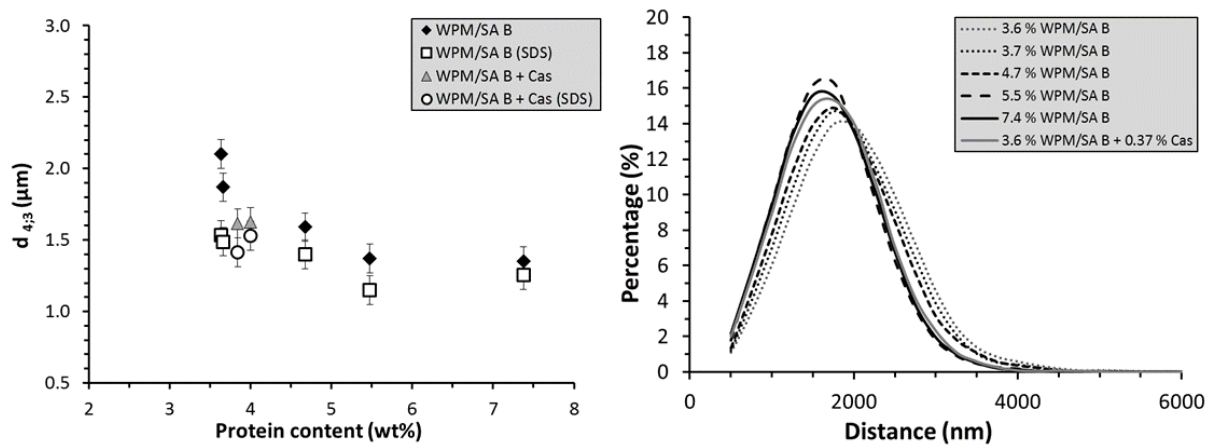


Figure 75: (A) Volume mean diameter ( $d_{4,3}$ ) determined by laser light scattering of the particles in the emulsions prepared with the WPM/SA B suspensions at various protein concentrations before heat treatment with (empty square) or without SDS (black diamond) and in the WPM/SA B emulsions with caseins adding with (empty circle) or without SDS (grey triangle) (B) Distribution of the distance between fat droplet surface for WPM/SA B emulsion as a function of protein concentration in the continuous phase: grey dotted line, 3.6 wt%; black dotted line, 3.7 wt%; small dashed line, 4.7 wt%; large dashed line, 5.5 %; black continuous line, 7.4 wt% and for WPM/SA B emulsion with 3.6 wt% and 0.37 wt% of sodium caseinate; grey continuous line.

Before heating, signs of fat droplet flocculation were observed at microscopic scale by light scattering measurements as well as by CLSM image analysis of the WPM/SA B emulsions at protein concentration below 5.5 wt% (Fig 75). Indeed, for these emulsions, the volume mean diameter of the fat droplets in the absence of SDS was slightly higher than in presence of SDS. In the presence of SDS the volume mean diameter was almost the same at  $1.4 \pm 0.1 \mu\text{m}$  for all emulsions (Fig 75A). By increasing the protein concentration, the volume mean diameter of the fat droplets in the absence of SDS decreased and was similar to the one in the presence of SDS at protein concentration above 5.5 wt%. Although the fat droplet distribution appeared to be homogeneous in the WPM/SA B emulsion by CLSM, a calculation of the distribution of the inter-fat droplet distances by CLSM image analysis revealed slight differences in the microstructure of the emulsions at low and high protein concentrations. The distribution of the inter-fat droplet distances was shifted toward the smaller size with the increase in the protein concentration indicating a more homogenous repartition of the fat droplets in the emulsions at high protein concentration. The repartition of the fat droplets was almost similar in the WPM/SA B emulsions at 5.5 wt% and 7.4 wt% proteins confirming light scattering measurements. It was worth noting that the addition of caseins to WPM/SA B emulsions at low protein concentration allowed to prevent fat droplet flocculation and to obtain the same fat droplet distribution as for the most concentrated emulsions (i.e. emulsions prepared with the 5.5 wt% and 7.4 wt% of protein concentration) (Fig 75A and 75B). This was confirmed by the CLSM image of the emulsions (Fig 76A, 76C and 76E).

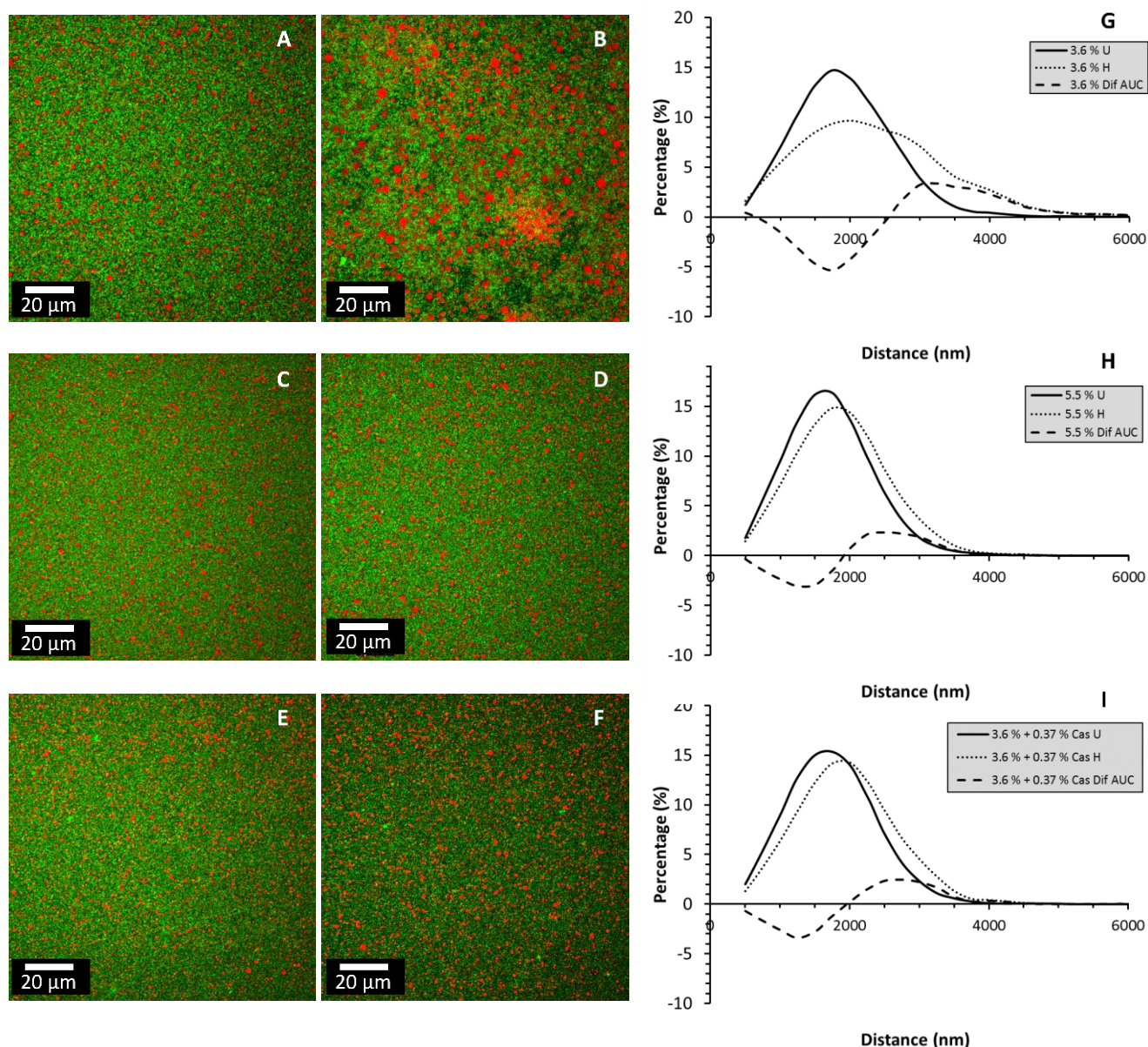


Figure 76: Microstructural analysis of emulsions prepared with the WPM/SA B suspensions. (A to F) Confocal Laser Scanning Microscopic (CLSM) images of the emulsions prepared with WPM/SA B suspension at 3.6 wt% (A, B) unheated (A) and heated 3 min (B); at 5.5 wt% (C, D) unheated (C) and heated 30 min (D); at 3.6 wt% and 0.37 wt% of caseins (E, F) unheated (E) and heated 30 min (F). Fat droplets were stained with Nile Red (red color on the images) and the proteins with Fast green (green color on the images). (G to I) Inter-fat droplet distance distribution for emulsions at (G) 3.6 wt%, (H) 5.5 wt% protein concentration and (I) at 3.6 wt% of protein concentration and 0.37 wt% of caseins before (continuous line) and after (small dotted line) heating as well as the Dif AUC (point by point difference of the area under the curve of the inter-particle distance distribution between the non-heated and heated emulsions; large dotted line).

After heating, the gelled emulsions exhibited fat droplet flocculation (Fig 76B). The distribution of the fat droplets in the emulsion became highly inhomogeneous (Fig 76G). Light scattering measurements confirmed the flocculation of the fat droplets before the gelation time. Indeed, the volume mean diameter of the fat droplets after 2 min of heat treatment was 4.9  $\mu\text{m}$  and 1.5  $\mu\text{m}$  without or with SDS,

CHAPTER 7: Impact of the whey protein aggregate morphology and the presence of caseins on the emulsion stability

respectively (Fig 77). These results suggested that the flocculation of the fat droplets was at the source of the emulsion gelation. In visually heat-stable emulsions, CLSM images showed a homogenous distribution of the fat droplets (Fig 76D) even if signs of flocculation and coalescence were evidence by CLSM image analysis (Fig 76H) and laser light scattering (Fig 77). The increase in the protein content in visually heat-stable WPM/SA B emulsions enhanced heat-induced fat droplet flocculation and coalescence as found in laser light scattering (Fig 77). It was confirmed by the CLSM image analysis that showed an evolution more important for the Dif AUC curve indicating a more heterogeneous distribution of the fat droplets in the emulsion at 7.4 wt% (Fig 78) compared to the emulsion at 4.7 wt%. As for the WPM A emulsions and the SA<sub>4</sub> emulsions an addition of caseins to WPM/SA B emulsion allowed improving its heat stability at low protein concentration ( $\leq 3.7$  wt%). After heating the distribution of the fat droplets was still homogeneous (Fig 76F), although some fat droplet flocculation and coalescence occurred but to a lesser extent than in other WPM/SA B emulsions (Fig 76I).

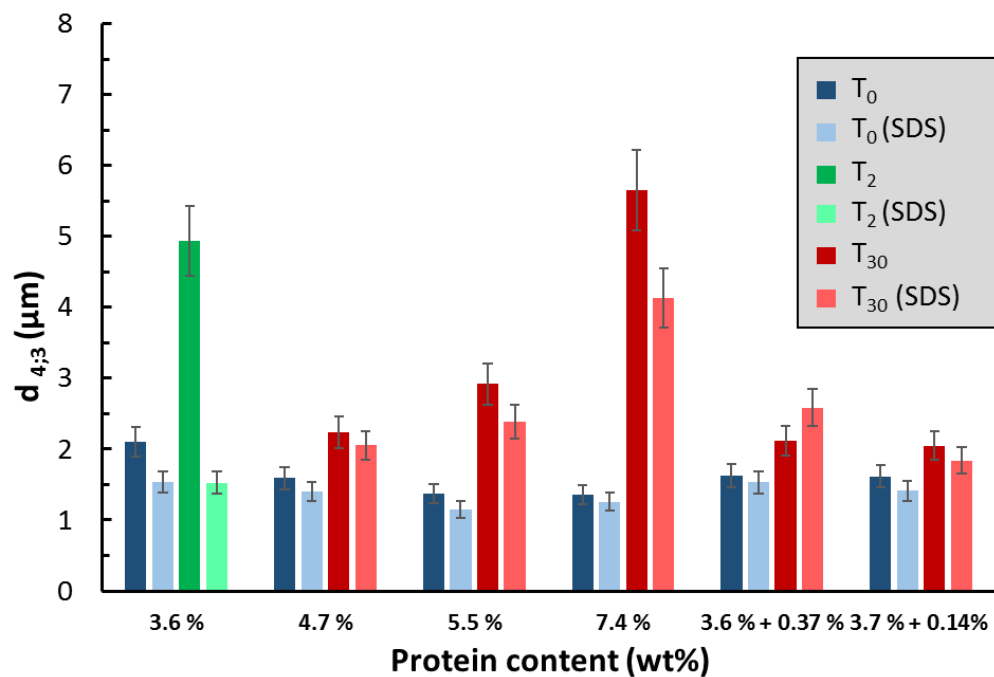


Figure 77: Volume mean diameter ( $d_{4,3}$ ) determined by laser light scattering of the particles in the heat stable emulsions prepared with the WPM/SA B suspensions at various protein concentrations and caseins concentrations before heat treatment with (light blue) or without SDS (dark blue), after 2 min of heat treatment for gelled emulsions with (light green) or without SDS (dark green) and after 30 min of heat treatment for stable emulsions with (light red) or without SDS (dark red).

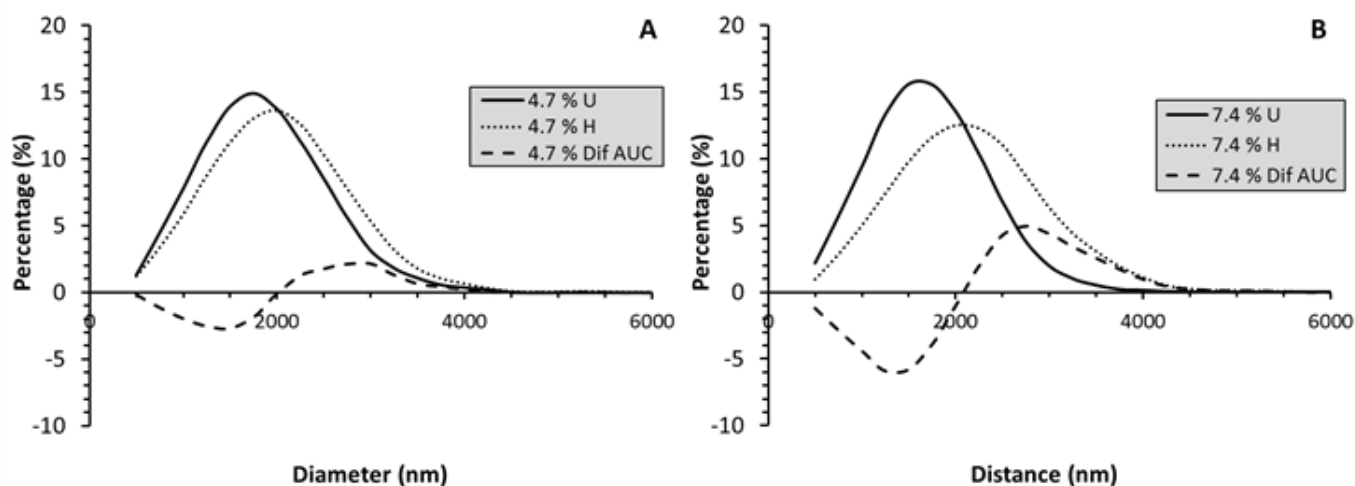


Figure 78: Inter-fat droplet distance distribution for emulsions at (A) 4.7 wt% and (B) 7.4 wt% protein concentration before (continuous line) and after (small dotted line) heating as well as the Dif AUC (point by point difference of the area under the curve of the inter-particle distance distribution between the non-heated and heated emulsions; large dotted line).

In order to explain the emulsion behavior difference on heating, an analysis of the fat droplet surface composition was performed (Fig 79). The heat-stable WPM/SA B emulsions had a lower surface protein load than the WPM/SA B emulsions that gelled on heating (i.e. above 8.5 mg/m<sup>2</sup>), but the difference of surface protein load between heat-stable emulsions and emulsions that gelled on heating is tiny. Presently, it is not clear what modifications in the protein composition of the fat droplet surface would be responsible for the changes of emulsion heat stability. Indeed, by SDS PAGE analysis it is not possible to distinguish between adsorbed WPM and adsorbed SA<sub>4</sub> at fat droplet surface (Fig 79B). Based on the previous characterization (WPM A emulsions and SA<sub>4</sub> emulsions) it is tempting to assign the decrease in the surface protein load to a lower amount of WPM at fat droplet surface. Indeed, WPM adsorbed to fat droplet surface only when the amount of caseins was insufficient to cover the fat droplet surface whereas an equilibrium between SA and caseins at fat droplet surface existed. Hence, by increasing casein concentration (for instance by increasing the protein concentration of the emulsions), WPM would be swept more easily than SA from the interface. The absence of WPM at fat droplet surface would be responsible for the increased emulsion heat stability. As the surface protein load was higher than what expected for a monolayer of proteins, we suggest the WPM/SA B emulsions were the most stable when both SA and caseins covered the fat droplet surface (Fig 77). This hypothesis has to be considered with caution and more studies were necessary to confirm it. The surface protein load was reduced in the presence of added caseins as already observed for WPM A emulsions and SA<sub>4</sub> emulsions. It is suggested that the caseins replaced WPM and strand-like aggregates at fat droplet interface as

# CHAPTER 7: Impact of the whey protein aggregate morphology and the presence of caseins on the emulsion stability

suggested by the decrease in surface protein load and the increase in the casein band intensity in the SDS-PAGE (Fig 79A and 79B). In order to give weight to these hypotheses, the surface protein load was calculated whether all the caseins and the sum of the caseins and the SA of the WPM/SA B suspension covered the fat droplet surface. In the WPM/SA B suspension, the proportion of caseins and SA on a weight protein basis was 4.75 % and 21.5 % respectively. Therefore, in the WPM/SA B suspension at 3.6 wt% protein, the concentration of caseins was 0.17 wt% and the concentration of SA was 0.77 wt%. Caseins and SA concentrations were at 0.35 wt% and 1.60 wt%, respectively, in the WPM/SA B suspension at 7.4 wt% protein. In chapter 6, we calculated that a concentration of 0.4 wt% of caseins was required to cover a total fat droplet surface of  $\sim 1000 \text{ m}^2$  (similar fat droplet surface here) at about  $3 \text{ mg/m}^2$ . The calculation indicates that the amount of caseins in the WPM/SA B suspensions was not enough to cover the total fat droplet surface. Consequently, the fat droplet surface was also covered by some WP aggregates explaining the surface protein load of the emulsions at 3.6 wt% ( $> 9 \text{ mg/m}^2$ ) and at 7.4 wt% ( $8 \text{ mg/m}^2$ ). In the WPM/SA B emulsion at 3.6 wt%, a theoretical surface protein load of  $9 \text{ mg/m}^2$  was calculated assuming all the caseins and SA were adsorbed at the fat droplet surface. This calculation explained the presence of the WPM at the fat droplet surface for the emulsion at low protein concentration (i.e.  $\leq 3.6 \text{ wt\%}$ ). For the emulsion at 7.4 wt% protein, the amount of caseins and SA was largely enough to cover the fat droplet surface at the experimental protein surface load ( $8 \text{ mg/m}^2$ ). Based on the competition between the caseins, SA and WPM for fat droplet surface we assume the caseins and strand-like aggregates mainly covered the fat droplet surface whereas the WPM were in the continuous phase.

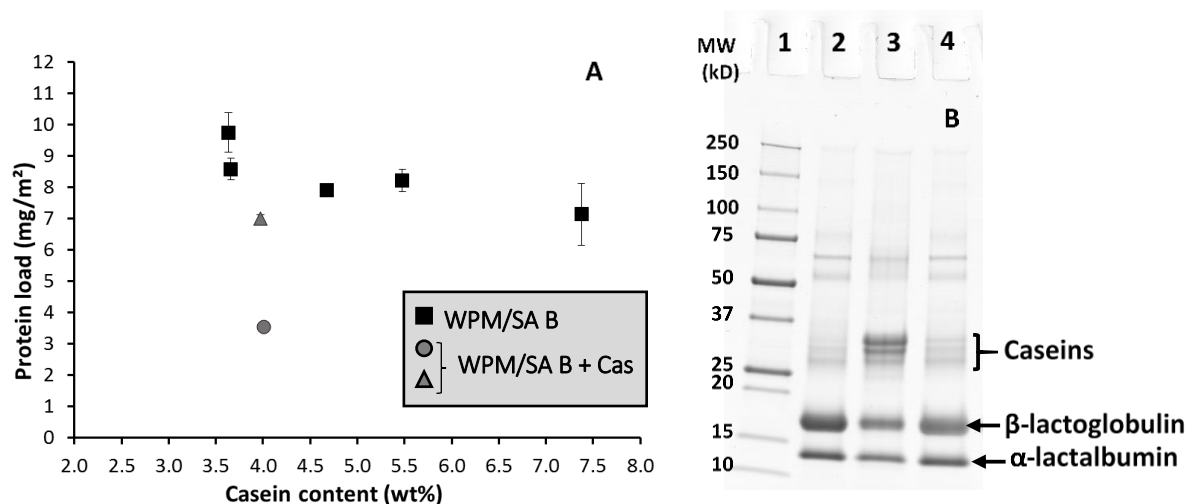


Figure 79: (A) Surface Protein load of the fat droplets of WPM/SA B emulsions prepared various amount proteins (black square) and WPM/SA B emulsions at 3.6 wt% and 0.37 wt% of sodium caseinate (grey circle) and at 3.7 wt% and 0.14 wt% of sodium caseinate (grey triangle). (B) SDS-PAGE of the adsorbed protein fraction onto fat droplet surface of emulsions prepared with WPM/SA B suspensions: 3.6 wt% (2), 7.4 wt% (4), 3.7 wt%; Molecular weight marker (1);



## CHAPTER 7: Impact of the whey protein aggregate morphology and the presence of caseins on the emulsion stability

The TEM observation of the fat droplets of the emulsion supported the hypothesis made above. At low protein concentration (i.e. below 3.7 wt%) before heat treatment, the TEM observation suggested that the fat droplet surface was mainly covered by strand-like aggregates (Fig 80A). The presence of strand-like aggregates at fat droplet surface gives thick interface as the one mainly observed in the present pictures (black arrow Fig 80). A fat droplet interface covered by caseins was thin as already observed in chapter 6 (Fig 54C). It is possible that some domains of fat droplets surface were covered by caseins but they were difficult to visualize (red arrow Fig 80). Some WPM were also adsorbed at the fat droplet surface (blue arrow Fig 80) but in low proportion compared to the emulsions prepared with WPM A suspensions (Chapter 6 Fig 54B). In WPM A emulsions, the WPM adsorbed to the surface of the fat droplet were responsible for the heat-gelation of the emulsion. They could also be responsible for the gelation of the WPM/SA B emulsions at low protein concentration.

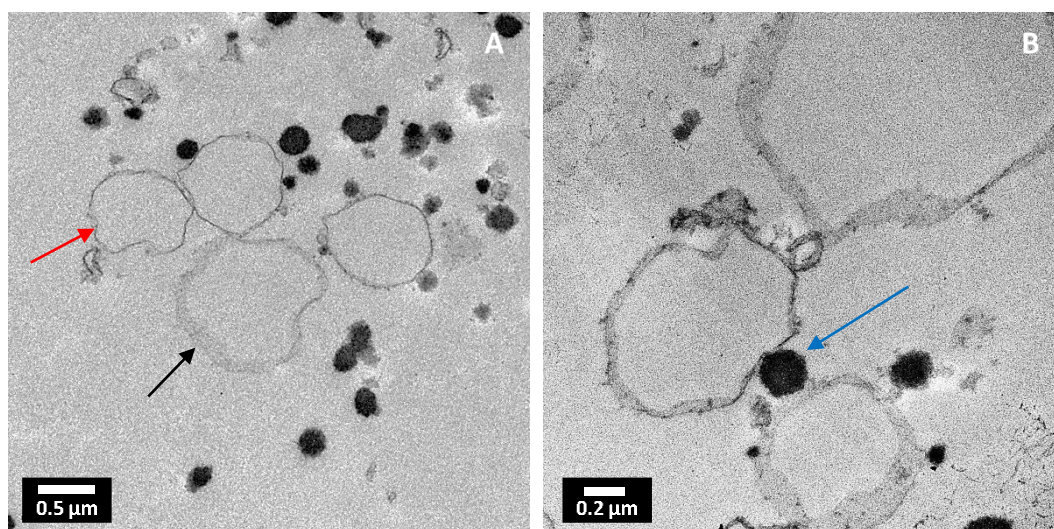


Figure 80: Transmission Electron Microscopy (TEM) images of the unheated emulsions prepared with WPM/SA B suspension at 3 wt% (A) unheated and (B) heated. Red arrow indicated thin interface, black arrow indicated thick interface and blue arrow indicated adsorbed WPM.

Based on previous observations, a scheme of the microstructure of the WPM/SA B emulsions could be proposed (Fig 81). The increase in the protein concentration or the adding of caseins conducted to a release of the WPM and some SA from the fat droplet surface (fig 81B). At low protein concentration, the presence of the WPM adsorbed at the fat droplet surface could be responsible for the gelation of the emulsion during heating (Fig 81A). At high protein concentration, WPM are in the continuous phase and are heat stable; the SA are either adsorbed at fat droplet surface with most of the caseins or dispersed in the continuous phase of the emulsion but below the critical concentration of gelation.

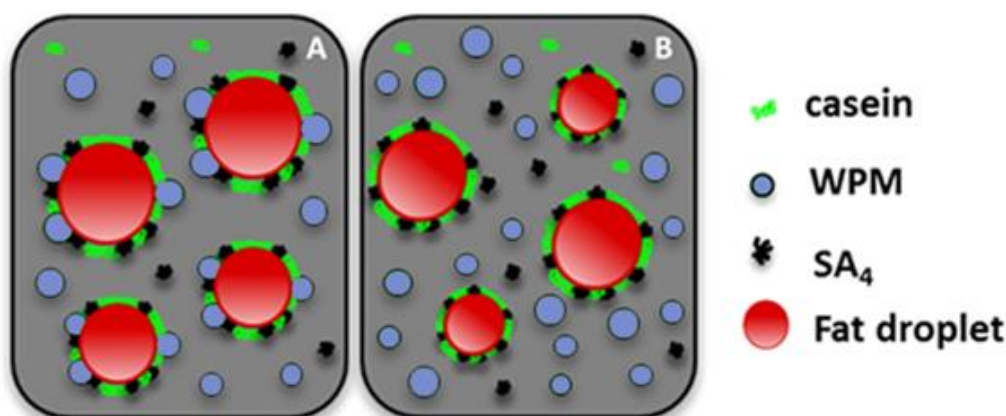


Figure 81: Proposition of scheme of the microstructure of the emulsions at two protein concentrations. (A) Interface for the emulsion prepared with WPM/SA B suspension at  $\leq 3.7$  wt% (B) Interface for the emulsion prepared with WPM/SA B suspension at  $\geq 4.4$  wt%

To conclude, heat-stable WPM/SA B emulsions were obtained when fat droplet surface was covered either by caseins or by both caseins and strand-like aggregates and when the WPM were dispersed in the continuous phase. Strand-like aggregates are probably in too low concentration to induce a destabilization of the emulsion as observed for SA<sub>4</sub> emulsions at similar protein concentration. The presence of WPM at fat droplet surface would be at the source of the heat-gelation of the emulsions at low protein concentration.

### 7.3.2 Freeze/thaw stability of the WPM/SA B emulsions

The WPM/SA B emulsions were also analyzed for the freeze/thaw stability. A blue symbol referred to a stable emulsion and a pink symbol to an emulsion with a creamed layer on the top of the tube after a freeze/thaw cycle. All the emulsions were creamed after one freeze/thaw cycle, except the emulsion with the highest quantity of sodium caseinate added (Fig 82).

The stable emulsion had the fat droplets with the lowest surface protein load. It is probable that the added caseins had reduced the amount of adsorbed aggregates to a level allowing the emulsion to stay stable during a freeze/thaw cycle. Previous experiments indicated a creamed layer of fat droplets was only observed at the top of the tube of WPM A emulsions containing adsorbed WPM at the fat droplet surface. The SA<sub>4</sub> emulsions exhibited fat droplets coalescence and trace of oiling off at the top of the tube when the amount of SA<sub>4</sub> was the highest at fat droplet surface. The present observation would suggest that the fat droplets having some adsorbed WPM were responsible for the formation of a creamed layer on the top of WPM/SA B emulsions after a freeze/thaw cycle. This assumption was in partly contradictory to the hypothesis formulated for the heat stability of the WPM/SA B emulsions.

Further experiments are required to link the structure of the emulsions to the stability during technological treatments.

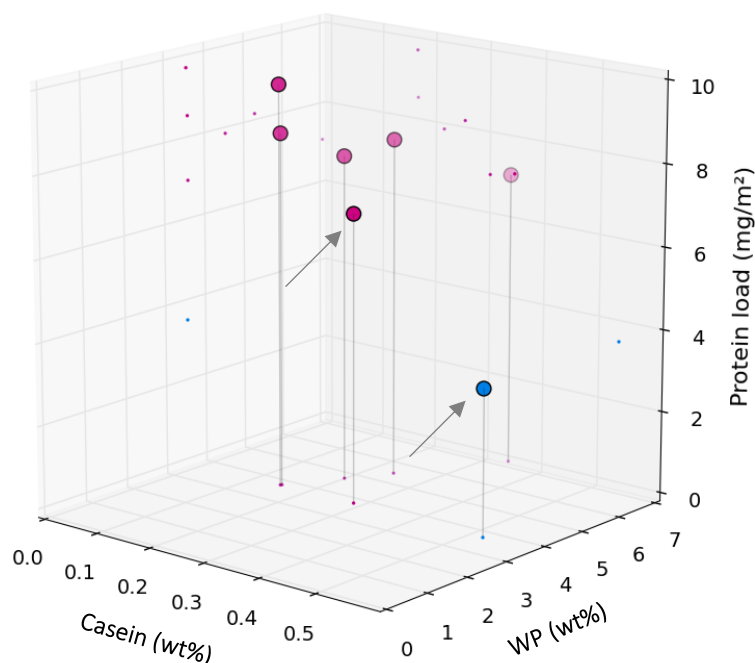


Figure 82: Freeze/thaw stability of the emulsions prepared with the WPM/SA<sub>4</sub> B suspensions and without or with added caseins: stable emulsion (blue circle) and emulsion showing a creamed layer at the top of the tube (pink circle). The grey arrows indicate the emulsion with added caseins.

## 7.4 Conclusion

This study showed that the presence of strand-like aggregates at the surface of fat droplets reduced the emulsion heat stability due to a rapid flocculation, the coalescence of the fat droplets and finally the released of fat (oiling off) on the top of the emulsions. They were also responsible for the destabilization of the emulsion during freeze/thaw cycle. These behaviors were observed for SA<sub>4</sub> emulsions at low protein concentrations (i.e. below 3 wt%). The reduction of the quantity of strand-like aggregates at the surface of the fat droplets, either by increasing the protein concentration or by adding caseins, considerably increased the SA<sub>4</sub> emulsion stability during technological treatments. However, when the SA<sub>4</sub> concentration in the continuous phase was above the critical gelation concentration (5.9 wt%), SA<sub>4</sub> emulsions gelled during heating. The fat droplets were entrapped in a gelled SA<sub>4</sub> network, but they did not contribute to its construction. Hence, it was possible to design heat-stable and freeze/thaw stable SA<sub>4</sub> emulsions by combining the use of SA<sub>4</sub> and a small amount of caseins. However, the heat stability of the emulsions was limited by the heat stability of the SA<sub>4</sub> in solution.



## CHAPTER 7: Impact of the whey protein aggregate morphology and the presence of caseins on the emulsion stability

Some stability tests were conducted on emulsions prepared with a mixture of aggregates but the results obtained indicated that the structure and the behavior of the emulsions were not a simple combination of the behaviors of the WPM emulsions and SA<sub>4</sub> emulsions. Such systems need to be further investigated. Nevertheless, it seemed to be possible to stabilize the emulsions during heat treatment with a higher surface protein load. The emulsions prepared with the suspensions containing a mixture of aggregates appeared to be more sensitive during the freeze/ thaw cycle.



## General Conclusion

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In the current consumer's expectation for more natural and healthy food products, the use of milk protein aggregates as ingredients with enhanced emulsifying, texturing or vectorization properties is of great interest to the dairy sector. The aim of the PROFIL project is to investigate the different properties of the milk protein aggregates in model food systems in order to allow French dairy companies to develop innovative food products adapted to new trends and to new consumptions habits.

My PhD research work focused on the use of the whey protein aggregates in collaboration with caseins to obtain whey protein-rich fluid oil-in-water emulsions, which remained stable after applying technological treatments i.e. heating and freeze/thaw cycle. Different types of whey protein aggregates were selected and their ability to stabilize emulsions during technological treatments in the absence and presence of caseins was investigated. The suspensions containing the whey protein aggregates and the emulsions were characterized by the combination of biochemical and structural approaches allowing multi scale observations. The full strategy developed in this research work allowed to increase knowledge and to bring answers to the 4 main objectives displayed earlier in the manuscript (see chapter 2):

### **1. What is the stability of the aggregates in the continuous phase of the emulsions during the technological treatments?**

The whey protein aggregates were prepared in water at defined pH values (i.e. pH 5.8, 6.6 and 7). In emulsions, the aggregates are dispersed in the soluble phase of milk, meaning a medium containing salts, lactose and at pH 7, which can be different to the pH of the suspension used for their formation. Therefore, their stability during the technological treatments could be modified. My research work showed that the structure and the concentration of the aggregates affected their heat stability in the soluble phase of milk. The influence of the aggregate size was clearly observed for the strand-like aggregates. Increasing the size of the aggregates decreased the heat stability of the aggregates suspensions. Above a critical concentration of strand-like aggregates (i.e.  $\geq 4.5$  wt%), the suspension gelled. Emulsions prepared with the strand-like aggregates could not be heat-stable above a concentration of strand-like aggregates in the continuous phase larger than the critical concentration of gelation. Spherical and dense aggregates (whey protein microgel) were extremely heat stable in the soluble phase of milk in the range of the protein concentration tested (i.e. 2 – 7.4 wt%). Therefore, the whey protein microgel in the continuous phase of the emulsions were heat stable in all the protein concentration range investigated. At the studied whey protein microgel/strand-like aggregates ratio

(i.e. ratio of 70/20) and in the protein concentration range investigated (i.e. 3.6 – 7.4 wt%), the mix suspension was heat stable at 120°C for at least 30 min. Based on previous observations, we suggest that heat stability of the strand-like aggregates governed the heat stability of the suspensions and emulsions containing a mixture of whey protein microgel and strand-like aggregates. However, in the experimental conditions used during my PhD work, the concentration of strand-like aggregates in the mix suspension was too low to induce a heat-destabilization of the continuous phase of the emulsions. All the suspensions of whey protein aggregates were stable after a freeze/thaw cycle. Therefore, the presence of whey protein aggregates in the continuous phase of the emulsions should not influence its freeze/thaw stability.

## **2. Do the whey protein aggregates adsorbed at the fat droplet surface influence the emulsion stability during the technological treatments?**

whey protein aggregates adsorbed at the fat droplet surface were responsible for the destabilization of the emulsions during heating, regardless the structure of these aggregates. The adsorbed whey protein microgel induced an extensive flocculation of the fat droplets leading to emulsion gelation. The gelled network was formed by interactions between neighboring whey protein microgel-covered fat droplets. We observed that a soft and self-supporting gelled network was formed with the fat droplet partially covered by the whey protein microgel. With the increase in fat droplets whey protein microgel coverage, the gelled network contracted and expelled the continuous phase of the emulsions. Strand-like aggregates adsorbed at fat droplet surface were responsible for the flocculation and the coalescence of the fat droplets. When the two types of aggregates were adsorbed simultaneously at the fat droplets surface, the behavior of the adsorbed whey protein microgel seemed to govern the emulsion heat stability.

The whey protein microgel adsorbed at the fat droplet surface was at the source of the emulsion destabilization after a freeze/thaw cycle. In contrast, stand-like aggregates adsorbed at the fat droplet surface did not negatively affect the emulsion stability during freeze/thaw cycle. The simultaneous presence of whey protein microgel and strand-like aggregates at the fat droplet surface reduce the freeze/thaw stability of the emulsions. Further experiments are needed to link the composition of the fat droplet surface and the emulsion stability.

## **3. Does the morphology of the whey protein aggregates affect the stability during the technological treatments of dairy emulsions?**

Even if both types of aggregates, i.e. whey protein microgel or strand-like aggregates, destabilize the emulsion once adsorbed at the fat droplet surface, the mechanism of destabilization was not the same. The adsorbed strand-like aggregates induced a rapid flocculation and coalescence of the fat droplets

along with the released of fat (oiling off) on the top of the emulsions during heating. The adsorbed whey protein microgel connected the fat droplets together, leading to the heat-induce gelation of the emulsions. Similarly, the structure of the aggregates influenced the heat stability of the continuous phase of the emulsions. Whey protein microgel remains mainly as individual particles during the heat treatment, while strand-like aggregates interacted to form larger aggregates or a gel above the critical concentration of gelation of the strand-like aggregates. It is highly possible that the heat induced gelation of the strand-like aggregates was due to the cation (i.e.  $\text{Ca}^{2+}$ ) present in milk permeate. In the absence of strand-like aggregates at the surface of the fat droplets, the gelled network of strand-like aggregates entrapped the fat droplets.

For the freeze/thaw stability, only the aggregates adsorbed at the fat droplet surface affect the emulsion stability. In co-adsorption with caseins at the fat droplet surface, the whey protein microgel and the strand-like aggregates were responsible for the coalescence and oiling off in the emulsions. Emulsions prepared with a mixture of aggregates had a structure and a behavior during technological treatments that is not directly related to the behaviors of the whey protein microgel emulsions and SA<sub>4</sub> emulsions. This system need to be further investigated.

#### **4. What is the combined effect of the whey protein aggregates and caseins on the stability of dairy emulsions?**

In this PhD project, a promising way to stabilize whey protein-rich emulsions was to use the combined properties of the whey protein aggregates and caseins. Caseins adsorbed preferentially to whey protein aggregates at the fat droplet surface. Once forming a protein monolayer at the fat droplet interface, caseins improve the emulsion stability during both freeze/thaw cycle and heat treatment by a protection of the fat droplet surface against flocculation and coalescence. Therefore, the amount of casein required to stabilize emulsions should be a function of the total protein surface area. In this study, the casein concentration in the whey protein aggregates suspension was increased by increasing the total protein concentration of the suspension due to the presence of trace of caseins in the whey protein powders or by adding a small amount of sodium caseinate. The amount of caseins required to cover the total fat droplet surface in our emulsions was about 0.4 wt%. In this research work, we showed the beneficial effect of adding caseins on the stability of the emulsions prepared with a mixture of whey protein microgel and strand-like aggregates. In such emulsions, a competition between the aggregates for the fat droplet surface seemed to occur.

Therefore, it is possible by a reasoned approach to control the stability of whey protein-rich emulsions during technological treatments by combining the functional role of the whey protein aggregates and the caseins. As soon as the caseins cover the fat droplet surface, the heat stability of the emulsions is only dependent on the heat stability of the aggregates in the continuous phase. In this case, the whey protein microgel appear to have more interesting properties. Whey protein-rich emulsions prepared with native whey proteins are extremely stable during freeze/thaw stability. However, in frozen dairy products, a heat treatment of the mix usually precedes the freezing step. Similarly, liquid dairy products (creams, milks) heat-treated for export can freeze during their shipments. Therefore, the presence of whey protein aggregates could affect the freeze/thaw stability of the emulsions. This latter can be controlled by a control of the composition of the fat droplet surface.

The global finding of the PhD thesis is shown in the Figure 83:

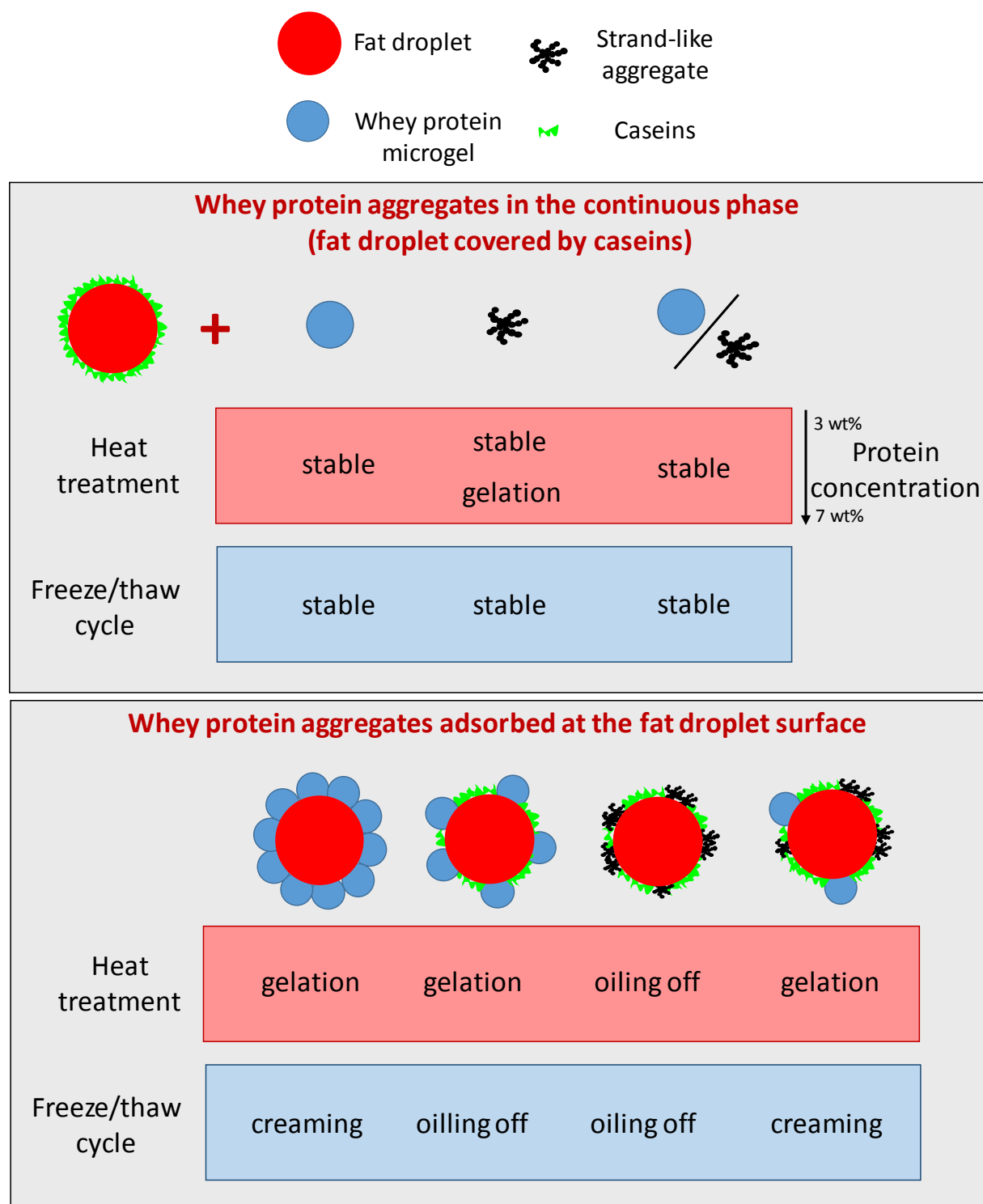


Figure 83: Highlights of the PhD project finding and conclusions.





## Further Outlook

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This PhD project highlighted the possibility of modulating the stability of the whey protein emulsions during the technological treatments by the use of a combination of whey protein aggregates and caseins. This work gives scientific elements for explaining some destabilizations occurring in real food emulsions during their manufacture and/or storage and indicates ways for a better control of their stability. The results obtained suggest that dairy emulsions with controlled stability can be formulated without the use of non-dairy additives. Therefore, this research work provides new and valuable conclusions in the field of dairy emulsions but also raises new research questions (Fig 84). On a scientific point of view, the influence of the whey protein powder composition (e.g. amount of residual caseins) and the identification of the key parameters for having reproducible whey protein aggregates (in terms of size, morphology and reactivity) have to be further explored. The mechanism of stabilization of the emulsions by combining whey protein aggregates and caseins depends on competition between proteins to cover fat surface and need to be confronted to emulsions prepared with various fat volume fractions and different fat droplet size distributions. This work showed that the whey protein aggregates adsorbed at the fat droplet surface induced a destabilization of the emulsions during heating or freeze/thaw cycle but the underlying mechanism is not completely understood. In a food context, the nutritional properties of emulsions prepared with whey protein aggregates have to be evaluated. In a technological point of view, this work addressed the influence of small amounts of “contaminants” on the functional properties of commercial ingredients. This could be exploited to create innovative functional ingredients. Lastly, the stability to technological treatments of real food emulsions prepared with a combination of whey protein aggregates and caseins have to be compared to emulsions already available on the market and prepared with non-dairy additives.

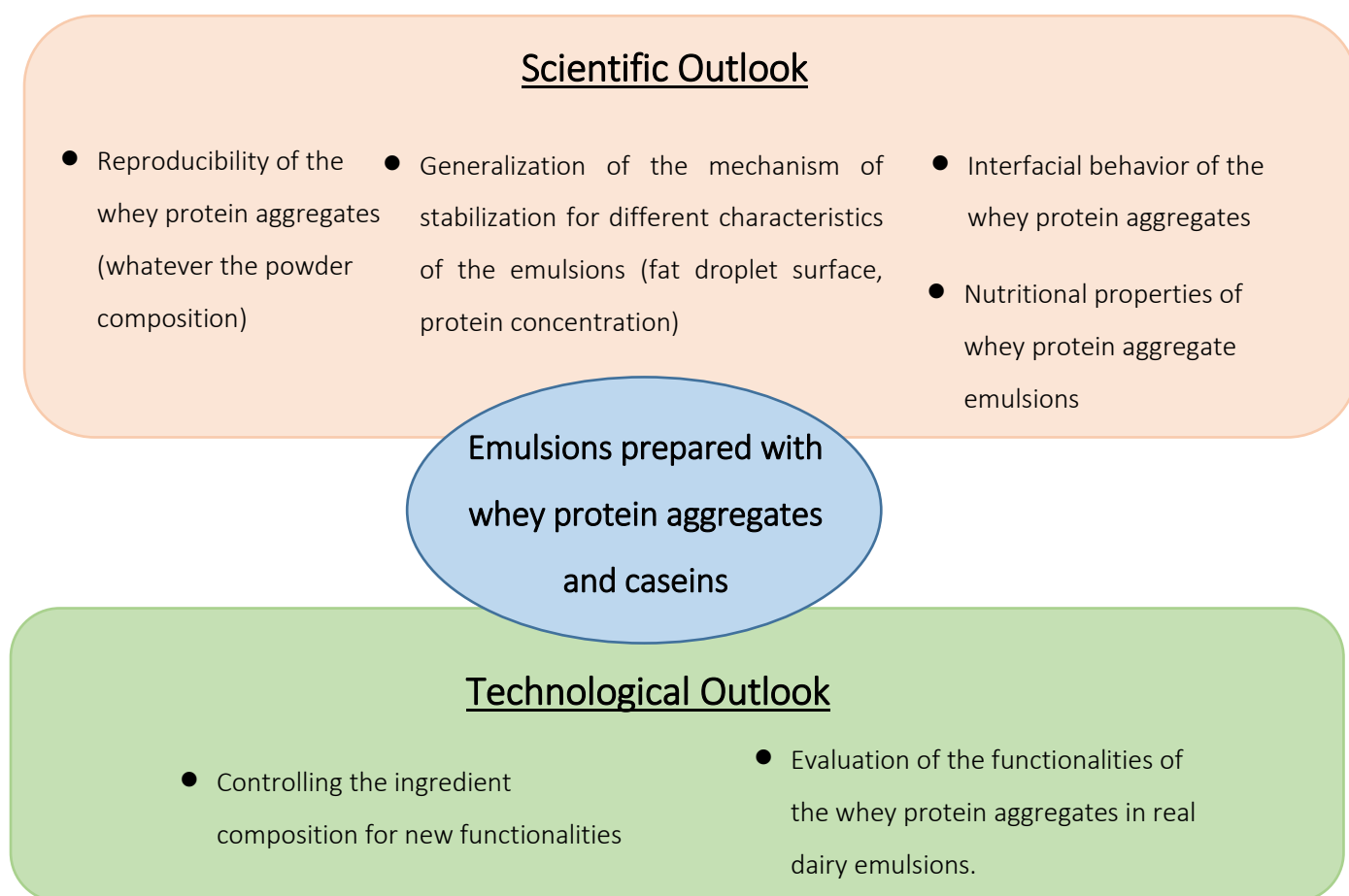


Figure 84: Scheme of proposed PhD project future outlook

## 7.5 Scientific outlook

The production of the whey protein aggregates is well described for purified ingredients under controlled conditions (pH, ionic strength, nature of salts). The present study shows that both the composition and the ageing of the whey protein powder affected the composition of the protein aggregate suspensions, as previously reported by Bovetto et al. (2007). The ageing or a variation of the powder composition changes the level of conversion of the proteins into a defined type of aggregates or leads to a mixture of aggregates in the suspension. Phan-Xuan et al. (2013) gave first information of the importance of the cation composition of the powder on whey protein strand-like aggregate or microgel formation. However, without significant difference in the quality and quantities of the cations in the two whey protein powders used in my PhD, we were unable to get a reproducible aggregate composition in the suspension using the same protocol. This clearly suggests that other factors that are still to be discovered influence the denaturation/aggregation of the whey proteins. Only this knowledge will allow to predict the denaturation/aggregation of the whey proteins and to correct the powder

composition as a function of the aggregate structure required. In our study, these aggregates were produced at bench scale but a scale-up of their production at industrial scale should also be considered.

The results obtained during my PhD project allow proposing a mechanism for the stability during technological treatments of emulsions prepared with whey protein aggregates and caseins. Although the conditions of stabilization of the emulsions was only defined for one type of emulsion (for defined fat volume fraction and mean fat droplet diameter), we suggest that the composition of the fat droplet surface can be reasoned for different total surface area and different whey protein aggregates to caseins ratio in the continuous phase of the emulsions. This could be validated in a near future. The competition between whey protein aggregates and caseins for fat droplet surface could explain some destabilizations observed in dairy products already present in the market. Notably, sterilized liquid milks and cream, which are exposed to temperatures below zero during their shipments. A modification of the scheduled heat treatment (sterilization) and/or of the scheduled homogenization pressure in the manufacture of the emulsions for export would modify the amount of whey protein aggregates and the total fat droplet surface area with consequences on their stability during shipments. The fat volume fraction and the fat droplet size also determine the inter-droplet distance, which influence the stability of the emulsions (McClements, 2005). For high fat volume fraction and/or small fat droplets, the emulsions prepared with whey protein aggregates are flocculated as soon as the homogenization step (PhD thesis T. Loiseleux, PROFIL). Indeed, if the distance between two fat droplets is small, the fat droplet interactions are facilitated and the emulsions are less stable especially to heat treatments. Further investigations still have to be conducted to evaluate this particular parameter on the emulsion heat stability.

The presence of whey protein aggregates at the fat droplet surface destabilizes the emulsions during technological treatments. The whey protein microgels bridge neighboring fat droplets during heating. The hypothesis indicating that the whey protein microgels spread and rearrange at the fat droplet surface (Destribats et al., 2013; Murphy et al., 2016) is a plausible explanation of the enhanced reactivity of whey protein microgels adsorbed at fat droplet surface compared to the ones dispersed in suspension. However, the TEM images presented in this PhD project do not give an unambiguously confirmation of the spreading of the whey protein microgels at the fat droplet surface. Further investigations are required to better understand the interfacial properties of the whey protein microgels and the structural changes at fat droplet surface responsible for this enhanced reactivity. We observed that the purified whey protein microgels tended to aggregate on heating compared to a mixture of whey protein microgels and trace of caseins. The caseins may have a protective action on the heat-induced aggregation of the whey protein microgels. The fact that the caseins adsorbed preferentially at fat

droplet surface induces a depletion of the caseins from the continuous phase of the emulsions. Consequently, whey protein microgels-coated fat droplets would have an enhanced sensitivity to heat-treatments in a similar way than purified whey protein microgels. The addition of caseins in emulsions containing fat droplets with adsorbed whey protein microgels could clarify the contribution of the non-adsorbed caseins on heat stability of emulsions containing whey protein microgels. In addition, the reactivity of aggregates adsorbed at fat droplet surface has to be considered in order to understand the mechanism by which they promote the fat droplet aggregation and coalescence during heat treatment. The protein functional groups responsible for the aggregation of the protein coated fat droplets can be deduced for instance by heating the emulsions in presence of thiol blocking agent (e.g. N-ethylmaleimide) to evaluate the contribution of the disulfide bonds. Further investigations are also needed on the emulsions prepared with a mixture of aggregates, as their stability during technological treatments could not be deduced from the behaviors of the aggregates taken individually.

The whey protein aggregates exhibit interesting properties for controlling the stability of the emulsions during technological treatments but they will also modify the nutritional properties of the emulsions. In the one hand, Sarkar et al. (2016) showed that heat-treated whey protein microgels emulsions modulated the rate of lipid digestion. In the other hand, the level of proteins denaturation/aggregation and the structure of the aggregates affected their digestion kinetics (Dupont et al., 2010; Nyemb et al., 2014; Peram, Loveday, Ye & Singh, 2013; Takagi, Teshima, Okunuki & Sawada, 2003; S. Zhang & Vardhanabhuti, 2014). Therefore, studies on the digestibility and nutritional properties of the emulsions prepared with whey protein aggregates have to be conducted in order to evaluate the potential benefits of such products compared to existing dairy products.

## 7.6 Technological outlook

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Although the whey protein powders are currently available on the market with different protein contents, manufacturers usually look for an increase in the whey protein/casein ratio. The PhD project brings back into question the usefulness of this extensive fractionation in order to produce functional ingredients. At least it shows there is a place for ingredients with an optimized protein composition. The presence of caseins in the powder in sufficient amount allows the recovery of the fat droplets with unreactive proteins and the release of the whey protein aggregates in the continuous phase leading to stable emulsions during technological treatments. Moreover, the protein purification is a time-consuming, energy-intensive procedure requiring the use of large amounts of water during the diafiltration and washing steps. Looking for lower levels of whey proteins purification could reduce the

production cost of the powder and for optimized whey protein aggregates/casein ratio, ingredients with innovative functionalities can be obtained. It is also possible to imagine a formulation of new ingredients by the assembly of different fractions of proteins, which structure is physically or chemically modified for target functionalities.

Finally, the use of a combination of whey protein aggregates and caseins for the stabilization of real food emulsions has to be evaluated. The food products are complex and contain numerous ingredients, which can be in competition with whey protein aggregates or caseins for the fat droplet surface (e.g. phospholipids). Firstly, we will limit the investigation to the product at neutral pH because our system has been tested only under these conditions. The use of whey protein aggregates and caseins in liquid infant formula and emulsion beverages for sportsmen and elderlies could be tested. Indeed, these products are rich in whey proteins and they can undergo destabilization during heat treatments. Their use in a mix for ice cream manufacture is also an issue. The use of the whey protein aggregates to texturize by heating a dairy matrix such as dessert or processed cheese is also a way to explore. At low protein concentration, the whey protein microgels are able to gel the emulsion during heating. The whey protein microgels can also texturize emulsions by the bridging neighboring fat droplets. Therefore, it was also possible imagine to use the whey proteins microgels in highly concentrated emulsion in order to obtain solid oil at room temperature. Hence, controlling the properties of the whey protein aggregate functionalities in emulsions offers new possibilities to create textures in dairy matrices.



## References

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- Adamcik, J., & Mezzenga, R. (2012). Proteins Fibrils from a Polymer Physics Perspective. *Macromolecules*, 45(3), 1137–1150. <https://doi.org/10.1021/ma202157h>
- Akkermans, C., Venema, P., van der Goot, A. J., Gruppen, H., Bakx, E. J., Boom, R. M., & van der Linden, E. (2008). Peptides are Building Blocks of Heat-Induced Fibrillar Protein Aggregates of  $\beta$ -Lactoglobulin Formed at pH 2. *Biomacromolecules*, 9(5), 1474–1479. <https://doi.org/10.1021/bm7014224>
- Alting, A. C., Hamer, R. J., de Kruif, C. G., Paques, M., & Visschers, R. W. (2003). Number of thiol groups rather than the size of the aggregates determines the hardness of cold set whey protein gels. *Food Hydrocolloids*, 17(4), 469–479. [https://doi.org/10.1016/S0268-005X\(03\)00023-7](https://doi.org/10.1016/S0268-005X(03)00023-7)
- Anema, S. G., Pinder, D. N., Hunter, R. J., & Hemar, Y. (2006). Effects of storage temperature on the solubility of milk protein concentrate (MPC85). *Food Hydrocolloids*, 20(2–3), 386–393. <https://doi.org/10.1016/j.foodhyd.2005.03.015>
- Arditty, S., Whitby, C. P., Binks, B. P., Schmitt, V., & Leal-Calderon, F. (2003). Some general features of limited coalescence in solid-stabilized emulsions. *The European Physical Journal E*, 11(3), 273–281. <https://doi.org/10.1140/epje/i2003-10018-6>
- Arganda-Carreras, I., Legland, D. & Schindelin, J. (2016). MorphoLibJ : Release v1.2.2. URL: <http://dx.doi.org/10.5281/zenodo.55995>.
- Aveyard, R., Binks, B. P., & Clint, J. H. (2003). Emulsions stabilised solely by colloidal particles. *Advances in Colloid and Interface Science*, 100–102, 503–546. [https://doi.org/10.1016/S0001-8686\(02\)00069-6](https://doi.org/10.1016/S0001-8686(02)00069-6)
- Ayache, J., Beaunier, L., Boumendil, J. P., Ehret, G., & Laub, D. (2007). Guide de préparation des échantillons pour la microscopie électronique en transmission: Techniques. Publications de l'Université de Saint-Étienne.
- Aymard, P., Gimel, J. C., Nicolai, T., & Durand, D. (1996). Experimental evidence for a two-step process in the aggregation of beta-lactoglobulin at pH 7. *Journal De Chimie Physique Et De Physico-Chimie Biologique*, 93(5), 987–997.
- Baines, D., & Seal, R. (2012). *Natural Food Additives, Ingredients and Flavourings*. Elsevier.

- Barford, N.M. & Krog, N., (1987). Destabilization and fat crystallization of whippable emulsions (toppings) studied by pulsed NMR. *Journal of the American Oil Chemists Society*, 64 (1), 112–119.
- Baussay, K., Bon, C. L., Nicolai, T., Durand, D., & Busnel, J.-P. (2004). Influence of the ionic strength on the heat-induced aggregation of the globular protein  $\beta$ -lactoglobulin at pH 7. *International Journal of Biological Macromolecules*, 34(1–2), 21–28. <https://doi.org/10.1016/j.ijbiomac.2003.11.003>
- Bernard, C., Regnault, S., Gendreau, S., Charbonneau, S., & Relkin, P. (2011). Enhancement of emulsifying properties of whey proteins by controlling spray-drying parameters. *Food Hydrocolloids*, 25(4), 758–763. <https://doi.org/10.1016/j.foodhyd.2010.08.011>
- Berton-Carabin, C. C., & Schroën, K. (2015). Pickering Emulsions for Food Applications: Background, Trends, and Challenges. *Annual Review of Food Science and Technology*, 6(1), 263–297. <https://doi.org/10.1146/annurev-food-081114-110822>
- Bijsterbosch, B., A. Bos, M. T., Dickinson, E., Opheusden, J. H. J. van, & Walstra, P. (1995). Brownian dynamics simulation of particle gel formation: from argon to yoghurt. *Faraday Discussions*, 101(0), 51–64. <https://doi.org/10.1039/FD9950100051>
- Binks, B. P. (2002). Particles as surfactants—similarities and differences. *Current Opinion in Colloid & Interface Science*, 7(1–2), 21–41. [https://doi.org/10.1016/S1359-0294\(02\)00008-0](https://doi.org/10.1016/S1359-0294(02)00008-0)
- Blijdenstein, T. B. J., Veerman, C., & van der Linden, E. (2004). Depletion–Flocculation in Oil-in-Water Emulsions Using Fibrillar Protein Assemblies. *Langmuir*, 20(12), 4881–4884. <https://doi.org/10.1021/la0497447>
- Bolder, S. G., Vasbinder, A. J., Sagis, L. M. C., & van der Linden, E. (2007). Heat-induced whey protein isolate fibrils: Conversion, hydrolysis, and disulphide bond formation. *International Dairy Journal*, 17(7), 846–853. <https://doi.org/10.1016/j.idairyj.2006.10.002>
- Boode, K., (1992). Partial coalescence in oil-in-water emulsions. Ph.D. Diss., Wageningen Agric. Univ., Wageningen, The Netherlands.
- Boode, K., & Walstra, P. (1993). Partial coalescence in oil-in-water emulsions 1. Nature of the aggregation. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 81, 121–137. [https://doi.org/10.1016/0927-7757\(93\)80239-B](https://doi.org/10.1016/0927-7757(93)80239-B)



## References

- Boode, K., Walstra, P., & de Groot-Mostert, A. E. A. (1993). Partial coalescence in oil-in-water emulsions 2. Influence of the properties of the fat. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 81, 139–151. [https://doi.org/10.1016/0927-7757\(93\)80240-F](https://doi.org/10.1016/0927-7757(93)80240-F)
- Bovetto, L., Schmitt, C. J. E., Beaulieu, M., Carlier, N., & Unterhaslberger, G. (2007). Nanoparticulated whey proteins. European Patent Office, EP 1 799 046 A2
- Bromley, E. H. C., Krebs, M. R. H., & Donald, A. M. (2006). Mechanisms of structure formation in particulate gels of  $\beta$ -lactoglobulin formed near the isoelectric point. *The European Physical Journal E*, 21(2), 145–152. <https://doi.org/10.1140/epje/i2006-10055-7>
- Buchheim, W., & Dejmek, P. (1997). Milk and dairy-type emulsions. *Food Emulsions*. Retrieved from <http://agris.fao.org/agris-search/search.do?recordID=US201302874689>
- Burin, L., Jouppila, K., Roos, Y., Kansikas, J., & Buera, M. del P. (2000). Color Formation in Dehydrated Modified Whey Powder Systems As Affected by Compression and Tg. *Journal of Agricultural and Food Chemistry*, 48(11), 5263–5268. <https://doi.org/10.1021/jf000240y>
- Çakır-Fuller, E. (2015). Enhanced heat stability of high protein emulsion systems provided by microparticulated whey proteins. *Food Hydrocolloids*, 47, 41–50. <https://doi.org/10.1016/j.foodhyd.2015.01.003>
- Cao, Y., Dickinson, E., & Wedlock, D. J. (1990). Creaming and flocculation in emulsions containing polysaccharide. *Food Hydrocolloids*, 4(3), 185–195. [https://doi.org/10.1016/S0268-005X\(09\)80151-3](https://doi.org/10.1016/S0268-005X(09)80151-3)
- Coppola, L. E., Molitor, M. S., Rankin, S. A., & Lucey, J. A. (2014). Comparison of milk-derived whey protein concentrates containing various levels of casein. *International Journal of Dairy Technology*, 67(4), 467–473. <https://doi.org/10.1111/1471-0307.12157>
- Coupland, J. N. (2002). Crystallization in emulsions. *Current Opinion in Colloid & Interface Science*, 7(5–6), 445–450. [https://doi.org/10.1016/S1359-0294\(02\)00080-8](https://doi.org/10.1016/S1359-0294(02)00080-8)
- Cramp, G. L., Docking, A. M., Ghosh, S., & Coupland, J. N. (2004). On the stability of oil-in-water emulsions to freezing. *Food Hydrocolloids*, 18(6), 899–905. <https://doi.org/10.1016/j.foodhyd.2003.10.007>
- Croguennec, T., Leng, N., Hamon, P., Rousseau, F., Jeantet, R., & Bouhallab, S. (2014). Caseinomacropeptide modifies the heat-induced denaturation–aggregation process of  $\beta$ -lactoglobulin. *International Dairy Journal*, 36(1), 55–64. <https://doi.org/10.1016/j.idairyj.2014.01.004>

- Croguennec, T., O’Kennedy, B. T., & Mehra, R. (2004). Heat-induced denaturation/aggregation of  $\beta$ -lactoglobulin A and B: kinetics of the first intermediates formed. *International Dairy Journal*, 14(5), 399–409. <https://doi.org/10.1016/j.idairyj.2003.09.005>
- Da Silva Pinto, M., Bouhallab, S., De Carvalho, A. F., Henry, G., Putaux, J.-L., & Leonil, J. (2012). Glucose Slows Down the Heat-Induced Aggregation of  $\beta$ -Lactoglobulin at Neutral pH. *Journal of Agricultural and Food Chemistry*, 60(1), 214–219. <https://doi.org/10.1021/jf2037664>
- Dalgleish, D. G. (1996). Conformations and structures of milk proteins adsorbed to oil-water interfaces. *Food Research International*, 29(5), 541–547. [https://doi.org/10.1016/S0963-9969\(96\)00065-8](https://doi.org/10.1016/S0963-9969(96)00065-8)
- Dalgleish, D. G. (1997). Adsorption of protein and the stability of emulsions. *Trends in Food Science & Technology*, 8(1), 1–6. [https://doi.org/10.1016/S0924-2244\(97\)01001-7](https://doi.org/10.1016/S0924-2244(97)01001-7)
- Dalgleish, D. G. (2006). Food emulsions—their structures and structure-forming properties. *Food Hydrocolloids*, 20(4), 415–422. <https://doi.org/10.1016/j.foodhyd.2005.10.009>
- Dalgleish, D. G., West, S. J., & Hallett, F. R. (1997). The characterization of small emulsion droplets made from milk proteins and triglyceride oil. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 123, 145–153. [https://doi.org/10.1016/S0927-7757\(97\)03783-7](https://doi.org/10.1016/S0927-7757(97)03783-7)
- Dave, A. C., Loveday, S. M., Anema, S. G., Jameson, G. B., & Singh, H. (2014a). Glycation as a Tool To Probe the Mechanism of  $\beta$ -Lactoglobulin Nanofibril Self-Assembly. *Journal of Agricultural and Food Chemistry*, 62(14), 3269–3278. <https://doi.org/10.1021/jf405441g>
- Dave, A. C., Loveday, S. M., Anema, S. G., Jameson, G. B., & Singh, H. (2014b). Modulating beta-Lactoglobulin Nanofibril Self-Assembly at pH 2 Using Glycerol and Sorbitol. *Biomacromolecules*, 15(1), 95–103. <https://doi.org/10.1021/bm401315s>
- Dave, A. C., Loveday, S. M., Anema, S. G., & Singh, H. (2016).  $\beta$ -Casein will chaperone  $\beta$ -lactoglobulin during nanofibril assembly, but prefers familiar company at high concentrations. *International Dairy Journal*, 57, 39–43. <https://doi.org/10.1016/j.idairyj.2016.02.035>
- David Julian McClements. (2005). *Food Emulsions: Principles, Practices, and Techniques*, Second Edition.
- de Wit, J. N. (1998). Nutritional and Functional Characteristics of Whey Proteins in Food Products. *Journal of Dairy Science*, 81(3), 597–608. [https://doi.org/10.3168/jds.S0022-0302\(98\)75613-9](https://doi.org/10.3168/jds.S0022-0302(98)75613-9)

## References

- Demetriades, K., Coupland, J. n., & McCLEMENTS, D. J. (1997a). Physical Properties of Whey Protein Stabilized Emulsions as Related to pH and NaCl. *Journal of Food Science*, 62(2), 342–347. <https://doi.org/10.1111/j.1365-2621.1997.tb03997.x>
- Demetriades, K., Coupland, J. n., & McCLEMENTS, D. j. (1997b). Physicochemical Properties of Whey Protein-Stabilized Emulsions as affected by Heating and Ionic Strength. *Journal of Food Science*, 62(3), 462–467. <https://doi.org/10.1111/j.1365-2621.1997.tb04407.x>
- Destribats, M., Ravaine, S., Heroguez, V., Leal-Calderon, F., & Schmitt, V. (2010). Outstanding Stability of Poorly-protected Pickering Emulsions. In *Trends in Colloid and Interface Science XXIII* (pp. 13–18). Springer, Berlin, Heidelberg. [https://doi.org/10.1007/978-3-642-13461-6\\_4](https://doi.org/10.1007/978-3-642-13461-6_4)
- Destribats, M., Rouvet, M., Gehin-Delval, C., Schmitt, C., & Binks, B. P. (2014). Emulsions stabilised by whey protein microgel particles: towards food-grade Pickering emulsions, 10(36), 6941–6954. <https://doi.org/10.1039/C4SM00179F>
- Destribats M., Véronique Lapeyre, Mélanie Wolfs, Elisabeth Sellier, Fernando Leal-Calderon, Valérie Ravaine, & Véronique Schmitt. (2011). Soft microgels as Pickering emulsion stabilisers: role of particle deformability - *Soft Matter* (RSC Publishing), pp. 7689–7698.
- Destribats, M., Wolfs, M., Pinaud, F., Lapeyre, V., Sellier, E., Schmitt, V., & Ravaine, V. (2013). Pickering Emulsions Stabilized by Soft Microgels: Influence of the Emulsification Process on Particle Interfacial Organization and Emulsion Properties. *Langmuir*, 29(40), 12367–12374. <https://doi.org/10.1021/la402921b>
- Dickinson, E. (1994). Emulsion Stability. In K. Nishinari & E. Doi (Eds.), *Food Hydrocolloids* (pp. 387–398). Springer US. [https://doi.org/10.1007/978-1-4615-2486-1\\_61](https://doi.org/10.1007/978-1-4615-2486-1_61)
- Dickinson, E. (1997). Properties of Emulsions Stabilized with Milk Proteins: Overview of Some Recent Developments. *Journal of Dairy Science*, 80(10), 2607–2619. [https://doi.org/10.3168/jds.S0022-0302\(97\)76218-0](https://doi.org/10.3168/jds.S0022-0302(97)76218-0)
- Dickinson, E. (1999). Caseins in emulsions: interfacial properties and interactions. *International Dairy Journal*, 9(3–6), 305–312. [https://doi.org/10.1016/S0958-6946\(99\)00079-5](https://doi.org/10.1016/S0958-6946(99)00079-5)
- Dickinson, E. (2001). Milk protein interfacial layers and the relationship to emulsion stability and rheology. *Colloids and Surfaces B-Biointerfaces*, 20(3), 197–210. [https://doi.org/10.1016/S0927-7765\(00\)00204-6](https://doi.org/10.1016/S0927-7765(00)00204-6)

- Dickinson, E. (2010a). Flocculation of protein-stabilized oil-in-water emulsions. *Colloids and Surfaces B: Biointerfaces*, 81(1), 130–140. <https://doi.org/10.1016/j.colsurfb.2010.06.033>
- Dickinson, E. (2010b). Food emulsions and foams: Stabilization by particles. *Current Opinion in Colloid & Interface Science*, 15(1–2), 40–49. <https://doi.org/10.1016/j.cocis.2009.11.001>
- Dickinson, E. (2012). Use of nanoparticles and microparticles in the formation and stabilization of food emulsions. *Trends in Food Science & Technology*, 24(1), 4–12. <https://doi.org/10.1016/j.tifs.2011.09.006>
- Dickinson, E. (2015). Microgels — An alternative colloidal ingredient for stabilization of food emulsions. *Trends in Food Science & Technology*, 43(2), 178–188. <https://doi.org/10.1016/j.tifs.2015.02.006>
- Dickinson, E., & Golding, M. (1997). Depletion flocculation of emulsions containing unadsorbed sodium caseinate. *Food Hydrocolloids*, 11(1), 13–18. [https://doi.org/10.1016/S0268-005X\(97\)80005-7](https://doi.org/10.1016/S0268-005X(97)80005-7)
- Dickinson, E., & McClements, D. J. (1995). *Advances In Food Colloids*. Springer Science & Business Media.
- Dickinson, E., & Parkinson, E. L. (2004). Heat-induced aggregation of milk protein-stabilized emulsions: sensitivity to processing and composition. *International Dairy Journal*, 14(7), 635–645. <https://doi.org/10.1016/j.idairyj.2003.12.005>
- Dickinson, E., Rolfe, S. E., & Dalgleish, D. G. (1989). Competitive adsorption in oil-in-water emulsions containing  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin. *Food Hydrocolloids*, 3(3), 193–203. [https://doi.org/10.1016/S0268-005X\(89\)80003-7](https://doi.org/10.1016/S0268-005X(89)80003-7)
- Dickinson, E & Stainsby, G. (1982). *Colloids in food*. Applied Science Publishers.
- Dissanayake, M., & Vasiljevic, T. (2009). Functional properties of whey proteins affected by heat treatment and hydrodynamic high-pressure shearing. *Journal of Dairy Science*, 92(4), 1387–1397. <https://doi.org/10.3168/jds.2008-1791>
- Donato, L., Schmitt, C., Bovetto, L., & Rouvet, M. (2009). Mechanism of formation of stable heat-induced beta-lactoglobulin microgels. *International Dairy Journal*, 19(5), 295–306. <https://doi.org/10.1016/j.idairyj.2008.11.005>
- Doxastakis, G. (1989). Milk proteins. In G. Charalambous & G. Doxastakis, *Food emulsifiers*, p 9–62. Amsterdam: Elsevier.

## References

- Dupont, D., Boutrou, R., Menard, O., Jardin, J., Tanguy, G., Schuck, P., ... Leonil, J. (2010). Heat Treatment of Milk During Powder Manufacture Increases Casein Resistance to Simulated Infant Digestion. *Food Digestion*, 1(1–2), 28–39. <https://doi.org/10.1007/s13228-010-0003-0>
- Durand, D., Christophe Gimel, J., & Nicolai, T. (2002). Aggregation, gelation and phase separation of heat denatured globular proteins. *Physica A: Statistical Mechanics and Its Applications*, 304(1–2), 253–265. [https://doi.org/10.1016/S0378-4371\(01\)00514-3](https://doi.org/10.1016/S0378-4371(01)00514-3)
- Dybowska, B. E. (2011). Whey protein-stabilized emulsion properties in relation to thermal modification of the continuous phase. *Journal of Food Engineering*, 104(1), 81–88. <https://doi.org/10.1016/j.jfoodeng.2010.11.030>
- Enomoto, H., Li, C.-P., Morizane, K., Ibrahim, H. R., Sugimoto, Y., Ohki, S., ... Aoki, T. (2007). Glycation and Phosphorylation of  $\beta$ -Lactoglobulin by Dry-Heating: Effect on Protein Structure and Some Properties. *Journal of Agricultural and Food Chemistry*, 55(6), 2392–2398. <https://doi.org/10.1021/jf062830n>
- Euston, S. R., Finnigan, S. R., & Hirst, R. L. (2000). Aggregation kinetics of heated whey protein-stabilized emulsions. *Food Hydrocolloids*, 14(2), 155–161. [https://doi.org/10.1016/S0268-005X\(99\)00061-2](https://doi.org/10.1016/S0268-005X(99)00061-2)
- Euston, S. R., & Hirst, R. L. (1999). Comparison of the concentration-dependent emulsifying properties of protein products containing aggregated and non-aggregated milk protein. *International Dairy Journal*, 9(10), 693–701. [https://doi.org/10.1016/S0958-6946\(99\)00138-7](https://doi.org/10.1016/S0958-6946(99)00138-7)
- Farrell Jr., H. M., Jimenez-Flores, R., Bleck, G. T., Brown, E. M., Butler, J. E., Creamer, L. K., ... Swaisgood, H. E. (2004). Nomenclature of the Proteins of Cows' Milk—Sixth Revision. *Journal of Dairy Science*, 87(6), 1641–1674. [https://doi.org/10.3168/jds.S0022-0302\(04\)73319-6](https://doi.org/10.3168/jds.S0022-0302(04)73319-6)
- Folter, J. W. J. de, Ruijven, M. W. M. van, & Velikov, K. P. (2012). Oil-in-water Pickering emulsions stabilized by colloidal particles from the water-insoluble protein zein, 8(25), 6807–6815. <https://doi.org/10.1039/C2SM07417F>
- Fyfe, K. N., Kravchuk, O., Le, T., Deeth, H. C., Nguyen, A. V., & Bhandari, B. (2011). Storage induced changes to high protein powders: influence on surface properties and solubility. *Journal of the Science of Food and Agriculture*, 91(14), 2566–2575. <https://doi.org/10.1002/jsfa.4461>

- Gaiani, C., Schuck, P., Scher, J., Ehrhardt, J. J., Arab-Tehrany, E., Jacquot, M., & Banon, S. (2009). Native phosphocaseinate powder during storage: Lipids released onto the surface. *Journal of Food Engineering*, 94(2), 130–134. <https://doi.org/10.1016/j.jfoodeng.2009.01.038>
- Gao, Z., Zhao, J., Huang, Y., Yao, X., Zhang, K., Fang, Y., ... Yang, H. (2017). Edible Pickering emulsion stabilized by protein fibrils. Part 1: Effects of pH and fibrils concentration. *LWT - Food Science and Technology*, 76, Part A, 1–8. <https://doi.org/10.1016/j.lwt.2016.10.038>
- Gao, Z.-M., Wang, J.-M., Wu, N.-N., Wan, Z., Guo, J., Yang, X.-Q., & Yin, S. (2013). Formation of Complex Interface and Stability of Oil-in-Water (O/W) Emulsion Prepared by Soy Lipophilic Protein Nanoparticles. *Journal of Agricultural and Food Chemistry*, 61(32), 7838–7847. <https://doi.org/10.1021/jf4018349>
- Gimel, J. C., Durand, D., & Nicolai, T. (1994). Structure and distribution of aggregates formed after heat-induced denaturation of globular proteins. *Macromolecules*, 27(2), 583–589. <https://doi.org/10.1021/ma00080a037>
- Giroux, H. J., Houde, J., & Britten, M. (2010). Preparation of nanoparticles from denatured whey protein by pH-cycling treatment. *Food Hydrocolloids*, 24(4), 341–346. <https://doi.org/10.1016/j.foodhyd.2009.10.013>
- Goff, H. D. (1997a). Colloidal aspects of ice cream—A review. *International Dairy Journal*, 7(6), 363–373. [https://doi.org/10.1016/S0958-6946\(97\)00040-X](https://doi.org/10.1016/S0958-6946(97)00040-X)
- Goff, H. D. (1997b). Instability and Partial Coalescence in Whippable Dairy Emulsions. *Journal of Dairy Science*, 80(10), 2620–2630. [https://doi.org/10.3168/jds.S0022-0302\(97\)76219-2](https://doi.org/10.3168/jds.S0022-0302(97)76219-2)
- Goff, H. D., Verespej, E., & Smith, A. K. (1999). A study of fat and air structures in ice cream. *International Dairy Journal*, 9(11), 817–829. [https://doi.org/10.1016/S0958-6946\(99\)00149-1](https://doi.org/10.1016/S0958-6946(99)00149-1)
- Griffin, W. G., Griffin, M. C. A., Martin, S. R., & Price, J. (1993). Molecular basis of thermal aggregation of bovine  $\beta$ -lactoglobulin A. *Journal of the Chemical Society, Faraday Transactions*, 89(18), 3395–3405. <https://doi.org/10.1039/FT9938903395>
- Gulzar, M., Bouhallab, S., Jardin, J., Briard-Bion, V., & Croguennec, T. (2013). Structural consequences of dry heating on alpha-lactalbumin and beta-lactoglobulin at pH 6.5. *Food Research International*, 51(2), 899–906. <https://doi.org/10.1016/j.foodres.2013.02.025>

## References

- Gulzar, M., Bouhallab, S., Jeantet, R., Schuck, P., & Croguennec, T. (2011). Influence of pH on the dry heat-induced denaturation/aggregation of whey proteins. *Food Chemistry*, 129(1), 110–116. <https://doi.org/10.1016/j.foodchem.2011.04.037>
- Guyomarc'h, F., Famelart, M.-H., Henry, G., Gulzar, M., Leonil, J., Hamon, P., ... Croguennec, T. (2014). Current ways to modify the structure of whey proteins for specific functionalities—a review. *Dairy Science & Technology*, 95(6), 795–814. <https://doi.org/10.1007/s13594-014-0190-5>
- Guyomarc'h, F., Nono, M., Nicolai, T., & Durand, D. (2009). Heat-induced aggregation of whey proteins in the presence of  $\kappa$ -casein or sodium caseinate. *Food Hydrocolloids*, 23(4), 1103–1110. <https://doi.org/10.1016/j.foodhyd.2008.07.001>
- Guyomarc'h, F., Warin, F., Donald Muir, D., & Leaver, J. (2000). Lactosylation of milk proteins during the manufacture and storage of skim milk powders. *International Dairy Journal*, 10(12), 863–872.
- Ha, E., & Zemel, M. B. (2003). Functional properties of whey, whey components, and essential amino acids: mechanisms underlying health benefits for active people (review). *The Journal of Nutritional Biochemistry*, 14(5), 251–258. [https://doi.org/10.1016/S0955-2863\(03\)00030-5](https://doi.org/10.1016/S0955-2863(03)00030-5)
- HadjSadok, A., Pitkowski, A., Nicolai, T., Benyahia, L., & Moulai-Mostefa, N. (2008). Characterisation of sodium caseinate as a function of ionic strength, pH and temperature using static and dynamic light scattering. *Food Hydrocolloids*, 22(8), 1460–1466. <https://doi.org/10.1016/j.foodhyd.2007.09.002>
- Haque, E., Bhandari, B. R., Gidley, M. J., Deeth, H. C., Møller, S. M., & Whittaker, A. K. (2010). Protein Conformational Modifications and Kinetics of Water–Protein Interactions in Milk Protein Concentrate Powder upon Aging: Effect on Solubility. *Journal of Agricultural and Food Chemistry*, 58(13), 7748–7755. <https://doi.org/10.1021/jf1007055>
- Haque, E., Bhandari, B. R., Gidley, M. J., Deeth, H. C., & Whittaker, A. K. (2011). Ageing-induced solubility loss in milk protein concentrate powder: effect of protein conformational modifications and interactions with water. *Journal of the Science of Food and Agriculture*, 91(14), 2576–2581. <https://doi.org/10.1002/jsfa.4478>
- Haque, E., Whittaker, A. K., Gidley, M. J., Deeth, H. C., Fibrianto, K., & Bhandari, B. R. (2012). Kinetics of enthalpy relaxation of milk protein concentrate powder upon ageing and its effect on solubility. *Food Chemistry*, 134(3), 1368–1373. <https://doi.org/10.1016/j.foodchem.2012.03.034>

- Hashizume, K., Kakiuchi, K., Koyama, E., & Watanabe, T. (1971). Denaturation of Soybean Protein by Freezing. *Agricultural and Biological Chemistry*, 35(4), 449–459. <https://doi.org/10.1080/00021369.1971.10859957>
- Havea, P. (2006). Protein interactions in milk protein concentrate powders. *International Dairy Journal*, 16(5), 415–422. <https://doi.org/10.1016/j.idairyj.2005.06.005>
- Hettiarachchi, C. A., Melton, L. D., Gerrard, J. A., & Loveday, S. M. (2012). Formation of  $\beta$ -Lactoglobulin Nanofibrils by Microwave Heating Gives a Peptide Composition Different from Conventional Heating. *Biomacromolecules*, 13(9), 2868–2880. <https://doi.org/10.1021/bm300896r>
- Hiller, B., & Lorenzen, P. C. (2010). Functional properties of milk proteins as affected by Maillard reaction induced oligomerisation. *Food Research International*, 43(4), 1155–1166. <https://doi.org/10.1016/j.foodres.2010.02.006>
- Hoffmann, M. A. M., & van Mil, P. J. J. M. (1999). Heat-Induced Aggregation of  $\beta$ -Lactoglobulin as a Function of pH. *Journal of Agricultural and Food Chemistry*, 47(5), 1898–1905. <https://doi.org/10.1021/jf980886e>
- Holt, C., Carver, J. A., Ecroyd, H., & Thorn, D. C. (2013). Invited review: Caseins and the casein micelle: Their biological functions, structures, and behavior in foods<sup>1</sup>. *Journal of Dairy Science*, 96(10), 6127–6146. <https://doi.org/10.3168/jds.2013-6831>
- Holt, C., McPhail, D., Nevison, I., Nylander, T., Otte, J., Ipsen, R. H., ... Chatterton, D. (1999). Apparent chemical composition of nine commercial or semi-commercial whey protein concentrates, isolates and fractions. *International Journal of Food Science & Technology*, 34(5–6), 543–556. <https://doi.org/10.1046/j.1365-2621.1999.00323.x>
- Horozov, T. S., & Binks, B. P. (2006). Particle-Stabilized Emulsions: A Bilayer or a Bridging Monolayer? *Angewandte Chemie International Edition*, 45(5), 773–776. <https://doi.org/10.1002/anie.200503131>
- Hunt, J. A., & Dalgleish, D. G. (1994). Adsorption behaviour of whey protein isolate and caseinate in soya oil-in-water emulsions. *Food Hydrocolloids*, 8(2), 175–187. [https://doi.org/10.1016/S0268-005X\(09\)80042-8](https://doi.org/10.1016/S0268-005X(09)80042-8)
- Hunt, J. A., & Dalgleish, D. G. (1995). Heat Stability of Oil-in-Water Emulsions Containing Milk Proteins: Effect of Ionic Strength and pH. *Journal of Food Science*, 60(5), 1120–1123. <https://doi.org/10.1111/j.1365-2621.1995.tb06306.x>



## References

- Hunter, T. N., Pugh, R. J., Franks, G. V., & Jameson, G. J. (2008). The role of particles in stabilising foams and emulsions. *Advances in Colloid and Interface Science*, 137(2), 57–81. <https://doi.org/10.1016/j.cis.2007.07.007>
- Ipsen, R., & Hansen, P. S. (1988). Factors affecting the storage stability of whole milk powder. Beretning. Statens Mejeriforsøg (Denmark). Retrieved from <http://agris.fao.org/agris-search/search.do?recordID=DK8920137>
- Jones, O. G., Adamcik, J., Handschin, S., Bolisetty, S., & Mezzenga, R. (2010). Fibrillation of  $\beta$ -Lactoglobulin at Low pH in the Presence of a Complexing Anionic Polysaccharide. *Langmuir*, 26(22), 17449–17458. <https://doi.org/10.1021/la1026619>
- Jost, R., Baechler, R., & Masson, G. (1986). Heat Gelation of Oil-in-Water Emulsions Stabilized by Whey Protein. *Journal of Food Science*, 51(2), 440–444. <https://doi.org/10.1111/j.1365-2621.1986.tb11150.x>
- Jung, J.-M., Gunes, D. Z., & Mezzenga, R. (2010). Interfacial Activity and Interfacial Shear Rheology of Native beta-Lactoglobulin Monomers and Their Heat-Induced Fibers. *Langmuir*, 26(19), 15366–15375. <https://doi.org/10.1021/la102721m>
- Jung, J.-M., Savin, G., Pouzot, M., Schmitt, C., & Mezzenga, R. (2008). Structure of Heat-Induced  $\beta$ -Lactoglobulin Aggregates and their Complexes with Sodium-Dodecyl Sulfate. *Biomacromolecules*, 9(9), 2477–2486. <https://doi.org/10.1021/bm800502j>
- Karbstein, H., & Schubert, H. (1995). Developments in the continuous mechanical production of oil-in-water macro-emulsions. *Chemical Engineering and Processing: Process Intensification*, 34(3), 205–211. [https://doi.org/10.1016/0255-2701\(94\)04005-2](https://doi.org/10.1016/0255-2701(94)04005-2)
- Kato, A., & Yutani, K. (1988). Correlation of surface properties with conformational stabilities of wild-type and six mutant tryptophan synthase  $\alpha$ -subunits substituted at the same position. *Protein Engineering*, 2(2), 153–156. <https://doi.org/10.1093/protein/2.2.153>
- Kaz, D. M., McGorty, R., Mani, M., Brenner, M. P., & Manoharan, V. N. (2012). Physical ageing of the contact line on colloidal particles at liquid interfaces. *Nature Materials*, 11(2), 138–142. <https://doi.org/10.1038/nmat3190>
- Kharlamova, A., Inthavong, W., Nicolai, T., & Chassenieux, C. (2016). The effect of aggregation into fractals or microgels on the charge density and the isoionic point of globular proteins. *Food Hydrocolloids*, 60, 470–475. <https://doi.org/10.1016/j.foodhyd.2016.04.013>

## References

- Kim, D. A., Cornec, M., & Narsimhan, G. (2005). Effect of thermal treatment on interfacial properties of  $\beta$ -lactoglobulin. *Journal of Colloid and Interface Science*, 285(1), 100–109. <https://doi.org/10.1016/j.jcis.2004.10.044>
- Kinsella, J. E., & Whitehead, D. M. (1989). Proteins in Whey: Chemical, Physical, and Functional Properties. In J. E. Kinsella (Ed.), *Advances in Food and Nutrition Research* (Vol. 33, pp. 343–438). Academic Press. Retrieved from <http://www.sciencedirect.com/science/article/pii/S1043452608601308>
- Knudsen, J. C., Øgden, L. H., & Skibsted, L. H. (2008). Droplet Surface Properties and Rheology of Concentrated Oil in Water Emulsions Stabilized by Heat-Modified  $\beta$ -Lactoglobulin B. *Langmuir*, 24(6), 2603–2610. <https://doi.org/10.1021/la703810g>
- Krebs, M. R. H., Devlin, G. L., & Donald, A. M. (2009). Amyloid Fibril-Like Structure Underlies the Aggregate Structure across the pH Range for  $\beta$ -Lactoglobulin. *Biophysical Journal*, 96(12), 5013–5019. <https://doi.org/10.1016/j.bpj.2009.03.028>
- Kroes-Nijboer, A., Venema, P., Bouman, J., & van der Linden, E. (2011). Influence of Protein Hydrolysis on the Growth Kinetics of  $\beta$ -lg Fibrils. *Langmuir*, 27(10), 5753–5761. <https://doi.org/10.1021/la104797u>
- Kroes-Nijboer, A., Venema, P., & Linden, E. van der. (2012). Fibrillar structures in food, 3(3), 221–227. <https://doi.org/10.1039/C1FO10163C>
- Lam, R. S. H., & Nickerson, M. T. (2015). The effect of pH and temperature pre-treatments on the physicochemical and emulsifying properties of whey protein isolate. *LWT - Food Science and Technology*, 60(1), 427–434. <https://doi.org/10.1016/j.lwt.2014.07.031>
- Lazzaro, F., Saint-Jalmes, A., Violleau, F., Lopez, C., Gaucher-Delmas, M., Madec, M.-N., ... Gaucheron, F. (2017). Gradual disaggregation of the casein micelle improves its emulsifying capacity and decreases the stability of dairy emulsions. *Food Hydrocolloids*, 63, 189–200. <https://doi.org/10.1016/j.foodhyd.2016.08.037>
- Le Bon, C., Nicolai, T., & Durand, D. (1999). Growth and structure of aggregates of heat-denatured  $\beta$ -Lactoglobulin. *International Journal of Food Science & Technology*, 34(5–6), 451–465. <https://doi.org/10.1046/j.1365-2621.1999.00310.x>

## References

- Le, T. T., Bhandari, B., & Deeth, H. C. (2011). Chemical and Physical Changes in Milk Protein Concentrate (MPC80) Powder during Storage. *Journal of Agricultural and Food Chemistry*, 59(10), 5465–5473. <https://doi.org/10.1021/jf2003464>
- Le, T. T., Bhandari, B., Holland, J. W., & Deeth, H. C. (2011). Maillard Reaction and Protein Cross-Linking in Relation to the Solubility of Milk Powders. *Journal of Agricultural and Food Chemistry*, 59(23), 12473–12479. <https://doi.org/10.1021/jf203460z>
- Le, T. T., Deeth, H. C., Bhandari, B., Alewood, P. F., & Holland, J. W. (2013). Quantification of lactosylation of whey proteins in stored milk powder using multiple reaction monitoring. *Food Chemistry*, 141(2), 1203–1210. <https://doi.org/10.1016/j.foodchem.2013.03.073>
- Leal-Calderon, F., & Schmitt, V. (2008). Solid-stabilized emulsions. *Current Opinion in Colloid & Interface Science*, 13(4), 217–227. <https://doi.org/10.1016/j.cocis.2007.09.005>
- Lee, D., Park, C. W., Paik, S. R., & Choi, K. Y. (2009). The modification of  $\alpha$ -synuclein by dicarbonyl compounds inhibits its fibril-forming process. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics*, 1794(3), 421–430. <https://doi.org/10.1016/j.bbapap.2008.11.016>
- Lee, L. L., Niknafs, N., Hancocks, R. D., & Norton, I. T. (2013). Emulsification: Mechanistic understanding. *Trends in Food Science & Technology*, 31(1), 72–78. <https://doi.org/10.1016/j.tifs.2012.08.006>
- Legland, D., Devaux, M.-F., Bouchet, B., Guillon, F., & Lahaye, M. (2012). Cartography of cell morphology in tomato pericarp at the fruit scale. *Journal of Microscopy*, 247(1), 78–93. <https://doi.org/10.1111/j.1365-2818.2012.03623.x>
- Li, J.-M., & Nie, S.-P. (2016). The functional and nutritional aspects of hydrocolloids in foods. *Food Hydrocolloids*, 53, 46–61. <https://doi.org/10.1016/j.foodhyd.2015.01.035>
- Li, Y., Lu, F., Luo, C., Chen, Z., Mao, J., Shoemaker, C., & Zhong, F. (2009). Functional properties of the Maillard reaction products of rice protein with sugar. *Food Chemistry*, 117(1), 69–74. <https://doi.org/10.1016/j.foodchem.2009.03.078>
- Li-Chan, E. (1983). Heat-Induced Changes in the Proteins of Whey Protein Concentrate. *Journal of Food Science*, 48(1), 47–56. <https://doi.org/10.1111/j.1365-2621.1983.tb14786.x>
- Liu, F., & Tang, C.-H. (2013). Soy Protein Nanoparticle Aggregates as Pickering Stabilizers for Oil-in-Water Emulsions. *Journal of Agricultural and Food Chemistry*, 61(37), 8888–8898. <https://doi.org/10.1021/jf401859y>

- Liu, F., & Tang, C.-H. (2014). Emulsifying Properties of Soy Protein Nanoparticles: Influence of the Protein Concentration and/or Emulsification Process. *Journal of Agricultural and Food Chemistry*, 62(12), 2644–2654. <https://doi.org/10.1021/jf405348k>
- Liu, G., Buldo, P., Greve, M. T., Nielsen, S. B., Nielsen, J. H., & Ipsen, R. (2016). Effects of added whey protein aggregates on textural and microstructural properties of acidified milk model systems. *International Dairy Journal*, 62, 43–52. <https://doi.org/10.1016/j.idairyj.2016.07.006>
- Loveday, S. M., Su, J., Rao, M. A., Anema, S. G., & Singh, H. (2011). Effect of Calcium on the Morphology and Functionality of Whey Protein Nanofibrils. *Biomacromolecules*, 12(10), 3780–3788. <https://doi.org/10.1021/bm201013b>
- Loveday, S. M., Wang, X. L., Rao, M. A., Anema, S. G., & Singh, H. (2012).  $\beta$ -Lactoglobulin nanofibrils: Effect of temperature on fibril formation kinetics, fibril morphology and the rheological properties of fibril dispersions. *Food Hydrocolloids*, 27(1), 242–249. <https://doi.org/10.1016/j.foodhyd.2011.07.001>
- Mackie, A.R., Mingins, J., Dann R., (1993). Preliminary studies of  $\beta$ -lactoglobulin adsorbed on polystyrene latex. *Food colloids and polymers: Stability and mechanical properties*. E. Dickinson, P. Walstra (Eds.), The Royal Society of Chemistry, Cambridge, 96–112.
- Madureira, A. R., Pereira, C. I., Gomes, A. M. P., Pintado, M. E., & Xavier Malcata, F. (2007). Bovine whey proteins – Overview on their main biological properties. *Food Research International*, 40(10), 1197–1211. <https://doi.org/10.1016/j.foodres.2007.07.005>
- Mahmoudi, N., Axelos, M. A. V., & Riaublanc, A. (2011). Interfacial properties of fractal and spherical whey protein aggregates, 7(17), 7643–7654. <https://doi.org/10.1039/C1SM05262D>
- Mahmoudi, N., Gaillard, C., Boué, F., Axelos, M. A. V., & Riaublanc, A. (2010). Self-similar assemblies of globular whey proteins at the air–water interface: Effect of the structure. *Journal of Colloid and Interface Science*, 345(1), 54–63. <https://doi.org/10.1016/j.jcis.2010.01.036>
- Mahmoudi, N., Mehalebi, S., Nicolai, T., Durand, D., & Riaublanc, A. (2007). Light-Scattering Study of the Structure of Aggregates and Gels Formed by Heat-Denatured Whey Protein Isolate and  $\beta$ -Lactoglobulin at Neutral pH. *Journal of Agricultural and Food Chemistry*, 55(8), 3104–3111. <https://doi.org/10.1021/jf063029g>

## References

- Marefati, A., Rayner, M., Timgren, A., Dejmek, P., & Sjöö, M. (2013). Freezing and freeze-drying of Pickering emulsions stabilized by starch granules. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 436, 512–520. <https://doi.org/10.1016/j.colsurfa.2013.07.015>
- Martinez-Alvarenga, M. S., Martinez-Rodriguez, E. Y., Garcia-Amezquita, L. E., Olivas, G. I., Zamudio-Flores, P. B., Acosta-Muniz, C. H., & Sepulveda, D. R. (2014). Effect of Maillard reaction conditions on the degree of glycation and functional properties of whey protein isolate – Maltodextrin conjugates. *Food Hydrocolloids*, 38, 110–118. <https://doi.org/10.1016/j.foodhyd.2013.11.006>
- Maskan, M. (2001). Kinetics of colour change of kiwifruits during hot air and microwave drying. *Journal of Food Engineering*, 48(2), 169–175. [https://doi.org/10.1016/S0260-8774\(00\)00154-0](https://doi.org/10.1016/S0260-8774(00)00154-0)
- McClement, D. J., (1999). *Food emulsions: principles, practices and techniques*. Boca Raton: CRC Press.
- McClements, D. J. (2000). Comments on viscosity enhancement and depletion flocculation by polysaccharides. *Food Hydrocolloids*, 14(2), 173–177. [https://doi.org/10.1016/S0268-005X\(99\)00065-X](https://doi.org/10.1016/S0268-005X(99)00065-X)
- McClements, D. J. (2004). Protein-stabilized emulsions. *Current Opinion in Colloid & Interface Science*, 9(5), 305–313. <https://doi.org/10.1016/j.cocis.2004.09.003>
- McClements, D.J., (2005) *Food emulsions: principles, practices, and techniques*. Second Edition. Boca Raton: CRC Press.
- McClements, D. J., & Gumus, C. E. (2016). Natural emulsifiers — Biosurfactants, phospholipids, biopolymers, and colloidal particles: Molecular and physicochemical basis of functional performance. *Advances in Colloid and Interface Science*, 234, 3–26. <https://doi.org/10.1016/j.cis.2016.03.002>
- McCrae, C. H., & Lepoetre, A. (1996). Characterization of dairy emulsions by forward lobe laser light scattering-Application to milk and cream. *International Dairy Journal*, 6(3), 247–256.
- Mehalebi, S., Nicolai, T., & Durand, D. (2008). Light scattering study of heat-denatured globular protein aggregates. *International Journal of Biological Macromolecules*, 43(2), 129–135. <https://doi.org/10.1016/j.ijbiomac.2008.04.002>
- Mimouni, A., Deeth, H. C., Whittaker, A. K., Gidley, M. J., & Bhandari, B. R. (2010). Investigation of the microstructure of milk protein concentrate powders during rehydration: Alterations during storage. *Journal of Dairy Science*, 93(2), 463–472. <https://doi.org/10.3168/jds.2009-2369>

- Moitzi, C., Donato, L., Schmitt, C., Bovetto, L., Gillies, G., & Stradner, A. (2011). Structure of  $\beta$ -lactoglobulin microgels formed during heating as revealed by small-angle X-ray scattering and light scattering. *Food Hydrocolloids*, 25(7), 1766–1774. <https://doi.org/10.1016/j.foodhyd.2011.03.020>
- Mollet, B., & Rowland, I. (2002). Functional foods: at the frontier between food and pharma. *Current Opinion in Biotechnology*, 13(5), 483–485. [https://doi.org/10.1016/S0958-1669\(02\)00375-0](https://doi.org/10.1016/S0958-1669(02)00375-0)
- Morgan, F., Mollé, D., Henry, G., Vénien, A., Léonil, J., Peltre, G., ... Bouhallab, S. (1999). Glycation of bovine  $\beta$ -Lactoglobulin: effect on the protein structure. *International Journal of Food Science & Technology*, 34(5–6), 429–435. <https://doi.org/10.1046/j.1365-2621.1999.00318.x>
- Morgan, P. E., Treweek, T. M., Lindner, R. A., Price, W. E., & Carver, J. A. (2005). Casein Proteins as Molecular Chaperones. *Journal of Agricultural and Food Chemistry*, 53(7), 2670–2683. <https://doi.org/10.1021/jf048329h>
- Moro, A., Baez, G. D., Ballerini, G. A., Busti, P. A., & Delorenzi, N. J. (2013). Emulsifying and foaming properties of beta-lactoglobulin modified by heat treatment. *Food Research International*, 51(1), 1–7. <https://doi.org/10.1016/j.foodres.2012.11.011>
- Moro, A., Gatti, C., & Delorenzi, N. (2001). Hydrophobicity of Whey Protein Concentrates Measured by Fluorescence Quenching and Its Relation with Surface Functional Properties. *Journal of Agricultural and Food Chemistry*, 49(10), 4784–4789. <https://doi.org/10.1021/jf001132e>
- Morr, C. V., & Ha, E. Y. W. (1993). Whey protein concentrates and isolates: Processing and functional properties. *Critical Reviews in Food Science and Nutrition*, 33(6), 431–476. <https://doi.org/10.1080/10408399309527643>
- Murphy, R., Cho, Y.-H., Farkas, B., & Jones, O. G. (2015). Control of thermal fabrication and size of  $\beta$ -lactoglobulin-based microgels and their potential applications. *Journal of Colloid and Interface Science*, 447, 182–190. <https://doi.org/10.1016/j.jcis.2014.09.067>
- Murphy, R. W., Farkas, B. E., & Jones, O. G. (2016). Dynamic and viscoelastic interfacial behavior of  $\beta$ -lactoglobulin microgels of varying sizes at fluid interfaces. *Journal of Colloid and Interface Science*, 466, 12–19. <https://doi.org/10.1016/j.jcis.2015.12.012>
- Murray, B. S., Durga, K., Yusoff, A., & Stoyanov, S. D. (2011). Stabilization of foams and emulsions by mixtures of surface active food-grade particles and proteins. *Food Hydrocolloids*, 25(4), 627–638. <https://doi.org/10.1016/j.foodhyd.2010.07.025>

## References

- Murray, B. S., & Phisarnchananan, N. (2016). Whey protein microgel particles as stabilizers of waxy corn starch + locust bean gum water-in-water emulsions. *Food Hydrocolloids*, 56, 161–169. <https://doi.org/10.1016/j.foodhyd.2015.11.032>
- Nguyen, B. T., Balakrishnan, G., Jacquette, B., Nicolai, T., Chassenieux, C., Schmitt, C., & Bovetto, L. (2016). Inhibition and Promotion of Heat-Induced Gelation of Whey Proteins in the Presence of Calcium by Addition of Sodium Caseinate. *Biomacromolecules*, 17(11), 3800–3807. <https://doi.org/10.1021/acs.biomac.6b01322>
- Nguyen, B. T., Nicolai, T., & Benyahia, L. (2013). Stabilization of Water-in-Water Emulsions by Addition of Protein Particles. *Langmuir*, 29(34), 10658–10664. <https://doi.org/10.1021/la402131e>
- Nicolai, T. (2015). Formation and functionality of self-assembled whey protein microgels. *Colloids and Surfaces B: Biointerfaces*. <https://doi.org/10.1016/j.colsurfb.2015.05.055>
- Nicolai, T. (2016). Formation and functionality of self-assembled whey protein microgels. *Colloids and Surfaces B: Biointerfaces*, 137, 32–38. <https://doi.org/10.1016/j.colsurfb.2015.05.055>
- Nicolai, T., Britten, M., & Schmitt, C. (2011). beta-Lactoglobulin and WPI aggregates: Formation, structure and applications. *Food Hydrocolloids*, 25(8), 1945–1962. <https://doi.org/10.1016/j.foodhyd.2011.02.006>
- Nicolai, T., & Durand, D. (2013). Controlled food protein aggregation for new functionality. *Current Opinion in Colloid & Interface Science*, 18(4), 249–256. <https://doi.org/10.1016/j.cocis.2013.03.001>
- Norwood, E.-A., Le Floch-Fouéré, C., Briard-Bion, V., Schuck, P., Croguennec, T., & Jeantet, R. (2016). Structural markers of the evolution of whey protein isolate powder during aging and effects on foaming properties. *Journal of Dairy Science*, 99(7), 5265–5272. <https://doi.org/10.3168/jds.2015-10788>
- Nyemb, K., Guérin-Dubiard, C., Dupont, D., Jardin, J., Rutherford, S. M., & Nau, F. (2014). The extent of ovalbumin in vitro digestion and the nature of generated peptides are modulated by the morphology of protein aggregates. *Food Chemistry*, 157, 429–438. <https://doi.org/10.1016/j.foodchem.2014.02.048>
- Oboroceanu, D., Wang, L., Magner, E., & Auty, M. A. E. (2014). Fibrillization of whey proteins improves foaming capacity and foam stability at low protein concentrations. *Journal of Food Engineering*, 121, 102–111. <https://doi.org/10.1016/j.jfoodeng.2013.08.023>

- O'Kennedy, B. T., & Mounsey, J. S. (2006). Control of Heat-Induced Aggregation of Whey Proteins Using Casein. *Journal of Agricultural and Food Chemistry*, 54(15), 5637–5642. <https://doi.org/10.1021/jf0607866>
- Palanuwech, J., & Coupland, J. N. (2003). Effect of surfactant type on the stability of oil-in-water emulsions to dispersed phase crystallization. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 223(1–3), 251–262. [https://doi.org/10.1016/S0927-7757\(03\)00169-9](https://doi.org/10.1016/S0927-7757(03)00169-9)
- Palatasa Havea, John Edward Grant, Michael Jiu Wai Hii, & Peter Gilbert Wiles. (2012). Dairy product and process. Retrieved from <http://www.google.com/patents/US20120114795>
- Palzer, S. (2009). Food structures for nutrition, health and wellness. *Trends in Food Science & Technology*, 20(5), 194–200. <https://doi.org/10.1016/j.tifs.2009.02.005>
- Panouillé, M., Nicolai, T., Benyahia, L., Durand D., (2005). Food Colloids Interactions, Microstructure and Processing, in E. Dickinson (Ed.), Royal Society of Chemistry, Cambridge, 194.
- Parkinson, E. L., & Dickinson, E. (2004). Inhibition of heat-induced aggregation of a beta-lactoglobulin-stabilized emulsion by very small additions of casein. *Colloids and Surfaces B-Biointerfaces*, 39(1–2), 23–30. <https://doi.org/10.1016/j.colsurfb.2004.08.011>
- Parkinson, E. L., & Dickinson, E. (2007). Synergistic stabilization of heat-treated emulsions containing mixtures of milk proteins. *International Dairy Journal*, 17(2), 95–103. <https://doi.org/10.1016/j.idairyj.2006.01.010>
- Patton, S., & Huston, G. (1986). A Method for Isolation of Milk-Fat Globules. *Lipids*, 21(2), 170–174. <https://doi.org/10.1007/BF02534441>
- Pelton, R., & Hoare, T., (2011). Microgels and their synthesis: an introduction. In A. Fernandez-Nieves, H. Wyss, J. Mattsson, & D. A. Weitz (Eds.), *Microgel suspensions*. Weinheim: WileyVCH, Chap. 1.
- Peng, J., Simon, J. R., Venema, P., & van der Linden, E. (2016). Protein Fibrils Induce Emulsion Stabilization. *Langmuir*, 32(9), 2164–2174. <https://doi.org/10.1021/acs.langmuir.5b04341>
- Peram, M. R., Loveday, S. M., Ye, A., & Singh, H. (2013). In vitro gastric digestion of heat-induced aggregates of  $\beta$ -lactoglobulin. *Journal of Dairy Science*, 96(1), 63–74. <https://doi.org/10.3168/jds.2012-5896>



## References

- Phan-Xuan, T., Durand, D., Nicolai, T., Donato, L., Schmitt, C., & Bovetto, L. (2011). On the Crucial Importance of the pH for the Formation and Self-Stabilization of Protein Microgels and Strands. *Langmuir*, 27(24), 15092–15101. <https://doi.org/10.1021/la203357p>
- Phan-Xuan, T., Durand, D., Nicolai, T., Donato, L., Schmitt, C., & Bovetto, L. (2013). Tuning the Structure of Protein Particles and Gels with Calcium or Sodium Ions. *Biomacromolecules*, 14(6), 1980–1989. <https://doi.org/10.1021/bm400347d>
- Phan-Xuan, T., Durand, D., Nicolai, T., Donato, L., Schmitt, C., & Bovetto, L. (2014). Heat induced formation of beta-lactoglobulin microgels driven by addition of calcium ions. *Food Hydrocolloids*, 34, 227–235. <https://doi.org/10.1016/j.foodhyd.2012.09.008>
- Pichot, R., Spyropoulos, F., & Norton, I. T. (2010). O/W emulsions stabilised by both low molecular weight surfactants and colloidal particles: The effect of surfactant type and concentration. *Journal of Colloid and Interface Science*, 352(1), 128–135. <https://doi.org/10.1016/j.jcis.2010.08.021>
- Pickering, S. U., (1907). Emulsions. *Journal of Chemical Society*, 91, 2001-2021.
- Pitkowski, A., Durand, D., & Nicolai, T. (2008). Structure and dynamical mechanical properties of suspensions of sodium caseinate. *Journal of Colloid and Interface Science*, 326(1), 96–102. <https://doi.org/10.1016/j.jcis.2008.07.003>
- Pouzot, M., Nicolai, T., Visschers, R. W., & Weijers, M. (2005). X-ray and light scattering study of the structure of large protein aggregates at neutral pH. *Food Hydrocolloids*, 19(2), 231–238. <https://doi.org/10.1016/j.foodhyd.2004.06.003>
- R Core Team (2016). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>.
- Ramsden, W., (1903). Separation of solids in the surface-layers of solutions and ‘suspensions’- preliminary account. *Proceedings of the royal Society of London*, 72, 156–164.
- Reiffers-Magnani, C. K., Cuq, J. L., & Watzke, H. J. (1999). Composite structure formation in whey protein stabilized O/W emulsions. I. Influence of the dispersed phase on viscoelastic properties. *Food Hydrocolloids*, 13(4), 303–316. [https://doi.org/10.1016/S0268-005X\(99\)00013-2](https://doi.org/10.1016/S0268-005X(99)00013-2)
- Relkin, P., Sourdret, S., Smith, A. K., Goff, H. D., & Cuvelier, G. (2006). Effects of whey protein aggregation on fat globule microstructure in whipped-frozen emulsions. *Food Hydrocolloids*, 20(7), 1050–1056. <https://doi.org/10.1016/j.foodhyd.2005.11.007>

- Renard, D., Axelos, M. A. V., Boué, F., & Lefebvre, J. (1996). "Ordered" structure in solutions and gels of a globular protein as studied by small angle neutron scattering. *Biopolymers*, 39(2), 149–159. [https://doi.org/10.1002/\(SICI\)1097-0282\(199608\)39:2<149::AID-BIP3>3.0.CO;2-P](https://doi.org/10.1002/(SICI)1097-0282(199608)39:2<149::AID-BIP3>3.0.CO;2-P)
- Roefs, S. P. F. M., & De Kruif, K. G. (1994). A Model for the Denaturation and Aggregation of  $\beta$ -Lactoglobulin. *European Journal of Biochemistry*, 226(3), 883–889. <https://doi.org/10.1111/j.1432-1033.1994.00883.x>
- Rullier, B., Axelos, M. A. V., Langevin, D., & Novales, B. (2009).  $\beta$ -Lactoglobulin aggregates in foam films: Correlation between foam films and foaming properties. *Journal of Colloid and Interface Science*, 336(2), 750–755. <https://doi.org/10.1016/j.jcis.2009.04.034>
- Rullier, B., Axelos, M. A. V., Langevin, D., & Novales, B. (2010).  $\beta$ -Lactoglobulin aggregates in foam films: Effect of the concentration and size of the protein aggregates. *Journal of Colloid and Interface Science*, 343(1), 330–337. <https://doi.org/10.1016/j.jcis.2009.11.015>
- Rullier, B., Novales, B., & Axelos, M. A. V. (2008). Effect of protein aggregates on foaming properties of  $\beta$ -lactoglobulin. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 330(2–3), 96–102. <https://doi.org/10.1016/j.colsurfa.2008.07.040>
- Ryan, K. N., Vardhanabhuti, B., Jaramillo, D. P., van Zanten, J. H., Coupland, J. N., & Foegeding, E. A. (2012). Stability and mechanism of whey protein soluble aggregates thermally treated with salts. *Food Hydrocolloids*, 27(2), 411–420. <https://doi.org/10.1016/j.foodhyd.2011.11.006>
- Ryan, K. N., Zhong, Q., & Foegeding, E. A. (2013). Use of Whey Protein Soluble Aggregates for Thermal Stability—A Hypothesis Paper. *Journal of Food Science*, 78(8), R1105–R1115. <https://doi.org/10.1111/1750-3841.12207>
- Sarkar, A., Murray, B., Holmes, M., Ettelaie, R., Abdalla, A., & Yang, X. (2016). In vitro digestion of Pickering emulsions stabilized by soft whey protein microgel particles: influence of thermal treatment, 12(15), 3558–3569. <https://doi.org/10.1039/C5SM02998H>
- Sawyer, W. H. (1968). Heat Denaturation of Bovine  $\beta$ -Lactoglobulins and Relevance of Disulfide Aggregation. *Journal of Dairy Science*, 51(3), 323–329. [https://doi.org/10.3168/jds.S0022-0302\(68\)86985-1](https://doi.org/10.3168/jds.S0022-0302(68)86985-1)

## References

- Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., ... Cardona, A. (2012). Fiji: an open-source platform for biological-image analysis. *Nature Methods*, 9(7), 676–682. <https://doi.org/10.1038/nmeth.2019>
- Schmidt, S., Liu, T., Rütten, S., Phan, K.-H., Möller, M., & Richtering, W. (2011). Influence of Microgel Architecture and Oil Polarity on Stabilization of Emulsions by Stimuli-Sensitive Core–Shell Poly(N-isopropylacrylamide-co-methacrylic acid) Microgels: Mickering versus Pickering Behavior? *Langmuir*, 27(16), 9801–9806. <https://doi.org/10.1021/la201823b>
- Schmitt, C., Bovay, C., & Rouvet, M. (2014). Bulk self-aggregation drives foam stabilization properties of whey protein microgels. *Food Hydrocolloids*, 42, Part 1, 139–148. <https://doi.org/10.1016/j.foodhyd.2014.03.010>
- Schmitt, C., Bovay, C., Rouvet, M., Shojaei-Rami, S., & Kolodziejczyk, E. (2007). Whey Protein Soluble Aggregates from Heating with NaCl: Physicochemical, Interfacial, and Foaming Properties. *Langmuir*, 23(8), 4155–4166. <https://doi.org/10.1021/la0632575>
- Schmitt, C., Bovay, C., Vuilliomenet, A.-M., Rouvet, M., & Bovetto, L. (2011). Influence of protein and mineral composition on the formation of whey protein heat-induced microgels. *Food Hydrocolloids*, 25(4), 558–567. <https://doi.org/10.1016/j.foodhyd.2010.05.010>
- Schmitt, C., Bovay, C., Vuilliomenet, A.-M., Rouvet, M., Bovetto, L., Barbar, R., & Sanchez, C. (2009). Multiscale Characterization of Individualized beta-Lactoglobulin Microgels Formed upon Heat Treatment under Narrow pH Range Conditions. *Langmuir*, 25(14), 7899–7909. <https://doi.org/10.1021/la900501n>
- Schmitt, C., Moitzi, C., Bovay, C., Rouvet, M., Bovetto, L., Donato, L., ... Stradner, A. (2010). Internal structure and colloidal behaviour of covalent whey protein microgels obtained by heat treatment. *Soft Matter*, 6(19), 4876–4884. <https://doi.org/10.1039/C0SM00220H>
- Schmitt, V., & Ravaine, V. (2013). Surface compaction versus stretching in Pickering emulsions stabilised by microgels. *Current Opinion in Colloid & Interface Science*, 18(6), 532–541. <https://doi.org/10.1016/j.cocis.2013.11.004>
- Schokker, E. P., Church, J. S., Mata, J. P., Gilbert, E. P., Puvanenthiran, A., & Udabage, P. (2011). Reconstitution properties of micellar casein powder: Effects of composition and storage. *International Dairy Journal*, 21(11), 877–886. <https://doi.org/10.1016/j.idairyj.2011.05.004>

- Schubert, H., Ax, K., & Behrend, O. (2003). Product engineering of dispersed systems. *Trends in Food Science & Technology*, 14(1–2), 9–16. [https://doi.org/10.1016/S0924-2244\(02\)00245-5](https://doi.org/10.1016/S0924-2244(02)00245-5)
- Serfert, Y., Lamprecht, C., Tan, C.-P., Keppler, J. K., Appel, E., Rossier-Miranda, F. J., ... Schwarz, K. (2014). Characterisation and use of  $\beta$ -lactoglobulin fibrils for microencapsulation of lipophilic ingredients and oxidative stability thereof. *Journal of Food Engineering*, 143, 53–61. <https://doi.org/10.1016/j.jfoodeng.2014.06.026>
- Sharma, R., Singh, H., & Taylor, M. W. (1996). Composition and Structure of Fat Globule Surface Layers in Recombined Milk. *Journal of Food Science*, 61(1), 28–32. <https://doi.org/10.1111/j.1365-2621.1996.tb14719.x>
- Shen, X., Fang, T., Gao, F., & Guo, M. (2017). Effects of ultrasound treatment on physicochemical and emulsifying properties of whey proteins pre- and post-thermal aggregation. *Food Hydrocolloids*, 63, 668–676. <https://doi.org/10.1016/j.foodhyd.2016.10.003>
- Silva, J. V. C., Legland, D., Cauty, C., Kolotuev, I., & Floury, J. (2015). Characterization of the microstructure of dairy systems using automated image analysis. *Food Hydrocolloids*, 44, 360–371. <https://doi.org/10.1016/j.foodhyd.2014.09.028>
- Sliwinski, E. L., Roubos, P. J., Zoet, F. D., van Boekel, M. A. J. S., & Wouters, J. T. M. (2003). Effects of heat on physicochemical properties of whey protein-stabilised emulsions. *Colloids and Surfaces B: Biointerfaces*, 31(1–4), 231–242. [https://doi.org/10.1016/S0927-7765\(03\)00143-7](https://doi.org/10.1016/S0927-7765(03)00143-7)
- Smithers, G. W. (2008). Whey and whey proteins—From “gutter-to-gold.” *International Dairy Journal*, 18(7), 695–704. <https://doi.org/10.1016/j.idairyj.2008.03.008>
- Srinivasan, M., Singh, H., & Munro, P. A. (1996). Sodium Caseinate-Stabilized Emulsions: Factors Affecting Coverage and Composition of Surface Proteins. *Journal of Agricultural and Food Chemistry*, 44(12), 3807–3811. <https://doi.org/10.1021/jf960135h>
- Srinivasan, M., Singh, H., & Munro, P. A. (2002). Formation and stability of sodium caseinate emulsions: influence of retorting (121°C for 15 min) before or after emulsification. *Food Hydrocolloids*, 16(2), 153–160. [https://doi.org/10.1016/S0268-005X\(01\)00072-8](https://doi.org/10.1016/S0268-005X(01)00072-8)
- Stapelfeldt, H., Nielsen, B. R., & Skibsted, L. H. (1997). Effect of heat treatment, water activity and storage temperature on the oxidative stability of whole milk powder. *International Dairy Journal*, 7(5), 331–339.

## References

- Surel, C., Foucquier, J., Perrot, N., Mackie, A., Garnier, C., Riaublanc, A., & Anton, M. (2014). Composition and structure of interface impacts texture of O/W emulsions. *Food Hydrocolloids*, 34(1), 3–9. <https://doi.org/10.1016/j.foodhyd.2013.06.016>
- Surroca, Y., Haverkamp, J., & Heck, A. J. R. (2002). Towards the understanding of molecular mechanisms in the early stages of heat-induced aggregation of  $\beta$ -lactoglobulin AB. *Journal of Chromatography A*, 970(1–2), 275–285. [https://doi.org/10.1016/S0021-9673\(02\)00884-1](https://doi.org/10.1016/S0021-9673(02)00884-1)
- Takagi, K., Teshima, R., Okunuki, H., & Sawada, J. (2003). Comparative Study of in Vitro Digestibility of Food Proteins and Effect of Preheating on the Digestion. *Biological and Pharmaceutical Bulletin*, 26(7), 969–973. <https://doi.org/10.1248/bpb.26.969>
- Thanasukarn, P., Pongsawatmanit, R., & McClements, D. J. (2004a). Impact of fat and water crystallization on the stability of hydrogenated palm oil-in-water emulsions stabilized by whey protein isolate. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 246(1–3), 49–59. <https://doi.org/10.1016/j.colsurfa.2004.07.018>
- Thanasukarn, P., Pongsawatmanit, R., & McClements, D. J. (2004b). Influence of emulsifier type on freeze-thaw stability of hydrogenated palm oil-in-water emulsions. *Food Hydrocolloids*, 18(6), 1033–1043. <https://doi.org/10.1016/j.foodhyd.2004.04.010>
- Thomas, M. E. C., Scher, J., Desobry-Banon, S., & Desobry, S. (2004). Milk Powders Ageing: Effect on Physical and Functional Properties. *Critical Reviews in Food Science and Nutrition*, 44(5), 297–322. <https://doi.org/10.1080/10408690490464041>
- Veerman, C., Baptist, H., Sagis, L. M. C., & van der Linden, E. (2003). A New Multistep  $\text{Ca}^{2+}$ -Induced Cold Gelation Process for  $\beta$ -Lactoglobulin. *Journal of Agricultural and Food Chemistry*, 51(13), 3880–3885. <https://doi.org/10.1021/jf0261396>
- Verheul, M., Roefs, S. P. F. M., & de Kruif, K. G. (1998). Kinetics of Heat-Induced Aggregation of  $\beta$ -Lactoglobulin. *Journal of Agricultural and Food Chemistry*, 46(3), 896–903. <https://doi.org/10.1021/jf970751t>
- Vilgis, T. A. (2015). Soft matter food physics—the physics of food and cooking. *Reports on Progress in Physics*, 78(12), 124602. <https://doi.org/10.1088/0034-4885/78/12/124602>
- Walstra, P. (1993). Principles of emulsion formation. *Chemical Engineering Science*, 48(2), 333–349. [https://doi.org/10.1016/0009-2509\(93\)80021-H](https://doi.org/10.1016/0009-2509(93)80021-H)

- Walstra P. (1996). Disperse system: Basic consideration, In Food Chemistry, 3rd ed. Fennema, O.R, Ed., Marcel Dekker, New York, NY, Chap 3.
- Walstra P. (2003). Physical Chemistry of Foods, Marcel Dekker, New York, NY.
- Walstra, P., & Smulders P.E.A., (1998). Emulsion formation. B Binks (Ed.), Modern Aspects of Emulsion Science, Royal Society of Chemistry, Cambridge, UK, 56–98.
- Wilde, P., Mackie, A., Husband, F., Gunning, P., & Morris, V. (2004). Proteins and emulsifiers at liquid interfaces. *Advances in Colloid and Interface Science*, 108–109, 63–71. <https://doi.org/10.1016/j.cis.2003.10.011>
- Yamauchi, K., Shimizu, M., & Kamiya, T. (1980). Emulsifying Properties of Whey Protein. *Journal of Food Science*, 45(5), 1237–1242. <https://doi.org/10.1111/j.1365-2621.1980.tb06529.x>
- Ye, A. (2008). Interfacial composition and stability of emulsions made with mixtures of commercial sodium caseinate and whey protein concentrate. *Food Chemistry*, 110(4), 946–952. <https://doi.org/10.1016/j.foodchem.2008.02.091>
- Ye, A. (2011). Functional properties of milk protein concentrates: Emulsifying properties, adsorption and stability of emulsions. *International Dairy Journal*, 21(1), 14–20. <https://doi.org/10.1016/j.idairyj.2010.07.005>
- Yost, R. A., & Kinsella, J. e. (1992). Microstructure of Whey Protein Isolate Gels Containing Emulsified Butterfat Droplets. *Journal of Food Science*, 57(4), 892–897. <https://doi.org/10.1111/j.1365-2621.1992.tb14317.x>
- Zhang, S., & Vardhanabhuti, B. (2014). Effect of initial protein concentration and pH on in vitro gastric digestion of heated whey proteins. *Food Chemistry*, 145, 473–480. <https://doi.org/10.1016/j.foodchem.2013.08.076>
- Zhang, X., Fu, X., Zhang, H., Liu, C., Jiao, W., & Chang, Z. (2005). Chaperone-like activity of  $\beta$ -casein. *The International Journal of Biochemistry & Cell Biology*, 37(6), 1232–1240. <https://doi.org/10.1016/j.biocel.2004.12.004>
- Zhang, Z., Arrighi, V., Campbell, L., Lonchamp, J., & Euston, S. R. (2016). Properties of partially denatured whey protein products: Formation and characterisation of structure. *Food Hydrocolloids*, 52, 95–105. <https://doi.org/10.1016/j.foodhyd.2015.06.009>

## References

Zhou, P., & Labuza, T. P. (2007). Effect of Water Content on Glass Transition and Protein Aggregation of Whey Protein Powders During Short-Term Storage. *Food Biophysics*, 2(2–3), 108–116.  
<https://doi.org/10.1007/s11483-007-9037-4>





## Table of Figures

<b>Figure 1:</b> Organization of the PROFIL project .....	3
<b>Figure 2:</b> Scheme of the micellar casein and individual monomers and small assemblies of caseins .....	7
<b>Figure 3:</b> Negative-staining TEM image of $\beta$ -lactoglobulin aggregates obtained upon heating a 1 wt% protein solution at different pH: (A) pH 2.0; (B) pH 5.8; (C) pH 7.0. Reproduced from Jung et al (2008) with permission of the America Chemical Society. ....	8
<b>Figure 4:</b> Scheme of the mechanism of amyloid fibrils formation. Reproduced from Adamcik & Mezzenga (2012) with permission of the America Chemical Society. ....	9
<b>Figure 5:</b> Scheme of the mechanism of formation of curved strand and fractal aggregates. Adapted from Nicolai, Britten & Schmitt (2011). ....	11
<b>Figure 6:</b> Concentration dependence of the weight-average molar mass of WPI (squares) and $\beta$ -Lg (circles) aggregates formed in the presence of 0.1 M NaCl (open symbols) or in the absence of added salt (solid symbols). The solid lines are guides to the eye. The dotted lines indicate the critical gelation concentration: $C_g \approx 100$ g/L for WPI and $\beta$ -Lg in the absence of added salt, $C_g \approx 25$ g/L for WPI, and $C_g \approx 15$ g/L for $\beta$ -Lg at 0.1 M NaCl. Adapted from Mahmoudi, Mehalebi, Nicolai, Durand and Riaublanc (2007) with permission of the America Chemical Society. ....	12
<b>Figure 7:</b> Physical instabilities occurring during technological treatments of oil-in-water emulsions.....	19
<b>Figure 8:</b> Number of publications related to (A) Pickering emulsions and (B) Pickering emulsions and food by year. Data from Thomson Reuters Web of Science. ....	22
<b>Figure 9:</b> Position of a spherical particle at a planar oil–water interface for a contact angle (measured through the aqueous phase) less than $90^\circ$ (A), equal to $90^\circ$ (B) and greater than $90^\circ$ (C). Putative particles positioning at a curved interface: for $\vartheta < 90^\circ$ , stabilization of o/w emulsions (D), for $\vartheta > 90^\circ$ , stabilization of w/o emulsions (E). Adapted from Aveyard, Binks & Clint (2003).....	23
<b>Figure 10:</b> Free oil (wt%) accumulating on the top of 20 wt% hydrogenated palm oil-in-water emulsions after being stored at different temperatures due to oiling off. The emulsions were stabilized by: (a) 2 wt% Tween 20; (b) 1 wt% whey protein isolate; (c) 1 wt% casein. Reproduced Thanasukarn, Pongsawatmanit & McClements (2004b) with permission of Elsevier. ....	29
<b>Figure 11:</b> Schematic representation of the effect of a small addition of sodium caseinate on the colloidal structure of a heat-treated WPI-B emulsion: (A) thin layer of globular whey protein molecules adsorbed at the oil–water interface; (B) thicker layer with disordered casein molecules incorporated; (C) reversibly flocculated heat-treated WPI-B emulsion (high viscosity); (D) non-flocculated emulsion (low viscosity) sterically stabilized by low density of dangling casein tails. Reproduced from Dickinson & Parkinson (2004) with the permission of Elsevier. ....	31
<b>Figure 12:</b> Heat stability at $120^\circ\text{C}$ of emulsions prepared with whey protein suspensions at various protein concentrations. The dotted lines are guides to the eye. ....	37
<b>Figure 13:</b> Picture of whey protein emulsion prepared in water (A) or in milk permeate solution (B) after a freeze/thaw cycle.....	38

## Table of Figures

<b>Figure 14:</b> Scheme representing the thesis strategy .....	42
<b>Figure 15:</b> Schema of the image analysis.....	56
<b>Figure 16:</b> Diagram summarizing the different steps of preparation and analysis of the protein suspensions and emulsions .....	58
<b>Figure 17:</b> Evolution of Browning Index for powders of aw 0.23 stored at 20°C (triangles) and 40°C (squares) as a function of storage time, with relative error < 0.7 %. (A-B) Images of powders after 1 month of storage at 4°C and 3, 6 and 12 months of storage at 40°C, respectively. ....	64
<b>Figure 18:</b> Amount of native (circles) and denatured/aggregated (squares) proteins for powders of aw 0.23 stored at 20°C (empty symbols) and 40°C (full symbols) as a function of storage time.....	65
<b>Figure 19:</b> Evolution of lactosylation of $\beta$ -Lg stored at 20°C (circles) and 40°C (squares) as a function of storage time. ....	66
<b>Figure 20:</b> Correlation between lactosylation and aggregation for Ref (empty circle) and powders with aw 0.23 stored at 40°C (empty square=1 month; light grey square = 3 months; grey square=6 months; dark grey square=9 months; black square = 12 months) as a function of storage time.....	67
<b>Figure 21:</b> Average aggregate sizes as a function of storage time for powders with aw 0.23 stored at 20°C (A) and 40°C (B) after heat treatment at pH 5.8 (circles), pH 6.2 (squares) and pH 6.6 (triangles). ....	68
<b>Figure 22:</b> TEM images of negatively stained whey protein Ref (A, C, E) and powder stored for 12 months at 40°C (B, D, F) when heat treated at pH 5.8 (A and B), pH 6.2 (C and D) and pH 6.6 (E and F). ....	69
<b>Figure 23:</b> Proposed scheme for formation of heat-induced aggregates of native and stored powders as a function of pH. From Norwood et al. (2016).....	71
<b>Figure 24:</b> Concentration dependence of the weight-average molar mass of WPI (squares) and $\beta$ -Lg (circles) aggregates formed in the presence of 0.1 M NaCl (open symbols) or in the absence of added salt (solid symbols). The solid lines are guides to the eye. The dotted lines indicate the critical gelation concentration: $C_g \approx 100$ g/L for WPI and $\beta$ -Lg in the absence of added salt, $C_g \approx 25$ g/L for WPI, and $C_g \approx 15$ g/L for $\beta$ -Lg at 0.1 M NaCl. (Mahmoudi, Mehalebi, Nicolai, Durand, & Riaublanc, 2007) .....	76
<b>Figure 25:</b> Heat stability of the emulsions (A) and pictures of emulsion after the freeze/thaw cycle (B).....	77
<b>Figure 26:</b> Confocal Laser Scanning Microscopic (CLSM) images of SA <sub>9</sub> emulsion (A) and SA <sub>Na</sub> emulsion (B) after a freeze/thaw cycle. Proteins are in green (labelled with fast green) and fat is in red (labelled with Nile red) .....	78
<b>Figure 27:</b> Proportion of the entities in the selected aggregates suspensions: WPM A suspension (green), SA <sub>4</sub> suspension (orange) and WPM/SA B suspension (grey). ....	79
<b>Figure 28:</b> SDS-PAGE of the proteins in the supernatant of WPM A suspension under non-reducing conditions (2) or under reducing conditions (3). Molecular weight marker (1). ....	80
<b>Figure 29:</b> Picture of the whey protein B suspension at different pH after heat treatment.....	81
<b>Figure 30:</b> SDS-PAGE of the protein in the supernatant WPM/SA B suspension under non-reducing conditions (2) or under reducing conditions (3). Molecular weight marker (1). ....	82
<b>Figure 31:</b> Particle size distribution of the WPM A suspensions at 3% (black line), of the WPM AC suspensions at 6.8 wt% (dotted black line), of the SA <sub>4</sub> suspensions at 3 wt% (grey line) and of the WPM/SA B suspensions at 3 wt% (dashed black line). ....	83

## Table of Figures

<b>Figure 32:</b> Separation profiles of the whey protein aggregates suspensions using asymmetrical flow field-flow fractionation coupled with multiangle laser light scattering (AF4-MALLS). Thin line, differential refractive index measurements; Thick line, online calculated molecular mass.....	84
<b>Figure 33:</b> Negative-staining Transmission Electronic Microscopy (TEM) image of (A) WPM A suspension and (B) SA <sub>4</sub> suspension.....	85
<b>Figure 34:</b> Heat stability of the protein suspensions: WP A suspension (black triangle), SA <sub>4</sub> suspension (black square), WPM A suspension (black circle), WPM AC suspension (open circle) and WPM/SA B suspension (grey circle). .....	86
<b>Figure 35:</b> (A) Particle size distribution in the WPM suspensions before heat treatment (black line) and after heat treatment (grey line) at various protein concentrations (3.1 wt%, dotted line; 6.5 wt%, dashed line) and in the WPM AC suspension (6.8 wt%, continuous line). (B) The evolution of the Z-average of the particles in the WPM A suspensions as at various proteins concentrations (3.1 wt%, open diamond; 6.5 wt%, open triangle) and in WPM AC suspension (6.8 wt%, grey circle) a function of heating time at 120°C. The dotted lines are guides to the eye. ....	87
<b>Figure 36:</b> Size of the particles before (full rectangle) and after freezing/thawing cycle (striped rectangle) in WPM A suspensions (blue), WPM AC suspension (yellow), SA <sub>4</sub> suspension (green) and WPM/SA B suspension (orange). .....	88
<b>Figure 37:</b> $\pi/A$ isotherm (A) and $\pi$ /Protein load isotherm (B) of WP A suspension (blue line), for SA <sub>4</sub> suspension (yellow line) and for WPM A suspension (green line) at air–water interface .....	89
<b>Figure 38:</b> Fat droplet size ( $d_{4,3}$ ) of WP A emulsions (black square), SA <sub>4</sub> emulsions (black triangle), WPM A emulsions (black circle), WPM AC emulsions (empty circle) and WPM/SA B emulsions (grey circle) at various protein concentrations in the dispersing phase .....	91
<b>Figure 39:</b> Pictures of 4 wt% of whey protein suspension B with an addition of CasNa (A) or an addition of MC (B) .....	92
<b>Figure 40:</b> Picture of the emulsion prepared with the WPM AC suspension at 3.2 wt% proteins. A creamed layer appeared at the top rapidly (less than one hour). .....	98
<b>Figure 41:</b> Volume mean diameter ( $d_{4,3}$ ) of the particles in the emulsions before heat treatment determined by laser light scattering: for the emulsions prepared with the whole WPM A suspensions at various protein concentrations with (empty square) or without SDS (black diamond) and for the emulsion prepared with the WPM AC suspension at 6.8 wt% proteins with SDS (empty circle).....	99
<b>Figure 42:</b> Confocal Laser Scanning Microscopic (CLSM) images of emulsions before heat treatment: (A) emulsion at 6.8 wt% proteins prepared with the WPM AC suspension (to remove the caseins and whey proteins not included in WPM); (B) emulsion at 6.5 wt% proteins prepared with the whole WPM A suspension. Co-localisation of fat (in red) and proteins (in green). Individual images showing the fat droplets repartition (C) and on the protein repartition (D) in the emulsion at 6.8 wt% proteins prepared with the WPM AC suspension; overlay of these 2 images corresponds to (A).....	100
<b>Figure 43:</b> Distribution of the inter-fat droplet distances of emulsions before heat treatment: for the emulsions prepared with the whole WPM A suspensions at 3.1 wt% (black dotted line), 4.1 wt% (black small dashed line), 4.8 wt% (black large dashed line), 6.3 wt% proteins (black continuous line), and for the emulsions prepared with the WPM AC suspensions at 3.2 wt% (grey dotted line) and 6.8 wt% proteins (grey continuous line). .....	101

- Figure 44:** Heat stability of emulsions prepared with the whole WPM A suspensions (black triangle), with the WPM AC suspensions (grey circle) and of WP A emulsions (grey diamond) as a function of protein concentration. The dotted lines are guides. .... 102
- Figure 45:** Volume mean diameter ( $d_{4,3}$  in  $\mu\text{m}$ ) of the particles in heated emulsions prepared with the whole WPM A suspensions at various protein concentrations as a function of heating time at  $120^\circ\text{C}$ : 2.7 wt% (square); 3.3 wt% (diamond); 6.5 wt% (circle). Fat droplet size measurements were performed by laser light scattering in the absence of SDS (black symbols) and in presence of SDS (empty symbols). The dotted lines are guides to the eye. .... 102
- Figure 46:** Microstructure analysis of emulsions reconstituted with the whole WPM A suspensions. (A to D) Confocal Laser Scanning Microscopy (CLSM) images of the emulsions reconstituted with the whole WPM A suspensions before (A, C) and after (B, D) heating. Unheated (A) and 5 min heated (B) emulsions at 4.5 wt% proteins; Unheated (C) and 30 min heated (D) emulsions at 3.1 wt% proteins. Fat droplets were stained with Nile Red (red color on the images) and the proteins with Fast green (green color on the images). (E and F) Distribution of the inter-fat droplet distances (distance existing between the fat droplets and its closest neighbor) for emulsions at (E) 4.5 wt% and (F) 3.1 wt% proteins before (continuous line) and after (small dotted line) heating as well as the Dif AUC (point by point difference of the area under the curve of the inter-fat droplet distances between the unheated and heated emulsions (large dotted line)). .... 103
- Figure 47:** (A) Fat droplet surface protein load in emulsions prepared with the whole WPM A suspensions as a function of protein concentration. The dotted line is guide to the eye. (B) SDS-PAGE of the protein fraction adsorbed onto fat droplet surface in emulsions at different protein concentration; Molecular weight marker (1); emulsions prepared with WPM A suspensions at the following protein concentrations 3.1 wt% (2), 4.1 wt% (3), 4.8 wt% (4), 6.3 wt% (5) and whey protein powder (6).  $10\ \mu\text{g}$  of protein was placed in each sample well. .... 104
- Figure 48:** Scheme of the structure of the emulsions prepared with the whole WPM A suspensions at low (below 3.5 wt% proteins) and high (above 4 wt% proteins) protein concentration before and after heat treatment. .... 106
- Figure 49:** (A) Fat droplet surface protein load in emulsions prepared with WPM A suspensions at  $\sim 2.7$  wt% proteins and various amount of caseins. The heat stability of the emulsion was indicated by colored symbols: gelled emulsions (red circle) and fluid emulsion (blue circle). (B) SDS-PAGE of the protein fraction adsorbed onto fat droplet surface in emulsions prepared with WPM A suspensions at  $\sim 2.7$  wt% various amount of caseins: Molecular weight marker (1); emulsions prepared with WPM A suspensions at 2.7 wt% proteins and 0.24 wt% (2), and 0.31 wt% (3), 0.39 wt% (4), 0.47 wt% (5) of caseins. .... 107
- Figure 50:** (A) particle size distribution of the emulsions and (B) distribution of the inter-fat droplet distances for emulsions prepared with WPM AC suspension at 5.3 wt% proteins as a function of casein concentration in the continuous phase: dotted line, 0 wt%; small dashed line, 0.2 wt%; large dashed line, 0.5 wt%; continuous line, 0.7 wt% casein. .... 108
- Figure 51:** (A to F) Confocal Laser Scanning Microscopy (CLSM) images of the heated emulsions prepared with WPM AC suspensions at 5.3 wt% and (A, B) 0 wt% of caseins (A: unheated and, B: heated 1 min at  $120^\circ\text{C}$ ); (C, D) 0.2 wt% of caseins (C: unheated and D: after 2 min of heating); (E, F) 0.7 wt% of caseins (E: unheated and F: after 30 min of heating). Fat droplets were stained with Nile Red (red color on the images) and the proteins with Fast green (green color on the images). (G to I) Inter-fat droplet distance distribution for emulsions prepared with the WPM

## Table of Figures

AC suspension at 5.3 wt% and 0 wt% (G), 0.2 wt% (H) and 0.7 wt% (I) of caseins before (continuous line) and after (small dotted line) heating as well as the Dif AUC (point by point difference of the area under the curve of the inter-particle distances between the unheated and heated emulsions; large dotted line). .....	109
<b>Figure 52:</b> Volume mean diameter ( $d_{4,3}$ ) of the particles in the unheated emulsions prepared with WPM AC suspension at 5.3 wt% and various concentrations of caseins determined by laser light scattering with (grey) or without SDS (black) .....	111
<b>Figure 53:</b> (A) Surface protein load versus casein content in the emulsions prepared with WPM AC suspension at 5.3 wt% and increasing amount of caseins. Red circles were for gelled emulsions during the course of heating and blue circles were for heat-stable emulsions during the full heating time. (B) Protein composition of the fat droplet surface of the emulsions prepared with WPM AC suspension at 5.3 wt% in the absence (1) and in the presence of 0.2 wt% (2), 0.5 wt% (3) and 0.7 wt% (4) of caseins determined by SDS-PAGE under reducing conditions. (5) whey protein powder; Mw, molecular weight marker. ....	112
<b>Figure 54:</b> Transmission Electron Microscopy (TEM) images of the unheated emulsions prepared with WPM A suspension after centrifugation at 2.8 wt% (A) and the unheated emulsions prepared with WPM A suspensions at 2.7 wt% (B) and 5.9 wt% (C). ....	114
<b>Figure 55:</b> Transmission Electron Microscopy (TEM) images of the heated emulsions prepared with WPM A suspension after centrifugation at 2.8 wt% (A) and the heated emulsion prepared with WPM A suspension at 2.7 wt% (B). ....	115
<b>Figure 56:</b> Phase diagram of WPM emulsions with or without added caseins: gelled emulsion (red circle) and stable emulsion (blue circle). ....	116
<b>Figure 57:</b> (A) Freeze/thaw stability of WPM emulsions prepared with WPM A or WPM AC suspensions with or without added caseins; Stable emulsions (blue circle) and emulsions exhibiting trace of oiling off (yellow circle). (B) Picture of an emulsion with a creamed layer of fat droplet at the top. (C) Picture of an emulsion with trace of fat release (oiling off). ....	118
<b>Figure 58:</b> Scheme of the emulsion structure before a freeze/thaw cycle. (A) Emulsion prepared with WPM AC suspension. (B) Emulsion prepared with WPM A suspension containing less than 0.3 wt% of caseins. (C) Emulsion prepared with WPM A suspension containing more than 0.35 wt% of caseins. ....	119
<b>Figure 59:</b> Volume mean diameter ( $d_{4,3}$ ) of the particles in the presence of SDS determined by laser light scattering for the emulsions prepared with the WPM AC suspensions at various protein concentrations before (black) or after freeze/thaw cycle (black). ....	120
<b>Figure 60:</b> Heat stability of the emulsion prepared with the SA <sub>4</sub> suspension and with or without the added caseins: stable emulsions (blue circle), gelled emulsions (red circle) and emulsions showing oiling off (yellow circle). ....	126
<b>Figure 61:</b> (A and B) Confocal Laser Scanning Microscopy (CLSM) images of SA <sub>4</sub> emulsions at 2.7 wt% (A) and 5.9 wt% (B) before heat treatment. Distribution of the distance between fat droplet surface for SA <sub>4</sub> emulsions as a function of protein concentrations in the continuous phase (A): small grey dotted line, 2.7 wt%; small black dotted line, 2.9 wt% dashed line, 3.8 wt%; long dashed line, 4.6 wt%; continuous line, 5.9 wt%. ....	128
<b>Figure 62:</b> Surface protein load of SA <sub>4</sub> emulsions (black triangle) and WP A emulsions (grey diamond) as a function of the protein concentration in the continuous phase. The dotted lines are guides for the eyes. ....	130

## Table of Figures

- Figure 63:** (A) SDS-PAGE of the protein fraction adsorbed onto the fat droplets of WP A emulsions and SA<sub>4</sub> emulsions at different protein concentrations in the continuous phase; Molecular weight marker (1); WP A emulsions at 2.9 wt% (2), 3.9 wt% (3), 4.7 wt% (4), and 5.8 wt% (5) protein content; SA<sub>4</sub> emulsions at 2.8 wt% (6), 3.8 wt% (7), 4.5 wt% (8) and 5.9 wt% (9) protein content; WP A powder (10). (B) Casein and (C) globular whey protein loads for WP A emulsions (grey diamond) and SA<sub>4</sub> emulsions (black triangle) as a function of protein concentration in the continuous phase. The dotted lines are guides for the eyes. .... 131
- Figure 64:** Heat stability of WP A emulsions (grey diamond) and SA<sub>4</sub> emulsions (black triangle) as a function of protein concentration in the aqueous phase. The dotted lines are guides for the eyes. .... 132
- Figure 65:** (A) Volume mean fat droplet diameter ( $d_{4,3}$ ) of WP A emulsions at various protein concentrations in the continuous phase (in the absence of SDS: 3.1 wt%, black diamond; 4 wt%, black square; 5 wt%, grey circle; 5.9 wt%, grey triangle; in the presence of SDS: 3.1 wt%, empty diamond; 4 wt%, empty square; 5 wt%, empty circle; 5.9 wt%, empty triangle) as a function of heating time at 120 °C. The dotted lines are guides for the eyes. (B) Volume mean fat droplet diameter ( $d_{4,3}$ ) of SA<sub>4</sub> emulsions at various protein concentrations in the continuous phase (in the absence of SDS: 3.4 wt%, black diamond; 4.5 wt%, black square; 5.5 wt%, grey circle; 6.6 wt%, grey triangle; in presence of SDS: 3.4 wt%, empty diamond; 4.5 wt%, empty square; 5.5 wt%, empty circle; 6.6 wt%, empty triangle) as a function of heating time at 120°C. The dotted lines are guides for the eyes. (C-H) Confocal Laser Scanning Microscopy (CLSM) images of WP A emulsions and SA<sub>4</sub> emulsions before and after heat treatment (heating times correspond to the time when emulsions were destabilized or to 30 min if the emulsion remained stable throughout heat treatment): (C) Unheated and (D) heated WP A emulsions at 3.1 wt% protein in the continuous phase; (E) heated WP A emulsion at 5.8 wt% protein in the continuous phase; (F) unheated and (G) heated SA<sub>4</sub> emulsions at 2.9 wt% protein in the continuous phase; (H) heated SA<sub>4</sub> emulsion at 5.9 wt% protein in the continuous phase. 134
- Figure 66:** Surface protein load of the WP A emulsions (empty diamond) and SA<sub>4</sub> emulsions (black triangle) at heating time just before a visual destabilization occurred according to the protein concentration in the aqueous phase. The dotted lines are guides for the eyes. .... 135
- Figure 67:** (A) Heat stability of WP A emulsions (grey diamond) as a function of non-adsorbed protein concentration in the continuous phase and WP A suspensions (black diamond) in the same range of protein concentration. (B) Heat stability of SA<sub>4</sub> emulsions (grey triangle) as a function of non-adsorbed protein concentration in the continuous phase and SA<sub>4</sub> suspensions (black triangle) in the same range of protein concentration. .... 136
- Figure 68:** Summary of the structure of the WP A emulsions (top) and SA<sub>4</sub> emulsions (bottom) before and after heat treatment as a function of protein concentration. .... 140
- Figure 69:** Distribution of the distances between fat droplet surface for the SA<sub>4</sub> emulsions at 2.8 wt% as a function of casein concentrations: small dotted line, 0.17 wt% ; dashed line, 0.24 wt%; large dashed line, 0.32 wt%; continuous line, 0.40 wt%. .... 141
- Figure 70:** (A) Protein surface load of the fat droplets of SA<sub>4</sub> emulsions (2.8 wt% of SA<sub>4</sub>) containing various amount of sodium caseinate: 0.17 wt% (empty triangle), 0.24 wt% (light grey triangle), 0.32 wt% (dark grey triangle) and 0.40 wt% (black triangle). (B) SDS-PAGE of the adsorbed protein fraction onto fat droplet surface of emulsions prepared with 2.8% of SA<sub>4</sub> suspension and various amount of sodium caseinate: 0.17 wt% (2), 0.24 wt% (3), 0.32 wt% (4) 0.40 wt% (5).; Molecular weight marker (1). .... 142

## Table of Figures

<b>Figure 71:</b> Heat stability of 2.8 wt% SA <sub>4</sub> emulsions (black triangle) as a function of casein concentration. The dotted lines are guides for the eyes. ....	143
<b>Figure 72:</b> Freeze/thaw stability of the emulsion prepared with the SA <sub>4</sub> suspension and with or without added caseins: stable emulsions (blue circle) and emulsions showing oiling off (yellow circle). ....	144
<b>Figure 73:</b> Volume mean diameter (d <sub>4,3</sub> ) determined by light scattering measurements with (full black symbol) or without SDS (open symbol) of the particles in the emulsions prepared with the SA <sub>4</sub> suspensions at various protein concentrations before (square) and after one freeze/thaw cycle (triangle) .....	145
<b>Figure 74:</b> Heat stability of the emulsions prepared with the WPM/SA B suspensions and with or without addition of sodium caseinate: stable emulsions (blue circle), and gelled emulsions (red circle). The grey arrows show the emulsions with added caseins. ....	146
<b>Figure 75:</b> (A) Volume mean diameter (d <sub>4,3</sub> ) determined by laser light scattering of the particles in the emulsions prepared with the WPM/SA B suspensions at various protein concentrations before heat treatment with (empty square) or without SDS (black diamond) and in the WPM/SA B emulsions with caseins adding with (empty circle) or without SDS (grey triangle) (B) Distribution of the distance between fat droplet surface for WPM/SA B emulsion as a function of protein concentration in the continuous phase: grey dotted line, 3.6 wt%; black dotted line, 3.7 wt%; small dashed line, 4.7 wt%; large dashed line, 5.5 %; black continuous line, 7.4 wt% and for WPM/SA B emulsion with 3.6 wt% and 0.37 wt% of sodium caseinate; grey continuous line. ....	147
<b>Figure 76:</b> Microstructural analysis of emulsions prepared with the WPM/SA B suspensions. (A to F) Confocal Laser Scanning Microscopic (CLSM) images of the emulsions prepared with WPM/SA B suspension at 3.6 wt% (A, B) unheated (A) and heated 3 min (B); at 5.5 wt% (C, D) unheated (C) and heated 30 min (D); at 3.6 wt% and 0.37 wt% of caseins (E, F) unheated (E) and heated 30 min (F). Fat droplets were stained with Nile Red (red color on the images) and the proteins with Fast green (green color on the images). (G to I) Inter-fat droplet distance distribution for emulsions at (G) 3.6 wt%, (H) 5.5 wt% protein concentration and (I) at 3.6 wt% of protein concentration and 0.37 wt% of caseins before (continuous line) and after (small dotted line) heating as well as the Dif AUC (point by point difference of the area under the curve of the inter-particle distance distribution between the non-heated and heated emulsions; large dotted line). ....	148
<b>Figure 77:</b> Volume mean diameter (d <sub>4,3</sub> ) determined by laser light scattering of the particles in the heat stable emulsions prepared with the WPM/SA B suspensions at various protein concentrations and caseins concentrations before heat treatment with (light blue) or without SDS (dark blue), after 2 min of heat treatment for gelled emulsions with (light green) or without SDS (dark green) and after 30 min of heat treatment for stable emulsions with (light red) or without SDS (dark red). ....	149
<b>Figure 78:</b> Inter-fat droplet distance distribution for emulsions at (A) 4.7 wt% and (B) 7.4 wt% protein concentration before (continuous line) and after (small dotted line) heating as well as the Dif AUC (point by point difference of the area under the curve of the inter-particle distance distribution between the non-heated and heated emulsions; large dotted line). ....	150
<b>Figure 79:</b> (A) Surface Protein load of the fat droplets of WPM/SA B emulsions prepared various amount proteins (black square) and WPM/SA B emulsions at 3.6 wt% and 0.37 wt% of sodium caseinate (grey circle) and at 3.7 wt% and 0.14 wt% of sodium caseinate (grey triangle). (B) SDS-PAGE of the adsorbed protein fraction onto fat droplet	

## Table of Figures

<i>surface of emulsions prepared with WPM/SA B suspensions: 3.6 wt% (2), 7.4 wt% (4), 3.7 wt%; Molecular weight marker (1); .....</i>	<i>151</i>
<b>Figure 80:</b> <i>Transmission Electron Microscopy (TEM) images of the unheated emulsions prepared with WPM/SA B suspension at 3 wt% (A) unheated and (B) heated. Red arrow indicated thin interface, black arrow indicated thick interface and blue arrow indicated adsorbed WPM. ....</i>	<i>152</i>
<b>Figure 81:</b> <i>Proposition of scheme of the microstructure of the emulsions at two protein concentrations. (A) Interface for the emulsion prepared with WPM/SA B suspension at <math>\leq 3.7</math> wt% (B) Interface for the emulsion prepared with WPM/SA B suspension at <math>\geq 4.4</math> wt%. ....</i>	<i>153</i>
<b>Figure 82:</b> <i>Freeze/thaw stability of the emulsions prepared with the WPM/SA<sub>4</sub> B suspensions and without or with added caseins: stable emulsion (blue circle) and emulsion showing a creamed layer at the top of the tube (pink circle). The grey arrows indicate the emulsion with added caseins. ....</i>	<i>154</i>
<b>Figure 83:</b> <i>Highlights of the PhD project finding and conclusions. ....</i>	<i>161</i>
<b>Figure 84:</b> <i>Scheme of proposed PhD project future outlook .....</i>	<i>164</i>



## Table of Tables

<b>Table 1:</b> Mains proteins in cow milk and their proportions	7
<b>Table 2:</b> Powders composition	47
<b>Table 3:</b> Z-average, size distribution, $\xi$ - potential of aggregates suspensions	82
<b>Table 4:</b> Molar mass and gyration radius calculated for the aggregates suspension in A4F	84
<b>Table 5:</b> Amount of caseins added and total casein content in WPM A suspension	107
<b>Table 6:</b> Theoretical Fat droplet size at different degree of surface coverage	113
<b>Table 7:</b> Theoretical concentration of WPM need to obtained a droplet size of $1.7 \mu\text{m}$	113
<b>Table 8:</b> Amount of caseins added and total casein content in SA4 suspension	141

## RÉSUMÉ

**Stabilisation des émulsions laitières aux cours des traitements technologiques : action combinée des agrégats de protéines de lactosérum et des caséines.**

Les émulsions laitières sont des systèmes thermodynamiquement instables qui doivent résister aux contraintes technologiques (chauffage, congélation) appliquées lors de leur fabrication ou usage. Les émulsions riches en protéines de lactosérum sont particulièrement sensibles et l'emploi d'additifs alimentaires est un moyen de ralentir leur déstabilisation. Dans l'objectif d'offrir des produits 100 % lait aux consommateurs, concevoir des émulsions, riches en protéines de lactosérum, sans additifs alimentaires et stables aux traitements technologiques, constitue un réel challenge. La stratégie employée dans ce projet de thèse a été de combiner les propriétés des agrégats de protéines de lactosérum et des caséines pour stabiliser des émulsions aux cours des traitements technologiques sur une large gamme de concentration.

Des émulsions ont été préparées avec des agrégats de protéines de lactosérum de structure différente et avec différents ratios agrégats/caséines. Quelle que soit leur structure, la présence d'agrégats à la surface des globules gras déstabilise l'émulsion (gélification /séparation de phase) alors que dans la phase dispersante ceux-ci sont stables aux traitements technologiques. A l'inverse, les émulsions dont la surface des globules gras est recouverte de caséines sont très stables aux traitements technologiques. Ainsi, il est possible de moduler la stabilité des émulsions riches en protéines de lactosérum aux cours des traitements technologiques en exploitant les propriétés des agrégats et des caséines et en contrôlant leur répartition entre la surface des globules gras et la phase continue.

**Mots-clés :** agrégats de protéines de lactosérum, caséines, émulsions, stabilité, traitements technologiques.

## ABSTRACT

**Combined effect of whey protein aggregates and caseins on dairy emulsions stability during technological treatments.**

Dairy emulsions are thermodynamically unstable systems, which have to be resistant to the technological treatments (heating, freezing/thawing) applied during their manufacture or use. Whey protein-rich emulsions are particularly sensitive to technological treatments and instabilities are currently tackled by the use of non-dairy additives. With aim to offer products that are more natural to consumers (additive-free), the preparation of whey protein-rich emulsions without additive and stable during technological treatments constitutes a major challenge for dairy companies. The strategy adopted during this thesis was to combine the properties of the whey proteins aggregates and caseins in order to stabilize emulsion during technological treatments in a large range of protein concentrations.

Emulsions were prepared with various whey protein aggregates and various whey protein aggregates/caseins ratio. Whatever the whey protein aggregates, their presence at the fat droplet surface destabilize the emulsions (gelation/phase separation) whereas they are stable in the continuous phase of the emulsions during technological treatments. In contrast, emulsions are extremely stable during technological treatments when caseins fully cover the fat droplet surface. The results obtained highlighted the possibility of modulating the stability during technological treatments of whey protein-rich emulsions by combining the properties of the whey protein aggregates and the caseins and by controlling their repartition between the fat droplet surface and the continuous phase of the emulsion.

**Keywords:** Whey protein aggregates, caseins, emulsions, stability, technological treatments.



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