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Application of ultra high-pressure homogenization (UHPH) in the production of submicron/nano-oil-in-water emulsions using vegetable oils and milk proteins as emulsifiers

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PhD THESIS

BELLATERRA, 2013

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Preface

The present thesis entitled "**Application of ultra high-pressure homogenization (UHPH) in the production of submicron/nano-oil-in-water emulsions using vegetable oils and milk proteins as emulsifiers**" concludes my PhD project carried out at the Centre Especial de Recerca Planta de Tecnologia dels Aliments (**CERPTA**), Department of Animal and Food Science, Faculty of Veterinary Medicine, Autonomous University of Barcelona (UAB). This project has been carried out as a part of several research projects as explained in the following:

1. Project **AGL2011-26766** entitled "**Application of ultra-high pressure homogenization for obtaining submicron emulsions containing bioactive compounds and their incorporation in dairy products**", financed by The Ministry of Economy and Competitiveness.
2. Project **EVALXaRTA 2011** entitled "**Aplicación de la ultra alta presión homogenización (UHPH) en la obtención de emulsiones**" financed by Xarxa de Referència en Tecnologia dels Aliments, Generalitat of Catalonia.
3. Project **CSD2007-00045** entitled "**Materia a alta presión (MALTA Consolider)**", financed by Ministry of Education and Science.

My PhD was under supervision of **Dr. Antonio-José Trujillo Mesa** (main supervisor), associate professor at the UAB, and **Dr. Martin Nicolas Buffa** (co-supervisor), superior technical support researcher at the UAB.

The project began on October 1, 2009 and continued until September 30, 2012 with personal financial support given by the Spanish Agency for International Cooperation for Development (**MAEC-AECID**). An additional 9-month extension was given by the **CERPTA (UAB)** due to project delay. In these 9 months I have worked at the University as a laboratory technical support.

This dissertation is a result of four years of study focused on the application of ultra high-pressure homogenization (UHPH) in the production of nano/submicron emulsions. I faced a lot of technical problems to carry out this research, especially with the UHPH prototype, and to fix the analytical methods used in this study because this line of investigation was a new line in the research center. Once the problems were solved, I have continued without stopping.

The present thesis is structured in eight chapters. The first is a short introduction to present to the reader the importance of carrying out this research, the aim of this study, as well as the working plans carried out to achieve these objectives. In the second chapter, an overview about emulsions, emulsifiers and emulsification techniques is given, with special emphasis on

emulsifiers and equipments utilized in the present work. In addition, important issues regarding physical and oxidative stability of emulsions are detailed. In the third chapter, information about the materials and analytical methods used at the present study is given. Additionally, the emulsion preparation and the emulsification systems used in its elaboration are also detailed.

From chapter four to chapter seven, all experiments fulfilled in the thesis to achieve its objectives are detailed. Every chapter contains a brief introduction, results and discussion and references section. Chapter four and five correspond to studying the effect of whey protein isolate concentration and oil-phase volume fraction on characteristics of emulsions elaborated by colloidal mill, conventional homogenization and ultra high-pressure homogenization, and chapter six and seven correspond to studying the effect of sodium caseinate concentration and oil-phase volume fraction on characteristics of emulsions elaborated by colloidal mill, conventional homogenization and ultra high-pressure homogenization.

Chapter eight highlights the final conclusions of the thesis.

Essam Hassan Emam Hebishy

Bellaterra, July 2013

DEDICATION

This dissertation is dedicated to my country ***Egypt***, my parents ***Hassan*** and ***Fathya***, my wife ***Asmaa*** and my daughters ***Habiba*** and ***Sara***. I am very appreciative of their love and support. Without their warm help, this achievement would not be possible.

Acknowledgements

Many people have supported me along the way, and these people deserve my sincere thanks.

First of all I am deeply grateful to my main supervisor Professor **Toni Trujillo** for always believing in me, for continuously encouraging me, for taking the time for discussions and advising me whenever needed, and for his critical "blue" approach to the work. Most importantly, for giving me the family feeling, teaching me that in research there is no such thing as problems, only consecutive challenges that have to be concurred. I really appreciate the support he has given to me, allowing me to assist in many congresses in food science to see what is happening in the scientific world.

I would also like to thank my co-supervisor **Dr. Martin Buffa**. I appreciate all our discussions on my experimental plans and results throughout the project.

Many thanks to the **AECID and CERPTA** for giving me the personal financial support to carry out my PhD work.

I owe my sincere thanks to **Joan Miquel** for his outstanding technical work in the pilot plant.

I also want to thank laboratory technicians **Sonia Llorens** and **Joan Gallardo** for being kind, helpful and always fun to spend time with.

My heartfelt thanks go to **Dra. Victoria Ferragut** for her invaluable help and guidance, especially from the rheological point of view, and for always being there.

My thanks go to **Dra. Anabel Blasco and Dra. Anna Zamora** for their helpful guidance in the statistical part of my thesis.

I would like also to thank **Dr. Ahmed Salama** for his personal advise which helped me adapt quickly to my research group, and for always being there to help in solving many problems.

Special thanks for **Ramón, Idoia, Roger, Natália, Bibiana and Begoña** for motivating me more and more and always being kind to me.

Furthermore, my thanks go to a great number of other people whom have made my years at the Division of Industrial Food Research a wonderful experience - you know who you are - I have enjoyed your company every single day.

iv Aknowledements

Finally, I would like to thank my family and my friends, for helping me out through the period of my thesis, and for listening to all my emulsion-talk.

My wife **Asmaa** and my daughters **Habiba and Sara** - you each have a special place in my heart.

My nieces and nephews - you are the stars in my life.

Essam Hassan Emam Hebishy

Bellaterra, July 12., 2013

Summary

Summary

The overall goal of the present PhD thesis was to study some factors related to the choice of emulsifier (whey protein isolate or sodium caseinate) concentration, oil-phase volume fractions (10-50%) and homogenization conditions (100-300 MPa) that could influence physical stability and lipid oxidation in nano/submicron oil-in-water emulsions by using a rotor-startor system (colloidal mill, CM, at 5000 rpm for 5 min) for obtaining the coarse emulsions and stabilized by ultra high-pressure homogenization (UHPH), in comparison to conventional homogenization (CH, 15 MPa).

Emulsions were characterized for their physical properties (droplet size distribution, microstructure, surface protein concentration, emulsifying stability against creaming and coalescence, and viscosity) and oxidative stability (hydroperoxide content and TBARs) under light (2000 lux/m^2 for 10 days).

The first study focused on using whey protein isolate (WPI) as emulsifier in different concentrations (1, 2 and 4%) with a fixed oil concentration (20%) of sunflower and olive oils (3:1). UHPH produced emulsions with lipid particles of small size in the sub-micron range (100-200 nm) and low surface protein with unimodal distribution in emulsions treated at 200 MPa using whey proteins at 4%. Long term physical stability against creaming and coalescence was observed in UHPH emulsions, compared to those obtained by CM and CH. Oxidative stability of emulsions was also improved by UHPH compared to CM and CH, especially when 100 MPa was applied. All emulsions exhibited Newtonian behavior ($n \approx 1$).

These results led us to use the best conditions obtained in the previous work (4% of protein concentration and pressure treatments of 100 and 200 MPa) to study the physical and oxidative stability of emulsions containing different oil-phase volume fractions (10, 30 and 50%). Increasing the oil concentration from 10 to 50%, in general, increased the particle size, decreased the surface protein concentration and resulted in a high degree of flocculation and coalescence, especially in emulsions treated at 200 MPa. All UHPH emulsions, except those treated at 100 MPa containing 10% oil, and CH emulsions with 50% oil displayed an excellent stability vs. creaming during storage at ambient temperature. The lowest oxidation rate was observed in UHPH emulsions, especially those containing 30% oil.

The third study was conducted on using sodium caseinate (SC) as emulsifier in different concentrations (1, 3 and 5%) with a fixed oil concentration (20%) of sunflower and olive oils (3:1). UHPH emulsions containing 1% protein presented a high particle size (especially in emulsions treated at 100 MPa) but increasing the protein content to 3 and 5% in UHPH emulsions reduced the particle size, and tended to change the rheological behaviour from Newtonian to shear thinning, improving the creaming and oxidative stabilities of emulsions.

From the previous study, the best droplet breakdown, physical and oxidative stability were obtained with pressures in the range of 200 and 300 MPa and sodium caseinate (5%). Therefore, the objective of the last study was to evaluating the emulsions containing different oil-phase volume fractions (10, 20, 30 and 50%) treated by UHPH in the conditions above mentioned, in comparison to CH emulsions containing 1 and 5% SC. Increasing the oil content to 50% tended to produce emulsions with a gel structure such as a mayonnaise type product so, the results of this study focused only on emulsions containing 10, 20 and 30% oil.

CM and CH emulsions containing 1% SC and different oil contents (10, 20 and 30%), exhibited a Newtonian flow behavior with a slow creaming rate, whereas the oxidation rate was faster in these emulsions. On the other hand, high degree of flocculation with a shear thinning behavior, higher creaming rates, but low oxidation rates were observed in CH emulsions containing 5%. UHPH-treated emulsions containing high oil contents (20 and 30%) exhibited excellent creaming stability, and with a shear thinning rheological behavior only in emulsions containing 30% oil. UHPH produced stable emulsions against oxidation, especially when high oil contents (20-30%) were used. Increasing the oil concentration from 10 to 30%, in general, resulted in an increase in the oxidative stability in all emulsions, except in CH emulsions containing 1% of SC.

Emulsions produced by both whey protein (4%) and caseinate (5%), and treated by UHPH have a good physical stability to flocculation, coalescence and creaming and also high stability to lipid oxidation, opening a wide range of opportunities in the formulation of emulsions containing bioactive components with lipid nature.

Resumen

El objetivo general de la presente tesis fue estudiar algunos factores relacionados con la elección de la concentración de emulsionante (aislado de proteína de suero de leche o caseinato sódico), el volumen de la fase lipídica (10-50%) y las condiciones de homogeneización (100-300 MPa) que podrían influir en la estabilidad física y oxidativa de las emulsiones aceite-en-agua nano/submicrón, utilizando un sistema rotor-estartor (molino coloidal, CM, 5000 rpm durante 5 min) para la producción de las emulsiones iniciales estabilizadas posteriormente por ultra alta presión de homogeneización (UHPH) en comparación a la homogeneización convencional (CH, 15 MPa).

Se caracterizaron las propiedades físicas (distribución del tamaño de partícula, microestructura, concentración de proteína en superficie, estabilidad frente al cremado y coalescencia, y viscosidad) y estabilidad oxidativa (contenido de hidroperóxidos y TBARs) inducida por la luz (2000 lux/m2 durante 10 días) de las emulsiones.

El primer estudio se centró en el uso del aislado de proteína del suero (WPI) como emulsionante a diferentes concentraciones (1, 2 y 4%) con una concentración fija de aceite (20%) consistente en una mezcla de aceite de girasol y oliva (3:1). La UHPH produjo emulsiones con partículas lipídicas de pequeño tamaño, en un intervalo inferior a la micra (100-200 nm), baja concentración de proteína en superficie y una distribución unimodal en emulsiones tratadas a 200 MPa con un 4% de WPI. Se observó una larga estabilidad física hacia el cremado y la coalescencia en las emulsiones UHPH, en comparación a las emulsiones obtenidas con CM y CH. La estabilidad oxidativa de las emulsiones fue también mejorada por la UHPH en comparación a los tratamientos de CM y CH, especialmente cuando se aplicaron presiones de 100 MPa. Todas las emulsiones exhibieron un comportamiento newtoniano ($n \approx 1$).

Estos resultados nos llevaron a utilizar las mejores condiciones obtenidas en el trabajo anterior (concentración de proteína del 4% y presiones de 100 y 200 MPa) para el estudio de la estabilidad física y oxidativa de emulsiones con diferentes volúmenes de fase oleosa (10, 30 y 50%). En general, el aumento de la concentración de aceite del 10 al 50% provocó un incremento del tamaño de partícula, una disminución en la concentración de proteína en superficie y un alto grado de floculación y coalescencia, especialmente en las emulsiones tratadas a 200 MPa. Todas las emulsiones UHPH, excepto aquellas formuladas con un 10% de aceite y tratadas a 100 MPa y las emulsiones CH con un 50% de aceite, mostraron una excelente estabilidad al cremado durante su almacenamiento a temperatura ambiente. El menor índice de oxidación fue observado en las emulsiones UHPH, especialmente en aquellas que fueron formuladas con un 30% de aceite.

El tercer estudio se llevó a cabo utilizando caseinato sódico (SC) como emulsionante a diferentes concentraciones (1, 3 y 5%), con un 20% de aceite consistente en una mezcla de aceite de girasol y oliva (3:1). Las emulsiones UHPH con un 1% de proteína mostraron un tamaño de partícula elevado (especialmente aquellas emulsiones tratadas a 100 MPa), pero al incrementar los niveles de proteína de 3 a 5% el tamaño de partícula disminuyó, cambiando el comportamiento reológico de las emulsiones UHPH de newtoniano a pseudoplástico, mejorando su estabilidad oxidativa y su tendencia al cremado.

A partir de los resultados del estudio anterior se pudo comprobar que la mejor estabilidad física y oxidativa de las emulsiones se obtuvo con presiones de 200 y 300 MPa y una concentración de SC del 5%. Por lo tanto, el objetivo del último estudio fue la evaluación de las emulsiones UHPH formuladas con diferentes volúmenes de aceite (10, 20, 30 y 50%), en las condiciones anteriormente mencionadas, y compararlas con las emulsiones CH preparadas con 1 y 5% de proteína. El aumento del contenido de aceite al 50% produjo emulsiones con una estructura de gel, similar a una mayonesa, por lo que los estudios se centraron solamente en las emulsiones con un 10, 20 y 30% de aceite.

Las emulsiones obtenidas con CM y CH con 1% de SC y diferentes contenidos de aceite (10, 20 y 30%) mostraron un comportamiento de flujo newtoniano con una velocidad lenta de cremado, mientras que su nivel de oxidación fue más elevado. Por otro lado, en las emulsiones obtenidas con CH y formuladas con un 5% de SC, se pudo observar un alto grado de floculación, un comportamiento reológico pseudoplástico, elevadas tasas de cremado, pero bajos niveles de oxidación. Las emulsiones UHPH formuladas con grandes volúmenes de aceite (20 y 30%) mostraron una excelente estabilidad al cremado, con un comportamiento reológico pseudoplástico en aquellas emulsiones producidas con un 30% de aceite. La UHPH produce emulsiones estables frente a la oxidación, especialmente cuando se utilizan elevados contenidos de aceite (20-30%). En general, el aumento de la concentración de aceite de 10 a 30% incrementó la estabilidad a la oxidación de todas las emulsiones, excepto en las emulsiones CH formuladas con un 1% de SC.

Las emulsiones producidas con WPI al 4% y SC al 5% y tratadas por UHPH mostraron una buena estabilidad física a la floculación, coalescencia y cremado, y también una alta estabilidad a la oxidación lipídica, abriendo una amplia gama de oportunidades en la formulación de emulsiones que contengan componentes bioactivos de naturaleza lipídica.

List of Publications

PAPER I

Hebishy E., Buffa M., Guamis B., Trujillo A. J. (2013). Stability of Sub-Micron Oil-in-Water Emulsions Produced By Ultra High Pressure Homogenization and Sodium Caseinate as Emulsifier. Chemical Engineering Transactions, 32, 1813-1818.

PAPER II

Hebishy E., Buffa M., Guamis B., Trujillo A. J. (2013). Production of physically and chemically stable sub-micron oil-in-water emulsions by ultra-high pressure homogenization and sodium caseinate as emulsifier. International Journal of Food and Biosystems Engineering (in press).

Oral communications

Hebishy, E., Buffa, M., Guamis, B., & Trujillo, A. J. "Effects of protein concentration and oil volume fraction on the physico-chemical stability of whey protein oil-in-water emulsions stabilized by ultra high pressure homogenization", 2nd EFFoST International Annual Meeting, Berlin, Germany, November 9-11th, 2011.

Hebishy, E., Buffa, M., Guamis, B., & Trujillo, A. J. "Effects of protein concentration and oil volume fraction on the physico-chemical stability of whey protein oil-in-water emulsions stabilized by ultra high pressure homogenization", Advances in UHPH processes (Funentech workshop), Barcelona, Spain, December 15th, 2011.

Hebishy, E., Buffa, M., Guamis, B., & Trujillo, A. J. "Effects of protein concentration and pressure on structure and stability of ultra high pressure and conventional homogenized sodium caseinate oil-in-water emulsions", Science and Technology of Food Emulsions Workshop, London, United Kingdom, June, 21-22th, 2012.

Hebishy, E., Buffa, M., Guamis, B., & Trujillo, A. J. "Production of physically and chemically stable sub-micron oil-in-water emulsions by ultra-high pressure homogenization and sodium caseinate as emulsifier", FaBE 2013 International Conference of Food Engineering, Skiathos Island, Greece, may 28th to June 3rd, 2013.

Poster presentations

Hebishy, E., Buffa, M., Guamis, B., & Trujillo, A. J. "Obtención de emulsiones submicrónicas con aceites vegetales por ultra alta presión homgenización", National Spanish Conference for Food Technology (CyTA), Valencia, Spain, June 8 – 10th, 2011.

Hebishy, E., Buffa, M., Guamis, B., & Trujillo, A. J. "Obtención de emulsiones submicrónicas con aceites vegetales por ultra alta presión homgenización", 5th High Pressure School, Tenerife, Canary Islands, Spain, June 27th – July 1st, 2011.

Hebishy, E., Buffa, M., Ferragut, V., Guamis, B., & Trujillo, A. J. "Physical and oxidative stability of whey protein oil-in-water emulsions stabilized by ultra high pressure homogenization: effect of pressure and protein concentration on emulsion characteristics", 2nd ISEKI International Food Conference, Milan, Italy, August 31st to September 2nd, 2011.

Hebishy, E., Buffa, M., Guamis, B., & Trujillo, A. J. "Effects of protein concentration and pressure on structure and stability of ultra high pressure and conventional homogenized sodium caseinate oil-in-water emulsions", Science and Technology of Food Emulsions Workshop, London, United Kingdom, June 21-22th, 2012.

Hebishy, E., Buffa, M., Juan, B., Guamis, B., & Trujillo, A. J. "Efectos de la concentración proteica y presión sobre la estructura y estabilidad de emulsiones de aceite en agua producidas con caseinato sódico y tratadas por ultra alta presión de homogenización", Spanish Conference of Food Engineering, Ciudad Real, Spain, November 7-9th, 2012.

Hebishy, E., Buffa, M., Guamis, B., & Trujillo, A. J. "Stability of sub-micron oil-in-water emulsions produced by ultra high pressure homogenization and sodium caseinate as emulsifier", 11th International Conference on Chemical & Process Engineering, Milan, Italy, June 2nd -5th, 2013.

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Chapter 1

Introduction, aims and working plan

In this chapter, an introduction to emulsions and the factors that may affect their stabilities is given, with special emphasis on the three main factors studied in the present PhD work. In addition, the aims of the present study and the working plan conducted to achieve these aims will be covered.

1.1. Introduction

A large variety of foods are emulsions, from the more natural, e.g. milk, creams, whipped creams, ice creams to the more sophisticated, e.g. sausages, mayonnaises.

An emulsion is prepared by dispersing one immiscible liquid in another using a process called homogenization where in one of the phases gets dispersed in the other by forming small droplets, and then stabilizing them using a third component, the emulsifier (Walstra, 1985).

Emulsion is a thermodynamically unstable system but it is possible to form a kinetically stable system for some period of time by adding emulsifiers. Emulsifiers are amphiphilic compounds with a hydrophilic and a hydrophobic head. Emulsifiers distribute at the interface and hydrophobic and hydrophilic heads are oriented towards oil and water, respectively, thereby not allowing oil droplets to coalesce together (McClements, 2005).

The milk protein products, whey protein isolate (WPI) and sodium caseinate (SC) are used as emulsifiers for oil-in-water emulsions because of their remarkable emulsifying properties. Furthermore, milk proteins have shown good antioxidative potential by inhibiting the oxidative deterioration of unsaturated fatty acids (see also section 2.5.4.4). WPI and SC therefore appear to be useful for the design of oil-in-water emulsions that serve as delivery systems for polyunsaturated fatty acids because of their dual

functionality as emulsifiers and antioxidants. Such emulsions may be incorporated into real food emulsion systems, e.g. milk, yoghurt, cheese or ice cream.

Sub-micron emulsions have a number of unique functional attributes that have led them to be utilized within an increasing number of industrial products, including foods, pharmaceuticals, cosmetics, personal care products and chemicals. Due to their size characteristics, sub-micron emulsions are expected to get high stability against creaming and coalescence.

The formation of sub-micron emulsions requires high energy inputs. Current equipment used for emulsion preparation includes colloid mills, microfluidizers, sonicators or high-pressure homogenizers (Stang, Schuchmann, & Schubert, 2001). The advantage of high-pressure homogenizers over other technologies is that more uniform droplet size distributions are obtained since the product is subjected to strong shear and cavitation forces that efficiently decrease the diameter of the original droplets (McClements, 2005; Perrier-Cornet, Marie, & Gervais, 2005).

High-pressure homogenization is an important process used in the preparation or stabilization of emulsions and suspensions, resulting in a decrease of the average droplet diameter and an increased interfacial area. The net result, from a practical point of view, is a much reduced tendency for creaming, contributing to an enhanced physical stability of the homogenized emulsions. The stabilization of emulsion may be partly attributed to the droplet's breakdown and the considerable increase of interaction between adsorbed proteins at the interface forming a more rigid interfacial layer (Lee, Lefèvre, Subirade & Paquin, 2009). Various oil-in-water (O/W) emulsions have been processed by UHPH: bovine whole milk, a natural emulsion (Pereda, Ferragut, Quevedo, Guamis, & Trujillo, 2007; Hayes & Kelly, 2003; Picart et al., 2006; Thiebaud, Dumay, Picart, Guiraud, & Cheftel, 2003; Zamora, Ferragut, Jaramillo, Guamis, & Trujillo, 2007); soymilk (Cruz, Capellas, Hernández, Trujillo, Guamis, & Ferragut, 2007); model emulsions prepared with vegetable oils and stabilized with surfactant of low molecular weight (MW) such as Tween 20® (Floury, Bellettre, Legrand, & Desrumaux, 2004), soybean proteins (Floury, Desrumaux, & Legrand, 2002) or whey proteins (Cortés-Muñoz, Chevalier Lucia, & Dumay, 2009; Floury, Desrumaux, & Lardiéres, 2000; Lee et al., 2009).

Although WPI and SC have shown high emulsifying and antioxidant properties in emulsions, a more detailed understanding of their effectiveness under certain conditions during emulsion formation is required. Three main factors during emulsion formation

may affect the effectiveness of milk proteins to form and stabilize the emulsion which are the following:

1. **The pressure of treatment** and the optimal pressure which can stabilize the emulsion without any adverse effect on the stabilizing protein molecules, especially protein molecules which are sensitive to temperature rise (WPI in our case), as a result of the temperature rise in the exit of the high-pressure valve during homogenization. When using an emulsifier during high-energy emulsification, the question is whether it can survive these harsh conditions and whether their emulsifying properties are affected or not. Increasing the temperature above a certain degree causes the whey proteins to be totally denatured and thus unable to play their role in stabilizing the emulsion.
2. **The choice of emulsifier and its concentration** can be used to modify droplet size (Dickinson, 2003). For a fixed emulsion composition, there is a maximum interfacial area, which can be completely covered by the emulsifier, and as emulsification continues, the interfacial area increases substantially (Friberg & Larsson, 1997). Below a certain droplet size, there is not enough emulsifier to cover the interface completely, and so droplets tend to coalesce with their neighbors. Some emulsification systems are not able to generate high-energy densities for droplet disruption and are unable to produce smaller droplet size, even though there might be sufficient emulsifier present (McClements, 2005).
3. **The choice of the oil concentration** which can stabilize the emulsion without any sign of flocculation or coalescence. At constant energy density (e.g. emulsification pressure), particle size rises with increasing oil content because the proteins available decreases, limiting the stabilizing benefits of the proteins. However, increasing the oil content may increase the emulsion viscosity and as a result, slow down the creaming rate (Sun and Gunasekaran, 2009). On the other hand, studies on protein stabilized O/W emulsions with varying volumes of the oil fraction have shown that a high oil fraction decreases lipid oxidation in safflower oil, (Sims, Fioriti, & Trumbetas, 1979), canola oil (Osborn & Akoh, 2004), menhaden oil (Sun and Gunasekaran, 2009) and walnut oil (Gharibzahedi, Mousavi, Hamedi, Khodaiyan, & Razavi, 2012). The effect of using high oil volume fractions on physical stability in emulsions produced using whey protein (Cortés muñoz et al., 2009; Floury et al., 2000) and sodium caseinate (San Martín-González, Roach, & Harte, 2009) by high-pressure homogenization could be

found in the literature. In contrast, little research has been conducted to study the oxidation behavior in simple O/W emulsions containing high oil contents.

1.2. Aim and Hypotheses

The overall aim of this PhD work was to study the effect of emulsifier type and concentration, homogenization conditions and oil concentration on the physical stability and lipid oxidation in sub-micron emulsions in order to produce emulsions that are physically stable against flocculation, coalescence and creaming and also oxidatively stable under the light. A possible strategy for protecting the fatty acids against oxidation could be to incorporate them in an emulsion (a delivery emulsion) prior to their addition to the food product (Let, Jacobsen, & Meyer, 2007).

To achieve this overall objective, the following specific objectives will also be undertaken:

1. Examination of the physical and oxidative stability after adding the whey protein isolate (WPI) at different levels (1, 2 and 4% w/w) as emulsifier to an-oil-in-water emulsion prepared with a fixed oil concentration (20%, sunflower oil 15% and olive oil 5%) and stabilized by conventional homogenization (CH) at 15 MPa and ultra high pressure homogenization (UHPH) at 100, 200 and 300 MPa (study I).
2. Selection of the best protein concentration of WPI and pressure to be used with different levels of sunflower and olive oils (10, 30 and 50% w/w) at the same ratio (3:1) using the same homogenization conditions (study II).
3. Examination of the physical and oxidative stability after adding the sodium caseinate (SC) at different levels (1, 3 and 5% w/w) as emulsifier to an-oil-in-water emulsion prepared with a fixed oil concentration (20% sunflower oil 15% and olive oil 5%) and stabilized by CH at 15 MPa and UHPH at 100, 200 and 300 MPa (study III).
4. Selection of the best protein concentration of SC and pressure to be used with different levels of sunflower and olive oils (10, 30 and 50% w/w) at the same ratio (3:1) using the same homogenization conditions (study IV).

1.3.Working plan

According to the objectives, Figures 1-4 schematically represent the experimental design and analysis of all experiments conducted in the present thesis.

The first step in the present study was to experiment with the effect of different concentrations of WPI (1, 2 and 4%) and SC (1, 3 and 5%) on the physical and oxidative stability of submicron emulsions treated at ultra high-pressures (100, 200 and 300 MPa) and additionally, conventional homogenization (15 MPa) was tested in these studies for comparison.

Additionally, a third step included the application of the best conditions obtained using different pressures and protein concentrations to produce emulsions with varying levels of oil concentrations from low (10%) to high (50%).

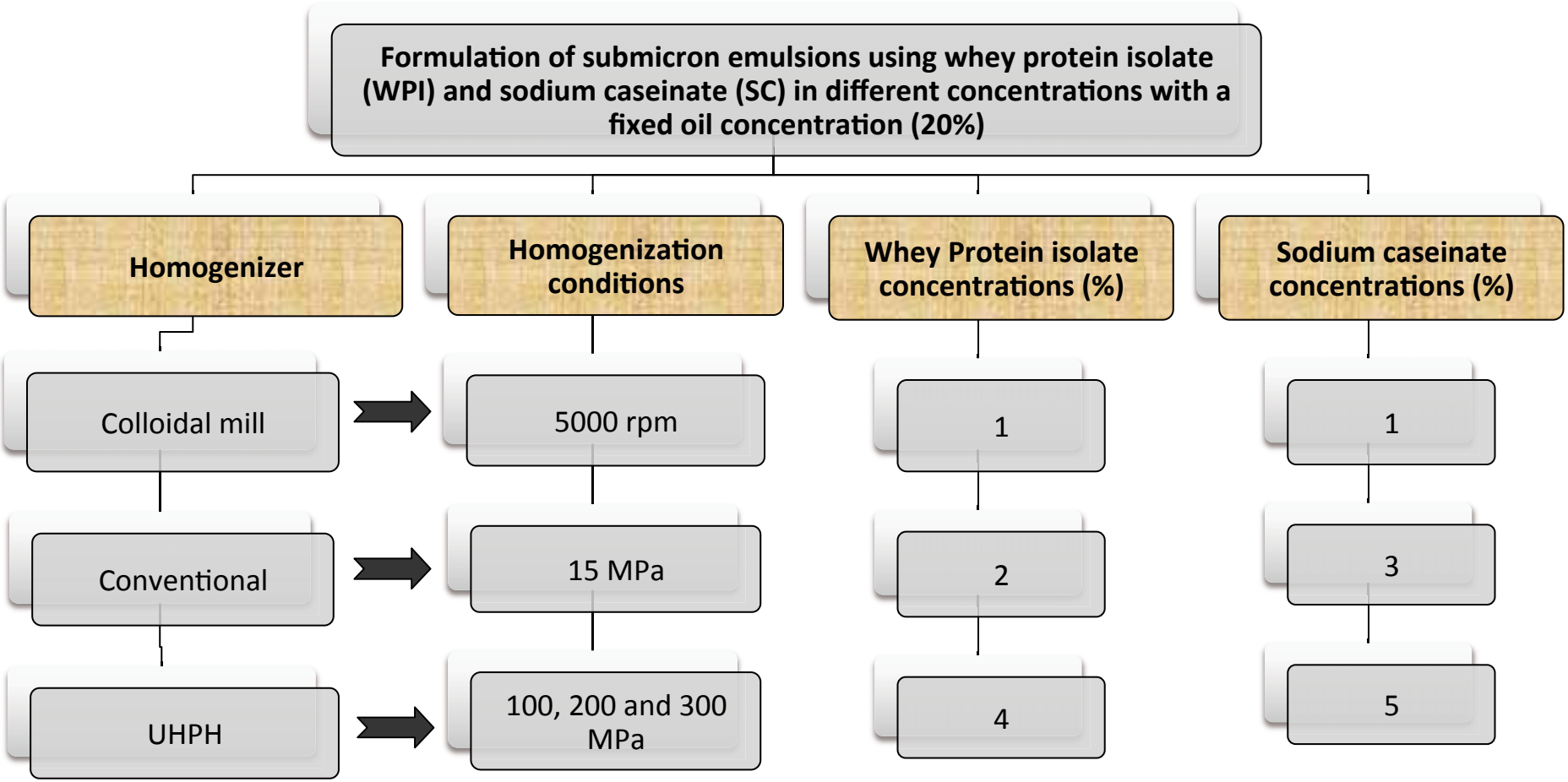

Figure 1. Working plan corresponding to the studies of the effect of pressure and protein (WPI and SC) concentrations on emulsion stability (studies 1 and 3).

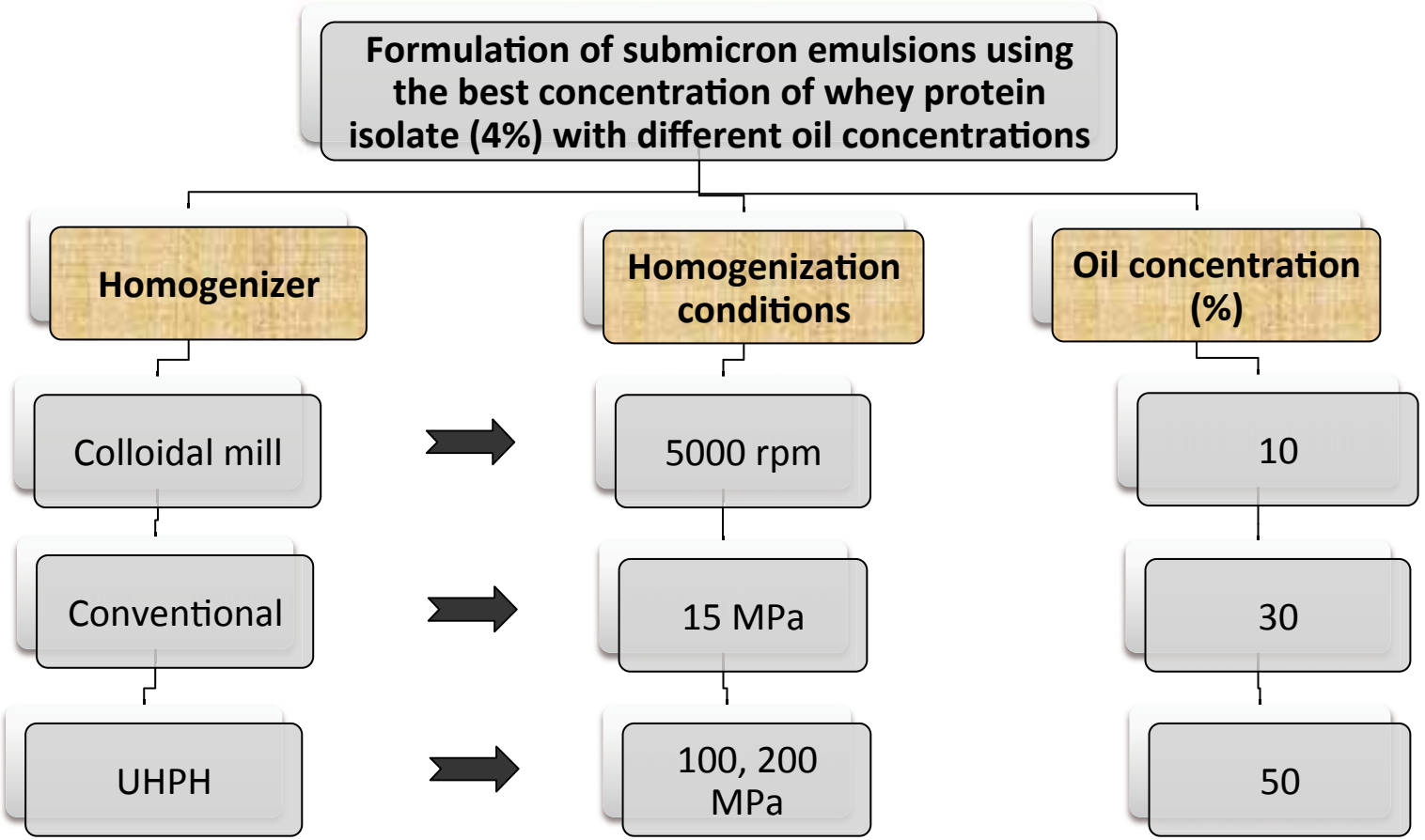

Figure 2. Working plan corresponding to the studies of the effect of pressure and oil concentrations on the stability of emulsions formulated using whey protein isolate (Study 2).

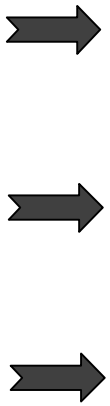

Figure 3. Working plan corresponding to the studies of the effect of pressure and oil concentrations on the stability of emulsions formulated using sodium caseinate (Study 4).

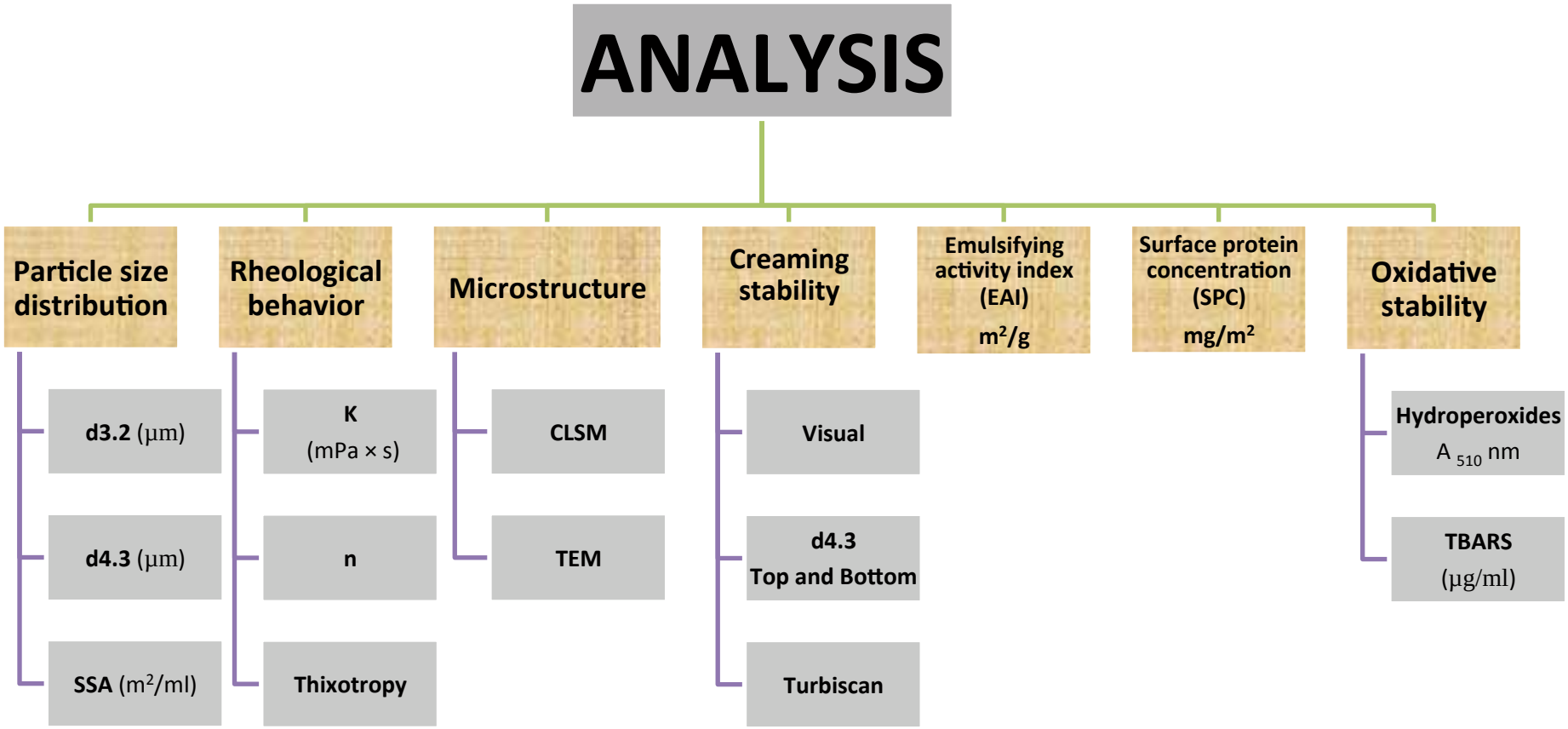

Figure 4. Physical and oxidative stability analysis of submicron emulsions.

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In this chapter, an introduction to emulsions, emulsifiers and emulsification techniques is given, with special emphasis on emulsifiers and equipments utilized in the present work. In addition, important issues regarding physical and oxidative stability of emulsions will be detailed.

2.1. Emulsions definition and types

2.1.1. Emulsion definition

A large variety of foods are emulsions, from the more natural, e.g. milk, creams, whipped creams, ice creams, to the more sophisticated, e.g. sausages and mayonnaises. Basically, an emulsion consists of three phases: a dispersed phase present as droplets in a continuous phase and separated by an interfacial region as can be seen in Figure 5.

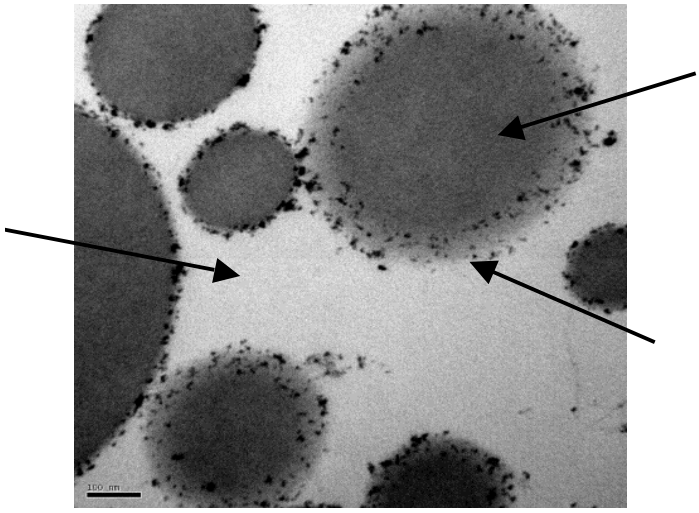

Figure 5. The three regions of an oil-in-water emulsion conventionally homogenized and stabilized by whey protein isolate.

Two separate immiscible liquids can be converted into an emulsion by mixing the two liquids followed by reducing the size of the droplets in the premix using a unit operation called homogenization. After homogenization, the oil and water dispersion generally have a thermodynamically driven tendency to phase separate. That is, the oil droplets merge with each other until the two phases rapidly separate into two distinct phases. This is because the presence of a large interfacial area between these molecules of different polarity is thermodynamically highly unfavorable. The emulsification of the two immiscible liquids, water, and oil also results in a considerable rearrangement of the oil-water interface (Walstra, 1983), where mainly two classes of molecules can be adsorbed: amphiphilic macromolecules (mainly proteins) and low molecular weight emulsifiers (lecithins, monoglycerides, tweens, spans, etc.), as reported by Burgaud, Dickinson, & Nelson (1990). Proteins and low molecular weight emulsifiers help the production and the stabilization of emulsions. Proteins play two major roles: on the one hand, they lower surface tension between the interfaces that are formed during the emulsification process, and on the other hand, they form a macromolecular layer surrounding the dispersed particles which structurally stabilizes the emulsions by reducing the rate of particle coalescence (Walstra, 1983). In food emulsions, stability is usually achieved by the application of proteins as the main stabilizer. This behaviour is attributed to the interactions of hydrophobic parts of proteins with the oil phase and hydrophilic parts with the aqueous phase.

Emulsions can either be O/W emulsions, where oil droplets are dispersed in an aqueous phase or water-in-oil (W/O) emulsions where water exists as droplets in an oil phase, as can be observed in Figure 6. In addition to simple O/W and W/O emulsions, multiple emulsions exist and are composed of dispersions in dispersions, such as for example an internal phase dispersed in a second internal phase that is dispersed in a continuous phase such as water-in-oil-in-water W/O/W and oil-in-water-in-oil O/W/O type emulsions. These emulsions may have improved properties compared to conventional emulsions but, they are typically less frequently used in the food industry due to the complexity and higher costs involved in their fabrication.

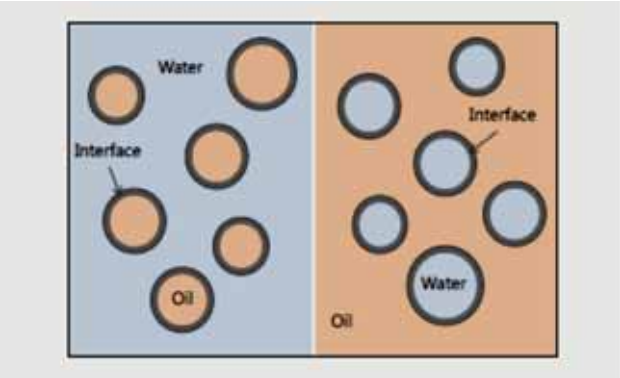

Figure 6. Schematic illustration of an oil-in-water emulsion with oil droplets dispersed in an aqueous phase (left) and a water-in-oil emulsion with water droplets dispersed in an oil phase (right). The oil and water are in both cases separated by an interface of emulsifier (Horn, 2012).

Depending on the emulsion droplet size, emulsions can be divided into micro- (10-100 nm), mini (nano)- (100-1000 nm), and macro-emulsions (0.5-100 mm) (Windhab, Dressler, Feigl, Fischer, & Megias-Alguacil, 2005). Some of the similarities and differences between these emulsions are presented in Table 1 (Jafari, Assadpoor, Bhandari, & He, Y, 2008).

2.1.2. Nano/submicron-emulsions

Most food emulsions are of the oil-in-water (O/W) type (Fennema, Parkin, & Damodaran, 2008). According to Dickinson & Patino (1999) in most food emulsions the diameters of the droplets usually range between 0.1 and 100 μm. These types of emulsions are thermodynamically unstable and tend to breakdown quickly over time.

Nanoemulsions are thermodynamically unstable but kinetically stable and require energy to be formed. The use of high-pressure valve homogenizers or microfluidizers often produces emulsions with droplet diameters between 100 to 500 nm. According to some other literatures, nano/submicron-emulsions are nanometric-sized emulsions with droplet sizes in the range of 20-300 nm (Anton, Benoit, & Saulnier, 2008; Jafari, Yinge & Bhandari, 2006). The food industry is highly interested in nano-emulsions because of their certain inherent advantages. The very small droplet size results in low gravity forces such as the Brownian motion, which may be sufficient to prevent creaming or sedimentation occurrence during storage. Weak flocculation is prevented and this enables the system to remain dispersed with no separation. The significant film

thickness prevents any thinning or disruption of the liquid film between the droplets (Tadros, Izquierdo, Esquena, & Solans, 2004). On the other hand, the strength of the net attractive forces acting between droplets usually decreases with decreasing droplet diameters (McClements, 2005).

According to Qian & McClements (2011), a key advantage of nano-emulsions is that they can be made to be optically transparent. Thus, nano-emulsions can be used to incorporate lipophilic functional components into transparent aqueous beverage products. Functional food components can be incorporated within the droplets, the interfacial region, or the continuous phase. Encapsulating functional components within the droplets often enables a slowdown of chemical degradation processes by engineering the properties of the interfacial layer surrounding them (McClements & Decker, 2000).

Some studies have also advised that the bioavailability of encapsulated non-polar components is higher in nano-emulsions than conventional emulsions due to the small particle size and high surface-to-volume ratio (Huang, Yu & Ru, 2010). McClements & Xiao (2012) have proposed a potential biological fate of ingested nano-emulsions. In recent years, nanoemulsions have been designed to deliver drugs by various administration routes such as intravenous, oral or ocular for therapeutic needs (Singh & Vingkar, 2008).

2.2. Emulsion formation

2.2.1. Emulsification process

Food colloids are very complicated systems because of the interactions and molecular properties of the three principal ingredients (oil, water, and emulsifier). Factors influencing emulsion properties include ingredient interaction, surfactant-surfactant, and surfactant-droplet interactions, lipid oxidation, and process condition such as temperature, pressure and mechanical agitation (McClements, 2005). It can thus be quite challenging to produce stable emulsions. Ultimately emulsion manufacturers need to understand the role that each of the parameters play in the overall property and stability of the emulsions in order to be able to design the desired emulsion system.

Emulsion formation is the process in which two separate liquids (oil and water) are converted into an emulsion. The liquid which broken up in this way is known as the dispersed, discontinuous or internal phase, while the other liquid is referred to as the dispersing, continuous or external phase.

To create an emulsion, an oil phase, a water phase and a stabilizing ingredient are needed. The stabilizing ingredient can be stabilizer polymer, stabilizer microparticle, surfactant, or both stabilizer and surfactant. Usually shear forces are also needed. Emulsification methods are divided between those with mechanical and non-mechanical processes. The need of shear amount and time as well as the need of additional surfactant varies between emulsion systems. The processes involved in emulsification can be seen in Figure 7.

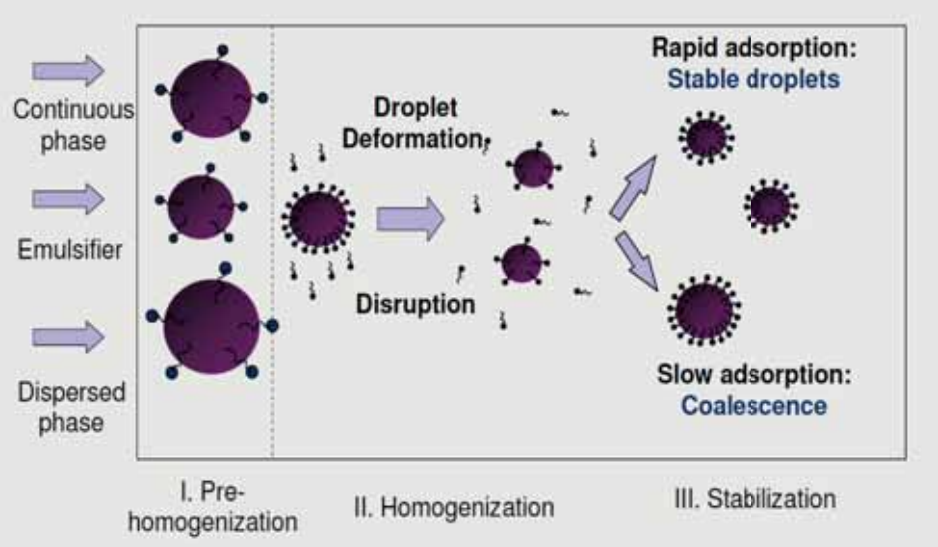

Figure 7. Physicochemical processes involved in emulsification of oil droplets in water phase with stabilizer (Weiss, 2008).

Table 1. Different emulsions (Jafari et al., 2008)

Property	**Macroemulsion**	**Nanoemulsion**	**Microemulsion**
Appearance	Formulation-dependent	Transparent to milky	Transparent
Preparation methods	Classic homogenization	High energy (Pressure)	Low-energy emulsification
Surfactant load	Fairly low	Medium (< 10%)	Fairly high (10-20%)
Droplet size	0.5-100 μm	100-1000 nm	10-100 nm
Thermodynamic stability	Unstable; kinetically stable	Unstable; kinetically stable	Stable

Stabilization of fine droplets requires mechanical deformation of coarse droplets accompanied by rapid effective adsorption of stabilizer and/or surfactant at the new oil-water interface. Collision of droplets with insufficient coverage of stabilizer and/or surfactant leads to flocculation and coalescence or one of these events (Dickinson, 2009). Three droplet deformation mechanisms can be distinguished. These include shear forces in laminar flow, shear forces in turbulent flow and inertial forces in turbulent flow (Stang, Schuchmann, & Schubert, 2001).

The emulsification process of macro-emulsions include two steps: first, shear stress leads to droplet deformation which increases their specific surface area up to disruption; second, the new interface is stabilized by stabilizer and/or surfactant (Perrier-Cornet, Marie, & Gervais, 2005).

2.2.2. Emulsification techniques

The emulsification technique can be simply a form of mixing. In fact, (pre) emulsions are prepared mostly by mixing equipment (Becher, 2001). Modern emulsions, however, can be produced by specially designed devices including high-pressure, ultrasonic, rotor–stator, and membrane systems (Schultz, Wagner, Urban, & Ulrich, 2004). In laboratory studies and most emulsion preparations, it is more efficient and convenient to produce an emulsion in two steps: (a) conversion of separate oil and water phases into a ‘‘coarse emulsion’’ with fairly large droplet size (usually by rotor–stator devices) and this process could be termed (primary homogenization) and then (b) final reduction of droplet size using another technique (e.g. high pressure systems) and this process could be termed (secondary homogenization) as can be seen in Figure 7.

The general principle on which all emulsifying equipment is based is to introduce energy into the system by subjecting the phases to vigorous agitation. In this way, droplets are deformed from their stable spherical shapes and break up into smaller units. If the conditions are suitable and the right type and quantity of emulsifying agent(s) is present, a stable emulsion will be formed. The homogenization equipments vary in their emulsification principles, the physical and chemical stress the product encounters during homogenization, the droplet sizes that can be obtained, and whether their distributions are mono- or poly-disperse (Schultz et al., 2004).

2.2.2.1. Produced particle sizes

Emulsion particle size and particle size distribution are the driving elements of emulsification. Cost-efficiency should also be taken into account in the energy efficiency of the device. Equipment producing finer emulsion particle sizes usually has wider particle size distribution, that is, more polydisperse emulsion. Roughly, emulsion particle sizes increase in the following order: high pressure or microfluidization and ultrasound < rotor-stator homogenizer < manual blending (Jafari, He, & Bhandari, 2007), as can be seen in Figure 8.

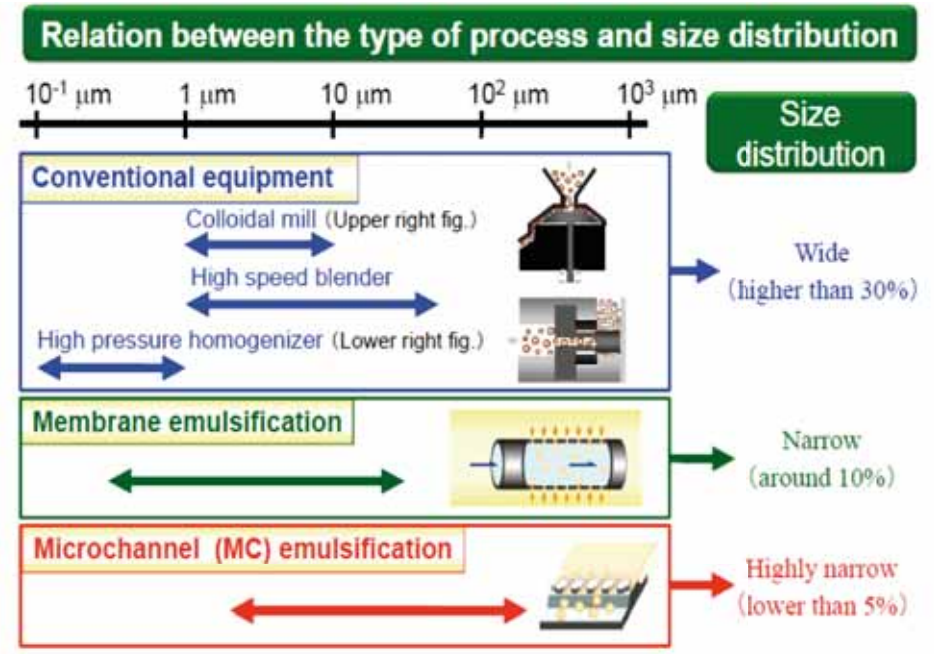

Figure 8. Relation between the type of process and size distribution (Nakajima, Neves, & Kobayashi, 2010).

Many laboratory to large scale emulsion forming equipments are commercially available. Each type of equipment has its advantages and disadvantages. Selection of emulsification equipment depends on many factors, such as the scale of production, the properties of starting material, the desired drop size distribution, physicochemical properties of final emulsion, and capital and operating costs. Main types of emulsification equipment are discussed below.

2.2.2.2. Emulsification equipments

2.2.2.2.1. High shear systems - blade and rotor/stator

These systems are widely used to emulsify liquids with medium to high viscosity (McClements, 2005). For discontinuous operations, agitators or gear-rim dispersion machines are usually used, while for continuous operations, colloid mills with smooth or toothed rotors and stators are available (Urban, Wagner, Schaffner, Roglin, & Ulrich, 2006).

Blade systems are the most simple homogenization systems, consisting of a mixing bowl and rotating blades to create high shear. One advantage of the blade mixer is that very viscous and oil-rich samples can be produced. The disadvantage of the blade mixer is that it produces fairly large oil droplets (> 1 μm) and rather broad droplet size distributions.

Another mechanical high shear homogenization system is the rotor-stator device. In the present PhD work, colloidal mill system with a rotor-stator head was used for primary homogenization prior to high-pressure homogenization. The liquid is fed into the colloid mill in the form of a coarse emulsion (Pinnamaneni, Das, & Das, 2003), or as separate phases, and flows through a narrow gap between a rotating disk (rotor) and a static disk (stator). The rotor/stator assembly consists of a rotor housed concentrically inside the stator with two or more blades and a stator with either vertical or slant slots (Fig. 9).

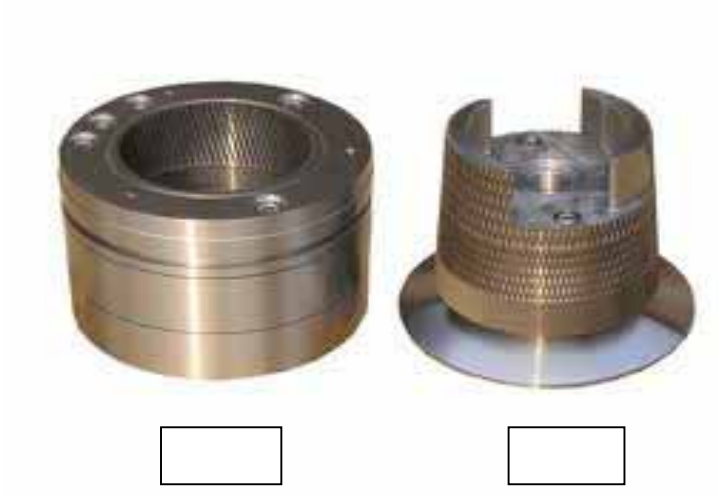

Figure 9. The two principle parts of the colloidal mill system

As the rotor rotates, it generates a lower pressure to draw the liquid in and out of the assembly, thereby resulting in circulation and emulsification (Maa & Hsu, 1996). One of the two major forces that can reduce the droplet size is mechanical impingement against the wall due to high fluid acceleration. Another force is the shear stress in the gap between rotor and stator, which is generated by the rapid rotation of the rotor. The intensity of the shear stress can be altered by varying the thickness of the gap (about 50-1000 mm), varying the rotation speed (about 1000-25,000 rpm), or by using disks that have toothed surfaces or interlocking teeth (Becher, 2001; McClements, 2005). In addition to increasing shear stress, increasing residence time also decreases droplet size, either by decreasing the flow rate or recycling the products. This method tends to produce droplets of emulsion which are larger than those produced by high-pressure homogenization, being of the order of 2 mm in diameter. Colloid mills are usually jacketed for temperature control because increasing the temperature due to the energy dissipation is unfavorable for emulsion stability.

Many factors affect the operation of a colloid mill. High rotation speed, smaller gap thickness or low flow rate will make finer droplets albeit at higher energy consumption. Geometry and material of rotator/stator also affect the energy consumption and emulsion quality.

2.2.2.2.2. High pressure systems

The high-pressure homogenizer is a continuous equipment used to produce fine emulsions. Like a colloid mill, it works at a much higher efficiency for pre-emulsions than for pure oil and liquid phases. Compared to the colloid mill, it is more suitable for low and intermediate viscosity fluids. The schematic of the high-pressure homogenizer is shown in Figure 10 (Brennan, Butters, Cowell, & Lilly, 1990).

In the last ten years homogenization technology has advanced so much that high-pressure homogenization devices are available from lab, pilot to production scales.

In the valve homogenizer a pump pulls the emulsion into a chamber on its backstroke, and then forces it through a narrow valve at the end of the chamber on its forward stroke. In the valve, intense disruptive forces cause the larger droplets to break into smaller ones. Droplets diameters of 0.1-0.2 μm are attainable in pressure homogenizers. The literature suggests that there is an approximately inverse linear relationship between

the logarithm of the homogenizing pressure and the logarithm of the droplet diameter produced by a pressure homogenizer. This high pressure flow through the valve creates turbulence, which pulls apart the oil droplets, during and after which the surfactant molecules adsorb to the newly created interface (Walstra, 1987). The combination of two theories, turbulence and cavitation, explain the droplet size reduction during the homogenization process (Tesch, Freudig, Schubert, 2003; Schultz et al., 2004). The pressure drop across the valve is a result of adjusting the size of the gap through which the emulsion is passed. Since the residence time in the homogenizer is usually very small, it is possible that the emulsifying agent is poorly distributed over the newly created liquid-liquid interface, especially when the emulsifying agent is protein. In such cases, the fine droplets that leave the homogenizer tend to cluster and clump. To overcome this, a "two-stage" homogenization process is applied in some commercial homogenizers (Brennen et al., 1990; Schultz et al., 2004). The processing performances not only depend on the valve design (geometrical characteristics of the needle and seat, height and shape of the valve gap), but also on the physicochemical characteristics of the fluid (density, viscosity, flow rate).

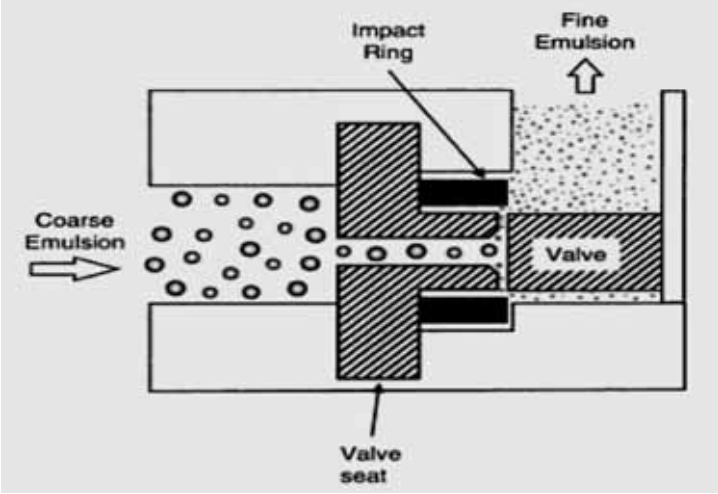

Figure 10. Schematic of high-pressure valve homogenizer.

Comparing to microfluidization, we can notice that microfluidizer devices (Microfluidics^{TM}) designed with a microchannel architecture set in a fixed reaction chamber operate quite differently. Indeed, the fluid stream is divided into two jets at the inlet of such devices then changes its flow direction leading to enhanced particle

collision and impingement on the chamber walls. At the chamber outlet, the fluid jets coming from two opposite microchannels collide, leading to enhanced particle disruption. Such devices combine laminar extensional flow at the chamber inlet to highly turbulent flow with cavitation and impact in and at the outlet of the chamber (Perrier-Cornet et al., 2005; Schultz et al., 2004; Stang et al., 2001).

In both high pressure systems the pressure applied and the number of passes through the homogenization chamber highly influence the resulting oil droplet size distributions, and in comparison to other homogenization devices the obtainable mean droplet size is very small (Jafari et al., 2007; Qian & McClements, 2011).

The advantage of high-pressure homogenizers over other technologies (laminar or turbulent rotor-stator systems, jet-dispersers, membrane or ultrasonic systems) is that more uniform droplet size distributions are obtained since the product is subjected to strong shear and cavitation forces that efficiently decrease the diameter of the original droplets (McClements, 2005; Perrier-Cornet et al., 2005). However, the disadvantages of using high pressure systems are the very high product stress due to the high pressure gradients and flow rates, as well as the possible generation of heat during homogenization (Schultz et al., 2004). In addition, this equipment cannot handle emulsions with a very high viscosity and intensive cleaning is also required after each run.

2.2.2.2.2.1. Ultra high pressure homogenization (UHPH)

UHPH is a novel technology recently studied in food, cosmetic and pharmaceutical areas. It is used to fragment particles in dispersions or emulsions, to produce fine and stable emulsions, to modify the viscous properties of fluids due to the particle size reduction, to facilitate metabolite extraction as well as to achieve inactivation of microorganisms, enzymes or even some viruses.

Depending on the nominal pressure level, the technology will be called high-pressure homogenization (HPH, up to 150 MPa) or ultra-high-pressure homogenization (UHPH, up to 350-400 MPa). For comparison, the standard homogenization operates with an upstream pressure of ~20-60 MPa, as in dairy industry. UHPH benefits from the latest developments in high pressure (HP) technology: development of HP-intensifiers, conception of materials resistant to high-pressure (stainless steel, ceramic, seals).

Sophisticated homogenization valves with seats and needles built in ceramic or coated with artificial diamond, and able to withstand pressure levels up to 350-400 MPa have been specially developed for UHPH equipment and are currently being studied.

2.2.2.2.2.2. Ultra high pressure homogenization (UHPH) equipment

In the case of HP-homogenization using piston-gap type homogenizers such as equipment developed by manufacturers Avestin™, APV™, Niro™, Stansted Fluid Power™, the processed liquid is brought to high pressure in few seconds in the pressure intensifier then forced through a very small orifice, the valve gap of few micrometres in width (Fig. 11).

HP-homogenizers of the piston-gap type used in the present PhD thesis (Fig. 12) are/were developed by Stansted Fluid PowerTM (Model/DRG number FPG 11300:400 Hygienic Homogenizer, Stansted Fluid Power Ltd., UK) with a flow rate of 120 l/h. This equipment consists of a high-pressure ceramic valve able to withstand 400 MPa, a pneumatic valve, located after the first one, able to withstand up to 40 MPa and two intensifiers, which were driven by a hydraulic pump. To minimize temperature retention after treatment, two spiral type heat-exchangers (Garvía, Barcelona, Spain) located behind the second valve were used. Emulsions were UHPH-treated at pressures of 100, 200 and 300 MPa (single-stage) with (Tin) at 25ºC (UHPH emulsions). Throughout the experiment, the inlet temperature, the temperature after the homogenization valve (T1) and the temperature of the outlet product (T2) were monitored. In such HP-homogenizers, the fluid under pressure is forced through a small orifice of some micrometers width to the HP-valve gap (Floury, Bellettre, Legrand & Desrumaux, 2004).

Recently, a group of researchers belonging to CERPTA (UAB) have patented a UHPH system which includes a HP-homogenizer able to sterilize vegetable liquids (Guamis, Trujillo, Ferragut, Quevedo, Lopez & Buffa, 2012).

2.2.2.2.2.3. Submicron emulsions processed by (ultra) high pressure homogenization

In recent years, a growing interest was particularly directed towards the specific innovative functionalities developed from the structural modifications that UHPH could induce.

The droplet size distribution is a key aspect of emulsion processing since the droplet size determines shelf-life stability, rheological and transport properties of emulsions. When the processed fluid is forced through the very narrow HP-valve gap, particles (emulsion oil droplets, fat globules, microorganisms) or polysaccharide macromolecules can be ruptured by the mechanical associated forces inducing a significant reduction of size down to the micron/submicron range (Cortés-Muñoz, Chevalier- Lucia, & Dumay, 2009; Floury et al., 2004). Thereby, emulsions processed by UHPH exhibit an excellent stability vs. time due to the narrow size distribution of the nano-/submicron droplets (Cortés-Muñoz et al., 2009). The nano-/submicron droplet sizes greatly reduce aggregation and gravitational separation phenomena during storage (Tadros et al., 2004).

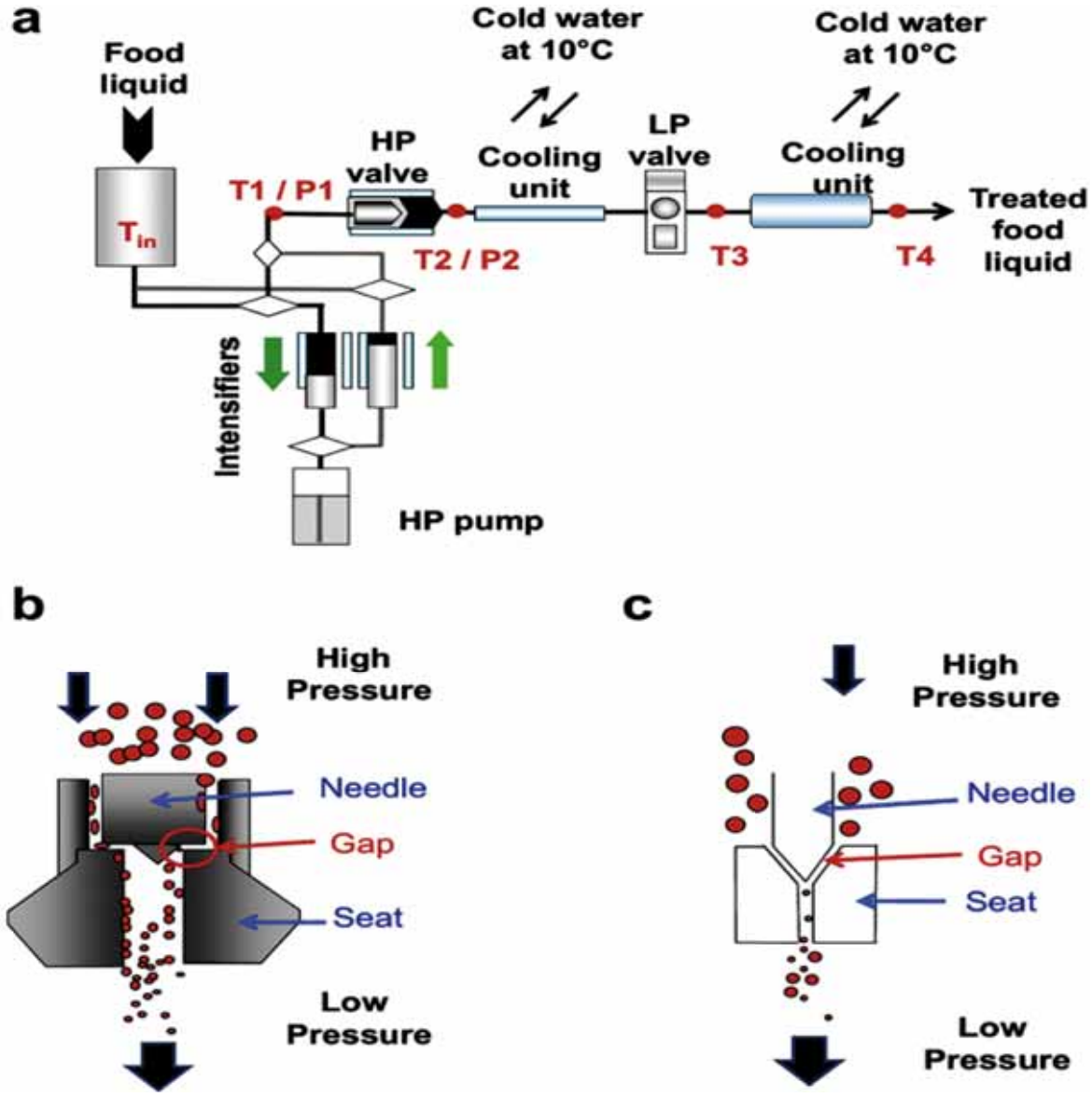

Figure 11. (a) Schematic representation of high-pressure homogenizer with twin-intensifiers. Tin, initial fluid temperature in the feeding tank; T1/ P1, temperature and pressure probes located at the HP-valve inlet; T2/P2, temperature and pressure probes located at the HP-valve outlet; T3 and T4, temperature probes after the first and the second cooling devices. (b) Schematic representation of a sharp-angle HP-valve from Stansted. (c) Schematic representation of a Y-shape HP-valve (Dumay et al., 2012).

Various oil-in-water (O/W) emulsions have been processed by UHPH: bovine whole milk, a natural emulsion (Pereda, Jaramillo, Quevedo, Ferragut, Guamis, & Trujillo, 2008; Zamora, Ferragut, Jaramillo, Guamis, & Trujillo, 2007; Picart et al., 2006; Thiebaud, Dumay, Picart, Guiraud, & Cheftel, 2003); soymilk (Cruz, Capellas,

Hernández, Trujillo, Guamis, & Ferragut, 2007); model emulsions prepared with vegetable oils and stabilized with surfactant of low molecular weight (MW) such as Tween 20® (Floury et al., 2004), soybean proteins (Floury, Desrumaux, & Legrand, 2002) or whey proteins (Cortés-Muñoz et al., 2009; Floury, Desrumaux, & Lardiéres, 2000; Lee, Lefévre, Subirade, & Paquin, 2009). Parameters of UHPH-processing such as the level of homogenization pressure, recycling, one or two-stage homogenization, inlet temperature (Tin) of the processed fluid, and some aspects of emulsion formulation have been investigated in the latter studies, in order to optimize the manufacturing of kinetically stable submicron emulsions.

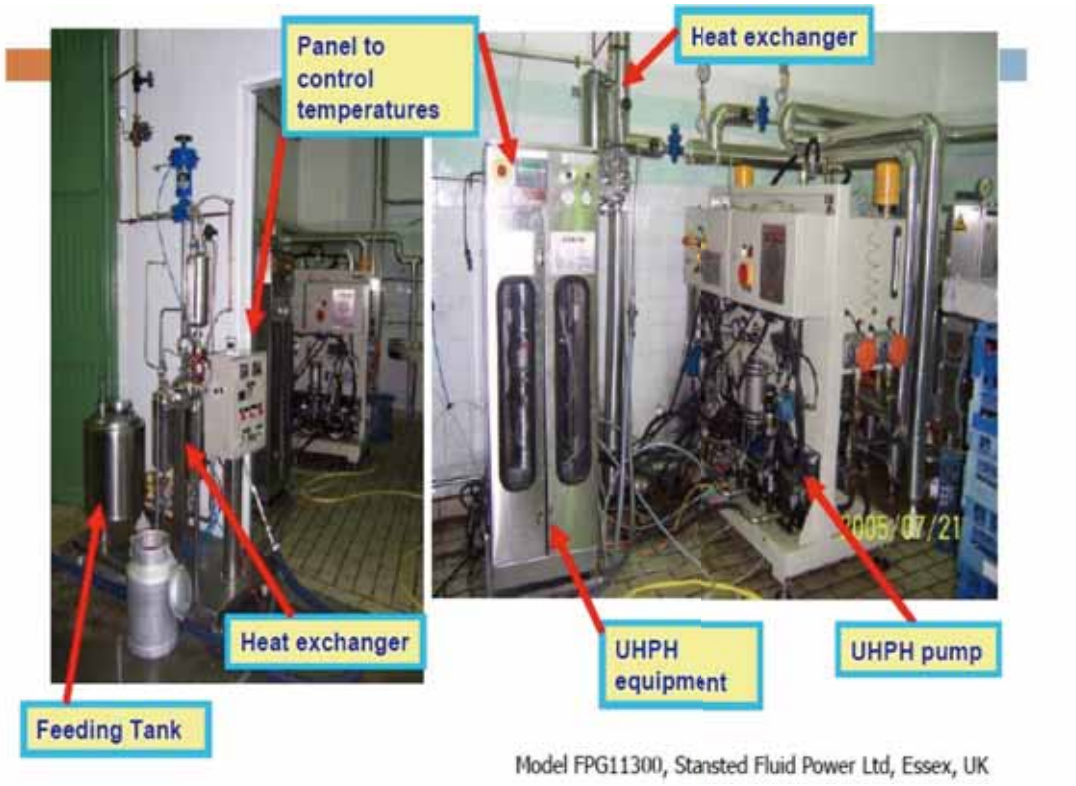

Figure 12. Piston-gap type HP-homogenizer used in the present PhD thesis developed by Stansted Fluid Power™, Essex, UK.

Comparing to small MW surfactants, proteins are more efficient stabilizing agents in UHPH-processed O/W emulsions due to the more compact and viscoelastic layer formed at the interface (Lee et al., 2009). However, some studies have reported over processing resulting in the increase of droplet/globule size at pressures more than 250-300 MPa as will be explained hereafter in details (refer to section 2.4.3.4).

Recycling the emulsion sample through the homogenizer once or twice is a way to improve UHPH-efficiency in oil droplet splitting, as a result of cumulated residence times of the fluid in the HP-valve. Consequently, several successive passes in the homogenizer at moderate high-pressure (~200 MPa) decreases both the mean diameter and the width of droplet/fat globule size distribution (Floury et al., 2000; Thiebaud et al., 2003).

Besides providing kinetically stable emulsions, UHPH also enables the production of emulsions with a large range of flow behaviors (i.e., from highly fluid to highly thick samples) when combining the pressure level of homogenization and the oil volume fraction. An increase has been generally observed of emulsion viscosity with the homogenization pressure and with the number of homogenization passes, parameters which induce an increase in the number of oil droplets (Cortés-Muñoz et al., 2009; Floury et al., 2003, 2002).

Furthermore, for peculiar UHPH conditions (200-225 MPa), O/W model emulsions stabilized by whey proteins showed a remarkable stability against coalescence, that thawing process could induce, because of sufficiently small droplets without (or with limited) protein denaturation (Cortés-Muñoz et al., 2009).

2.3. Emulsifiers - composition and adsorption behavior

If a crude emulsion is formed by mixing two immiscible liquids, the internal phase will take the form of spherical droplets, representing the smallest surface area per unit volume. If the mixing is stopped, the droplets coalesce to form larger ones and eventually the two phases will completely separate. To form an emulsion, this interfacial tension has to be overcome. The greater the interfacial tension between the two liquids, the more energy is required to disperse the internal phase. Emulsifiers are active surface compounds whose molecules have hydrophilic and hydrophobic groups. Emulsifiers are classified as ionic (anionic, cationic, amphoteric) and non-ionic.

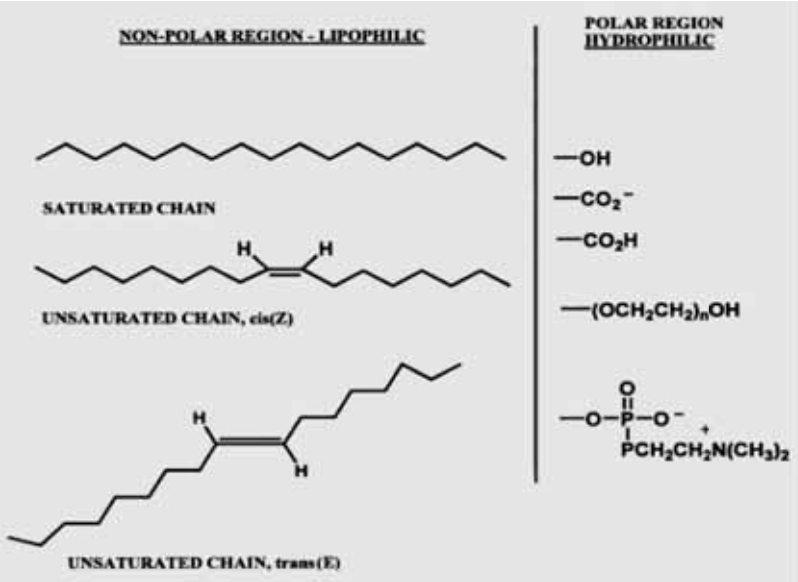

Figure 13. Polar and nonpolar functional groups of an emulsifier (Hasenhuettl, 2008).

An effective emulsifier should have three general characteristics: 1) rapidly adsorb to the oil/water interface of newly formed droplets during homogenization; 2) substantially reduce the interfacial tension; and 3) form an interfacial membrane to stabilize the emulsion by steric or electrostatic interactions between droplets.

Proteins and low-molecular-weight (LMW) surfactants are key components of many foodstuffs. Some dairy products, for example ice cream, contain both proteins and LMW surfactants in their formulation. Both types of molecules can adsorb at fluid interfaces, reducing the interfacial tension and so facilitating the formation of emulsions and foams and providing stability to droplets and bubbles. However, their molecular properties are very different.

LMW surfactants are small molecules each consisting of a hydrophilic head group and one or several hydrophobic tails. When such molecules reach an air–water or oil–water interface, they tend to adsorb by arranging the hydrophobic tails within the non-aqueous phase and the hydrophilic head in the water phase (Fig. 14 A). LMW surfactants are very mobile and they are particularly efficient at reducing the interfacial tension. As a result, they rapidly coat the newly created oil-water and air-water interface during emulsification and foaming.

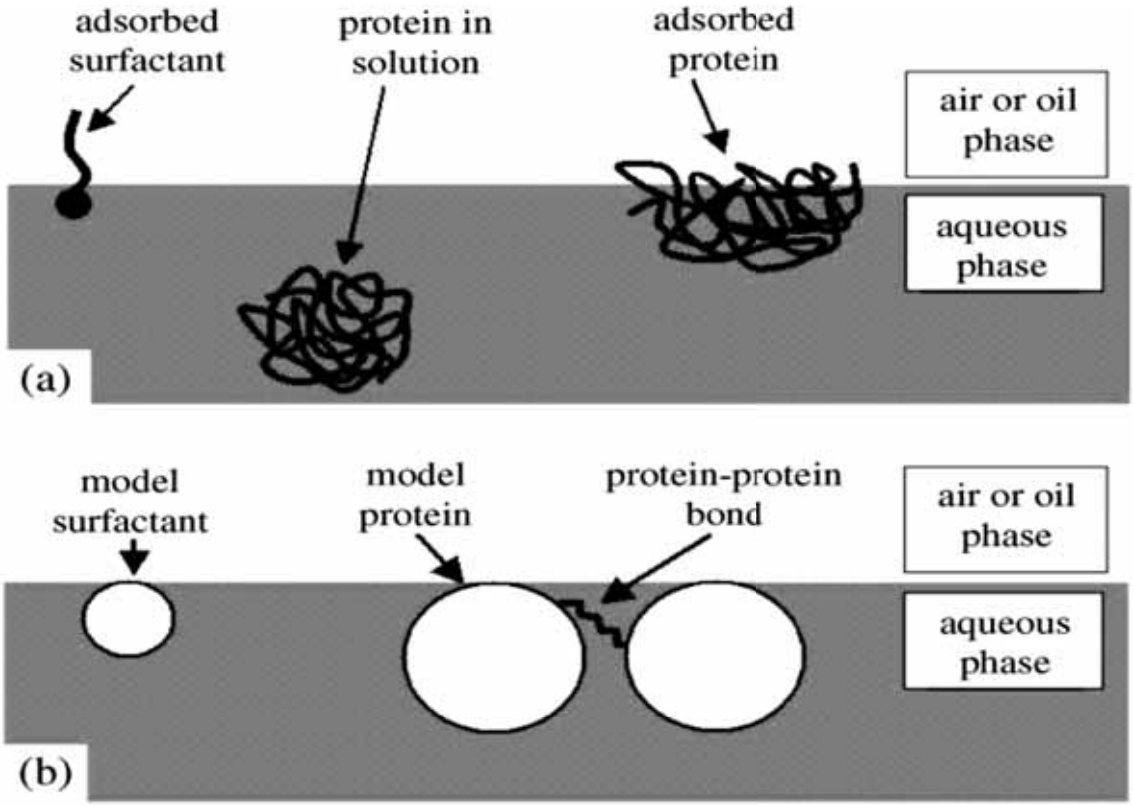

Figure 14. Schematic representation of surface-active species. (a) Protein and surfactant molecules. (b) Representation of the proteins and surfactants as particles (Pugnaloni Dickinson, Ettelaie, Mackie, & Wilde, 2004).

Proteins are high-molecular-weight molecules each consisting of a chain of amino acids. As there are polar, non-polar and ionic amino acids, proteins contain a mixture of hydrophilic and hydrophobic groups. In aqueous solution, a protein molecule will tend to fold in a coil-like structure in order to expose the most hydrophilic groups to the water and hide the most hydrophobic segments in the centre of the coil (Fig. 14 A). However, when a protein molecule reaches an air-water or oil–water interface, the molecule will partially unfold orientating its hydrophobic groups towards the non-aqueous phase (Fig. 14 A). Proteins are very slow at diffusing and adsorbing as compared with LMW surfactants; and they do not normally lower the interfacial tension so efficiently. However, proteins form thick protective layers at the surface of oil droplets and gas bubbles which, under appropriate conditions, can prevent coalescence after an emulsion or foam has been formed thereby conferring long-term stability to the system (Pugnaloni et al., 2004).

The emulsifiers included in the present thesis are milk proteins. These emulsifiers are described in section 2.3.1, and their properties related to lipid oxidation are outlined in section 2.5.3.

2.3.1. Milk protein as emulsifiers

Proteins can act as emulsifiers because of the mixture of hydrophilic and hydrophobic functional groups in their component amino acids (McClements, 2004).

Bovine milk contains approximately 3.2% proteins, whereof around 80% are caseins and 20% are whey proteins (Fox & Mulvihill, 1982). Both of these milk protein classes are quite heterogenous as the protein groups within each class have different molecular, physical and chemical properties (Fox & Kelly, 2003). Depending on how they are processed, various protein products can be produced with varying emulsifying and stabilizing properties. The whey protein products used in the present PhD thesis are very gently prepared, thus they have not been denatured and they have structural properties very similar to the original whey proteins in milk. However, the caseinate used is a sodium salt, which will most likely behave differently in an emulsion than the original casein does in milk.

Caseins occur as casein (CN) micelles in milk, which are complexes of colloidal calcium phosphate and caseins (Schmidt, 1982). These micelles are spherical aggregates with diameters ranging from 40 to 300 nm and show considerable variation in composition, structure and size distribution (Swaisgood, 1996).

Bovine milk caseins consist mainly of four different proteins, (α_{s1}, α_{s2}, β and κ-CN), and all four are present in sodium caseinate in an almost similar ratio as in the original milk. The four components differ in their number and composition of amino acid residues and thereby in their structural abilities (Table 2). Due to absence of the higher levels of secondary and tertiary structures, caseins are flexible and unstable structures, thus, they are considered very flexible molecules with a high surface activity (Creamer, 2003).

It was reported that unlike caseins, caseinates do not aggregate in the form of micelles and can be manufactured by precipitating caseins from milk. Caseinates are more functional than caseins in various food applications (Srinivasan, Singh, & Munro, 2003) in terms of most important functional properties such as viscosity and solubility (Fox & Mullvihill, 1982; Hooker et al., 1982).

Whereas whey proteins are compact, globular proteins and contain disulphide bonding to stabilize their structure with major proteins including α-La, β-Lg, bovine serum albumin and immunoglobulins (Fox, 2001). These proteins have a more organized secondary and tertiary structure due to less proline and more cysteine residues (Table 2). About 20% of the total protein of bovine milk is whey or serum proteins. These proteins are soluble at pH 4.6 and in saturated NaCl, and they are not sensitive to calcium ions (Fox & McSweeney, 2003).

2.3.2. Adsorption of milk proteins and formation of interfacial membrane

During homogenization, the milk protein, in the form of individual molecules or protein aggregates, becomes rapidly adsorbed at the surface of the newly formed oil droplets to prevent droplet coalescence. The amount of protein present at the interface per unit surface of dispersed phase is defined as the protein load, which is usually expressed as milligrams of protein per unit area of the dispersed phase (mg/m^2). The protein load determines the amount of protein required to make an emulsion with a desired oil volume and droplet size and is dependent on the concentration and the type of protein as well as on the conditions used for emulsion formation. The factors that affect the protein load include protein concentration, volume of oil, energy input, state of protein aggregation, pH, ionic strength, temperature and calcium ions (Dickinson & Stainsby, 1988).

Proteins and peptides often form a monolayer at oil/water interfaces, yielding a maximum surface excess. In some cases, multilayer adsorption occurs and no maximum surface excess is found. These multilayers are usually readily removed by lowering the protein concentration of the continuous phase (Graham & Phillips, 1979).

Jackson & Pallansch (1961) found the interfacial activity of the milk proteins to be in the decreasing order as: β-CN > casein micelles > serum albumins > α-La > α_{s1}- casein = κ-CN > β-Lg.

Table 2. Characteristics of the amino acid compositions of major proteins in bovine milk (Ng-Kwai-Hang, 2003).

		Approximate concentration in milk (g/L)	∑ amino acid residues	Proline	Cysteine (sulfhydryl residues)	Phosphoseryl residues
Caseins	**α_{s1}**	10	199	17	0	8
	α_{s2}	3	207	10	2	10-13
	β	9	209	35	0	5
	κ	3	169	20	2	1
Whey proteins	**α-La**	1	123	2	8	0
	β -Lg	3	162	8	5	0

When adsorbed at an interface, milk proteins as well as other proteins form a strong viscoelastic film around the oil droplets by arranging themselves in “trains”, “loops” and “tails” as visualized in Figure 15.

Hence, upon adsorption at an oil-water interface, the hydrophilic amino acid domains will project into the water phase, whereas the hydrophobic amino acid domains will face the oil phase (Krog, 2004). Consequently, the structurally disordered caseins are expected to possess a higher surface activity and emulsifying capacity than the compact and highly ordered whey proteins. However, the structural conformations and the emulsifying properties of whey proteins are very sensitive towards different treatments, such as homogenization, a change in pH or heating (Fang & Dalgleish, 1998; Lee, Lefèvre, Subirade, & Paquin, 2007; Stapelfeldt & Skibsted, 1999).

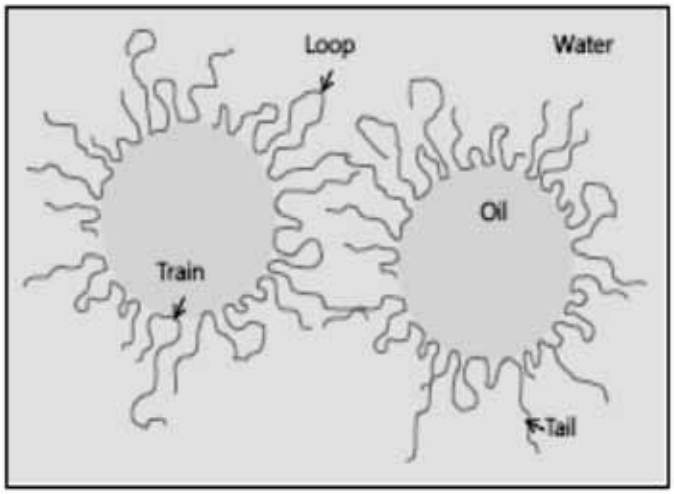

Figure 15. Schematic illustration of the adsorption of milk proteins at an oil droplet interface in a loop and train manner due to distinct hydrophilic and hydrophobic domains in the amino acid structure of the protein (Horn, 2012).

In comparison, the droplet surface adsorption behaviour of whey proteins was suggested to be a little different, owing to the globular nature of the whey proteins (Hunt & Dalgleish, 1994). Since more whey protein was needed to obtain a stable emulsion (1.5 mg/m^2 compared to 1 mg/m^2 for the casein), these proteins were not expected to be able to stretch over the droplet surface to the same extent as caseins.

In a study on emulsions containing β-lg, the authors suggested that in low concentrations (1% β-Lg to 20% oil) the proteins were stretched over the interface whereby they changed conformation. In contrast, when proteins were present in excess (2% β-Lg to 20% oil) they did not have to stretch to cover the interface, and therefore did not differ in conformation from the native protein in solution (Fang & Dalgleish, 1997). Thus, a concentration dependent conformational behaviour of whey proteins was suggested similarly to the one suggested for caseins.

In 30% soy oil emulsions prepared with a combination of sodium caseinate and whey protein concentrate (1:1), whey proteins were adsorbed in preference to caseins at total protein concentrations below 3%, whereas the opposite was observed at total protein concentrations above 3% (Ye, 2008). In homogenization studies on milk, the adsorption

of the different milk proteins and their conformations at the interface have been shown to depend on the homogenization equipment used (Dalgleish, Tosh, & West, 1996).

2.4. Stability of oil-in-water emulsions

The term "emulsion stability" refers to the ability of an emulsion to resist any alteration in its properties over the timescale of observation (Dickinson, 2003; McClements, 2005). An emulsion is thermodynamically unstable as the free energy of mixing is positive because of the large interfacial area between the oil and the aqueous phase. Therefore, the kinetic stability, i.e. the time period for which the emulsion is stable, is important (Dickinson, 2003; McClements, 2005). For instance, an emulsion can be considered to be "stable" if the inevitable process of separation has been slowed to an extent that it is not of practical importance during the shelf life of the product. An emulsion may become unstable because of a number of different types of physical and chemical processes.

Physical instability refers to the change in spatial arrangement or size distribution of emulsion droplets, such as creaming, flocculation or coalescence, whereas chemical instability includes change in the composition of the emulsion droplet itself, such as oxidation, hydrolysis, etc. (McClements & Decker, 2000; McClements, 2005).

The physical stability of an O/W emulsion is highly dependent on the emulsifier and the droplet size distribution. Emulsion instability includes different processes such as droplet aggregation or gravitational separation.

Emulsions are stable if sufficiently large repulsive forces act between the dispersed droplets, and the mobility of the dispersed phase is adequately restricted. Repulsive forces prevent droplet aggregation and coalescence; the limited mobility inhibits creaming or sedimentation (Walstra, 1983).

2.4.1. Mechanisms of emulsions instability

Creaming is the movement of oil droplets, under gravity or in a centrifuge, to form a concentrated layer at the top of an oil-in-water emulsion sample, with no accompanying change in the droplet size distribution. If the density of droplets is higher than that of the continuous phase, droplets will tend to move in the direction of the gravitational field, a

process known as sedimentation. Gravitational separation strongly affects the appearance and texture of food emulsions often resulting in unacceptable product qualities. Creaming is reversible and the original uniform distribution of droplets can usually be obtained by gentle mixing. The creaming process can be explained by Stokes' Law (McClements, 2005):

$$U_{stokes} = 2r^2(\rho 1 - \rho 2) / \eta$$

where U_{stokes} = velocity of creaming, **r** = emulsion droplet radius, $\rho 1$ and $\rho 2$ = density of the continuous phase and the dispersed phase, respectively and η = shear viscosity of the continuous phase.

The creaming rate can be reduced by lowering the radius, increasing the continuous phase viscosity or decreasing the difference in density between the two phases. However, this law often fails to define the rate of creaming due to flocculation or coalescence.

Submicron/nano-emulsions typically have much better stability to gravitational separation than conventional emulsions because the relatively small particle size means that Brownian motion effects dominate gravitational forces (Floury et al., 2002; Lee et al. 2009). It has been shown that when the particle sizes are smaller than 100 nm, creaming would be greatly reduced and aggregation becomes a dominant mechanism for emulsion instability (McClements, 2005). The stabilization of emulsion may be partly attributed to the considerable increase of interaction between adsorbed proteins at the interface of the emulsion, because strong interactions between adsorbed proteins at the interface lead to the formation of a more rigid interfacial layer at higher pressure, so that it may effectively better protect emulsion droplets against destabilizing processes (Lee et al., 2009). The greater droplet size reduction and the rigid interfacial layers around oil droplets of UHPH emulsions which in turn increase the density of particles may enhance creaming stability. Creaming itself does not destabilize an emulsion, but the high concentration of oil droplets in the creamed layer promotes interactions that lead to flocculation, aggregation or coalescence, as will be explained in the following section.

Droplet aggregation covers two processes: coalescence and flocculation.

Coalescence is the process where two droplets meet, and merge into a bigger droplet, which gradually results in separation of the oil and the aqueous phase and is always irreversible, as illustrated in Figure 16 (McClements, 2005).

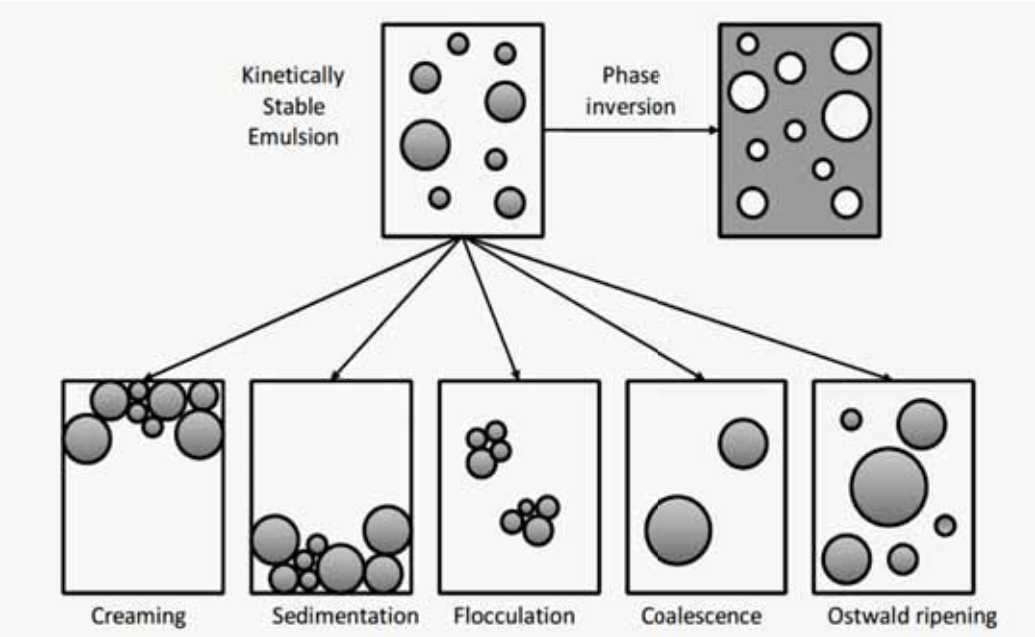

Figure 16. Overview of mechanisms that induce emulsions instability.

Coalescence occurs if the emulsifier concentration is not high enough whereby oil droplets become large, or if the emulsifier used does not have the properties to sufficiently stabilize the emulsion. Coalescence requires rupture of the stabilizing film at the oil-water interface, but this occurs only when the layer of continuous phase between the droplets has thinned to a certain critical thickness (Dickinson & Stainsby, 1988).

It is well known that, final particle size is the result of equilibrium between droplet break-up and re-coalescence. Between new droplet formation and its subsequent encounter with surrounding droplets, emulsifiers adsorb onto the created interface to prevent re-coalescence. If the timescale of emulsifier absorption is longer than the timescale of collision, the fresh interface will not be completely covered and will lead to

over processing phenomenon (re-coalescence), i.e., particle size increase (Perrier-Cornet et al., 2005).

Since the energy input by high-pressure homogenization is very high and re-coalescence of newly formed droplets is inevitable, there should be an optimization of the process along with appropriate selection of the emulsifier type and concentration in order to reduce "over-processing" and produce a stable submicron emulsion with the optimum size distribution. Floury et al. (2004) showed that whatever the oil or surfactant content, re-coalescence increased sharply with emulsification pressure (re-coalescence rate was about 5% at 20 MPa up to almost 70% at 350 MPa), and they concluded that ultra-high pressures did not appear to cause real benefits to emulsification efficiency because of high re-coalescence rates. Hence, extensive polymer interactions at the interface when applying ultra-high pressures may lead to the formation of an interfacial membrane, which may therefore provide better protection against droplet re-coalescence and bigger droplets. The effect of energy input during homogenization on the particle size and droplet re-coalescence will be explained in details in section 2.4.3.4.

Similar to coalescence, ***flocculation*** is also a process where two droplets collide, but instead of merging, the droplets maintain their individual integrity (Fig. 16) (McClements, 2005). Flocculation has been defined as the reversible aggregation mechanism that arises when droplets associate as a result of unbalanced attractive and repulsive forces (Dalgleish, 1997). Over time the flocculation led to a gravitational separation (creaming and sedimentation).

Generally, two types of flocculation are distinguished, i.e. depletion flocculation and bridging flocculation (Dickinson, 2003). The type of mechanism prevailing depends upon the interaction between the interfacial layer and the emulsion droplets. Depending on the concentration of the ingredients in the emulsions, protein-coated droplets may be destabilized by bridging or depletion flocculation (Gu, Decker, & McClements, 2004).

Bridging flocculation normally occurs when a high MW biopolymer at a significantly low concentration adsorbs to two or more emulsion droplets, forming bridges (Dickinson, 2003; McClements, 2005; Fellows & Doherty, 2006).

Depletion flocculation occurs as a result of the presence of unadsorbing biopolymer in the continuous phase, which can promote association of oil droplets by inducing an

osmotic pressure gradient within the continuous phase surrounding the droplets (Tuinier & de Kruif, 1999; McClements, 2005). Both depletion flocculation and bridging flocculation cause an emulsion to cream more rapidly. It can be concluded that the protein concentration has a high impact on the emulsion stability towards flocculation, which will be detailed in the section 2.4.3.5.

Ostwald ripening is the instability process by which, larger droplets grow at the expense of smaller ones due to higher solubility of smaller droplets and molecular diffusion through the continuous phase (Capek, 2004). In other words, large droplets become bigger and small droplets become smaller. This process is different from coalescence since no film rupture is happening between flocculated droplets (Damodaran, 2005). The rate of Ostwald ripening depends on the mean droplet size, e.g. the smaller the droplet size such as nano-emulsions, the higher the Ostwald ripening rate. Coalescence phenomena due to Ostwald ripening can affect nanoemulsions stability, leading to a significant growth in droplet size over time.

Phase inversion is the process whereby the two phases of an emulsion invert e.g. the emulsion changes from an O/W emulsion to a W/O emulsion or vice versa. Phase inversion typically occurs if the composition or environmental conditions of a colloidal system are altered, for example, disperse phase volume fraction, emulsifier type, emulsifier concentration, solvent conditions, temperature, or mechanical agitation.

2.4.2. Factors affecting emulsion physical stability

2.4.2.1. Particle size

Control of the droplet size is one of the most critical parameters required to produce a desirable food colloidal system since the size of the droplets contributes to the stability, appearance, texture, and taste of the emulsion. Droplet size is controlled by the volumetric energy input during the homogenization, number of passes through the homogenizer, composition of component phases, temperature, viscosity of the

suspension, and the amount of emulsifier present (Dickinson, 2003; McClements, 2005; Dickinson, 2009).

The ideal droplet size to enhance emulsion stabilization is small with a narrow distribution as these factors prevent agglomeration (creaming) and coalescence (Dalgleish, 2004; McClements, 2005; Dickinson, 2009). Typically within food emulsions, the droplet diameters range from 0.1-100 μm (McClements, 2005). When droplet radii are less than 10 nm creaming should be retarded almost completely due to Brownian motion (Damodaran, 2005; McClements, 2005; Dickinson, 2010). Droplets that are larger (radius > 1 μm), and with broad distribution tend to agglomerate and coalesce more rapidly.

2.4.2.2. Interfacial tension

Interfacial tension is the measure of the free energy that is stored in the interface. Interfacial tension is created by the imbalance of molecular interactions between molecules located at the interface. However, the introduction of a surface active agent (e.g., protein) can reduce the interfacial tension because the agent/emulsifier minimizes the thermodynamically unfavorable interactions between the various molecules at the interface (Dickinson, 2003; Damodaran, 2005; McClements, 2005). This interfacial energy is important in the formation of emulsions because it plays a role in determining the amount of mechanical energy needed via homogenization to break up system droplets (Damodaran, 2005; McClements, 2005). The resistance of droplets towards coalescence and Ostwald ripening, and the packing of large droplets in concentrated emulsions are also affected by interfacial tension (Damodaran, 2005; McClements, 2005). Therefore, the value of the interfacial tension of a system can provide valuable information about the emulsifier and the interface including: excess surface concentration, surface activity, adsorption rates, and interfacial rheology (McClements, 2005). In order to reduce the droplet size, the pressure required by the homogenizer must increase with increasing interfacial tension (Damodaran, 2005; McClements, 2005).

2.4.2.3. Initial temperature of the processed fluid (Tinlet)

One important condition which influences emulsion formation is temperature. Interfacial tension and viscosity are temperature-dependent, both decreasing with increase in temperature. Thus, raising the temperature of the liquids usually facilitates emulsion formation. However, for any system there will be an upper limit of temperature depending on the heat sensitivity of the components.

2.4.2.4. Energy input and temperature rise during high- or ultra-high pressure processing (HPH or UHPH)

Droplet size can be reduced by increasing the amount of energy supplied during emulsification (as long as there is sufficient emulsifier to cover any new interface and re-coalescence is prevented as much as possible). This can be achieved in a number of different ways depending on the nature of the emulsification system. Under a given set of emulsification conditions (energy input, emulsion composition), there is a certain size below which droplet size cannot be reduced with repeated emulsification, and therefore emulsifying the system any longer would be inefficient (McClements, 2005), or sometimes leads to an increase in droplet size because of poor stabilization of the newly formed droplets and is referred to as ‘‘over-processing’’ as will be discussed hereafter in this section.

During HPH or UHPH, the fluid is forced to pass through a narrow gap in the homogenizer valve, where it is submitted to a rapid acceleration (Floury et al., 2004). As a consequence, phenomena such as cavitation, shear and turbulence are simultaneously inducted (Freudig, Tesch, & Schubert, 2003) leading to a short-life heating phenomena and a liquid temperature jump depending on the intensity of the applied pressure. A total jump in temperature of 17-21°C per 100 MPa at the exit of the HP-valve is measured when processing whole milk or O/W emulsions processed at an initial temperature of 4-24°C (Pereda et al., 2008; Zamora et al., 2007; Cortés-Muñoz et al., 2009; Picart et al., 2006; Thiebaud et al., 2003). Moreover, the temperature increase is proportional to the pressure applied. At a fixed oil concentration (i.e. 50%), the evolution of temperature, the difference between T2 and T1, after applying a pressure of 100 MPa is 30 ºC, while 54 ºC of difference can be achieved after treating the emulsions at 200 MPa.

A temperature rise reduces the viscosities of emulsion phases, and lowers the interfacial tension and Laplace pressure, thereby reducing the minimum thermodynamic energy necessary for emulsification that facilitates production of smaller droplets (McClements, 2005), but taking into consideration the presence of heat-sensitive biomolecules in the system, the temperature must be measured and controlled by efficient cooling devices to avoid over-processing. A study by Marie, Perrier-Cornet, Gervais, (2002) found that with cooling, the droplet diameter during high-pressure jet emulsification was smaller and more uniform than higher temperatures: for 10% sunflower oil content, a droplet diameter reduction of 39% was obtained when cooling was applied.

When using an emulsifier during high-energy emulsification, the question is whether it can survive these harsh conditions and whether its emulsifying properties are affected or not. There is a lot of controversy in the literature regarding this issue. Emulsifiers vary in the degree of sensitivity and resistance. In the following, we will focus on the effect of ultra-high pressures and temperature rise in emulsions stabilized by milk proteins.

With respect to emulsions stabilized using globular whey proteins, there is a lot of controversy in the literature regarding the high pressure effect. Desrumaux & Marcand (2002) by a differential scanning calorimetry analysis, showed that during ultra high-pressure emulsification, the conformation of whey proteins was changed (denaturation happened), which probably affected their emulsifying properties, but their molecular weight was not changed significantly (confirmed by electrophoresis). They found an optimum pressure of about 100 MPa, in which d3.2 and Span values reached a minimum. On the other hand, some authors claimed that high microfluidizing pressures can facilitate interface adsorption of proteins by modifying their 3D structures (a better unfolding) and resulting in smaller particle size. For example, Perrier-Cornet et al. (2005) proved that at pressures above 200 MPa, the adsorption rate of whey proteins significantly increased (60%), corresponding to a very narrow particle size of sunflower oil. Bouaouina et al. (2006) showed that dynamic high-pressure treatment did not affect the conformation of whey proteins but enhanced their stabilizing properties because of increased exposure of their hydrophobic sites. Lee et al. (2009) also found that homogenization at high pressures (50 to 200 MPa) of emulsions (10% soybean oil and 0.5% whey proteins) resulted in partial denaturation of protein adsorbed at the (O/W) interface with the exposure of its hydrophobic groups in a similar way to that observed

in heating processes. Thus, the improved adsorption of proteins at the interface, caused by high-pressure homogenization, contributed to the stability of the system.

On the other hand, emulsions stabilized by caseinates have shown greater stability to high- pressure than those stabilized by whey proteins (Britten & Giroux, 1991), although the disruption of casein micelles by high pressure homogenization has been reported (Roach & Harte, 2008; Sandra & Dalgleish, 2005). Perrechil & Cunha (2010) characterized coarse and fine neutral emulsions stabilized by locust bean gum (LBG) plus acidified caseinate emulsions. They found that high-pressure homogenization produced a reduction in the droplet size and therefore a decrease in the creaming velocity. San Martin-Gonzalez et al. (2009) reported the development of a gel-like structure in emulsions containing 30% oil and 2 - 3.5% casein when homogenized between 20 and 100 MPa. The authors hypothesized that homogenization resulted in the exposure of hydrophobic sites within the micelle core which allowed micelle-coated oil droplets to interact with neighboring particles, creating an elastic three-dimensional structure that becomes fairly strong at a threshold casein concentration. However, applying the UHPH at high pressures tended to create emulsions with a low creaming index; the highest pressure (300 MPa) resulted in a creaming index of 0 regardless of oil and casein concentration for up to 10 days, suggesting that extensive disruption of the casein micelles occurs at the highest pressure, thus increasing the availability of emulsifying protein molecules.

2.4.2.5. Protein type, concentration and adsorption rate

The choice of emulsifier and its concentration can be used to modify droplet size (Dickinson, 2003). For a fixed emulsion composition, there is a maximum interfacial area, which can be completely covered by the emulsifier, and as emulsification continues, the interfacial area increases substantially (Friberg & Larsson, 1997). Below a certain droplet size, there is not enough emulsifier to cover the interface completely, and so droplets tend to coalesce with their neighbors.

Some emulsification systems are not able to generate high-energy densities for droplet disruption and are unable to produce smaller droplet size, even though there might be sufficient emulsifier present (McClements, 2005; Urban et al., 2006). The emulsion must also spend sufficient time within the emulsification zone for all droplets to be

completely disrupted. If an emulsion passes through this zone too rapidly, some droplets may not be disrupted (Walstra, 1983).

The other important effect of an emulsifier is its interfacial adsorption rate which determines to a large degree the stabilization of the newly broken up droplets against coalescence (Schultz et al., 2004; Stang et al., 2001). Depending on the residence time of droplets in the dispersing zone of the emulsification systems, emulsifiers with different interfacial adsorption rates can be used. The fresh interfaces must be occupied as soon as possible, before the emulsion leaves the emulsification zone and arrives in zones of laminar flow.

Slow emulsifiers, like biopolymers and high MW surfactants, can only be used effectively in emulsification systems with long residence times, such as colloid-mills, or multistage high-pressure systems because they get the chance to stabilize newly broken up droplets more than once. Linear small-molecule emulsifiers such as Tween 20 stabilize new interfaces in milliseconds, so that droplets are unlikely to re-coalesce.

The protein type is very important in determining the physical stability of an emulsion towards flocculation and re-coalescence, where the sensitivity of the emulsifiers to high pressures is type dependent. In the literature agreement exists that emulsions stabilized with caseins are more stable against partial coalescence than those stabilized with whey proteins Zhao, Zhao, Wang, Wang, & Yang, (2008).

When a mixture of emulsifiers is present, different molecules compete to adsorb at oil-water interface and lower the interfacial tension (Arboleya & Wilde, 2005; Dickinson, 2003; Klinkesorn, Sophanodora, Chinachoti, & McClements, 2004; McClements, 2004). Experimental evidence indicates that proteins will adsorb to the oil interfaces in proportion to their concentrations in the aqueous phase (Hunt & Dalgleish, 1994). This statement is further strengthened by recent studies (Ye, 2008), which indicate that the interfacial composition of emulsions made with mixtures of sodium caseinate and whey protein concentrate depend on the protein concentration. Caseins adsorb preferentially at the oil-water interface at high protein concentrations, whereas at low protein concentrations (< 3%), whey proteins adsorb in preference to caseins. The opposite was found by Dickinson & Golding (1997) who observed that emulsions made with 2% sodium caseinate were more unstable towards creaming than emulsions made with lower caseinate concentrations. This destabilization was attributed to depletion

flocculation caused by the presence of high concentrations of non-adsorbed caseinate. The stability of emulsions stabilized by milk protein is protein concentration dependent. Jafari et al. (2007) found that emulsions produced at 40 MPa for one cycle had a d3.2 equal to 573 and 268 nm, for lower and higher emulsifier contents, respectively. They attributed the decrease in the d3.2 when the protein concentration increased to the covering of more interfacial area, the higher rate of surface coverage and the lower rate of droplet collisions. All these reasons will lead to a lower re-coalescence and consequently, smaller droplet size.

At low caseinate/oil ratios, there is insufficient protein present to saturate the oil-water interface fully during emulsification, and so the resulting emulsion is unstable to bridging flocculation. Conversely, at high caseinate/oil ratios, the presence of excess (non adsorbed) protein in the form of small caseinate aggregates (submicelles) may lead to poor creaming stability caused by depletion flocculation (Dickinson, Flint, & Hunt, 1989). Optimum stability is attained at intermediate concentrations of caseinate, just enough to allow full protein saturation coverage at the oil-water interface (Dickinson, 1999).

In O/W emulsions containing 30% soy oil and stabilized by sodium caseinate (0.5% to 3.0%), a concentration dependent tendency for oil droplets to flocculate was observed (Srinivasan, Singh, & Munro, 2001). When prepared with 2.0% caseinate, large irregular flocs appeared in the emulsion, and a further increase in caseinate concentration resulted in a network structure of flocs.

Dickinson, (2006) found that emulsions formulated with 0.5 and 1% SC destabilized mainly by creaming. For the 2% SC emulsion, both creaming and flocculation mechanisms, were involved. They found that concentrations below 0.5% SC seemed to be below the ones required for saturation monolayer coverage since creaming rate was greater for 0.5% than for 1% SC and further addition of protein led to high instability. At 2-4% SC range, there was flocculation and migration of flocculates. Indeed, it is known that at concentrations of protein of more than about 0.5% (with oil concentration of 20%), some of the protein remains unadsorbed, even after powerful homogenization where the concentration of protein is the limiting factor in the determination of the sizes of the droplets (Fang & Dalgleish, 1993). If homogenization is less extensive, then the proportion of protein which is adsorbed decreases.

According to Wang, Li, Wang, & Özkan (2010), the reasons for the decrease in particle diameter with increasing protein concentration could be due to: (1) the increase in protein concentration, which would increase the coverage of oil droplets thereby inhibiting the droplet aggregation; and (2) the collisions decrease of droplets covered with proteins due to the increase in the emulsion viscosity. Viscous effects taking place at the entrance and in the high-pressure valve gap could explain the better droplet splitting at higher protein contents. The frictional loss coefficient, that predicts mechanical shearing effects in the valve gap increases with the viscosity of the inlet fluid (Stevenson & Chen, 1997).

2.4.2.6. Dispersed-phase (oil) concentration

At constant energy density (e.g. emulsification pressure), particle size rises with increasing oil content. Some experiments by high-pressure valve homogenization (Phipps, 1985) or ultrasound emulsification (Abismail, Canselier, Wilhelm, Delmas, & Gourdon, 1999) have confirmed this trend.

There are a number of possible reasons: (1) higher oil contents increase the emulsion viscosity, and thereby droplet disruption becomes more difficult (Kolb, Herrera, Ferreyra, & Uliana, 2001); Moreover, during homogenization, the residence time of emulsifying molecules might not be sufficient in the valve of the homogenizer, because of the high viscosity, to allow their adsorption on the available droplet surface before droplet-droplet collisions occurred; (2) at constant emulsifier concentration, there may be an insufficient amount of protein present to completely cover the new droplets. An inadequate amount of protein in the aqueous phase could cause some aggregation of fat globules. Mohan & Narsimhan (1997) demonstrated that, in protein-stabilized emulsions, the rate of coalescence during homogenization is reduced due to repulsive interactions between droplets, and droplet coalescence is only significant when there is insufficient protein to completely cover the droplet interface. According to Desrumaux et al. (2000), as the fat content increases, the available proteins decrease, which favors oil droplet coalescence and therefore increases the mean droplet diameters; and (3) the rate of collision frequency and thus coalescence frequency is increased as the oil content increases. Tornberg, Olsson, & Persson (1990) and Srinivasan, Singh, & Munro (1996) attributed the increase of particle size with increasing the oil concentration to the greater

incidence of coalescence and bridging at higher oil concentrations, both of which lead to reduction in total fat surface area. Sun & Gunasekaran (2009) indicated that increasing oil phase volume fraction enhances collision frequency among oil droplets, and consequently the rate of flocculation.

Cortés Muñoz (2009) using different oil concentrations (15, 30 and 45%) and pressures up to 300 MPa in O/W emulsions stabilized by whey protein isolate (4%), reported that optimal droplet breakup was observed for 30% oil (w/w) and homogenization pressure ≥ 200 MPa. Floury et al. (2000) reported that ultra high-pressure homogenizing conditions with high oil content emulsions (> 40%) from shear-thinning behaviors (at 20 MPa) to Newtonian behaviors (at 300 MPa). A higher percentage of oil in the emulsions resulted in a larger mean droplet diameter for the same homogenizing conditions. They attributed this to the limitation on surface-active agents in the most oil concentrated emulsions.

2.4.2.7. pH and ionic strength

Protein stabilized emulsions are especially susceptible to pH and ionic strength changes due to the fact that the interfacial membranes that are formed by proteins are thin and electrically charged, so the major mechanism that will prevent/delay droplet aggregation is electrostatic repulsion (Dickinson, 2010).

Protein stabilized emulsions tend to flocculate at pH values that are close to their isoelectric point (pI) and when the ionic strength of the medium exceed droplet size of a certain level. This is because the electrostatic repulsion between the droplets becomes insufficient to overcome droplet attractive forces (McClements, 2004; Dickinson, 2010). Therefore, if the pH of the aqueous phase is altered such that the overall protein charge is lost, or if salt is added to screen the electrostatic interactions among droplets, the repulsive forces will be insufficient to prevent droplet aggregation (McClements, 2005).

2.4.2.8. Viscosity of the emulsion

Proteins and polysaccharides are commonly used together in oil-in-water food emulsions. Proteins are widely used as an emulsifier because they have an ability to

adsorb at the oil-water interface and stabilize the oil droplets, while polysaccharides are usually added to increase the viscosity of emulsion (Dickinson, 1995).

Cortés Muñoz et al. (2009) reported that the increase in the inlet fluid viscosity could (1) favour droplet breakup in the valve gap due to higher extensional stress that occurs in the UHPH process, meaning it could result from higher viscosity of the inlet fluid and/or from an overcrowding of the whole matrix, and (2) limit droplet collision and thus droplet re-agglomeration/coalescence downstream of the valve gap, where usually a turbulent flow prevails. In the case of continuous phase, with increasing viscosity, re-coalescence is reduced because the drainage time between droplets is extended while the collision time remains the same. Tesch & Schubert (2002) found that for O/W emulsions stabilized with protein (slow adsorption rate), at a sufficiently high continuous phase viscosity (by adding some stabilizers), the same d3.2 was obtained as by using a fast stabilizing surfactant. Dalgleish & Hollocou (1997) studied the interaction between pectin and sodium caseinate in emulsions. It was found that very low concentrations of pectin can protect sodium caseinate coated droplets against aggregation at pH $\leq$ 5.0 and can bind to the surface of emulsion droplets at pH value above the isoelectric point.

2.5. Lipid oxidation in food emulsions

Many lipid containing processed foods are either oil-in-water or water-in-oil emulsions such as milk, infant formula, salad dressing, mayonnaise, sauces, soups, beverages, cream, and some desserts (McClements & Decker, 2000; Okuda et al., 2005). As well as the food industry, the cosmetics, pharmaceutical and medical industries also utilize oil-in-water emulsions as a means to encapsulate, protect, and release bioactive lipids in their products. However, these industries face a major problem regarding utilizing an oil-in-water emulsion because they can undergo lipid oxidation which then causes a deterioration of the product.

Lipid oxidation is a great concern for the food industry because it causes deterioration to lipid containing food products, even in foods that contain only small amount of lipids such as vegetable products. Not only does lipid oxidation cause undesirable changes in appearance, texture and development of rancidity that shortens product shelf life, but it also causes losses in important nutrients and formation of potentially toxic reaction

products (such as aldehydes and ketones) which cause important health concern for consumers (McClements & Decker, 2000; Chaiyasit, Elias, McClements, & Decker, 2007). Therefore, retarding lipid oxidation is necessary in order to extend the shelf life of the products as well as to maintain nutrition functionality of the lipid with a benefit of reduction of raw material wastes (Chaiyasit et al, 2007).

It is very important for the lipid chemists to understand the mechanisms of the lipid oxidation thoroughly to be able to utilize it as a basic fundamental to develop the proper methods to retard lipid oxidation.

The oil-in-water emulsions can be differentiated into three different regions; the emulsion droplet's lipid core, the interfacial membrane of the emulsion droplet, and the continuous phase (Fig. 17). It has been suggested that the polar molecules are located in the aqueous phase while non-polar molecules are mostly located in the oil droplets and surface active or amphiphillic molecules are accumulated at the interface (McClements and Decker, 2000; Chaiyasit et al., 2007). The reactants that influence in lipid oxidation can partition in these different regions, resulting in different lipid oxidation rates and mechanisms in oil-in-water emulsions than in bulk oils (Nuchi, McClements, & Decker, 2001).

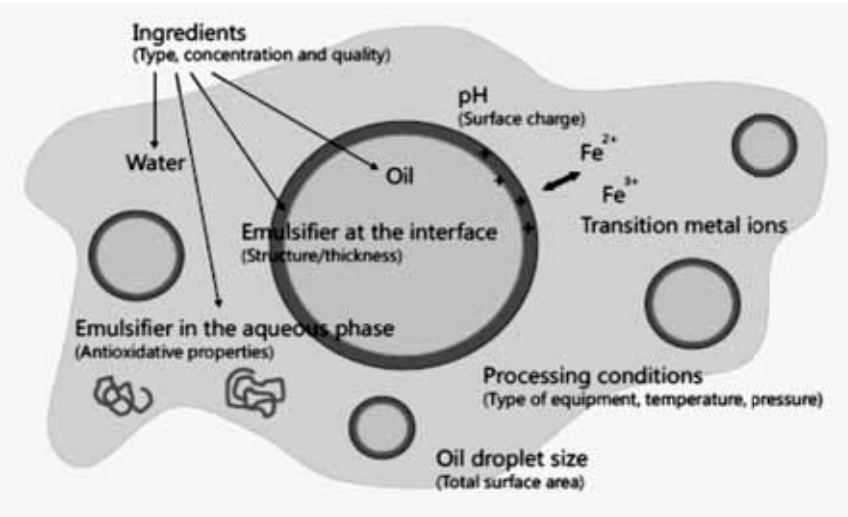

Figure 17. Parameters influencing lipid oxidation in emulsions (Horn, 2012).

2.5.1. General oxidation chemistry and mechanisms

Traditionally, lipid oxidation is assumed to be an autocatalytic "free radical chain reaction". However, food products often contain pro-oxidants that can initiate lipid oxidation reactions, such as transition metals (e.g., iron and copper), photo-sensitizers,

and enzymes (e.g., lipoxygenases). In addition, food products are often exposed to harsh environmental conditions that can initiate lipid oxidation reactions, such as thermal processing or exposure to UV light.

Lipid oxidation is a complex series of reactions that can be summarized in an initiation stage, a propagation stage and a termination stage (Fig. 18).

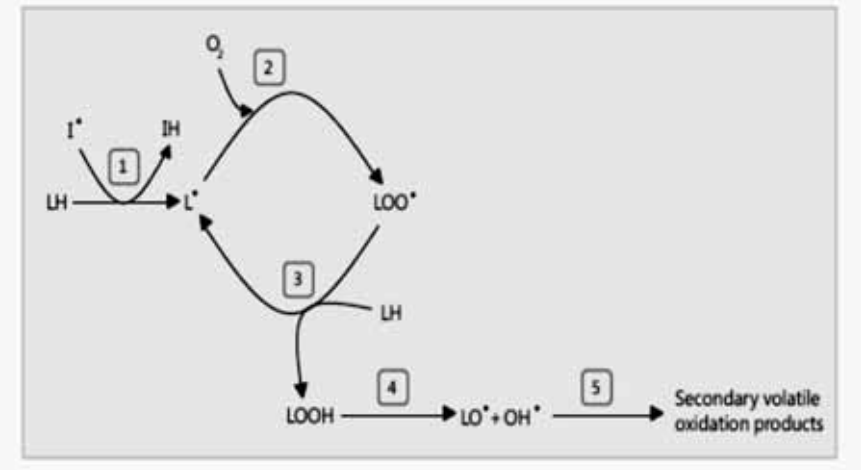

Figure 18. Oxidation steps (initiation, propagation and termination stages).

The initiation of autoxidation is dependent upon an initiator such as a free radical or a transition metal ion. By extraction of hydrogen from an unsaturated lipid (LH), a lipid radical (L•) is formed (Fig. 18.1). This lipid radical immediately reacts with atmospheric oxygen and generates a lipid peroxyl radical (LOO•) (Fig. 18, 2), and onsets the propagation of lipid autoxidation. The reaction between the lipid peroxyl radical and an unsaturated lipid leads to the formation of a new lipid radical (Fig. 18, 3), whereby the propagation can be continuously repeated. The other product of propagation is a lipid hydroperoxide (LOOH), which is recognized as a primary oxidation product. The type of lipid hydroperoxides generated is dependent on the initial lipid subjected to autoxidation. Thus, the autoxidation of e.g. α-linolenic acid (C18:3n-3) leads to the formation of four 9-, 12-, 13- and 16-hydroperoxides.

2.5.2. Measurement of lipid oxidation

Numerous tests exist to evaluate the oxidative stability of an emulsion. Oxidation measurements are typically carried out under standardized conditions to a suitable end point (Frankel, 2005).

Primary lipid oxidation compounds are the first oxidation products produced by the initiation and propagation steps of lipid oxidation. They can appear early in the oxidative deterioration of lipids. In the later stages of oxidation the concentrations of primary compounds decrease because their formation rates become slower than their decomposition rates. Peroxide value is one of the most commonly used methods for measuring the extent of oxidation in oils. The ferric thiocyanate method is more sensitive than other peroxide methods and requires a smaller sample size. This method is based on the oxidation of ferrous to ferric ions, which are determined colorimetrically as ferric thiocyanate (Shantha & Decker, 1994). In bulk oils, the peroxide value can be analyzed directly. In food systems, such as emulsions and muscle tissues, the lipid must first be extracted by mixing with solvents (Frankel, 2005). The peroxide value is an empirical measure of oxidation which is useful for samples that are oxidized to relatively low levels under mild conditions so that the hydroperoxides are not appreciably decomposed. During oxidation, the peroxide value reaches a maximum peak and then begins to decrease at more advanced stages of oxidation (Nawar, 1996; Frankel, 2005). The maximum peroxide value can occur at earlier or later stages depending on the fatty acid composition of the oil and the conditions of oxidation. For most polyunsaturated oils, such as fish oils, the peroxide value maximum will occur at an earlier stage because their hydroperoxides decompose more rapidly. Hydroperoxides will also rapidly decompose during oxidation conditions involving temperatures over 100°C, exposure to light and the presence of metals (Frankel, 2005).

Secondary lipid oxidation products are compounds that are formed from the decomposition of fatty acid hydroperoxides by means of β-oxidation reactions. The thiobarbituric acid reactive substances, or TBARS, method is used to measure the extent of secondary lipid oxidation products. During lipid oxidation, malonaldehyde (MA), a minor component of fatty acids with 3 or more double bonds, is formed as a result of the degradation of polyunsaturated fatty acids. It is usually used as an indicator of the lipid oxidation process, both for the early appearance as oxidation occurs and for the sensitivity of the analytical method (Cesa, 2004). In this assay, the MA is reacted with

thiobarbituric acid (TBA) to form a pink MA-TBA complex under thermal acidic conditions (Nawar, 1996; Frankel, 2005), that is measured spectrophotometrically at its absorption maximum at 530–535 nm (De las Heras, Schoch, Gibis, & Fischer, 2003). It must, however, be noted that alkenals and alkadienals also react with the TBA reagent and produce a pink color. Thus, the term thiobarbituric acid reactive substances (TBARS) is now used instead of MA. The TBA test is used frequently to assess the oxidative state of a variety of food systems, despite its limitations, such as lack of specificity and sensitivity (De las Heras et al., 2003). As already noted many other substances may react with the TBA reagent and contribute to absorption, causing an overestimation of the intensity of color complex. Despite its limitations, the TBA test provides an excellent means for evaluating lipid oxidation in foods, especially on a comparative basis.

Since these reactions can generate hundreds of volatile and nonvolatile compounds which would be impossible to measure simultaneously, methods such as GC detection are necessary and generally focus on analyzing a single compound or class of compounds.

Various ***chromatographic techniques***, including gas, liquid and thin-layer, have been used to determine oxidation in oil and lipid containing foods. These methods are based on the separation and quantification of specific fractions or individual components that are typically known to be produced during autoxidation (Nawar, 1996). The dynamic headspace method, commonly known as purge-and-trap, includes the following steps: 1) adding a sample to a sealed tube or vessel, 2) trapping the vaporized volatiles into a short column without cooling, 3) desorbing the volatiles from the trap and transferring by back flushing with a carrier gas into the capillary inlet of the gas chromatograph (GC), and 4) separating the compounds by GC. In this method, the recovery of a suitable internal standard subjected to the same conditions as the sample is the basis for the quantification of the volatile compounds. Volatile profiles can be greatly affected by the sampling temperature. Lower temperatures can yield a smaller percentage of volatiles contributing to the total peak area, whereas, higher temperatures yield larger percentages of volatiles contributing to the total peak area (Frankel, 2005).

In the present study, hydroperoxides as primary oxidation products and thiobarbituric acid reactive substances (TBARS) as secondary oxidation products have been determined.

2.5.3. Oxidative stability of milk protein stabilized emulsions

The emulsifier influences the oxidative stability of emulsions in two ways. Firstly through its ability to create a protective membrane around the oil droplets that can help inhibit lipid oxidation when they are positively charged (pH < protein pI) and can repel cationic transition metals when they form thick interfacial layers, and secondly by having different reactive groups with antioxidative properties.

Sodium caseinate and whey protein isolate in the continuous phase or as emulsifiers can enhance the oxidative stability of oil-in-water emulsions (Hu, McClements, & Decker, 2003; Faraji, McClements, & Decker, 2004).

McClements & Decker (2000) reported that WPI inhibits lipid oxidation in oil-in-water emulsions either at the emulsion droplet interface or in the aqueous phase. Osborn & Akoh (2004) reported that WPI was a better antioxidative surfactant than the low-MW surfactant. The relatively thick and viscoelastic interfaces formed by proteins around lipid droplets were accordingly suggested to be at least partly responsible for the highest oxidative stability of protein-stabilised emulsions, as compared to surfactant-stabilised emulsions (Fomuso et al., 2002; McClements & Decker, 2000).

Casein (CN) has been shown to be a more effective antioxidant than whey protein (Diaz & Decker, 2004; Hu et al., 2003a). When comparing emulsions prepared using the same homogenization equipment with different milk proteins (Horn, Nielsen, Jensen, Horsewell, & Jacobsen, 2012), WPI emulsions oxidized more than CN emulsions, as will be explained hereafter in section 2.5.4.7. However, some reports found similar lipid oxidation levels in emulsions containing casein and whey protein, and also showed that the level of oxidation was dependent on the total concentration of protein in the system (Hu et al., 2003b).

Several physicochemical mechanisms have been proposed for the antioxidant activity of these proteins. It has been shown that milk protein components absorb to the surface of the lipid droplet until the droplet surface is saturated with excess protein partitioning into the continuous phase (Faraji et al., 2004), creating interfacial layers of different thicknesses (Fang & Dalgleish, 1993; Hunt & Dalgleish, 1994). For instance, Berton, Ropers, Viau, & Genot, (2011) hypothesized that the best protection against oxidation in β-CN-stabilised emulsions probably resulted from the most important surface concentration of β-CN at the interface of oil droplets.

Some proteins contain appreciable amounts of amino acids that act as free radical scavengers, e.g., tyrosine, cysteine, and tryptophan (Taylor & Richardson, 1980). In general, the specific antioxidative feature of casein appears to be its chelating capacity as a result of its phosphoseryl groups (Gaucheron, Famelart, & LeGraet, 1996), and in the case of WPI appears to be its free-radical-scavenging activity as a result of free sulfhydryl groups (Faraji et al., 2004; Hu et al., 2003a; McClements & Decker, 2000). Caseins do not provide any free sulphydryl group, so its free-radical-scavenging activity would be expected to be lower than that of WPI. On the other hand, WPI has a limited ability to chelate metal ions, due to its lack of phosphoseryl groups. Nevertheless, these characteristics, phosphoseryl groups and free sulphydryl groups, do not contribute solely to the total antioxidative capacity of the respective protein.

Virtanen, Pihlanto, Akkanen, & Korhonen, (2007) reported the liberation of antioxidant peptides with high radical scavenging ability from both β- and αs-CN, whereas Peňa-Ramos, Xiong, & Artega, (2004) demonstrated inhibition of lipid peroxidation by whey protein-derived peptides with high prevalence of histidine and other hydrophobic amino acids.

The metal chelating properties of caseins have mainly been associated with their presence in the continuous phase (Berton et al., 2011; Faraji et al., 2004). It could potentially shield the oil against lipid oxidation if the metal ions are considered to be sufficiently far from the lipid surface. The radical scavenging properties of whey proteins have been shown to be highly dependent on the unfolding of proteins, since the reactive groups might otherwise be deeply buried within the core of the protein molecule (Elias, McClements, Decker, 2007). In the case of caseins adsorbed onto the fat droplets, they give physical protection to some external factors, but also these proteins have the ability of binding pro-oxidant metals to their phosphoserine residues.

2.5.4. Factors that impact lipid oxidation in emulsions containing milk protein

Several studies have been carried out to investigate how different factors affect the oxidation of lipids in emulsions. These factors are visualized in Figure 17, and elaborated upon in the following sections.

2.5.4.1. Droplet size and interfacial area

The interfacial area of an emulsion depends on the droplet concentration and particle size: $A = \phi / 6\, d3.2$, where A is the interfacial area per unit volume of emulsion, ϕ is the disperse phase volume fraction, and d3.2 is the surface-weighted mean diameter. The size of the droplets in a food emulsion, and therefore the interfacial area, vary in different food products. Droplet diameters can vary from larger than 100 μm in salad dressings and mayonnaise to less than 0.2 μm in cream liqueurs and soft drinks. Since lipid oxidation reactions in emulsions are greatly influenced by surface interactions between metals and hydroperoxides, one would expect droplet surface area also to be an important factor (McClements & Decker, 2000; Lethuaut, Metro, & Genot, 2002). Nevertheless, studies of the effect of droplet size on lipid oxidation in O/W emulsions are conflicting.

Some studies have found that the rate of lipid oxidation increased when the surface area increased (Gohtani, Sirendi, Yamamoto, Kajikawa, & Yamano, 1999). In mayonnaise, lipid oxidation was observed to progress faster in smaller droplets than in larger ones in the initial part of the storage period, whereas no dependence on droplet size was observed in oxidative flavor deterioration in the later part of the storage period (Jacobsen et al., 2000). Similarly, smaller droplets were observed to oxidize faster than larger droplets in the initial part of the storage of O/W emulsions stabilized by bovine serum albumin when the oxygen was not limited (Lethuaut et al., 2002). However, after 24 hours no difference was observed in the development of volatile secondary oxidation products. In accordance with these studies an increase in the oil volume fraction of caseinate and Tween20 stabilized O/W emulsions, resulted in a better oxidative stability (Kargar, Spyropoulos, & Norton, 2011). This observation was explained by a concomitant decrease in oil droplet surface area through an increase in droplet size and thereby a reduction in the exposure to iron in the aqueous phase.

In contrast, other studies have shown no correlation between oil droplet size and lipid oxidation (Gohtani et al., 1999; Sun & Gunasekaran, 2009).

Lipid oxidation might not only exist as a result of oil droplet size, but rather as a result of a combination of factors involved in the macrostructure of the emulsion. For example in milk, where oil droplet size was decreased by an increase in homogenization pressure, the protein composition at the interface was shown to be influenced by the pressure as well (Let, Jacobsen, & Meyer, 2007b; Sørensen et al., 2007). Thus, lipid oxidation was shown to be more influenced by the protein composition at the interface than by the actual droplet size. For instance, the radius of a droplet, if regarded as a sphere, is proportional to the ratio between its volume and its surface area. Therefore, the ratio between the volume of the lipid core and the surface covered by caseinate decreases with decreasing droplet size or, in other words, more caseinate covers fewer lipids per droplet. At the same time, the total droplet surface increases and, overall, more caseinate can interact with the emulsion lipid. This close interaction could enhance the antioxidative effect of caseinate. In the study of Shen, Udabage, Burgar, & Augustin, (2005) in microfluidized WPI-based emulsions, the total particle surface area was about seven times that of the corresponding homogenized emulsion, whereas the amount of propanal formed by the microfluidized emulsions was only about 1.5 times that formed by homogenization during 8 wk of storage.

2.5.4.2. Droplet charge and emulsion pH

The oxidative stability of O/W emulsions depends on the electrical charge on the droplet surfaces (Silvestre, Chaiyasit, Brannan, McClements, Decker, 2000; Boon et al., 2008). The impact of droplet surface charge has also been observed in O/W emulsions stabilized by proteins, where the rate of lipid oxidation was faster when the protein-coated droplets were anionic (pH > pI) than when they were cationic (pH < pI) (Hu et al., 2003a; Trunova et al., 2007; Djordjevic et al., 2008). Hu et al. (2003a) found that the oxidation of cationic emulsion droplets produced by emulsifying oil with proteins at pH 3.0 varied as a function of protein type. In this experiment, oxidative stability was in the order sodium caseinate > whey protein isolate > soy protein isolate. Several studies have shown that anionic surfactants (such as sodium dodecyl sulfate, SDS) at droplet surfaces promote lipid oxidation by attracting cationic transition metals to the surfaces

(e.g., Fe^{2+} or Fe^{3+}), whereas cationic surfactants (such as dodecyl trimethyl ammonium bromide, DTAB) retard lipid oxidation by repelling these transition metals away from the surface (Silvestre et al., 2000; Boon et al., 2008).

The density of the cationic charge of the emulsion droplets did not correlate with oxidative stability, suggesting that other factors such as droplet interfacial thickness and/or the antioxidant properties of the protein were also involved in the ability of the interfacial proteins to inhibit oxidation at pH 3.0. The impact of negative surface charge on the rate of lipid oxidation in protein-stabilized emulsions was reported by Villiere, Viau, Bronnec, Moreau, & Genot, (2005). This study compared stripped sunflower O/W emulsions (30 vol %) stabilized by sodium caseinate or bovine serum albumin at pH 6.5. The droplets in the sodium caseinate-stabilized emulsions had a substantially higher negative charge than those in the bovine serum albumin-stabilized emulsions. Presumably the transition metals ions were more strongly attracted to the surfaces of the lipid droplets in the NaCas-stabilized emulsions.

Controlling the electrical charge on emulsion droplets is therefore one of the most important potential means of impacting lipid oxidation in O/W emulsions. If the droplets in an emulsion can be made to be neutral or positive, then they are less likely to attract the cationic transition metal ions that frequently catalyze lipid oxidation in emulsions.

2.5.4.3. Interfacial thickness

Emulsion droplets are surrounded by the continuous phase in which the droplets are formed and dispersed. As droplets move close to each other, a thin layer, usually called thin film, of the continuous phase is formed between the droplets. As long as this film exists, there is no droplets contact, due to hydrodynamic resistance induced by the presence of the thin film (Sanfeld & Steinchen, 2008).

Proteins in the emulsion decrease the interfacial tension; it tends to be higher than in the case of other surfactants. They form a thick protective interfacial film at the droplets interface and may provide strong electrostatic repulsive forces between droplets.

Interfacial thickness can be manipulated by selecting emulsifiers with different molecular dimensions (e.g., molecular weights, conformations, head group sizes, or tail group sizes), or by using the layer-by-layer (LbL) deposition method to deposit one or

more biopolymer layers around droplets (Shaw, McClements, & Decker, 2007; Djordjevic, Cercaci, Alamed, McClements, & Decker, 2007).

Emulsifiers with large molecular dimensions can be used to form thick interfacial coatings around droplets that may protect against lipid oxidation. For example, the coating could form a barrier that decreases interactions between lipids and hydroperoxides or between lipids and aqueous phase prooxidants e.g. transition metals (Silvestre, et al., 2000; Chaiyasit, Silvestre, McClements, & Decker, 2000). The influence of surfactant head-group size on lipid oxidation in salmon O/W emulsions was studied using Brij 76 and Brij 700 as surfactants (Silvestre et al., 2000). The results showed that Fe^{2+}-promoted decomposition of cumene hydroperoxide was lower in emulsions made with Brij 700 (10 times more polyoxyethylene groups than Brij 76), which was attributed to a thicker interfacial layer on the emulsion droplets. The effect of surfactant tail group size has also been studied using Brij-lauryl (contains 12 carbon atoms) and Brij-stearyl (contains 18 carbon atoms) by Chaiyasit et al. (2000). This study suggested that surfactant tail group size played a minor role in lipid oxidation in O/W emulsions, with increasing tail group size slightly increasing oxidative stability.

The thickness of the interfacial membrane may be affected by the concentration of protein available in the emulsion elaboration. Not only can the protein concentration affect the interfacial thickness of O/W emulsions, but the protein type which determines the amino acid composition may also affect the adsorption rate and in turn the interfacial thickness. Cornacchia & Roos (2011) reported that despite the fact that WPI forms a multilayer structure at the O/W interface, the sodium caseinate monolayer was more effective in protecting β-carotene from degradation. This was presumably due to the different amino acid composition, which resulted in a different radical scavenging property, and the different thickness of the interfacial membrane.

2.5.4.4. Emulsifier type and concentration

Since droplet sizes are about the same for most emulsions, it may be deduced that emulsifier concentration, rather than droplet size distribution, causes changes in oxidation properties. At higher surfactant concentrations, the packing of surfactant molecules at the oil-water interface is tighter; hence, the membrane acts as an efficient barrier to the diffusion of lipid oxidation initiators into the oil droplets (Coupland,

McClements, 1996). The presence of oligo- or multilayers of surfactants at the oil/water interface at high surfactant concentration may play a role in reducing the entry of pro-oxidants into the oil droplets.

Studies in the literature indicated that high concentration of whey protein in emulsion systems decreases the oxidation rate. This increase of WPI is expected to be adsorbed at droplet surfaces, which renders better protection against oxidation since WPI can act as an effective antioxidant in the emulsions (Sun, Wenbin, Dejun, Yunping, Shangyin, & Shuiyan, 2007). Sun & Gunasekaran, (2009) reported that a higher concentration of WPI was more effective than a lower concentration of WPI for protection against oil oxidation, as evidenced by a significantly higher concentration of propanal in 1% WPI emulsions compared with 5% WPI emulsions produced by the same emulsification procedure. They attributed this oxidative stability, when high protein concentrations (5%) were used, to more excess protein in the continuous phase than would occur with 1% WPI emulsions. Hu et al. (2003b) suggested that the interface of the emulsion droplets became saturated with 0.2% (w/w) WPI in an O/W emulsion at pH 7 containing 5% oil and homogenized at 34.4 MPa, and that further increases in WPI left an excess of WPI in the continuous phase. Since WPI has antioxidative properties, it is possible that the excess WPI in the continuous aqueous phase contributed to the better oxidative stability of the oil (Falch, Anthonsen, Axelson, & Aursand, 2004). Another potential factor that could have contributed to the oxidative stability of the emulsions stabilized by 5% WPI was the thicker barrier of protein at the interface.

Several studies conducted on the oxidation of O/W emulsions, with casein, have shown that the rate of lipid oxidation tends to decrease with increasing levels of casein (Faraji et al., 2004; Kargar et al., 2011). O'Dwyer, O'Beirne Eidhin, & O'Kennedy, (2013) reported that particle size decreased as a result of increasing microfluidization pressure and sodium caseinate concentration, which in turn decreased lipid oxidation product formation. The reasoning behind this was suggested to be that sodium caseinate was available to surround the high surface area. The authors reported that the emulsions stabilized using lower levels of sodium caseinate had higher oxidation products (hydroperoxides and ansidine value), probably because emulsions did not have enough sodium caseinate to surround the droplets and cover such a large surface area. At higher surfactant concentrations, the packing of surfactant molecules at the oil-water interface is tighter; hence, the membrane acts as an efficient barrier to the diffusion of lipid

oxidation initiators into the oil droplets (Coupland, McClements, 1996). Casein can form a thick interfacial layer around dispersed oil droplets of up to 10 nm, which is a high packing rate compared to other emulsifiers i.e. whey proteins (1-2 nm) (Dalgleish, Srinivasan, & Singh, 1995). Furthermore, at concentrations of protein of more than about 0.5% (with oil concentration of 20%), some of the protein remains unadsorbed, even after powerful homogenization, which may be considered as another reason behind the high oxidative stability in sodium caseinate emulsions containing high amounts of protein in the aqueous phase, which in turn may act as antioxidants with metal ions, or by scavenging free-radicals in the aqueous phase (Sun & Gunasekaran, 2009).

2.5.4.5. Oil type, concentration and quality

Studies on protein stabilized O/W emulsions with varying volumes of the oil fraction have shown that a high oil fraction decreases lipid oxidation in safflower oil, (Sims, Fioriti, & Trumbetas, 1979), canola oil (Osborn & Akoh, 2004), Menhaden oil (Sun and Gunasekaran2009) and Walnut oil (Gharibzahedi, Mousavi, Hamedi, Khodaiyan, & Razavi, 2012). These findings have been related to differences in oil droplet size and thereby the protein availability for each oil droplet (Kargar et al., 2011; Sun & Gunasekaran, 2009). Sun & Gunasekaran (2009) reported that lipid hydroperoxides were significantly decreased as the oil content increased from 5 to 20%, but further increase in the oil content to 40% affected the oxidative stability in a bad way. They attributed this increase in the oxidation rate in emulsions containing lower oil fraction to the increase in the number of radicals produced per oil droplet. However, at higher oil fraction more unsaturated fatty acids may have moved into the interior of the oil droplet, and thus these fatty acids became less accessible to direct interaction with the pro-oxidants at the interface. A more intensive oxidation process was measured in the emulsions with 30% olive oil than in the emulsions with 70% oil. The increase of the oil phase resulted in closely packed droplets and delay in oxidation. An increase in the oil volume fraction of caseinate and Tween 20 stabilized O/W emulsions, resulted in a better oxidative stability (Kargar et al., 2011). This observation was explained by a concomitant decrease in oil droplet surface area through an increase in droplet size and thereby a reduction in the exposure to iron in the aqueous phase.

In addition, oil quality might have an influence on the oxidative stability of emulsions, since low quality oil with a high concentration of lipid hydroperoxides already present will oxidize faster than good quality oil. In fish oil enriched milk, even a slightly increased peroxide value in the fish oil added during production resulted in a less oxidatively stable final product (Let, Jacobsen, & Meyer, 2005).

2.5.4.6. Emulsion viscosity

Several research groups (Sims, 1994; Imagi et al. 1992; Hsieh, and Harris, 1987) have supported the view that elevated viscosities of the continuous aqueous phases of emulsions containing dissolved polyols inhibit oxygen diffusion and thereby cause a suppression of the oxidation of disperse phase lipids. According to Serferta et al. (2011), the higher viscosity of the multilayer emulsion, as a direct result of adding lecithin/chitosan, contributed to the higher oxidative stability of emulsions. They reported that the viscosity of the continuous phase in emulsions influences the diffusion of pro-oxidants to the droplet interface. However, Let, Jacobsen, Sørensen, & Meyer, (2007a) when studying the oxidative stability of milk, yoghurt and salad dressings observed that the least viscous milks were most oxidized; the dressings were more viscous than the yoghurts but were also more oxidized than the yoghurts. They reported that no direct relationship between viscosity and oxidation was indicated in the data.

2.5.4.7. Homogenization equipment and processing conditions

As mentioned previously the obtainable droplet sizes in different homogenization equipments vary. Hence, the choice of homogenization device might indirectly affect lipid oxidation through the oil droplet sizes produced as explained here before. Furthermore, different high-pressure homogenization equipments have been shown to differ with respect to their generation of heat (Mao, Yang, Xu, Yuan, & Gao, 2010), which is another factor that can potentially influence lipid oxidation. Finally, studies in milk have shown that the protein structure at the interface differs depending on the type of high-pressure homogenizer used, due to differences in the geometries of the interaction chambers (Dalgleish et al., 1996). Hence, lipid oxidation studies in this area are needed.

Apart from the emulsification principle homogenization conditions could also potentially influence lipid oxidation. In mechanical homogenization devices the only parameter that can be varied is the speed of rotation, which will eventually influence the resulting oil droplet size and may also lead to oxygen inclusion in the emulsion. In addition, droplet disruption by cavitation and subsequent rearrangement of oil droplets during homogenization promote distribution of oxygen, catalysts and lipid oxidation products among the newly arranged oil droplets and may thus accelerate the lipid oxidation.

In high-pressure homogenizers the main parameters that can be varied are the pressure applied and the inlet temperature. Increasing the pressure or the number of passes through the interaction chamber reduces droplet size (Qian & McClements, 2011). Nevertheless, lipid oxidation studies on emulsions prepared with caseinate, Tween 20 or whey protein concentrate have not been able to confirm a relationship between oxidative stability, pressure and droplet size (Dimakou, Kiokias, Tsaprouni, & Oreopoulou, 2007; Kiokias, Dimakou, & Oreopoulou, 2007). Serra, Trujillo, Quevedo, Guamis, & Ferragut (2007) observed a strong increase in some methyl ketones such as 2-propanone and 2-butanone at the end of storage, especially in yogurts from milk treated at 200 MPa and 300 MPa, as a result of the β-oxidation of saturated FFA, which could interfere in the quantification of lipid peroxidation. Pereda et al. (2008) observed low primary (hydroperoxides) and high secondary (TBARS and hexanal) oxidation products immediately after milk treatment by UHPH at 300 MPa compared to UHPH samples treated at 200 MPa and pasteurized milk. They related the lower hydroperoxide value in combination with the higher levels of TBARS and hexanal to the progression of oxidation from a primary to a secondary state. The authors attributed this high sensitivity to oxidation in emulsions treated at 300 MPa to the temperature rise during UHPH; milk achieved temperatures around 100°C when it was treated at inlet temperatures of 30-40°C. In a recent study using high-pressure homogenization (20 and 80 MPa) up to 7 homogenization cycles to stabilize whey protein emulsions (3% protein) containing flaxseed oil (30%), Kuhn & Cunha (2012) showed that increasing the pressure to 80 MPa produced an increase in the formation of primary oxidation products in the emulsions in relation to the emulsions homogenized at 20 MPa. They attributed this increase in the oxidation to the increase in temperature observed in these emulsions.

In contrast, Let et al. (2007a) hypothesized that increasing the homogenization temperature from 50 to 72°C may lead to improved physical coverage of the oil droplets by proteins, most likely β-Lg, which starts to unfold above 65°C (Ye, Singh, Taylor, & Anema, 2004) and also contains amino acids with sulfhydryl groups, which have been shown to have antioxidant properties such as radical scavenging properties (Hu et al, 2003b). In a very recent study by Horn et al. (2012), working with sodium caseinate and whey protein isolate to produce submicron emulsions using different homogenization equipments (microfluidizer and high-pressure valve homogenizer), the homogenization pressure is not suggested to play a major role, since the same differences in oxidative stability were observed when emulsions were prepared on the two systems, using similar pressures. Neither peroxide value nor secondary volatile oxidation products differed between the two emulsions prepared with sodium caseinate when homogenized by the two different equipments. In contrast, the emulsion with WPI prepared on the valve homogenizer oxidized faster during storage than the similar emulsion prepared on the microfluidizer. The contents of secondary volatile oxidation products especially were significantly higher in the emulsion prepared on the valve homogenizer. In milk, where both casein and whey proteins are present, a competition occurs between the two types of milk proteins to reach the surface of the oil droplets during homogenization. Thus, it has previously been reported that when milk is homogenized in a conventional homogenizer, both casein and whey proteins are present at the oil droplet interface, whereas when milk is homogenized in a microfluidizer only casein is present at the interface (Dalgleish et al., 1996). Thus, a preferential adsorption of one type of milk protein (whey proteins and caseins) over the other is found, depending on the homogenizer used.

Some studies have shown that, it can be beneficial to pre-treat milk proteins such as whey proteins by heating prior to homogenization, as this could potentially unfold the protein, increase its emulsifying capacity and potentially expose antioxidative components that would otherwise be buried within the core of the protein (Kiokias et al., 2007). These authors showed a decrease in conjugated diene formation as a result of using pre-heated whey protein as emulsifier in 30% sunflower O/W emulsions. Accordingly, Elias et al. (2007) also observed a decrease in lipid hydroperoxides and TBARS formation upon heating of β-lg to 95°C when added to the aqueous phase of 5% Brij-stabilized menhaden O/W emulsions in a concentration of 500 μg protein/g oil.

Interestingly, heating to 95°C reduced the ability of β-lg to bind iron, but increased the ability of β-lg to scavenge peroxyl radicals. Regarding amino acid exposure an increase in tryptophan was observed, while a reduction in cysteine exposure was observed when the protein was heated above 70°C. Thus, it was suggested that the observed enhancement in the antioxidant activity of thermally denatured β-lg (95°C, 15 min) is related to an improved accessibility of radical scavenging amino acids.

2.6. References

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Chapter 3

Material and Methods

In this chapter, information about the materials used in the present study and their composition and characteristics will be given. Additionally, the emulsion preparation and the emulsification systems used in its elaboration are also detailed. Finally, the physical and oxidative stability analysis of emulsions will be established.

3.1. Ingredients

Milk proteins (mainly whey protein isolate and sodium caseinate) were selected due to their common use in food products, their milk origin and their potential antioxidative effects. A mixture of sunflower and olive oils in the ratio of 3:1 was used as a source of unsaturated and polyunsaturated fatty acids.

3.1.1. Characteristics of proteins and oils

Refined sunflower and olive oils were purchased from Gustav Heess Company (Barcelona, Spain). The characteristics and composition of oils are described in Table 1.

The concentration of oil used for preparing the emulsions was in the range of 10-50%. Emulsions with low oil concentrations (i.e. 10-20%) were selected, as these are well described in the literature and considered good systems for investigating a wide range of factors related to their production conditions. However, knowledge of emulsions containing high oil concentrations (i.e. 50%) is scarce. When the aim is to use the emulsion as a delivery emulsion, a high oil concentration is preferable, particularly in food products where addition of water changes its texture in an unwanted way.

Table 3. Chemical composition of sunflower and olive oils.

Chemical characteristics	Sunflower oil	Olive oil
Density at 20 ºC	0.921	0.913
Acid value	0.09 (mg KOH/g)	0.11 % (oleic)
Peroxide value (meqO_2/kg)	0.02	0.5
Absorbance at 270 nm	---	0.29
Unsaponifiable (% m/m)	<0.05	<1.5
Fatty acid composition (%)		
C 16 : 0	6.34	11.97
C 18 : 0	3.97	3.30
C 18 : 1	26.65	75.23
C 18 : 2	61.02	6.75
C 18 : 3	---	0.38

Whey protein isolate (WPI) was obtained from Lactalis (Prolacta 90, Retiers, France). The WPI contained 95.9 g dry solids per 100 g powder, and in dry basis (w/w), 1.04% non-protein nitrogen (NPN), 89.3% protein [(total N - NPN) × 6.38], 1.1% ash (including 0.27% calcium) and 1.6% lactose, as given by the producer. Protein constituents in the WPI corresponded mainly to β-lactoglobulin (β-Lg) and α-lactalbumin (α-La) (i.e. 68.5% β-Lg and 21.5% α-La per 100 g soluble protein) plus small amounts or traces of immune globulins, bovine serum albumin (BSA) and lactoferrin.

Sodium Caseinate (SC) was obtained from Zeus Quimica (sodium caseinate 110 Barcelona, Spain). The protein physicochemical and microbiological analysis, as indicated by the producer, is shown in Table 2.

3.2. Preparation of emulsions

3.2.1. Preparation of protein dispersions

WPI and SC dispersions containing 1, 2 and 4% and 1, 3 and 5%, respectively, were prepared using decalcified water by agitation with high speed mechanical blender (Frigomat machine) with two blenders at room temperature avoiding foam formation (Guardamiglio, Italy). Protein dispersions were stored overnight at 4ºC to allow protein hydration.

Table 4. Physicochemical and microbiological analysis of Sodium Caseinate 110

		Physical and chemical analysis		
Analysis	**Unit**	**Target value**	**Tolerance**	**Result**
Moisture	%	6	---	5.73
Granulometry	% < 300 μm	98	---	99.99
Cleanness	---	A	B	A
pH	---	6.7 ± 0.3	6.7 ± 0.4	6.7
Sediment (70°C)	%	< 0.2	< 0.5	0.05
Minerals	%	≤ 3.8	≤ 4	3.52
MAT (N X 6.38)	%	≥ 88	---	90
Fat	%	≤ 1.5	---	1
Density	---	0.40 to 0.50	---	0.42
Antibiotics	---	Absence	---	Absence
		Microbiological analysis		
Total plate count	cfu/g	≤ 1000	≤ 5000	100
Thermophillic count	cfu/g	≤ 500	≤ 2500	100
Enterobacteriaceae	cfu/g	≤ 10	---	< 10
Yeasts and Molds	cfu/g	≤ 20	≤ 50	< 10
Salmonella spp.	cfu/25g	Absence	---	Absence
Asian soybean rust (ASR) (37°C)	Spores cfu/g	≤ 10	≤ 30	< 10
***Staphylococcus* coag +**	cfu/g	Absence	---	Absence
Listeria monocytogenes	cfu/25g	Absence	---	Absence

3.2.2. Homogenization

After rehydration, protein dispersions and oil (10, 20, 30 and 50%) were equilibrated at 20ºC before mixing. Pre-emulsions (or coarse emulsions) were prepared by mixing the above protein dispersions with the oil mix (3 sunflower : 1 olive oil) using the colloidal mill homogenizer (E. Bachiller B. S.A, Barcelona, Spain) at 5000 rpm during 5 min at room temperature (CM emulsions).

The secondary emulsions were produced by the use of various equipments. Two homogenizers were used for obtaining the final emulsions, a Stansted high-pressure homogenizer and a pilot scale APV conventional homogenizer.

3.2.2.1. Ultra high pressure homogenization (UHPH)

Pre-emulsions were treated by UHPH using a Stansted high-pressure homogenizer (Model/DRG number FPG 11300:400 Hygienic Homogenizer, Stansted Fluid Power Ltd., UK) with a flow rate of 120 l/h. This equipment consisted of a high-pressure ceramic valve able to withstand 400 MPa, a pneumatic valve, located after the first one, able to withstand up to 40 MPa, and two intensifiers which were driven by a hydraulic pump. To minimize temperature retention after treatment, two spiral type heat-exchangers (Garvía, Barcelona, Spain) located behind the second valve were used. Emulsions were UHPH-treated at pressures of 100, 200 and 300 MPa (single-stage) with (Tin) of 25ºC (UHPH emulsions). Throughout the experiment, the inlet temperature, the temperature after the homogenization valve (T1) and the temperature of the outlet product (T2) were monitored.

3.2.2.2. Conventional homogenization

The conventional homogenization of the pre-emulsions was performed using APV Rannie Copenhagen Series Homogenizer (Model 40.120H, single stage hydraulic valve assembly, Copenhagen, Denmark) with Tin of 60ºC at 15 MPa for a single stage (CH emulsions).

The homogenization conditions used were optimized for the purpose of each experiment. The experimental designs for studies of simple emulsion systems are summarized in Table 3. The experiment in each study was repeated on three independent occasions.

Sodium azide (0.1% w/w) was added to the final emulsions in order to prevent microbial growth in the samples, which were used to assess the physical characteristics. All emulsions (CM, CH and UHPH) were characterized using the following analyses.

Table 5. Overview of the experimental approach in the studies of simple oil-in-water emulsions.

	Oil (%)	Protein type	Protein (%)	Homogenization
Study 1	20	WPI	1, 2, 4	CM, 5000 rpm / 5 min
			1, 2, 4	CH, 15 MPa
			1, 2, 4	UHPH, 100, 200 and 300 MPa
Study 2	10	WPI	4	CM, 5000 rpm / 5 min
	30		4	CH, 15 MPa
	50		4	UHPH, 100 and 200 MPa
Study 3	20	SC	1, 3, 5	CM, 5000 rpm / 5 min
			1, 3, 5	CH, 15 MPa
			1, 3, 5	UHPH, 100, 200 and 300 MPa
Study 4	10	SC	5	CM, 5000 rpm / 5 min
	30		5	CH, 15 MPa
	50		5	UHPH, 200 and 300 MPa

3.3. Emulsion analyses

3.3.1. Particle Size Distribution

The particle size distribution in the emulsion samples was determined using a Beckman Coulter laser diffraction particle size analyzer (LS 13 320 series, Beckman Coulter, Fullerton, CA, USA). Emulsion samples were diluted in distilled water until an

appropriate obscuration was obtained in the diffractometer cell. Emulsion samples were also diluted in sodium dodecyl sulphate (SDS) 1 g/l at least 30 min before light scattering analyses as a dissociation medium to check for the possible presence of aggregated or coalesced droplets that could be (if aggregated) or not (if coalesced) dissociated by SDS (Pearce & Kinsella, 1978). An optical model based on the Mie theory of light scattering by spherical particles was applied by using the following conditions: real refractive index of the oil mixture (15% sunflower oil + 5% olive oil) which was obtained by refractometric measurement (Spectronic Instruments, Inc. Rochester, New York, USA), 1.471; refractive index of fluid (water), 1.332; refractive index of the protein was assumed to be 0 (Hemar, Tamehana, Munro, & Singh, 2001); imaginary refractive index, 0; pump speed, 20%. The volume-weighted mean diameter (d4.3, µm), in the presence and absence of SDS, the surface-weighted mean diameter (d3.2, µm) and the specific surface area (SSA, m^2/ml) were determined. Each diluted sample was analyzed, at least, four successive times in the diffractometer to obtain a mean size distribution curve and the corresponding mean values.

3.3.2. Surface protein concentration

Surface protein concentration of oil droplets was determined according to the method of Desrumaux & Marcand (2002). Emulsion samples were centrifuged at 20000 × *g* for 30 min in a temperature controlled centrifuge at 25ºC (Sigma laboratory centrifuge, 4K-15, SN. 93250, Osterode am Harz, Germany) in order to separate the droplets from the aqueous serum phase. The cream layer was carefully removed from the aqueous phase using a spatula. The cream layer was re-suspended in ultra-pure water to wash away any protein trapped between droplets, and the resulting emulsion was centrifuged again at 20000 × *g* for 30 min. The protein content in the isolated purified protein layers was determined in triplicate by the Dumas method with a Leco FP-528 nitrogen/protein instrument (Leco Corp., St. Joseph, MI, USA), calculating crude protein content as N × 6.38. Protein coverage (mg/m^2) was calculated by dividing the amount of protein per gram of washed cream by the SSA of fat globules (Lee & Sherbon, 2002).

3.3.3. Rheological measurements

Rheological measurements were performed using a controlled stress rheometer (Haake Rheo Stress 1, Thermo Electron Corporation, Karlsruhe, Germany) using a con (1º, 60 mm diameter) and plate geometry probe at 25 ºC. Prior to analysis, the sample placed in the rheometer cell rested for 5 min, allowing the stress induced during loading to relax and thus avoiding any structure destruction. Flow curves were fitted to the Ostwald de Waele rheological model: $\tau = K (\gamma^{\cdot})^n$ and the consistency coefficient (K, mPa × s) and flow behavior index (n) were obtained. All viscosity parameters were the mean of three measurements per sample.

3.3.4. Emulsifying properties

Emulsifying activity index (EAI) was determined according to the method of Pearce and Kinsella (1978) with a minor modification by Tang et al. (2005), and it expresses the emulsifying properties of proteins in the oil/water interface area (m^2) (Pearce and Kinsella, 1978; Moure et al., 2005). Aliquots (100 µl) were taken from the emulsion and then added to 10 ml of 0.1% (w/v) SDS solution to give absorbance from 0.01-0.6. After vortex mixing, the absorbance of the diluted mixture solution was recorded at 500 nm in the UV-visible spectrophotometer (CECIL model 9000 series, Cambridge, UK). EAI value was calculated using the following equation,

$$\text{EAI } (m^2/g) = \frac{2 \times 2.303 \times A_0 \times DF}{C \times Ø \times (1-\theta) \times 1000}$$

where (DF) is the dilution factor which could be changed from emulsion to another depending on the particle size and particles concentration, taking into account that the absorbance should not exceed 0.6 (i.e. 250 times for CM emulsions and 2500 times for CH and UHPH emulsions), (C) is the initial concentration of sample (g/ml), (θ) is the fraction of oil used to form the emulsion (0.2 for 20% oil), and (Ø) is the optical path

(0.01m). Emulsions were analyzed immediately after production expressing the emulsifying activity index (EAI). Measurements were performed in triplicate.

3.3.5. Physical stability

Physical stability was visually measured in the emulsions by storing the samples in 50 ml plastic tubes for 20 days at 20°C and observing any cream layer at the top of the tubes.

Physical stability was also assessed in the emulsions, measuring the d4.3 value at the top or at the bottom of the emulsion tubes stored at room temperature for 9 days and under the same conditions for comparison. Physical stability was determined in the homogenized emulsions (conventional and UHPH), but not in the CM emulsions where oily or creamy phases were clearly separated from the aqueous phases on the same day of preparation.

The stability of emulsions was also determined through the use of the vertical scan analyzer Turbiscan MA 2000 (Formulaction, Toulouse, France). This equipment allows the optical characterization of any type of dispersion (Mengual, Meunier, Cayré, Puech, & Snabre, 1999). The reading head is composed of a pulsed near-IR light source (λ=850 nm) and two synchronous detectors (Fig. 1).

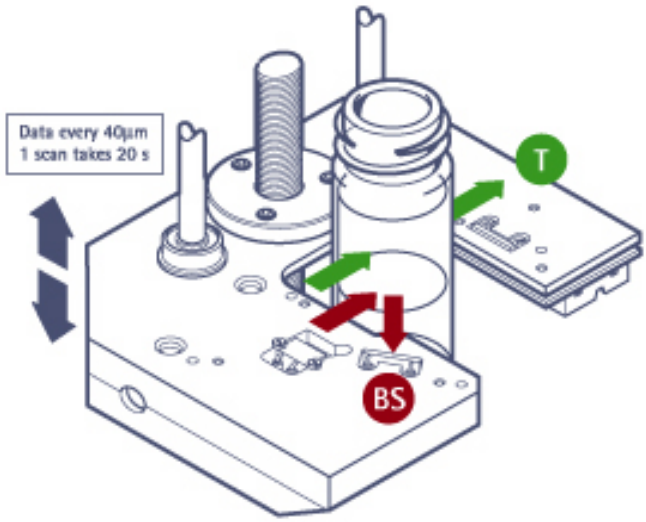

Figure 19. The reading of a pulsed near-IR light source and two synchronous detectors.

The transmission detector receives the light, which goes through the sample (0°), while the back-scattering detector receives the light back scattered by the sample (135°). The intensity of the light backscattered by the sample depends on three parameters: the diameter of the particles, their volume fraction and the relative refractive index between the dispersed and continuous phases. Therefore, any change due to a variation of the particle size (flocculation, coalescence) or a local variation of the volume fraction (migration phenomena: creaming, sedimentation) is detected by the optical device as could be seen in Figure 2.

The measurement protocol using the Turbiscan should be set depending on the expected shelf life of the products tested, taking the reference samples as the base. According to the application note of Turbiscan, if a product is designed to be stable for several hours/days, it is recommended to leave the sample inside the Turbiscan and run analyses for a few hours (i.e. 1 scan every minute for 1 h + 1 scan every 10 min for 5 h). If a product is expected to be stable for several months, it is better to store the samples in a thermo-regulated chamber and analyze them manually (e.g. 2 scans a day for 15 days). In the present study, the Turbiscan Lab, in the backscattering mode, measured the light backscattered by the sample, which is directly dependent on the particle mean diameter, at preset intervals (30 min for CM emulsions, 3 days for CH and UHPH emulsions) over a predetermined period of time (5 h for CM emulsions and 17 days for CH and UHPH emulsions). Any particle aggregation would cause a change in the amount of back-scattered light (ΔBS), which is taken as a measure of the stability of the emulsion (Lemarchand, Couvreur, Vauthier, Costantini, & Gref, 2003). Creaming was detected using the Turbiscan as it induced a variation of the concentration between the top and the bottom of the cell. The migration velocity V (t) (μm/min) of the clarification front was also calculated in order to follow the kinetics of the creaming phenomenon.

3.3.6. Emulsions microstructure

In order to assess the microstructure of emulsions, laser confocal scanning and transmission electron microscopes were used.

Confocal laser scanning microscopy observations were performed in fluorescence mode essentially as Michalski, Michel, & Geneste (2002) described. The protein was stained by the fluorescent dye, fluorescein isothiocyanate (FITC; Fluka, Steinheim, Germany), and the fat globules were stained by Nile red (Sigma, Steinheim, Germany). The FITC and Nile red were dissolved in ethanol at a concentration of 2 and 1 mg/ml, respectively. Emulsions (10 ml) warmed at 32 ºC were dyed with 2 drops of FITC and 3 drops of Nile red. Then, 3 to 4 drops of the labeled emulsions were transferred to microscope slides with concave cavities, covered with a cover slip, sealed to prevent evaporation. The confocal microscope (Leica TCS SP2 AOBS, Heidelberg, Germany) was equipped with an oil-coupled Leica objective with a 63× augmentation and a numerical aperture of 1.4. Fluorescence from the samples was excited by the 488 nm line of an argon laser. Images were acquired in 2 channels simultaneously (501 to 549 nm and 574 to 626 nm) as 1,024 × 1,024 pixel slices in the horizontal x–y plane along the z plane at constant gain and offset.

To examine the changes in emulsion microstructure, emulsion samples were observed by transmission electron microscopy, preparing samples as described by Cruz et al. (2007). Emulsions were mixed with warm 2% low-temperature gelling agarose (type VII, Sigma Aldrish) at a 1:1 ratio. The mixture was allowed to gel and was chopped into 1 mm^3 cubes. The cubes were fixed using glutaraldehyde (3% final concentration) and were then washed as follows: with 0.1 M sodium cacodylate buffer pH 7.2 for 30 min, then again twice for 1 h with 1 ml of a solution containing 50% osmium tetroxide (2% solution) and 50% cacodylate/HCL buffer for 2 h, with 1 ml of 1% uranium acetate for 30 min, followed by two washes with deionized water and a sequential dehydration in ethanol. Samples were embedded in Eponate 12™ resin (Ted Pella Inc., Redding, California) and polymerized at 60ºC for 48 h. Semithin sections (0.03-0.05 μm thick) were cut with a Reichert ultracut microtone, placed on non-coated 200 mesh copper grids and contrasted with conventional uranyl acetate (30 min) and Reynolds lead citrate (5 min) solutions. Sections were observed with a Jeol 1400 transmission electron microscope (Jeol Ltd, Tokyo, Japan) equipped with a Gatan Ultrascan ES1000 CCD Camera.

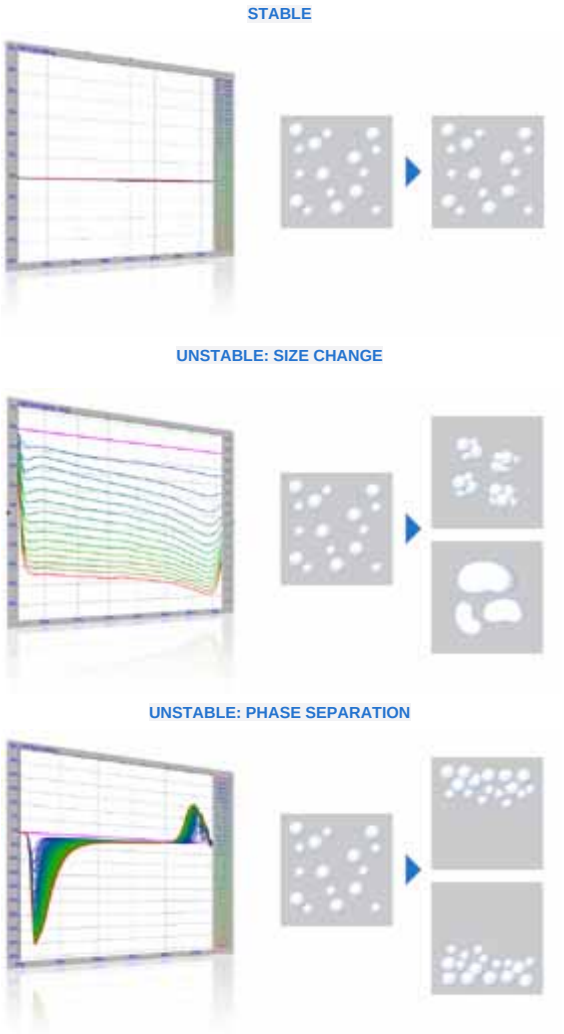

Figure 20. destabilization phenomena predicted by the Turbiscan lab.

3.3.7. Oxidative stability

Emulsions were stored at 10 ºC in glass transparent bottles for 10 days under light (2000 lux/m^2) in order to analyze the samples on the first and the last day of storage.

For the determination of primary oxidation products, lipid hydroperoxides were measured by mixing 0.3 ml of emulsion with 1.5 ml of isooctane/2-propanol (3:1, v/v) by vortexing (10 s, three times) and isolation of the organic solvent phase by centrifugation at 1000 × *g* for 2 min. The organic solvent phase (200 µl) was added to 2.8 ml of methanol/1-butanol (2:1, v/v), followed by 15 µl of 3.97 M ammonium thiocyanate and 15 µl of ferrous iron

solution (prepared by mixing 0.132 M $BaCl_2$ and 0.144 M $FeSO_4$). The absorbance of the solution was measured at 510 nm, 20 min after addition of the iron (Shantha and Decker, 1994). Hydroperoxide content was expressed as absorbance (A_{510}).

For the determination of secondary oxidation products, thiobarbituric acid-reactive substances (TBARs) were determined according to an adapted method of McDonald and Hultin (1987). The emulsion (1.0 ml) was combined with 2.0 ml of TBA solution (prepared by mixing 15 g of trichloroacetic acid, 0.375 g of thiobarbituric acid, 1.76 ml of 12 N HCl, and 82.9 ml of H_2O) in test tubes and placed in a boiling water bath for 15 min. The tubes were cooled to room temperature for 10 min and then the colored solution was separated by filtration through a glass wall. The absorbance was measured at 532 nm. Concentrations of TBARS were calculated from a standard curve prepared with 1, 1, 3, 3-tetraethoxypropane.

3.3.8. Statistical analyses

3.3.8.1. General statistical analysis

Descriptive statistics, mean and standard deviation, were listed for each variable in this study. In order to evaluate the physical and oxidative stability of emulsions among type of emulsion (CM, CH or UHPH) and concentration of protein (1, 2 and 4%), a General Lineal Model with repeated measures was performed. Variables of interest related to physical and oxidative stability needed to be transformed using log-transformation in order to stabilize the variance.

The statistical analysis was performed using SAS System ® v9.2 (SAS Institute Inc., Cary, NC, USA), using a nominal significance level of 5% ($p < 0.05$) and Tukey adjustment was performed for multiple comparisons of the means.

3.3.8.2. Response surface methodology

For each type of protein, whey protein isolate and sodium caseinate, and for each design type (different pressures combined with different concentrations of protein and oil), a

response-surface model was performed. Accordingly, four different models were conducted for each variable of interest. The variables of interest were related to physical and oxidative stability of emulsions as particle size indices (d3.2 and d4.3), specific surface area (SSA), consistency coefficient (K), emulsifying activity index (EAI), hydroperoxide content and TBARs concentration.

Response-surface model was conducted to discover which values of given factor variables (pressure and concentration of protein or oil) optimize the response variable (d3.2, d4.3, SSA, etc). Since each factor was measured at three or more values, a quadratic response surface could be estimated by least-squares regression and the predicted optimal value could be found from the estimated surface. If the estimated surface was more complicated, or if the predicted optimum was far from the region of experimentation, then the shape of the surface could be analyzed to indicate the directions in which new experiments should be performed.

For one of the interest response variables (y) measured at combinations of values of the two factor variables, pressure (x1) and protein concentration (x2) for example, the quadratic response-surface model for this variable is written as

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_1^2 + \beta_4 x_2^2 + \beta_5 x_1 x_2 + \epsilon$$

The steps in the analysis for such data are: model fitting and analysis of variance to estimate parameters, canonical analysis to investigate the shape of the predicted response surface and ridge analysis to search for the region of optimum response.

These kinds of models treat the factors as continuous variables, and this allows us to estimate the response surface. Therefore, it was possible to obtain the optimum response based on optimum levels of the two factors.

3.4. References

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Chapter 4

Physical and oxidative stability of whey protein oil-in-water emulsions stabilized by conventional and ultra-high pressure homogenization: effects of pressure and protein concentration on emulsion characteristics

4.1. Introduction

The stability and the emulsion formation become easier when using an emulsifier, which is adsorbed at the interface between oil and water and can lower the interfacial tension and prevent coalescence of droplets by increasing repulsion forces between droplets. Globular proteins derived from milk are widely used as natural emulsifiers to enhance the formation and stability of oil-in-water emulsions, e.g., whey proteins such as β-Lg, α-La, and bovine serum albumin (Dickinson, 2003; Livney, 2010).

Whey protein isolate (WPI) is an excellent emulsifier and widely used in food emulsions due to its surface-active property. WPI is adsorbed at the oil-water interface during homogenization to form a protective film and provides structural support for oil droplets through a combination of electrostatic and steric interactions (Djordjevic, Kim, McClements & Decker, 2004).

Contrasting results in whey protein-stabilized emulsions subjected to high-pressure homogenization can be found in the literature. Desrumaux & Marcand (2002) showed that during ultra high-pressure emulsification, the conformation of whey proteins was changed (denaturation occurred), which probably affected their emulsifying properties. They found an optimum pressure of about 100 MPa, in which d3.2 and Span values reached a minimum. Perrier-Cornet, Marie, & Gervais, (2005) proved that at pressures above 200 MPa, the adsorption rate of whey proteins significantly increased (60%) corresponding to a very narrow particle size of sunflower oil. Furthermore, Bouaouina, Desrumaux, Loisel & Legrand, (2006) showed that dynamic high-pressure treatment (up

to 300 MPa) did not affect the conformation of whey proteins but enhanced their stabilizing properties due to increased exposure of their hydrophobic sites.

Although several researchers have examined the effect of homogenization pressure on the physical stability of emulsion (Desrumaux & Marcand, 2002; Floury, Desrumaux, Axelos & Legrand, 2003; Cortés-Muñoz, Chevalier-Lucia & Dumay, 2009; Kiokias, Reiffers-Magnani & Bot, 2004), there is not much literature evidence regarding any association of homogenization pressure with oxidative deterioration of the emulsions. A further elucidation of the effect of varying homogenization pressure (that generated varying droplet sizes) on oxidative deterioration of whey protein-stabilized O/W emulsions could contribute to control processing parameters for the production of high quality emulsified foods. The methodology applied for this purpose is described in Chapter 3 and includes the study of physical properties including: particle size distribution, surface protein concentration, rheological behavior, microstructure (CLSM and TEM microscopy) and stability to creaming, measured visually and by two light scattering techniques (particle size at the top and the bottom of emulsions and Turbiscan lab); and the stability to oxidation, determining the hydroperoxides and thio barbituric acid reactive substances (TBARS).

4.2. Results and discussion

4.2.1. Temperature rise during UHPH processing

In dynamic high-pressure systems, forced-induced phenomena of cavitation, shear and turbulence are involved. The temperature rise is often fairly small, but it can become appreciable if the emulsion is recirculated or particularly high-pressures are used. In these cases it may be necessary to keep the emulsion cool by using a water jacketed homogenization chamber to minimize the heating effect.

Table 6 shows the rise of temperature with the homogenization pressure. Temperatures of processed emulsions were measured before the HP-valve (T1) and at the HP-valve outlet (T2). Fluid temperatures T1 and T2 increased linearly with the homogenization pressure, in agreement with other authors working with emulsions treated by UHPH (Desrumaux & Marcand, 2002; Floury et al., 2003, & Cortés-Muñoz et al., 2009). The increase in temperature, in general, corresponds to the short-life heating of the fluid passing through the valve gap and is mainly related to conversion of kinetic energy into

heat. The rise in temperature depends on the fluid composition (Cortés-Muñoz et al., 2009) and on the configuration and heat capacity of the homogenizer itself (Sandra & Dalgleish, 2005).

Table 6. Mean ± SD values of temperature measured before (T1) the high-pressure valve and at the outlet (T2) of the high-pressure valve for emulsions containing different protein concentrations (1, 2 and 4%), and treated by ultra-high pressure homogenization at 100 and 200 MPa (Tin = 25°C).

Protein concentration (%)	Pressure (MPa)	T1 (°C)[a]	T2 (°C)[b]
1	100	38 ± 2.00	59 ± 3.00
	200	43 ± 1.15	82 ± 3.21
2	100	39 ± 0.57	56 ± 4.00
	200	43 ± 0.57	83 ± 5.13
4	100	32 ± 1.00	55 ± 2.46
	200	41 ± 2.08	84 ± 2.64

Temperature (T2), measured after the high-pressure valve, increased by 23, 27 or 29 ºC per 100 MPa for the three respective protein concentrations (1, 2 or 4%). The temperature increase after the high pressure valve, with both the pressure and protein content being increased, has also been observed by Gràcia-Juliá et al. (2008) with WPI dispersions containing 6 and 10% protein content and treated at pressures varying from 100-300 MPa, where the temperature was slightly higher in dispersions containing 10% than in those containing 6% protein. They attributed this increase to the higher frictional stress in the high-pressure valve (followed by heat dissipation), in relation with the higher viscosity of the initial dispersion. The same may have occurred in our emulsions, in which the viscosity of the emulsions increased in proportion to the protein concentration, as will be explained hereafter (see the rheological section).

4.2.2. Particle size distribution

The droplet size distribution of an emulsion-based food product often has a major impact on its physicochemical and sensory properties, e.g., shelf life, appearance, flavor, and texture (McClements, 2005).

On passing through the homogenizing valve, emulsion droplets simultaneously encounter several processes: deformation and disruption of droplets, adsorption of surfactant at the newly formed interface, and collision and possible recoalescence of droplets. The balance between breakup and recoalescence of droplets determines droplet size.

Droplet size indices (d3.2 and d4.3 μm) and SSA (m^2/ml) for all emulsions containing 20% oil and different WPI concentrations (1, 2 and 4%) are shown in Table 7. Colloidal mill (CM) emulsions, at all protein concentrations had the largest particle size (d3.2 and d4.3) followed by emulsions stabilized by conventional homogenization (CH) and the minimum droplet size was obtained in emulsions stabilized by UHPH. These results were also confirmed by TEM microscopy (Fig. 21 A-L).

The protein concentration affected the particle size of all emulsions treated by CM, in which increasing the protein concentration from 1 to 4% resulted in a decrease of d3.2 value, although this decrease was only significant when the protein concentration increased from 2 to 4% protein. This decrease in the particle size of CM emulsions could be clearly seen in the CLSM images (Fig. 22 A-D). On the contrary, the protein concentration had no effect on the d3.2 in CH emulsions, but a significant decrease in the d4.3 value was observed when the protein was increased from 1 to 4%.

In UHPH emulsions, the droplet size decreased with increased pressure and the concentration of WPI. However, it seems that 4% of WPI is necessary to decrease the droplet size in each UHPH treatment, especially in emulsions treated at 100 MPa, indicating that the protein started to become limited for surface coverage in UHPH emulsions containing 1 and 2% of WPI. When protein is limited, there is no longer sufficient protein to fully stabilize the droplet interface, and therefore larger particles may be formed as a result of coalescence or bridging flocculation. Similar results have been observed in emulsions stabilized by whey protein isolates and other proteins, such as fish gelatin and bovine serum albumin (Lizarraga, Pan, Añon, & Santiago, 2008).

Table 7. Mean ± SD of particle size distribution indices (d3.2 and d4.3), specific surface area (SSA, m^2/ml), surface protein concentration (SPC, mg/m^2) and rheological characteristics (flow and consistency indices) of O/W emulsions containing 20% (w/w) of sunflower and olive oils plus whey protein isolate (1, 2 and 4%), and prepared by colloidal mill (CM), conventional homogenization (15 MPa) and ultra-high pressure homogenization at 100 and 200 MPa.

Pressure (MPa)	Protein Content (%)	Particle size distribution indices			Surface protein concentration SPC (mg/m^2)	Rheological behavior		
		*d*3.2 (μm)	*d*4.3 (μm)	Specific surface area SSA (m^2/ml)		Consistency coefficient K (mPa × s)	Flow behavior index (n)	r^2
CM	**1**	6.22 ± 0.016^{a}	16.7 ± 0.37^{a}	0.96 ± 0.0004^{e}	2.62 ± 0.25^{cd}	1.49 ± 0.291^{f}	1.06 ± 0.031	0.998
	2	5.74 ± 0.601^{a}	20.2 ± 2.70^{a}	1.06 ± 0.117^{e}	5.16 ± 0.58^{b}	1.80 ± 0.304^{ef}	1.06 ± 0.039	0.998
	4	3.79 ± 0.855^{b}	20.1 ± 2.43^{a}	1.65 ± 0.332^{e}	11.16 ± 0.13^{a}	2.36 ± 0.152^{cde}	1.05 ± 0.024	0.999
15	**1**	0.85 ± 0.072^{c}	2.35 ± 0.48^{b}	7.24 ± 0.516^{d}	2.11 ± 0.10^{d}	5.03 ± 0.882^{a}	0.97 ± 0.008	0.999
	2	0.79 ± 0.044^{c}	1.99 ± 0.89^{bc}	7.67 ± 0.510^{d}	3.36 ± 0.49^{c}	2.85 ± 0.209^{cd}	0.97 ± 0.023	1.000
	4	0.73 ± 0.016^{c}	1.42 ± 0.21^{c}	8.16 ± 0.203^{d}	3.58 ± 0.39^{c}	2.27 ± 0.226^{def}	0.98 ± 0.027	0.999
100	**1**	0.20 ± 0.024^{d}	0.29 ± 0.01^{d}	30.43 ± 3.88^{c}	0.51 ± 0.01^{f}	2.04 ± 0.198^{def}	0.98 ± 0.014	0.999
	2	0.16 ± 0.032^{de}	0.24 ± 0.03^{de}	38.76 ± 6.67^{bc}	0.86 ± 0.14^{e}	2.53 ± 0.331^{cde}	0.96 ± 0.026	0.997
	4	0.13 ± 0.011^{e}	0.21 ± 0.01^{e}	44.44 ± 3.24^{ab}	0.83 ± 0.11^{e}	3.40 ± 0.475^{bc}	0.98 ± 0.022	0.999
200	**1**	0.15 ± 0.044^{de}	0.23 ± 0.003^{de}	39.42 ± 1.88^{bc}	0.48 ± 0.07^{f}	2.43 ± 0.221^{cde}	0.95 ± 0.014	0.998
	2	0.13 ± 0.016^{e}	0.19 ± 0.040^{ef}	46.65 ± 5.85^{ab}	0.53 ± 0.14^{ef}	2.78 ± 0.160^{cd}	0.98 ± 0.037	0.998
	4	0.11 ± 0.002^{e}	0.14 ± 0.001^{f}	55.02 ± 0.60^{a}	0.84 ± 0.09^{e}	4.02 ± 0.731^{ab}	1.00 ± 0.013	1.000

$^{a-f}$ Different letters at the same column indicate significant differences *($P < 0.05$)* between treatments.

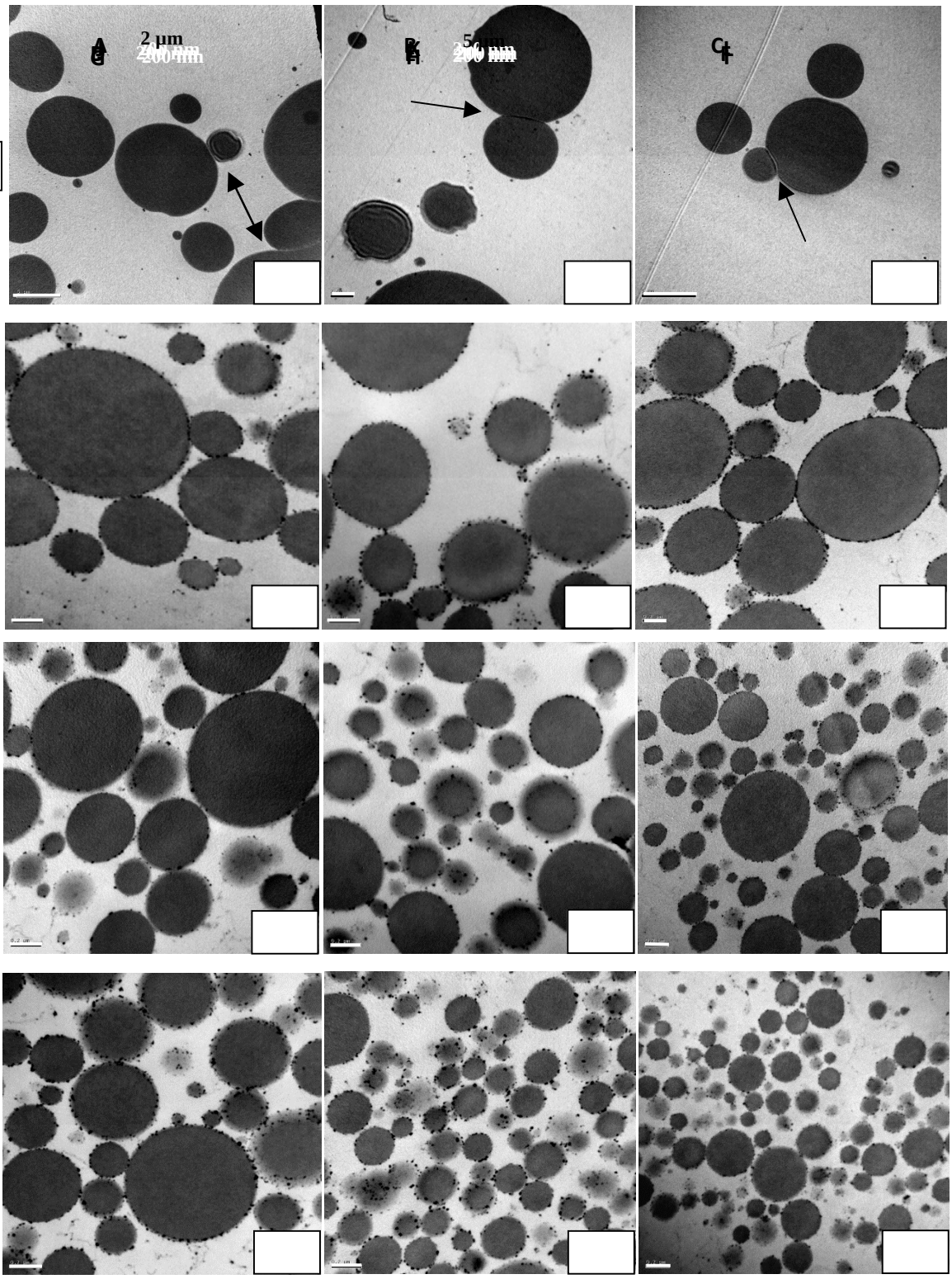

Figure 21. TEM images of O/W emulsions containing whey protein isolate (1, 2 and 4%), and prepared by (A-C) colloidal mill (CM) ×5000, (D-F) conventional homogenization (CH) at 15 MPa ×50000, and by ultra high-pressure homogenization at 100 MPa (G-I) and 200 MPa (J-L) ×50000.

According to Wang, Li, Wang, & Özkan (2010), the reasons for the decrease in particle diameter with increasing protein concentration could be due to: (1) the increase in protein concentration, which would increase the coverage of oil droplets thereby inhibiting the droplet aggregation, and forming a smaller droplet, a fact that was confirmed in our study by the surface protein concentration results (Table 7); and (2) due to the high protein surface coverage, which could result in an increase in the emulsion viscosity (Table 7), the collisions of droplets decrease. Viscous effects taking place at the entrance and in the high-pressure valve gap could explain the better droplet splitting at higher protein contents. The frictional loss coefficient, that predicts mechanical shearing effects in the valve gap increases with the viscosity of the inlet fluid (Stevenson & Chen, 1997). The viscosity of our pre-emulsions (CM emulsions) significantly increased from 1.49 ± 0.291 at 1% to 2.36 ± 0.152 at 4% of protein (Table 7). The increase in the inlet fluid viscosity could thus (1) favour droplet breakup in the valve gap due to higher extensional stress that occurs in UHPH process, which could result from higher viscosity of the inlet fluid and/or from an overcrowding of the whole matrix, and (2) limit droplet collision and thus droplet re-agglomeration/coalescence downstream of the valve gap where usually a turbulent flow prevails (Diels, Callewaert, Wuytack, Masschalck, & Michiels, 2005).

In the present study, other pressures of more than 200 MPa (i.e. 300 MPa) were also tested. At pressure of 300 MPa, the particle size increased in comparison to those obtained at 200 MPa (i.e. 0.158 µm vs. 0.177 µm in emulsions containing 2% WPI and treated at 200 and 300, respectively). This behavior was also observed by Floury et al. (2003) in emulsions treated up to 350 MPa and made with 20% sunflower oil plus 0.75% methylcellulose as an emulsifier. They attributed this increase in the droplet size with increased pressure to the heat generated by this high-pressure process. In our study, the emulsion at the output of the high-pressure homogenizer valve reached a temperature higher than 100 °C for emulsions treated at 300 MPa, while the temperature did not exceed 75 and 85 °C for the emulsions treated at 100 and 200 MPa, respectively for all protein concentrations, as shown in Table 6. This heating which results from the degradation of the high process energy input, may have a negative influence on the size of the particles; above a certain critical temperature, the whey protein molecule unfolds and exposes non-polar side chains, thereby conferring hydrophobic character on the surface of the emulsion droplets and enhancing protein-protein interactions

(Demetriades, Coupland, & McClements, 1997). Desrumaux, Loisel & Marcand (2000) suggested that if the homogenizing pressure is too high, emulsifying whey proteins are denatured and then do not play their stabilizing role in the corresponding emulsion, because of the high shear stress and temperature they have to support in the homogenizing valve. In the present study, the TEM images obtained for emulsions UHPH-treated at 300 MPa (Fig. 23 A) showed protein aggregates in the medium and in the oil-water interface, corresponding to denatured whey proteins, a fact that could explain the higher particle size determined in emulsions treated at 300 MPa in comparison to those treated at 200 MPa.

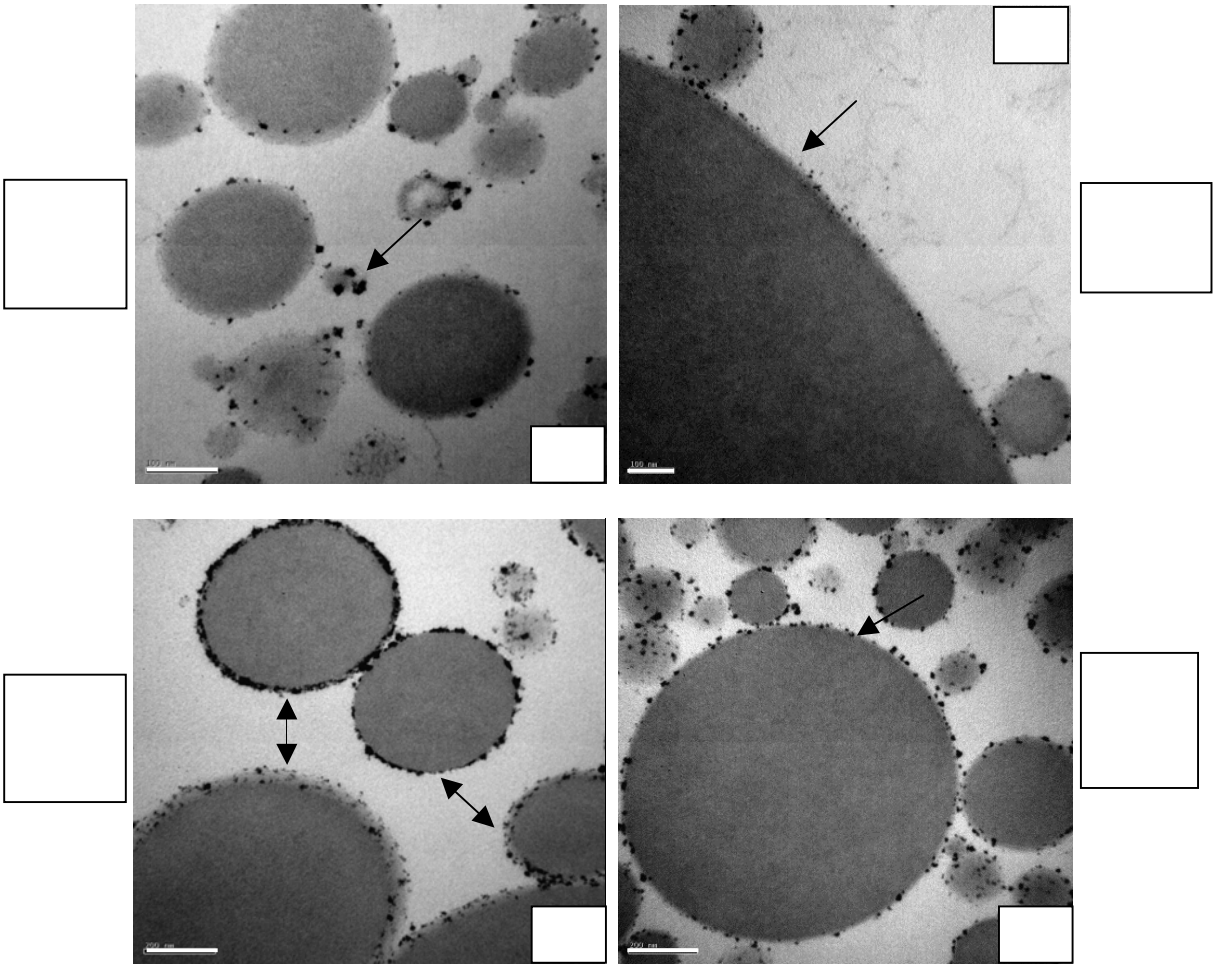

Figure 23. TEM images (×100000) of O/W emulsions prepared by (B) colloidal mill (CM) containing 4 % of whey protein isolate (WPI), (C) conventional homogenization (CH) at 15 MPa containing 4% of WPI and by ultra high pressure homogenization (UHPH) at 300 MPa containing 1% WPI (A) (see whey protein aggregations in the medium) and at 200 MPa containing 2% of WPI (D).

According to Qian & McClements (2011), β-lactoglobulin denaturation could lead to an increase in measured droplet size through a number of mechanisms: (1) unfolded proteins may have formed multilayers around each droplet; (2) unfolded proteins may have promoted droplet flocculation by increasing droplet surface hydrophobicity; (3) unfolded proteins may have formed protein aggregates in the continuous phase that contributed to the light scattering signal.

The d4.3 parameter allows detecting coalescence and flocculation process with more sensibility than the d3.2 value. However, even if the d4.3 values could be absolutely reliable, a large increase in d4.3 reflects the association of the emulsion droplets into large aggregates. Such a method has been largely used by Anton, Beaumal, Brossard, Llamas, & Le Denmat (2002) in order to compare flocculation degree of different emulsions placed in similar conditions. Table 7 shows a decrease in the d4.3 diameter in all emulsions, except in CM emulsions, as the protein concentration increased from 1 to 4%, which can also be evidently observed in the TEM images (Fig. 21 A-L), where the emulsions containing 1% of protein presented larger droplets than those containing 2 and 4% of proteins.

Emulsions prepared with CM exhibited the highest d4.3 values, which could be attributed to the incapability of the homogenizer to create particles with small size and to the droplets coalescence as can be observed in Figure 21 (A-C). Jafari, He, & Bhandari (2007b) studied O/W emulsions containing D-limonene, maltodextrin and modified starch made by mechanical agitation or ultrasounds. They observed that d3.2 and d4.3 resulted three times higher for emulsions prepared by mechanical agitation than for ultrasounds, ascribing the differences to the energy delivered to the system. When high-pressure homogenizers are used, the cavitation phenomena is the main cause for the droplets rupture during emulsification; however, when a mechanical agitator is used, the force mainly involved is shear stress in laminar flow, which does not produce a good rupture of the droplets, and this could explain why the presence of populations of droplets in our study is higher than 10 μm in CM emulsions (Table 7).

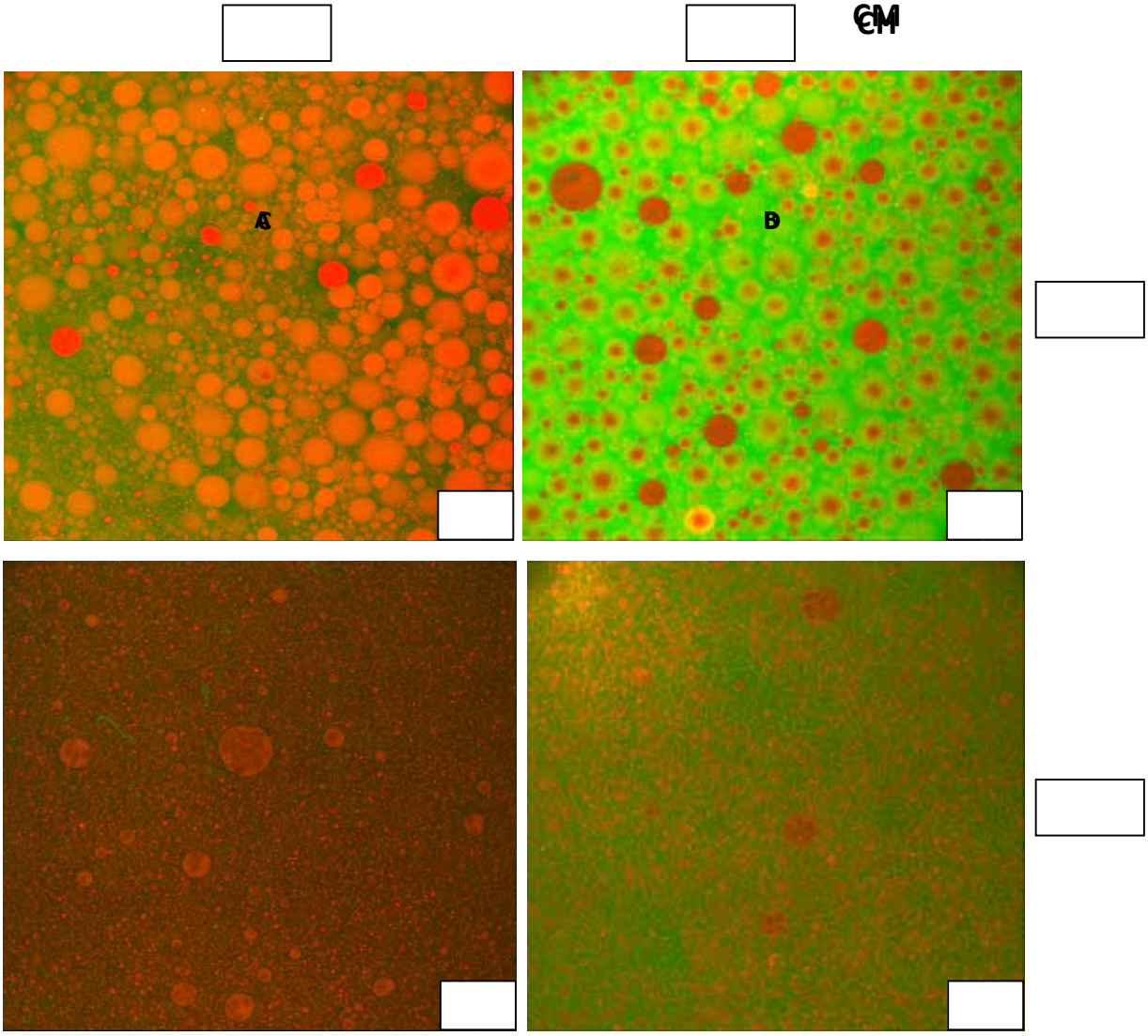

Figure 22. Confocal laser scanning microscope images of O/W emulsions containing 1 and 4% of whey protein isolate, prepared by (A-B) colloidal mill (CM) and (C-D) conventional homogenization (CH).

CH emulsions exhibited much smaller d4.3 values than CM emulsions, especially when higher protein concentrations were used. A decrease in the viscosity of CH emulsions was observed when the protein concentration increased from 1 to 4%, which confirms the presence of flocculated particles in emulsions containing a low protein concentration and that increasing the protein concentration results in less flocculated emulsions. CLSM images (Fig. 22 C, D) may confirm these results, in which a greater number of large particles and a lower protein coverage (reddish color) were observed in emulsions containing 1% of protein in comparison to those containing 4% of protein, which resulted in lower particle size and higher emulsion surface protein concentration and

subsequently droplets that were well covered with protein (yellowish color). These results are in agreement with Lizarraga et al. (2008) working with 50% O/W emulsions produced by Ultra Turrax and valve homogeniser using different whey protein concentrations (0.37–2.93% w/w), and with Palazolo, Sobral, & Wagner (2011) in 25% O/W emulsions produced by Ultra Turrax and a two-stage valve high-pressure homogenizer using different protein concentrations (0.5, 1 and 2% w/w) of native and thermally denatured soy protein and sodium caseinate, where increasing the protein concentration decreased the degree of flocculation.

UHPH emulsions showed minimum d4.3 values and clearly decreased when increasing the protein concentration from 1 to 4% and increasing the pressure from 100 to 200 MPa. In general, a certain degree of flocculation could be observed in the majority of the UHPH emulsions containing 1% of protein (Fig. 21 G, J); however, increasing the protein concentration to 2 and 4% decreased the degree of flocculation as can be seen in Figure 21 (H, I, K, L).

Figure 24 shows the particle size distributions in volume as obtained by light scattering in the case of emulsions stabilized by different concentrations of WPI (1, 2 and 4%) with 20% oil after emulsion dilution in distilled water (Fig. 24 A-C) and the same emulsions after dilution using SDS solution 0.1% as a disaggregating material (Fig. 24 D-F) after running the emulsion through the colloidal mill, conventional homogenizer and UHPH.

CM emulsions displayed large droplets with a monomodal distribution without changes in the size distribution after increasing the protein concentration or adding the SDS. This is not a result of the stability of CM emulsions, but may be a result of the change from the flocculation phase to coalescence phase, in which two droplets might be merged into one droplet, increasing the particle size, a fact that was confirmed by the transmission microscope (Fig. 21 A-C). The increase of particle size is reversible in case of flocculation, but once aggregation, coalescence or Ostwald ripening happens, the increase of particle size becomes irreversible (Silvestre, Decker & McClements, 1999). The coalescence probability increases as the fluctuations in the membrane shape becomes large enough to form a hole, which extends from one droplet to another. The magnitude of the shape fluctuations is governed by the interfacial tension, film rheology and mechanical applied forces (McClements, 2005).

Emulsions may become stable when the thickness of the continuous phase around the droplets (interstitial continuous phase) is enough to avoid contact between droplet films, and when interfacial fluctuations do not lead to the exclusion of interstitial water (McClements, 2005). Applying the homogenization pressure leads to a decrease in the interfacial tension between particles and forms a protective layer around the oil droplets leading to a repulsion force that protects the particles to be coalesced. This may explain the high coalescence rate in CM emulsions, as no complete thin protein layer around oil droplets could be observed (Fig. 23 B) in comparison to CH and UHPH emulsions (Fig. 23 C, D), where a protective mono or multi-layers of protein could be observed. This result was also confirmed by the change in the particle size distribution curves after adding the SDS in CH and UHPH emulsions, while this value did not change in the case of CM emulsions, indicating the coalescence of particles.

Conventional homogenization reduced the median of the particle size distribution considerably, but was unable to achieve the narrow particle size distribution achieved by UHPH, and it presented a bimodal distribution at all protein concentrations.

In UHPH emulsions, monomodal and narrow distribution was observed in the emulsions as the pressure increased to 200 MPa when the WPI concentration used was 4%, whereas bimodal distribution was observed in emulsions treated at 100 MPa at all WPI concentrations used (Fig. 24 A-C), a fact that was also observed by TEM images (Fig. 21 G-L).

A bimodal distribution in oil-in-water emulsions treated by high-pressure homogenization can be obtained due to the over processing phenomena caused by droplets flocculation when the energy input or the number of homogenization passes increase, and/or when the surfactant concentration is no longer sufficient to cover the newly created interface (Jafari, He, & Bhandari, 2007a). After adding SDS (Fig. 24 D-F), the distribution curves of UHPH emulsions generally changed to monomodal distribution, showing the reversible flocculation of oil particles in these emulsions. However, in case of emulsions containing 4% of WPI and treated at 200 MPa, the same distribution before and after SDS addition was observed, which confirms the absence of flocculation and the stability of these emulsions, a fact that was confirmed by transmission microscopy as Figure 21L shows.

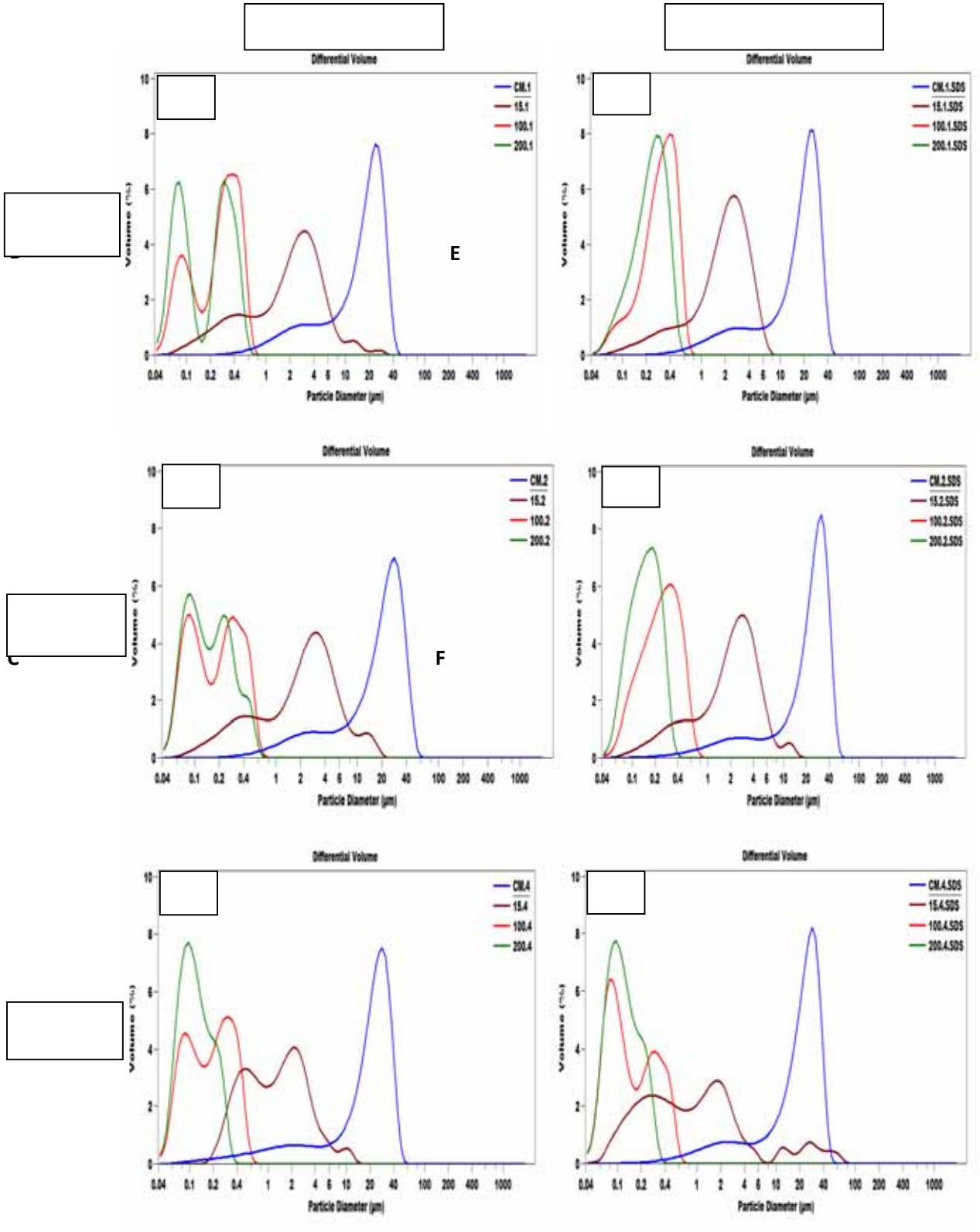

Figure 24. Droplet size distribution curves of O/W emulsions prepared by colloidal mill (CM), conventional homogenization (CH) and ultra high-pressure homogenization at 100 and 200 MPa measured by light scattering with 1% (A, D), 2% (B, E) or 4% (C, F) of whey protein isolate in the absence (A-C) or presence (D-F) of 0.1% SDS.

4.2.3. Surface protein concentration

The droplets in emulsions are usually surrounded by a thin coating of material (typically 1-50 nm thick) that contains a mixture of various types of molecules, e.g., oil, water, emulsifier, biopolymers, and minerals, which is usually expressed as milligrams of protein per unit area (mg/m^2) of the dispersed phase (Dickinson, 2003). The composition, structure, thickness, rheology and responsiveness of this interfacial layer often play major roles in determining the overall properties of emulsions (McClements, 2005).

Table 7 shows the amount of protein adsorbed at the emulsions interface. In general, higher protein amounts (mg/m^2) were observed in CM emulsions, especially at 2 and 4% of protein, as a result of the high particle size and the lower SSA in comparison to conventional homogenized and UHPH-treated emulsions.

Increasing protein concentration from 1 to 4% increased the amount of adsorbed protein in CM and CH emulsions as a result of decreasing the particle size, a fact that was confirmed by the LCSM images (Fig. 22 A-D), as described before in the particle size section. These results are in agreement with those of Guo & Mu (2011) who inferred that increasing the protein concentration in the emulsion from 1 to 2% w/v protein, increased the surface concentration, which could be explained by the closer packing of the adsorbed proteins in the monomolecular layer.

All UHPH emulsions, which showed higher SSA, exhibited significantly lower protein concentrations at the interface, compared to CM and CH emulsions, which may be attributed to the increased spreading and rearrangement of adsorbed protein molecules at the interface. However, taking into account the SSA of UHPH emulsions (30.4 and 39.4 m^2/ml for emulsions produced by 1% of WPI at 100 and 200 MPa, respectively) and comparing with those of CM and CH emulsions produced at the same concentration of WPI (0.96 and 7.24 m^2/ml, respectively), the amount of surface protein per milliliter was higher in the UHPH emulsions (18.49 and 19.32 mg/ml) compared to CM and CH emulsions (6.79 and 13.25 mg/ml, respectively).

A relatively high surface concentration at low homogenization pressure, in medium SSA, such as CH samples in our case, might indicate that multilayers of proteins were formed at the interface (Fig. 23 C), whereas at high homogenization pressure there are strong interactions between adsorbed proteins at the interface due to the unfolding and

exposure of hydrophobic sites of proteins, leading to the formation of a more rigid and thinner interfacial layer which probably approaches a compacted monolayer (Fig. 23 D) decreasing the protein load (Lee, Lefèvre, Subirade, & Paquin, 2009; Shukat & Relkin, 2011). Partial unfolding of globular whey proteins by UHPH can result in more compact adsorbed layers, exposing reactive sulphydryl groups, resulting in sulfhydryl disulfide interchange reactions between protein molecules adsorbed at the interface (Dickinson, Rolfe, Dalgleish, 1990; McClements, Monahan, & Kinsella, 1993).

Increasing the protein concentration from 1 to 2% in UHPH-treated emulsions at 100 MPa tended to increase the surface protein concentration ($P < 0.05$) but, no further increase was observed when the protein content increased to 4%. After adsorption on the surface, the protein probably became partially unfolded at the interface and the increase of pressure treatment caused no further conformational changes. On the other hand, 4% of protein was necessary for a significant increase in the surface protein concentration in emulsions treated at 200 MPa, which may be explained by the reduced particle size observed in these emulsions which need more protein for a complete surface coverage.

4.2.4. Rheological Behavior

Table 7 shows the consistency coefficient (K) value, which corresponds to the viscosity when the fluid is Newtonian, and the flow behavior index (n) of emulsions obtained by colloidal mill, conventional homogenization and UHPH. All emulsions showed a flow Newtonian behavior ($n \approx 1$) with viscosity being less than ~ 6 mPa × s. Floury, Desrumaux & Lardieres (2000) reported that emulsions containing less than 20% of dispersed phase follow Newtonian behaviors ($n \approx 1$) in the pressure range 20-300 MPa. The low particle-particle interactions in these emulsions are supposed to be responsible for the Newtonian behaviors of the fluids. This theory is supported by Samavati, Emam-djomeh, Mohamedifar, Omid, & Mehdinia (2012), who reported that WPI concentration has no significant effect on the flow curves of emulsions, which suggests that viscosity is insensitive to the amount of unadsorbed WPI in the aqueous phase.

Viscosity of emulsions stabilized by CM and UHPH increased with increased protein concentration, but not in the case of CH emulsions. Emulsions obtained by CM showed low consistency coefficients compared to the other treatments, possibly due to the large

particle size distribution of these emulsions, which means that there was a low interaction between particles. The viscosity of CM emulsions increased ($P < 0.05$) as the protein content increased from 1 to 4%, however this increase was only significant in emulsions containing 4% of WPI.

In CH emulsions, the viscosity decreased as the protein concentration increased from 1 to 4%. The decrease in the viscosity could be related to the level of particle floculation (see d4.3 values in Table 7) being lower in the case of emulsions with high protein concentration (4%) in comparison to those containing low protein concentration (1%), which presented higher d4.3 values.

Viscosity in UHPH emulsions increased as the protein concentration increased especially when 4% protein was used (Table 7). This could be attributed to the reduced droplet size and to the increased number of fat globules in these emulsions, which increases the hydrodynamic interactions between the droplets, since the mean separation distance between the droplets decreases when the droplet size is reduced (Pal, 2000). According to Cortés-Muñoz et al. (2009), for a given oil volume fraction, the viscosity of UHPH-processed emulsions is inversely correlated to d4.3 values, indicating an increase of emulsion viscosity with the number of oil droplets.

The change in the emulsion rheology caused by UHPH could not be only related to the change in the emulsion droplet size, as initially thought, but might also be related to the properties of the stabilizing molecules and the simultaneous adsorption of milk proteins on the increased fat globule surface (Hayes, Lefrancois, Waldron, Goff, & Kelly, 2003). Protein adsorbed into the interface plays a key role in increasing emulsion viscosity. Desrumaux & Marcand (2002) suggested that UHPH treatment further reduces the droplet size (increasing the SSA), enhancing the amount of adsorbed protein fraction, leading to greater viscosity.

4.2.5. Emulsion stability against creaming and coalescence

The term “emulsion stability” refers to the ability of an emulsion to resist any alteration in its properties over the timescale of observation (McClements, 2005; Dickinson, 2003). Submicron emulsions are reported to be more stable to creaming during storage, compared to microemulsions, due to the effects of Brownian motion being stronger than gravitational forces. They are also more stable to flocculation and coalescence due to

the lowering of the interfacial tension when the particle size decreases which in turn decreases the stress required to break up the droplets (Maher, Fenelon, Zhou, Haque, & Roos, 2011).

Physical stability of emulsions was assessed by measuring the d4.3 values at the top or at the bottom of the emulsion tubes stored at the room temperature (Fig. 25 A-F). In addition, emulsions stability was also evaluated using the Turbiscan Lab. The biggest advantage of this technique is that it can detect changes in the particle size of emulsions long before they become visible. Figure 26 (A-D) shows the backscattering profiles for all emulsions containing 4% of protein prepared with CM, CH and UHPH at 100 and 200 MPa, respectively. A great variation with time could be observed in the backscattering profiles for CM emulsions; the emulsion was totally separated in one hour, due to the high particle size, high interfacial tension between oil droplets, low viscosity and high coalescence rate as described previously in the size distribution section.

The CH emulsions were more stable against creaming in comparison to CM emulsions, although creaming could be detected in all CH emulsions by Turbiscan Lab, which agrees with the d4.3 values obtained at the top or the bottom of the CH emulsions tubes: 1.93, 2.03 and 1.50 µm and 1.14, 1.31 and 0.667 µm of emulsion tubes containing 1, 2 and 4% WPI, respectively; however, CH emulsions were visually comparable to the UHPH emulsions and they remained almost turbid and stable during 17 days of storage without any visual changes. CH emulsions containing 4% WPI seem to be the most stable, as shown in the size distribution curves, compared to emulsions containing 1% of protein (Fig. 25 A, B). This result was also confirmed by calculating the migration or creaming velocity V (t) in the clarification layer using the Turbiscan software, obtaining a value much lower in 4% protein emulsions (17.7 µm/min) compared to emulsions containing 1% of protein (56.6 µm/min). These results are consistent with the decrease in the viscosity in 4% protein emulsions, which may be related to the level of particle flocculation (see d4.3 values in Table 7). The higher d4.3 values (ability to flocculate) in 1% emulsions speeded up the creaming rate in comparison to those containing high protein concentration (4%), which presented lower d4.3 values. The higher stability of CH emulsions stabilized by 4% of protein may also be explained by the significant higher protein amount on the interface of the droplets ($P < 0.05$) as discussed before

(see the Surface Protein Concentration section), which may protect the oil droplets from collision and coalescence.

The d4.3 values measured at the top and the bottom of UHPH treated emulsions indicated the high physical stability of these emulsions against creaming. No significant changes in the d4.3 value between the two positions were observed in all emulsions whatever the protein concentration was, with values of 0.281, 0.256 and 0.196 µm (at the top) and 0.272, 0.245 and 0.202 µm (at the bottom) of emulsions treated at 100 MPa and containing 1, 2 and 4% WPI respectively, and with values of 0.221, 0.199 and 0.188 µm (at the top) and 0.213, 0.192 and 0.188 µm (at the bottom) of emulsions tubes treated at 200 MPa and containing 1, 2 and 4% of WPI, respectively (Fig. 25 C-F).

All UHPH emulsions remained fully turbid throughout the tube during the 17 days of storage without any visual changes of phase separation. Turbiscan analysis of UHPH emulsions hardly showed changes in the backscattering curves (Fig. 26 C, D), with no differences between treatments (pressure and protein concentration), which is a result of the significant decrease in the particle size.

Findings in the literature suggest that high homogenization pressure improves the creaming stability of emulsions by five mechanisms:

1. Decreasing the droplet diameters (Floury, Desrumaux, Axelos & Legrand, 2002; Lee et al. 2009), because larger droplets result in less stable emulsions. Smaller particles have a lesser tendency to cream, but a greater tendency to aggregate because they are more numerous at a given phase ratio and more susceptible to the influence of Brownian motion, both of which would lead to greater chance of collision.It has been shown that when the particle sizes are smaller than 100 nm, creaming is greatly reduced and aggregation becomes a dominant mechanism for emulsion instability (McClements, 2005).
2. An increase in the homogenization pressure not only decreases the particle size, but also decreases the difference in densities between the fat globules and bulk phase because protein adsorbs at the interface of the fat globules.
3. The low particle sizes in emulsions also increase the emulsion viscosity, limiting the movements of the oil particles and then lowering the creaming rate. In the case of our emulsions, the average migration velocity value calculated by Turbiscan for all UHPH emulsions (13.82 µm/min) was much lower than that calculated for the CM and CH emulsions (278.68 and 37 µm/min).

4. By extensive disruption of the biopolymer, thus increasing the availability of emulsifying protein molecules (San Martin-González, Roach, & Harte, 2009).
5. Forming a gel-like particulate network, caused by droplets aggregation (Dickinson, 1989).

The stability results obtained for the UHPH emulsions are in agreement with the results found by Cortés-Muñoz et al. (2009). These authors, determining the d4.3 values at the top and at the bottom of emulsions, indicated a slight creaming effect in few cases only, and mainly after processing emulsions with 15% (w/w) oil treated at 100-150 MPa, while emulsions treated at 200 MPa led to excellent oil droplet stability vs. creaming and coalescence.

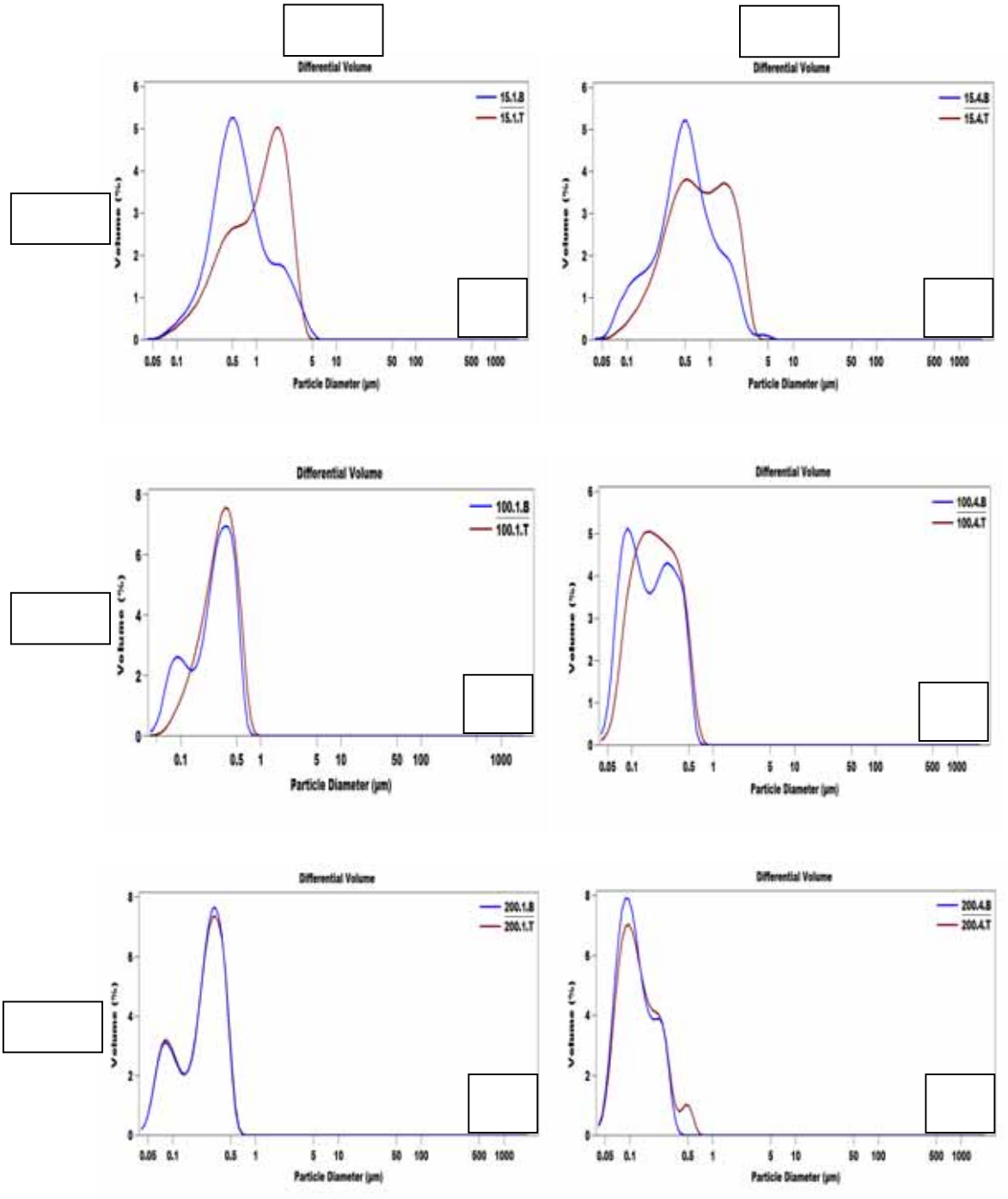

Figure 25. Droplet size distribution curves at the top (T) and the bottom (B) of O/W emulsions containing 1 and 4% of whey protein isolate processed by (A-B) conventional homogenization (CH), and by ultra high pressure homogenization at 100 (C-D) and 200 MPa (E-F) after 9 days of storage at room temperature.

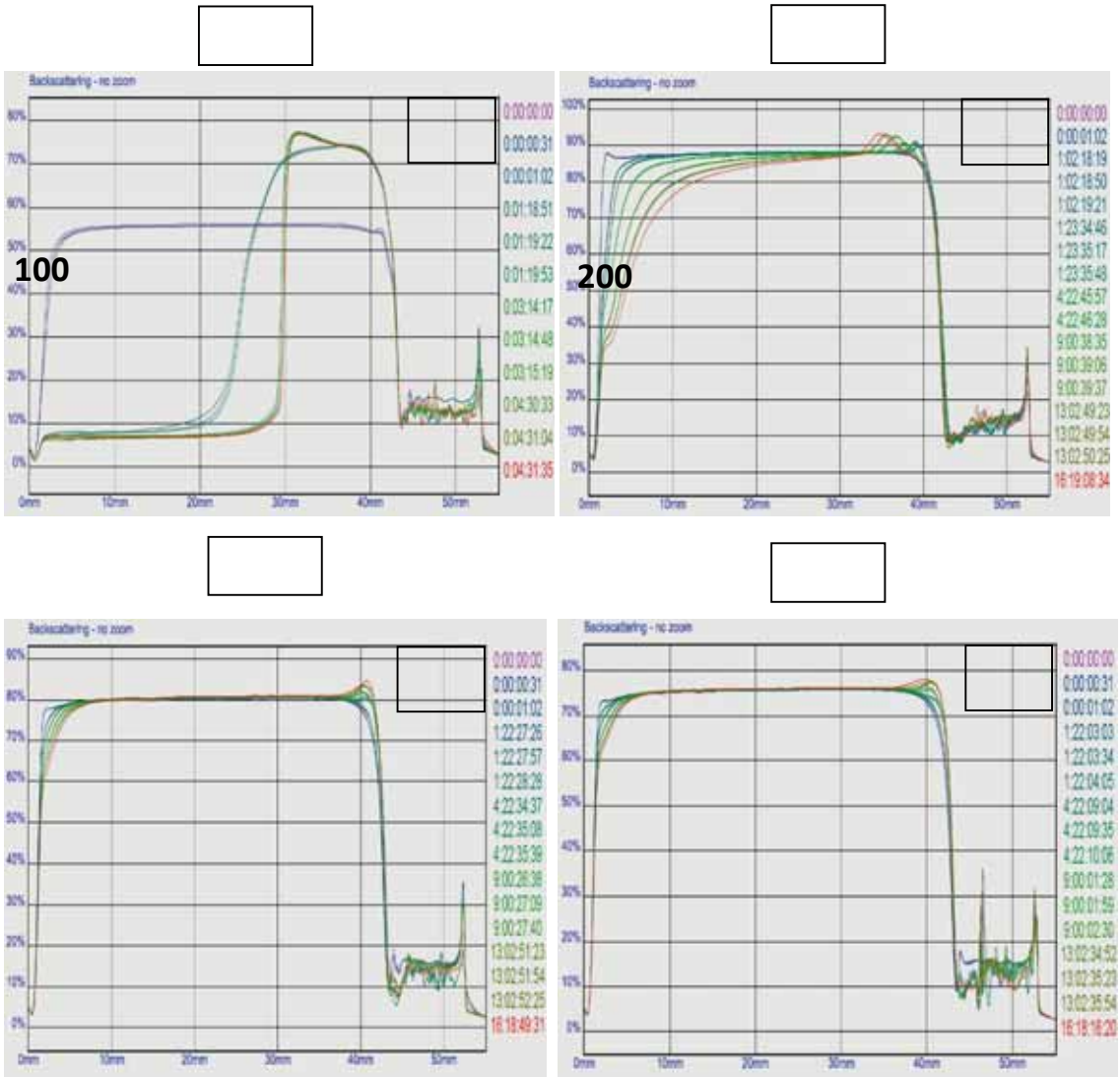

Figure 26. Changes in backscattering profiles of O/W emulsions containing 4% of whey protein isolate (WPI) prepared by (A) colloidal mill (CM), (B) conventional homogenization (CH) and by ultra high pressure homogenization at 100 (C) and 200 MPa (D), as a function of storage time (5 h for CM emulsions and 17 days for both CH and UHPH emulsions).

4.2.6. Oxidative stability of WPI emulsions

Hydroperoxides are primary oxidation components that have a lower half-life than secondary oxidation components (Fomuso, Corredig, & Akoh, 2002). The peroxides in oxidized oil are transitory intermediates that decompose into various carbonyl and other compounds (Rossell, 1986). Measuring secondary oxidation products is important in the determination of lipid oxidation in food products for human consumption, because they

are generally odour-active, whereas primary oxidation products are colourless and flavourless.

Lipid oxidation is accelerated by reactions that take place at the surface of O/W emulsion droplets. Based on this principle alone and as expected, the rate of lipid oxidation should increase in the UHPH emulsions rather than CM and CH emulsions, as the droplet size decreases, because of the increased SSA that is exposed to the aqueous phase. However, it is interesting to note that the CM and CH emulsions with the larger droplets and lower SSA oxidized more or in the same manner (depending on the treatment) than the emulsions with the smaller droplets and higher SSA (UHPH emulsions) as shown in Table 8. This fact was also evidenced by Let, Jacobsen, & Meyer (2007) who compared oxidative stability of salad dressing, yoghurt and milk enriched with neat fish oil or fish oil-in-water emulsion (50% oil) prepared with whey protein as emulsifier and produced by homogenization (22.5 MPa). They observed that the yoghurts had much larger interfacial areas than the dressings, which nonetheless were more oxidized than the yoghurts. No direct relationship between droplet size or interfacial surface area and the degree of oxidation could be drawn from these data, indicating that factors other than the SSA itself are important determinants for oxidative stability.

CM emulsions, in general, contained the lower amounts of the primary and the higher amounts of secondary oxidation products on the first day of production in comparison with the other emulsions. Lipid oxidation is a free-radical chain reaction involving initiation, propagation, and termination stages. The lower hydroperoxide value in combination with the higher levels of TBARs obtained in CM samples indicates the progression of oxidation from a primary to a secondary state, showing the high sensitivity of these emulsions to lipid oxidation. The possible reason of the high sensitivity of CM emulsions may be the limited amount of protein at their interface as indicated in the Surface Protein Concentration section and as shown in the TEM image (Fig. 23 B).

CH emulsions presented the highest levels of hydroperoxides at day 10 of storage, especially in those containing 4% of protein, and levels of TBARs between those observed in CM and UHPH-treated emulsions.

UHPH-treated emulsions exhibited the highest oxidative stability in comparison to CM and CH emulsions, although multilayers were present in CH emulsions (Fig. 23 C). The

possible explanation for the lower primary and secondary oxidation products of UHPH emulsions may be the relatively thick and viscoelastic interfaces formed by proteins around lipid droplets by the partial denaturation of whey proteins due to the UHPH treatment, which results in more charges and interactions between particles. Due to this denaturation, whey protein exposes the hydrophobic regions, which makes the protein adsorb even better to the oil-water interface (see the Surface Protein Concentration section). These interactions have been accordingly suggested to be at least partly responsible for the highest oxidative stability of protein-stabilized emulsions, as compared to surfactant-stabilized emulsions (Fomuso et al., 2002; Haahr & Jacobsen, 2008; McClements & Decker, 2000).

Emulsions treated at 100 MPa were the most stable emulsions at the oxidative level even more stable than the emulsions treated at 200 MPa. UHPH emulsions treated at 100 MPa containing 1 and 2% of protein presented similar TBARs levels during storage. Among the emulsions treated at 200 MPa, those containing 4% of WPI, were also emulsions with a very low level of oxidation. However, emulsions produced at 200 MPa containing 1 and 2% of WPI had similar levels of hydroperoxides but, higher levels of TBARs than emulsions treated at 100 MPa. These results indicate that other factors other than particle size are assumed to influence lipid oxidation. Similar results were found by Pereda, Jaramillo, Quevedo, Ferragut, Guamis, & Trujillo, (2008) applying UHPH to milk (200 and 300 MPa, Tin = 30-40 °C). They reported that 300 MPa produced milk samples with less hydroperoxides compared to 200 MPa. However, in this case, the secondary oxidation, studied through malondialdehyde and hexanal formation, was higher in milk samples treated at 300 MPa, which indicated an evolution in the oxidation process during storage to final products.

Table 8. Mean ± SD of hydroperoxides and TBA reactive substances (µg/ml) of O/W emulsions containing 20% (w/w) of sunflower and olive oils plus whey protein isolate (1, 2 and 4%), and prepared by colloidal mill (CM), conventional homogenization (15 MPa) and ultra-high pressure homogenization at 100 and 200 MPa.

Pressure (MPa)	Protein (%)	Hydroperoxides (A510 nm)			TBARS (µg/ml)		
		Day 1	Day 10	Difference (day 10 – day 1)	Day 1	Day 10	Difference (day 10 – day 1)
CM	**1**	0.018 ± 0.003^{a}	0.051 ± 0.0093^{bc}	$0.032 \pm 0.008^{b*}$	0.092 ± 0.012^{a}	0.145 ± 0.017^{ab}	$0.054 \pm 0.024^{ab*}$
	2	0.021 ± 0.004^{a}	0.065 ± 0.016^{bc}	$0.044 \pm 0.010^{abc*}$	0.058 ± 0.002^{bc}	0.079 ± 0.002^{cde}	$0.021 \pm 0.003^{abcd*}$
	4	0.025 ± 0.001^{a}	0.079 ± 0.007^{bc}	$0.054 \pm 0.007^{abc*}$	0.098 ± 0.008^{a}	0.157 ± 0.022^{a}	$0.059 \pm 0.014^{a*}$
15	**1**	0.034 ± 0.014^{a}	0.143 ± 0.052^{ab}	$0.110 \pm 0.063^{ab*}$	0.054 ± 0.001^{bc}	0.076 ± 0.008^{cde}	$0.022 \pm 0.007^{abcde*}$
	2	0.044 ± 0.015^{a}	0.121 ± 0.031^{ab}	$0.078 \pm 0.036^{ab*}$	0.057 ± 0.004^{bc}	0.093 ± 0.010^{cd}	$0.036 \pm 0.008^{abcd*}$
	4	0.047 ± 0.020^{a}	0.197 ± 0.086^{a}	$0.150 \pm 0.074^{a*}$	0.066 ± 0.004^{b}	0.114 ± 0.040^{bc}	$0.048 \pm 0.042^{abc*}$
100	**1**	0.066 ± 0.035^{a}	0.105 ± 0.005^{bc}	0.039 ± 0.031^{bc}	0.052 ± 0.005^{bc}	0.044 ± 0.004^{f}	$-0.008 \pm 0.002^{f*}$
	2	0.058 ± 0.027^{a}	0.083 ± 0.005^{bc}	0.025 ± 0.030^{bc}	0.050 ± 0.005^{bc}	0.044 ± 0.006^{f}	$-0.006 \pm 0.003^{f*}$
	4	0.032 ± 0.016^{a}	0.066 ± 0.018^{bc}	$0.034 \pm 0.005^{bc*}$	0.040 ± 0.011^{c}	0.052 ± 0.007^{ef}	$0.012 \pm 0.006^{ef*}$
200	**1**	0.046 ± 0.021^{a}	0.071 ± 0.016^{bc}	0.025 ± 0.020^{bc}	0.053 ± 0.008^{bc}	0.094 ± 0.008^{cd}	$0.041 \pm 0.007^{abcd*}$
	2	0.030 ± 0.012^{a}	0.057 ± 0.010^{bc}	$0.027 \pm 0.021^{bc*}$	0.046 ± 0.013^{bc}	0.065 ± 0.011^{de}	$0.020 \pm 0.012^{ef*}$
	4	0.037 ± 0.008^{a}	0.038 ± 0.008^{c}	0.002 ± 0.007^{c}	0.067 ± 0.010^{b}	0.072 ± 0.003^{de}	0.005 ± 0.009^{f}

[a-f] Different letters at the same column indicate significant differences *(P < 0.05)* between treatments.

[*] Sign indicates that the differences between day 10 and day 1 (oxidation evolution) is significant *(P < 0.05)*.

The possible reason for the higher oxidation rate observed in UHPH emulsions homogenized at 200 MPa, and containing 1 and 2% of WPI, with respect to those treated at 100 MPa, could be the decrease in the efficiency of whey proteins to protect the oil droplets when the pressure was increased. This may be due to the over processing phenomenon caused by the increase in the product temperature at the outlet of the homogenization valve which affects the emulsifying properties of whey proteins. In a recent study using high-pressure homogenization (20 and 80 MPa), up to 7 homogenization cycles were used to stabilize whey protein emulsions (3% protein) containing 30% flaxseed oil (Kuhn & Cunha, 2012). The authors showed that increasing the pressure to 80 MPa produced an increase in the formation of primary oxidation products in the emulsions in relation to the emulsions homogenized at 20 MPa. They attributed this increase in the oxidation to the increase in temperature observed in these emulsions. Let et al. (2007a) hypothesized that increasing the homogenization temperature from 50 to 72 °C (like our emulsions treated at 100 MPa), may lead to improved physical coverage of the oil droplets by proteins, most likely β-Lg, which starts to unfold above 65 °C (Cano-Ruiz, & Richter, 1997; Ye, Singh, Taylor, & Anema, 2004) and also contains amino acids with sulfhydryl groups, which have been shown to have antioxidant properties, such as radical scavenging properties (Hu, McClements, & Decker, 2003).

It can be observed that emulsions containing 4% of protein and treated at 200 MPa had a lesser tendency to oxidation, which agrees with the high physical stability of these emulsions (i.e. high viscosity, high protein concentration at the interface and monomodal distribution) while, the higher oxidation rate observed in emulsions containing 1 and 2% of WPI and treated at 200 MPa may be related to the bimodal distribution (see the Particle Size Distribution section), which can be obtained due to the over processing phenomena when the energy input or the number of homogenization passes increase, and/or when the surfactant concentration is no longer sufficient to cover the newly created interface.

With respect to the protein concentration effect, and for each treatment applied (CM, CH and UHPH), in general, emulsions were not affected, in terms of oxidation, by the protein concentration since for the same treatment applied they contained similar amounts of

primary and secondary oxidation products either at the first or the last day of storage, except for different treatments that did not follow any particular pattern.

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Chapter 5

Characterization of whey protein oil-in-water emulsions stabilized by ultra high-pressure homogenization: effect of pressure and oil concentration on emulsion characteristics

5.1. Introduction

Protein concentration and the oil volume fraction perform profound effects on the physicochemical and viscoelastic properties of the emulsions, such as droplet size distribution, creaming, oxidative stability, and rheology (Dickinson & Chen, 1999; Hemar, Tamehana, Munro, & Singh, 2001). Few data are available concerning the effects of dynamic high-pressure and oil volume fraction on emulsion stability. Cortés-Muñoz, Chevalier-Lucia, & Dumay (2009) using oil concentrations of 15, 30 and 45%, and pressures up to 300 MPa in O/W emulsions stabilized by whey protein isolate (4%), reported that optimal droplet breakup was observed for 30% oil (w/w) and homogenization pressure ≥ 200 MPa. Floury, Desrumaux, & Lardieres (2000) reported that a higher percentage of oil in the emulsions resulted in a larger mean droplet diameter for the same homogenizing conditions, due to the limitation of surface-active agents in the most oil concentrated emulsions. They revealed that the emulsions containing less than 20% of dispersed phase follow Newtonian behaviors ($n \approx 1$) whatever the homogenizing pressure applied. However, emulsions containing more than 20% of oil and issued from homogenizations at 20 or 150 MPa showed shear-thinning behaviors ($n < 1$), but higher pressures brought about the high oil content emulsions (>40%) from shear-thinning behaviors (at 20 MPa) to Newtonian behaviors (at 300 MPa).

A further elucidation of the effect of varying homogenization pressure, which generates variations of droplet sizes, on oxidative deterioration of protein-stabilized O/W emulsions could contribute to control processing parameters for the production of high

reduces the droplet size and consequently increases the total interfacial area of the emulsion. One would therefore expect that the rate of lipid oxidation increases as the droplet size of emulsion decreases because a greater amount of lipid would be exposed to the aqueous phase. Although some studies in emulsions support this hypothesis, other studies have found no dependence of the lipid oxidation rate on droplet size (Sørensen, Baron, Let, Bruggemann, Pedersen, & Jacobsen, 2007).

Although several researchers have examined the effect of ultra-high pressure homogenization on the physical stability of emulsions, there is not much literature evidence regarding any association with oxidative deterioration of the emulsions, including those containing large oil volumes.

As was concluded from the previous section, the best droplet breakdown, physical and oxidative stability were obtained when pressures of 100 and 200 MPa, and protein content of 4% were used. Therefore, the objective of this study was to evaluate the effect of homogenization pressures (100-200 MPa) and oil-phase volume fraction (10, 30 and 50%) on emulsion structure, rheological properties, physical and oxidative stability of emulsions containing 4% of whey protein isolate, in comparison with those produced by colloidal mill and conventional homogenization. The methodology applied for this purpose is described in Chapter 3 and includes the study of physical properties including: particle size distribution, surface protein concentration, rheological behavior, emulsifying activity, microstructure (CLSM and TEM microscopy) and stability to creaming, measured visually and by two light scattering techniques (particle size at the top and the bottom of emulsions and Turbiscan lab); and the stability to oxidation, determining the hydroperoxides and thio barbituric acid reactive substances (TBARS).

5.2. Results and Discussion

5.2.1. Temperature elevation during UHPH treatment

Temperatures of processed emulsions were measured before (T1) and at the outlet (T2) of the high-pressure valve. Fluid temperatures T1 and T2 increased linearly with the homogenization pressure (Table 9). The increase in temperature, in general, corresponds to the short-life heating of the fluid passing through the valve gap and is mainly related to conversion of kinetic energy into heat. At a fixed oil concentration (i.e. 50%), the

100 MPa was 30 ºC, while 54 ºC of difference was achieved after treating the emulsions at 200 MPa. These results are in line with the findings of Floury, Desrumaux, Axelos, & Legrand, (2003), Desrumaux & Marcand (2002); & Bouaouina, Desrumaux, Loisel, & Legrand, (2006), who reported a strong linear relationship between operating pressure and temperature rise at the exit of the high-pressure valve.

With respect to the effect of different oil volume fractions, it can be seen from Table 9 that the temperature after the valve (T2) was increased by 22, 26 or 29 ºC for the three respective oil contents (10, 30 or 50%). The same trend was observed by Cortés-Muñoz et al. (2009) working on emulsions containing 4% of WPI and different oil contents (15, 30 and 45%) who attributed this increase to the fluid compression in the intensifier during the pressure build up. In addition, the strong warming up of the fluid would be attributed to the viscous stress caused by the high velocity of the fluid flow, which is then impinged on the ceramic valve. This mechanical energy is almost fully dissipated as heat in the fluid (McClements, 2005). The outlet temperature (T3), which was measured after the final cooling, did not exceed 25 ºC in all cases, even with varying the oil concentrations.

Table 9. Mean ± SD values of temperature measured before (T1) the high-pressure valve and at the outlet (T2) of the high-pressure valve for emulsions containing different oil concentrations (10, 30 and 50%) treated by ultra high-pressure homogenization at 100 and 200 MPa (Tin = 25°C).

Oil content (%)	Pressure (MPa)	T1 (ºC)[a]	T2 (ºC)[b]
10	100	36 ± 1.15	60 ± 3.05
	200	42 ± 2.08	82 ± 5.85
30	100	35 ± 2.25	63 ± 1.00
	200	40 ± 2.02	89 ± 4.50
50	100	38 ± 2.08	68 ± 2.08
	200	43 ± 2.08	97 ± 2.64

5.2.2. Particle size distribution

Droplet size distribution is an important parameter for some emulsion properties such as shelf life and texture, and thus its control and measurement is necessary (McClements, 2005). During homogenization processes, there is usually a dynamic equilibrium between droplet break-up and coalescence which determines the final droplet size distribution.

Droplet size (d3.2 and d4.3 values) and specific surface area (SSA, m^2/ml) for emulsions containing 4% of whey protein isolate and different oil concentrations (10, 30 and 50%) are shown in Table 10. CM emulsions, at all oil concentrations, had the largest particle size (~ 6 µm); however, the particle size was drastically decreased to less than 1 µm in CH and to less than 0.25 µm in UHPH emulsions.

The oil concentration significantly affected the particle size in CM emulsions when the oil concentration increased from 10-30 to 50% decreasing the d3.2, a fact that was also evidenced from the size distribution curves (Fig. 27 A). The decrease in the particle size and the good protein coverage (yellowish color) can be observed in the CLSM images (Fig. 28 A-B) as the oil concentration increased from 30 to 50%. The decrease in the droplet size was accompanied with a significant increase in the SSA and a significant decrease in the d4.3 value, which indicates that 4% of WPI is sufficient to stabilize the 50% oil emulsion, despite the high oil content in the emulsion. The high particle size observed in CM emulsions, in comparison to CH and UHPH emulsions, could be attributed to the incapability of the homogenizer to create particles with small size and to the droplet coalescence as demonstrated in the TEM image (Fig. 29 A). When high-pressure homogenizers are used, the cavitation phenomena is the main cause for droplet rupture during emulsification; however, when a mechanical agitator is used, the force mainly involved is shear stress in laminar flow, which does not produce a good rupture of the droplets (Jafari, He, & Bhandari, 2007).

Increasing the oil concentration in CH and UHPH emulsions, in general, resulted in a significant increase in the particle size accompanied by a decrease in the SSA value, especially in emulsions containing 50% oil. A significant increase in the d3.2 was observed in CH emulsions when the oil content increased from 10 to 30% as also shown by the TEM images (Fig. 30 D, E), but further increase had no significant effect on d3.2 value (Fig. 30 F). Furthermore, the increase in the oil concentration was accompanied

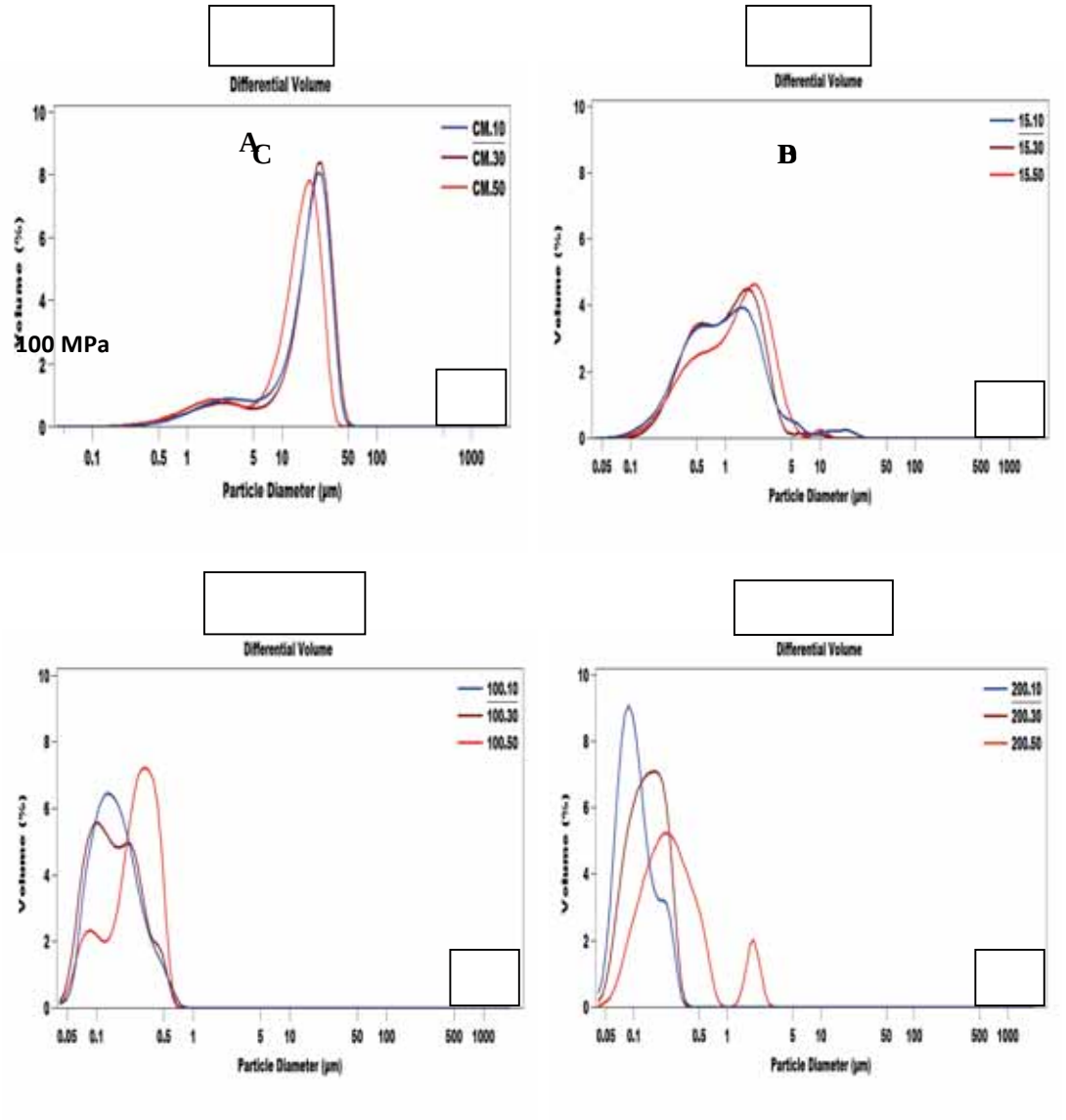

Figure 27. Droplet size distribution curves measured by light scattering of O/W emulsions containing 10, 30 and 50% oil plus 4% WPI and processed by (A) colloidal mill (CM), (B) conventional homogenization (CH) and ultra-high pressure homogenization at 100 (C) and 200 MPa (D).

Table 10. Mean ± SD of particle size distribution indices (d3.2 and d4.3), specific surface area (SSA, m^2/ml) and surface protein concentration (mg/m^2) of O/W emulsions containing 4% (w/w) of whey proteins plus sunflower and olive oils (10, 30 and 50%) and prepared by colloidal mill (CM), conventional homogenization (15 MPa) and ultra high-pressure homogenization at 100 and 200 MPa

Pressure (MPa)	Oil content (%)	Particle size distribution			Surface protein concentration SPC (mg/m^2)
		d3.2 (µm)	d4.3 (µm)	Specific surface area SSA (m^2/ml)	
CM	10	6.656 ± 0.654^{a}	17.58 ± 1.063^{a}	0.862 ± 0.061^{f}	27.04 ± 7.17^{a}
	30	6.132 ± 0.166^{a}	18.30 ± 1.560^{a}	0.979 ± 0.027^{ef}	12.87 ± 0.17^{b}
	50	5.151 ± 0.215^{b}	13.67 ± 1.343^{b}	1.193 ± 0.015^{e}	6.85 ± 0.95^{c}
15	10	0.559 ± 0.055^{d}	1.394 ± 0.237^{c}	10.89 ± 1.139^{d}	2.37 ± 0.41^{f}
	30	0.746 ± 0.107^{c}	1.308 ± 0.021^{c}	8.853 ± 0.521^{d}	4.69 ± 0.44^{d}
	50	0.699 ± 0.036^{c}	1.464 ± 0.162^{c}	8.537 ± 0.450^{d}	3.65 ± 0.13^{e}
100	10	0.134 ± 0.006^{gh}	0.181 ± 0.005^{ef}	45.00 ± 2.072^{b}	0.92 ± 0.04^{g}
	30	0.141 ± 0.007^{fg}	0.174 ± 0.013^{f}	43.52 ± 1.836^{b}	0.85 ± 0.01^{g}
	50	0.188 ± 0.022^{ef}	0.258 ± 0.010^{de}	34.24 ± 2.259^{c}	1.16 ± 0.02^{g}
200	10	0.103 ± 0.006^{i}	0.120 ± 0.005^{g}	60.81 ± 1.903^{a}	0.88 ± 0.07^{g}
	30	0.123 ± 0.010^{hi}	0.151 ± 0.004^{f}	51.38 ± 2.380^{b}	1.06 ± 0.14^{g}
	50	0.214 ± 0.033^{e}	0.294 ± 0.034^{d}	31.55 ± 0.339^{c}	1.18 ± 0.13^{g}

$^{a\text{-}i}$ Different letters in the same column indicate significant differences *($P < 0.05$)* between treatments.

UHPH emulsions treated at 100 MPa containing 10% oil exhibited low particle size and monomodal distribution (Fig. 27 C). As the oil content increased, the particle size increased and the SSA decreased; however, these changes were only significant in emulsions containing 50% oil, where the distribution curves changed to bimodal. The increase in particle size may be attributed to the high degree of flocculation as can be observed in Figure 30 I. As the oil content increased to 30% in emulsions treated at 200 MPa, the UHPH was able to produce monomodal distribution, but was unable to achieve a narrow size distribution. Increasing the oil content to 50% in emulsions treated at 200 MPa completely shifted the curve to bimodal with a wider size distribution (Fig. 27 D). In the TEM images (Fig. 30 J-L), developed phenomena of flocculation or coalescence in emulsions treated at 200 MPa containing 50% oil can be observed, which is strongly associated with the high particle size observed in these emulsions, in comparison to emulsions containing 10 and 30% oil.

A bimodal distribution in an O/W emulsion treated by high-pressure homogenization can be obtained due to the over processing phenomena caused by droplet flocculation when the energy input or the number of homogenization passes increase, and/or when the surfactant concentration is no longer sufficient to cover the newly created interface (Jafari et al., 2007). The bimodal distribution in our UHPH emulsions may be due to some recoalescence of the newly created fine droplets in the homogenization chamber or very shortly afterwards. Our results are in agreement with the results of Cortés-Muñoz et al. (2009), who studied the submicron emulsion characteristics (15-45%) using pressures up to 300 MPa. They reported that the best droplet breakdown was achieved when the pressure used was less than 225 MPa with 30 % of oil. They attributed this result to the increase in the fluid viscosity at the valve gap outlet since it may shift the flow pattern from turbulent to transitional, reducing therefore cavitation and impact phenomena and limiting droplet re-agglomeration and coalescence. Similar trends have been reported in emulsions containing different oil contents and stabilized by whey protein (Lizarraga, Pan, Añón, & Santiago, 2008), bovine serum albumin (Rangsansarid & Fukada, 2007), sweet potato protein (Guo & Mu., 2011) and flax seed protein concentrate containing mucilage (Lin, Tsai, & Lai, 2009).

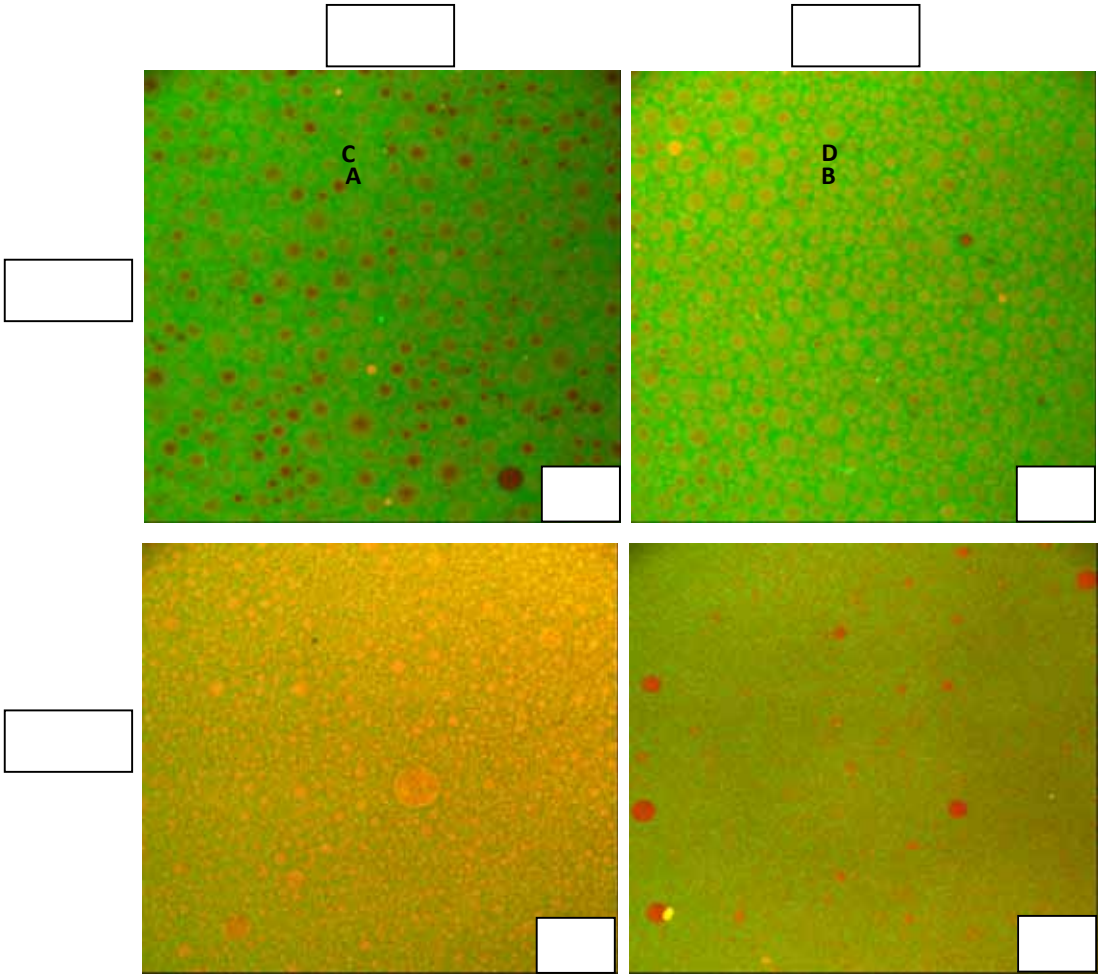

Figure 28. Confocal laser scanning microscope images of emulsions containing 30 and 50% oil and 4% WPI stabilized by (A, B) colloidal mill (CM) and (C, D) conventional homogenization (CH).

At constant energy density (e.g. emulsification pressure), particle size rises with increasing oil content. Some experiments by high-pressure valve homogenization (Phipps, 1985; Tesch, Gerhards, & Schubert, 2002) or ultrasound emulsification (Abismail, Canselier, Wilhelm, Delmas, & Gourdon, 1999) have confirmed this trend. There are a number of possible reasons to explain this trend: (1) higher oil contents increase the emulsion viscosity, and thereby droplet disruption become more difficult (Kolb, Herrera, Ferreyra, & Uliana, 2001; Seekkuarachchi, Tanaka, & Kumazawa, 2006); moreover, during homogenization, the residence time of emulsifying molecules might not be sufficient in the valve of the homogenizer, because of the high viscosity, to allow their adsorption on available droplet surface before droplet-droplet collisions occurred; (2) at constant emulsifier concentration, there may be an insufficient amount

of protein present to completely cover the new droplets. An inadequate amount of protein in the aqueous phase could cause some aggregation of fat globules, as reported by Tomas, Paquet, Courthaudon, & Lorient (1994). Mohan & Narsimhan (1997) demonstrated that, in protein-stabilized emulsions, the rate of coalescence during homogenization is reduced due to repulsive interactions between droplets, and droplet coalescence is only significant when there is insufficient protein to completely cover the droplet interface. According to Desrumaux, Loisel, & Marcand (2000), as the fat content increases, the proteins available decrease, limiting the stabilizing benefits of the proteins, which favors oil droplet coalescence and therefore, increases the mean droplet diameters; and (3) the rate of collision frequency and thus coalescence frequency is increased as the oil content increases. Tornberg, Olsson, & Persson (1990) and Srinivasan, Singh, & Munro (1996) attributed the increase of particle size with increasing the oil concentration to the greater incidence of coalescence and bridging at higher oil concentrations, both of which lead to reduction in total fat surface area. Sun & Gunasekaran (2009) indicated that increasing oil phase volume fraction enhances collision frequency among oil droplets, and consequently the rate of flocculation.

Concerning the effect of the homogenization pressure, generally, applying UHPH at 200 MPa was more effective to reduce the particle size than 100 MPa (Fig. 30 G-L). The tendency generally observed in our study, strongly indicates that the particles tend to be smaller when the homogenization pressure increases, which permits the separation of clusters into individual particles. Similar results have been obtained by Cruz, Capellas, Hernández, Trujillo, Guamis, & Ferragut, (2007) and Pereda, Ferragut, Quevedo, Guamis, & Trujillo (2007) when applying similar homogenization pressures to soymilk and cow milk systems, respectively.

In the present study, pressures of more than 200 MPa (i.e. 300 MPa) were also tested (data not shown). High particle size was obtained as the pressure increased to 300 MPa and this increase was accompanied by a high degree of coalescence (Fig. 29 C). This increment may be due to the over processing phenomena caused by the temperature increase at the outlet of the HP-valve. The emulsion at the outlet of the high-pressure homogenizer valve reached a temperature higher than 100°C for the emulsions treated at 300 MPa, which would influence the particle size in a bad way. Desrumaux et al. (2000) suggested that if the homogenizing pressure was too high, emulsifying whey

proteins are denatured and then do not play their stabilizing role in the corresponding emulsion.

Some studies reported that over processing in emulsions results in an increase of droplet size at 250-300 MPa, which could be attributed to re-coalescence (with or without particle aggregation) phenomena over droplet disruption when the energy input increased, or to the concentration of surfactant being no longer sufficient to cover the newly created O/W interface (Floury et al., 2000; Jafari, Assadpoor, He, & Bhandari, 2008).

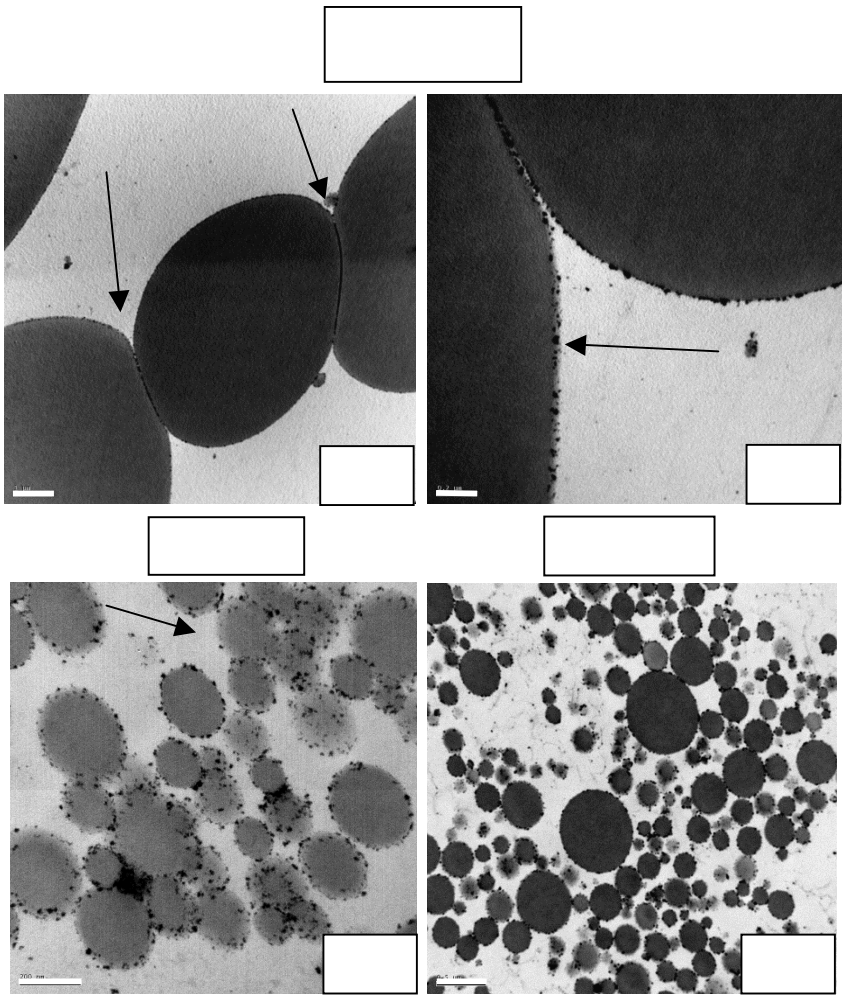

Figure 29. TEM images of O/W emulsions stabilized by colloidal mill (CM) containing 30% oil (A) ×10000 and (B) ×50000, and emulsions containing 50% oil treated by ultra-high pressure homogenization at 300 MPa (C) ×200000 and at 100 MPa (D) ×25000, respectively.

5.2.3. Surface protein concentration

The ability of proteins to form and stabilize emulsions is dependent on their ability to adsorb to interfaces and on the amount of protein required to saturate the interface (McClements, 2005). The factors that affect the protein load include protein concentration, volume of oil, energy input, state of protein aggregation, pH, ionic strength, temperature and calcium ions (Dickinson & Stainsby, 1988).

Table 2 shows the amount of protein adsorbed at the emulsion droplet interface. Significantly higher protein amounts (mg/m^2) were observed in CM emulsions, as a result of the higher particle size and the lower SSA in comparison to CH and UHPH-treated emulsions.

The amount of protein adsorbed on the interface of an emulsion droplet suggests the state of the protein adsorbed at the interface. If the protein load is ~1 mg/m^2 (as UHPH emulsions in our case), it suggests that the protein molecules are fully unfolded. If the protein load is 1-3 mg/m^2 (as our CH emulsions), a monolayer of globular proteins may be present or unfolded molecules may be adsorbed in the conformation of trains, loops and tails. Above 5 mg/m^2 (as our CM emulsions), it suggests the adsorption of aggregates of proteins or multilayers of proteins (McClements, 2005).

As shown in Table 10, increasing oil concentration in CM emulsions tended to a linear and drastic decrease in the SPC, probably due to the decrease in the particle size and the increase in the SSA. The higher the surface area formed during homogenization, the higher the amount of protein needed for the full coverage of the created particles, as explained before (see the Particle Size section).

In CH emulsions, increasing the oil content from 10 to 30% resulted in a significant increase in the SPC, possibly due to the significant increase in the particle size and the decrease in the SSA, but further increase in the oil content to 50% decreased significantly the SPC, and this might be due to the limited protein availibility to cover the newly created interface as could be observed in the CLSM images (Fig. 28 C-D), with 50% oil emulsions presenting lower yellowish color than emulsions containing 30% oil. The reduction in the SPC as the oil content increased from 30 to 50% may be the factor that was most strongly associated with the increase in the particle flocculation, as it was approved by the shear thinning behaviour (n □ 0.596) of emulsion containing 50% oil. Another reason for the low surface coverage of CH

emulsions containing 50% oil may be the high viscosity of emulsion, due to the high flocculation rate, which may make the time to cover the new interface insufficient. CH emulsions prepared with high protein concentrations and low oil phase volume possibly reflect the presence of thick and viscoelastic layers, possibly due to the increased probability of protein-protein interactions, as shown in the TEM images (Fig. 30 A-B) while, emulsions containing 50% oil clearly displayed low surface protein concentration (Fig. 30 C).

All UHPH emulsions, which showed higher SSA, exhibited significantly lower protein concentration at the interface in comparison to CM and CH emulsions, which may be attributed to the increased spreading and rearrangement of adsorbed protein molecules at the interface.

From a superficial look at these results, one might say that CM emulsions had the higher surface protein amount but, taking into consideration the SSA of UHPH emulsions (45 and 60.81 m^2/ml for emulsions produced by 10% of oil and treated at 100 and 200 MPa, respectively) in contrast to those treated by CM and CH treatments and produced using the same oil concentration (0.862 and 10.89 m^2/ml, respectively), the amount of surface protein per milliliter was higher in the UHPH emulsions (41 and 53.51 mg/ml) set against CM and CH emulsions (23.30 and 25.80 mg/ml, respectively). The high homogenization pressure can modify the protein properties and especially serum proteins by modifying their 3D-structures (Denda & Hayashi, 1992; Shibauchi, Yamamoto, & Sagara, 1992) and thus facilitate their adsorption at the interface, as reported by Dalgleish (1996).

In emulsions treated at 100 MPa, the increase in the oil concentration from 10 to 50% tended to the formation of multi protein layers at the interface (Fig. 30 G-I). In the case of emulsions treated at 200 MPa, and from TEM images (Fig. 30 J-L), it is evident that the amount of protein adsorbed onto the particle surface decreased as the oil content increased from 30 to 50% oil, indicating the insufficiency of covering protein, and subsequently the high rate of coalescence. This may be the reason for the high particle size, especially d4.3, the low surface area, and the low value of flow behaviour index of emulsions containing 50% of oil.

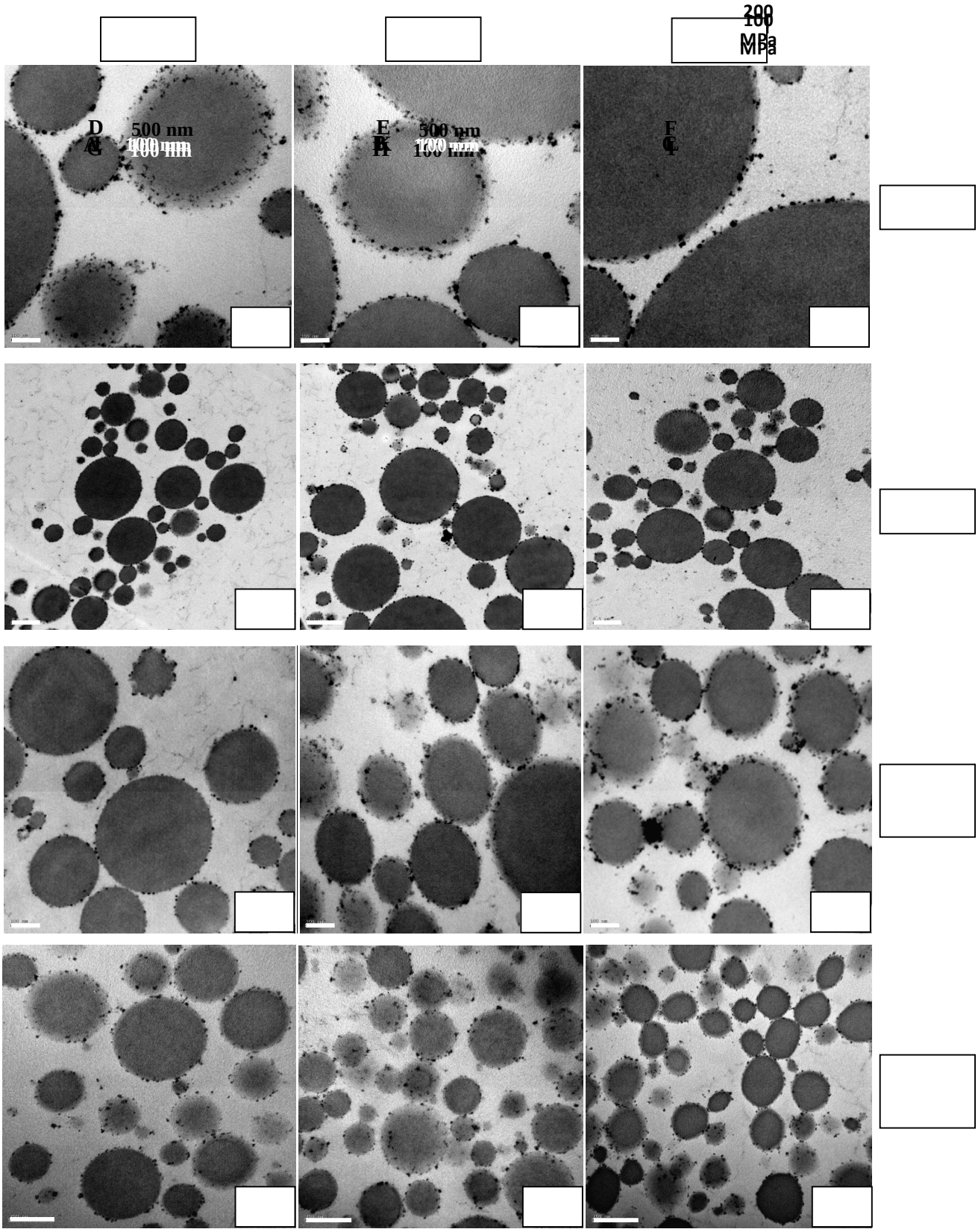

Figure 30. TEM images of emulsions containing 10, 30 and 50% oil and WPI 4% stabilized by conventional homogenization (CH) at 15 MPa (A-C) ×100000 and (D-F) ×25000, and by ultra high-pressure homogenization (UHPH) at 100 MPa (G-I) and 200 MPa (J-L) ×100000.

5.2.4. Rheological behaviour

Low viscosities and Newtonian behavior were observed for CM emulsions including those of 50% oil, and for CH emulsions containing 10 and 30% oil, because of the low interaction between particles; however, high consistency coefficient (K) and shear thinning behavior or pseudo-plasticity was observed in CH emulsions when the oil concentration increased to 50% (Table 11). Finally, when high homogenization pressure was used (UHPH), apparent viscosity of the emulsion increased, a fact that could be explained by the increase of particle interactions.

Besides providing kinetically stable emulsions, UHPH also enables the production of emulsions with a large range of flow behaviors (i.e., from highly fluid to highly thick samples) when combining the pressure level of homogenization and the oil volume fraction. Newtonian behavior ($n \approx 1$) and low viscosities were observed in the UHPH emulsions containing 10% (w/w) oil; however, increasing the oil content to 30% (w/w) significantly increased K values with a slight change in the rheological behavior toward the shear-thinning behavior in emulsions homogenized at 200 MPa ($n = 0.88$), but not in those treated at 100 MPa. The increase of oil concentration to 50% resulted in a huge increase in the K value, especially in emulsions treated at 200 MPa, and the flow index was completely changed to a high degree of pseudo-plasticity. Similar trends in the rheological characteristics of emulsions have been reported by Floury et al. (2000) using UHPH and the same emulsifier (1.5% whey proteins) with oil contents varying between 10 and 50%, and by Cortés-Muñoz et al. (2009) using 4% whey proteins and 15-45% oil. In a study realized by Bellaltaa, Troncosob, Zúñigac, & Aguilerab (2012), WPI emulsions sonicated at a nominal power level of 100 W for 180 s and containing 50-55% oil showed a Newtonian behavior, but further increase in the oil content to 60% changed the rheological behavior to shear-thinning. They reported that, in emulsions with lower oil contents, the particles are far apart and the inter-particle interactions are relatively weaker. As oil content increases, the particles are closer, which leads to packing of the oil droplets and the inter-particle interactions are stronger, giving a non-Newtonian behavior. The attractive forces between droplets drive the formation of aggregates, which can normally evolve into a space-filling particulate network.

Table 11. Mean ± SD of rheological characteristics (flow and consistency indices) and emulsifying activity index (EAI, m^2/g) of O/W emulsions containing 4% (w/w) of whey proteins plus sunflower and olive oils (10, 30 and 50%) and prepared by colloidal mill (CM), conventional homogenization (15 MPa) and ultra-high pressure homogenization at 100 and 200 MPa.

Pressure (MPa)	Oil content (%)	Rheological behavior			
		Consistency coefficient (K) mPa × s	Flow behavior index (n)	r^2	Emulsifying activity index EAI (m^2/g)
CM	10	0.0016 ± 0.0001^{h}	0.968 ± 0.020	0.998	407 ± 121^{g}
	30	0.0025 ± 0.0008^{gh}	1.105 ± 0.087	0.999	1566 ± 216^{ef}
	50	0.0185 ± 0.0051^{f}	1.045 ± 0.038	1.000	4715 ± 331^{d}
15	10	0.0017 ± 0.0001^{h}	0.984 ± 0.012	0.999	6674 ± 527^{cd}
	30	0.0051 ± 0.0018^{g}	0.973 ± 0.021	1.000	25571 ± 1434^{b}
	50	0.5299 ± 0.0696^{d}	0.596 ± 0.152	0.938	59968 ± 3433^{a}
100	10	0.0017 ± 0.0001^{h}	0.984 ± 0.013	0.999	1912 ± 174^{e}
	30	0.4037 ± 0.0008^{de}	0.973 ± 0.020	1.000	8029 ± 648^{c}
	50	2.8961 ± 0.7420^{b}	0.437 ± 0.086	0.984	5965 ± 168^{d}
200	10	0.0020 ± 0.0003^{h}	0.983 ± 0.016	0.999	1285 ± 85^{f}
	30	1.1960 ± 0.0168^{c}	0.882 ± 0.087	1.000	5522 ± 926^{d}
	50	8.3300 ± 1.108^{a}	0.284 ± 0.076	0.988	5412 ± 388^{d}

$^{a-h}$ Different letters in the same column indicate significant differences *($P < 0.05$)* between treatments.

Shear-thinning behavior is observed in flocculated emulsions because of deformation and breakdown of aggregates as shear stresses increase. The decrease in apparent viscosity of the emulsions with increasing shear rate could be attributed to the deformation and disruption of clusters or aggregates of droplets, and their ordering within the flow field (McClements, 2005).

UHPH emulsions prepared with 50% (w/w) oil presented not only a shear-thinning behavior, but also a thixotropic behavior (hysteresis) as shown in Figure 5. Thixotropic behavior of an emulsion indicates the existence of a structure that breaks down while shearing at a constant shear rate, in function of time followed by a gradual recovery when the shear is removed (Petrovic, Sovilj, Katona, & Milanovic, 2010). In this type of time dependent fluids, hysteresis loop could be observed when the sample is subjected to increasing and then reducing shear. CH and UHPH emulsions with 50% oil content had a thixotropic behavior, so a loop is visually seen between the up and down curves, i.e. the samples behave differently before and after shearing in the order 200 MPa > CH > 100 MPa (Fig. 5).

It is evident from Figure 5 that the emulsion treated at 200 MPa had a very strong aggregated structure (Fig. 4 L), in which the structure continued to disflocculate even after the shear rate was removed.

When such highly concentrated emulsions are subjected to small shear deformation, they exhibit a strong elastic response (characterized by a high value of the storage modulus); they also exhibit a yield stress. The emulsions treated at 200 MPa not only presented shear thinning and thixotropic behavior, but also possessed a yield stress, where the storage modulus versus stress plots exhibit a linear response (constant value of the storage modulus independent of the stress) up to a certain critical shear stress (Fig. 5, see the start of the violet line); however, this constant value did not observe in the rest of emulsions. With further increase in shear stress, the storage modulus drops sharply.

The yield stress, where a sharp reduction in storage modulus occurs, increases with an increase in the volume fraction of the dispersed phase (ϕ). Pal, (1999) reported that, when the volume fraction of the dispersed phase (ϕ) of the emulsion exceeds the maximum packing volume fraction (ϕ max, where the droplets just touch each other), the emulsion is referred to as a high internal phase ratio emulsion (HIPRE). The dispersed droplets of the emulsions are generally of spherical shape when ϕ is less than

ϕ max. When ϕ is greater than ϕ max, the droplets are no longer spherical; they are deformed against their neighbors and take the shape of a polyhedron, as was occurred in our emulsions containing 50% oil and treated at 200 MPa, see TEM image (Fig. 4 L).

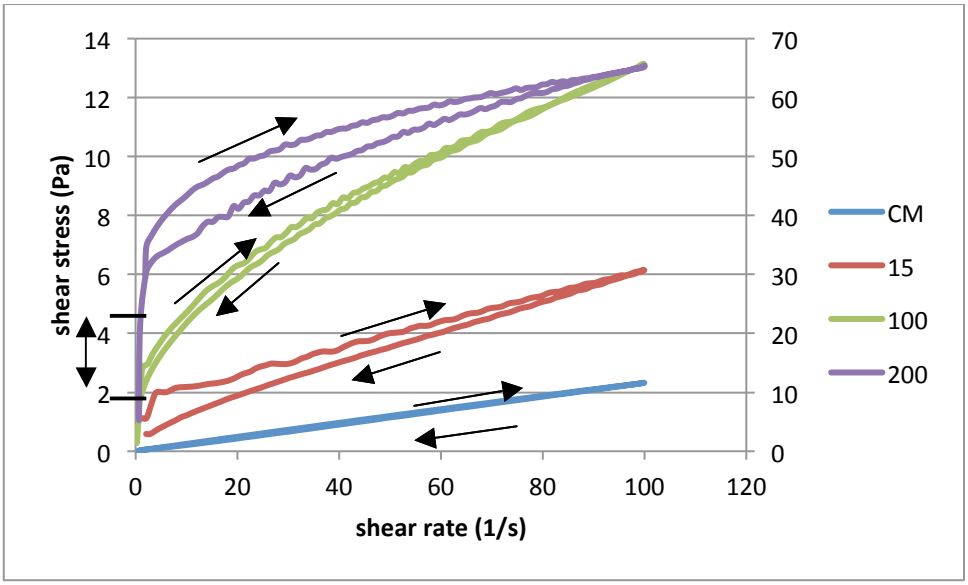

Figure 31. Hysteresis loops of O/W emulsions containing 50% oil and WPI 4% stabilized by colloidal mill ( ), conventional homogenization at 15 MPa ( ) and by ultra high-pressure homogenization at 100 MPa ( ) and 200 MPa ( ).

5.2.5. Emulsifying Activity Index (EAI)

Normally, EAI is a measure of the ability of the protein to aid the dispersion of the oil phase and to quickly provide sufficient coating of the interfacial area to avoid immediate coalescence (Dagorn-Scaviner, Guegan, & Lefebvre, 1987).

CM emulsions exhibited low EAI, but when applying low homogenization pressure (CH treatment) the EAI increased in comparison to CM emulsions, and further increase in the homogenization pressure (100 and 200 MPa) decreased the emulsifying activity of whey proteins. This could be due to the high surface area in UHPH emulsions, comparing with those treated by CM and CH, and the necessity of high protein amounts to cover the new/newly created interface.

It is important to note that the emulsifying activity of emulsions treated at 100 MPa was higher than their homologous emulsions treated at 200 MPa. Whey proteins are excellent foaming and emulsifying agents; however, to obtain optimum foaming and

emulsifying characteristics, the protein must be substantially soluble, to diffuse to the newly formed interface, to unfold and reorient in ways that lower interfacial tension, and to form cohesive and viscoelastic films by polymerization, mainly via disulfide bonds and hydrophobic interactions (Bouaouina et al., 2006). Partial denaturation of WPI usually improves its emulsifying capacity, due to an increase in the surface hydrophobicity and molecular flexibility (Kato, Osako, Matsudomi, & Kobayashi, 1983). This is consistent with many previous literature reports showing that the major protein (β-Lg) in WPC usually unfolds and denatures at a temperature which is close to its temperature of denaturation (Qi, Brownlow, Holt, & Sellers, 1995). The partially unfolded and denatured proteins, as could occur in the UHPH emulsions treated at 100 MPa (T2 = 60-68°C), would be more easily adsorbed and associated to form a viscoelastic film on the oil-water interfaces. However, extensive denaturation, as could occur in UHPH emulsions treated at 200 and 300 MPa, may result in poor interfacial mechanical properties, which would be detrimental to long-term stability of emulsions (Kim, Cornec, & Narsimhan, 2005).

Focusing on how the oil volume fraction affects the EAI, it is evident that EAI values of WPI emulsions produced by CM increased as the oil volume fraction did (Table 3), indicating that per gram of WPI the emulsions could form larger oil surface areas in high than in low oil volume fractions, as was explained before in the Surface Protein Concentration (SPC) section. As elucidated in the SPC section, increasing the oil concentration while maintaining a constant protein level, leads to a reduced surface concentration of protein, thus suggesting the spreading of protein at an interface to form a thinner layer (Srinivasan et al., 1996). A similar trend was observed by Al-Malah, Azzam, & Omari (2000) in emulsions stabilized by 0.1% w/v bovine serum albumin in corn, soybean, sunflower and olive oils when the oil volume fraction increased from 25 to 56%, and by Gu, Decker, & McClements (2009), increasing oil concentrations from 10 to 20% in sunflower and soy oil emulsions.

Increasing the oil content from 10 and 30% to 50% in the CH emulsions tended to a considerable increase in the EAI values. As described before, the EAI is a measure of the ability of proteins to adsorb at the interface. Considerable increase in the surface protein concentration (Table 2) and visual multilayers could be observed in the TEM images of CH emulsions as oil content increased to 30% (Fig. 4 A, B) whereas, this amount of proteins significantly decreased as oil content increased to 50% (Fig. 4 C).

We hypothesize that the observed increase in the EAI value when the oil content increased to 50% may be attributed to the bridging flocculation due to the insufficient protein but not to the high ability of protein to cover the oil interface as can normally be expected. Flocculation increases the apparent volume of dispersed phase and leads to the formation of nonspherical aggregates (Tadros, Izquierdo, Esquena, & Solans, 2004), which may enhance the turbidity of sample during the EAI measurements, and thus increase the EAI value.

In the case of the UHPH emulsions, the EAI increased as the oil content increased from 10 to 30% but, the EAI was decreased or maintained when the oil content further increased to 50%, which may be attributed to the high rate of flocculation of oil particles in UHPH-treated emulsions containing 50% oil, as explained before (see the Surface Protein Concentration section).

5.2.6. Stability of emulsions to creaming

An emulsion is thermodynamically unstable because of the large interfacial area between the oil and the aqueous phase (McClements, 2005). Nano/submicron emulsions typically have much better stability to gravitational separation than conventional emulsions because the relatively small particle size means that Brownian motion effects dominate gravitational forces. They also tend to have better stability against droplet flocculation and coalescence because the range of the attractive forces acting between the droplets decreases with decreasing particle size, while the range of the steric repulsion is less dependent on particle size (McClements, 2005; Tadros et al., 2004).

The light scattering fingerprints obtained by Turbiscan lab of the emulsion samples, with oil concentrations of 10 and 50% w/w and treated by CM, CH and UHPH are shown in Figure 6.

Low creaming stability could be observed in CM emulsions, in comparison to CH and UHPH emulsions. A great variation with time could be observed in the backscattering profiles for CM emulsions containing 10%, the emulsion being totally separated in ~ 1 h. The possible reasons for the low creaming stability of CM emulsions may be the high particle size, the high interfacial tension between oil droplets, because no protective protein layer exists surrounding the oil droplets, the low viscosity and the high coalescence rate, as was described previously in the size distribution section. CM

emulsions containing 50% of oil exhibited higher stability to creaming and lower separation rate with the time. These results were validated by calculating the migration velocity V (t) (µm/min) of particles to the top of the tube using the Turbiscan. The migration velocity was reduced in emulsions containing 50% of oil (37.1 µm/min), in comparison to those containing 10 and 30% of oil (273.6 and 79.3 µm/min, respectively). The high stability of emulsions containing 50% of oil may be due to the significant lower particle size, the high viscosity, and the good protein coverage as shown in CLSM image (Fig. 2 C).

Table 4 and Figures 7 (A-F) and 6 (C-H) illustrate the physical stability against creaming in CH and UHPH emulsions. The CH emulsions exhibited lower tendencies to creaming in comparison to CM emulsions, especially those containing 30 and 50% oil, presenting lower migration velocity of particles (120.4, 18.6 and 17.7 µm/min for CH emulsions containing 10, 30 and 50% oil, respectively). Creaming was visually observed in CH emulsions containing 10 and 30% of oil, but not in emulsions containing 50% oil. These results were also confirmed by the d4.3 value at the top or at the bottom of emulsions, as shown in Table 4, where differences were significant in emulsions containing 10 and 30% oil, but not in those containing 50% oil. It can also be observed in Figure 7 (A, B) that no changes in the distribution curves in emulsions containing 50% oil were observed, while the distribution curves were notably changed in the case of emulsions containing 10% oil. Conventional emulsions are thermodynamically unstable systems because of the positive free energy associated with the contact of oil and water phases, as manifested by a relatively high positive interfacial tension (Friberg, Larsson, & Sjobolom, 2004).

Table 12. Mean ±SD of d4.3 values at the top or at the bottom of samples stored at room temperature for 9 days under the same conditions for comparison, of O/W emulsions containing 4% (w/w) of whey proteins plus sunflower and olive oils (10, 30 and 50%) and prepared by conventional homogenization (15 MPa) and ultra-high pressure homogenization 100 and 200 MPa.

Pressure (MPa)	Oil content (%)	Emulsion creaming stability after 9 days	
		d4.3 (Top)	d4.3 (Bottom)
15	**10**	0.907 ± 0.037^{b}	**0.487 ± 0.061^{c}***
	30	1.204 ± 0.053^{ab}	**0.775 ± 0.046^{b}***
	50	1.411 ± 0.194^{a}	1.436 ± 0.191^{a}
100	**10**	0.175 ± 0.018^{d}	**0.145 ± 0.015^{fg}***
	30	0.186 ± 0.016^{d}	0.183 ± 0.014^{ef}
	50	0.267 ± 0.021^{cd}	0.240 ± 0.039^{de}
200	**10**	0.117 ± 0.009^{d}	0.121 ± 0.004^{g}
	30	0.191 ± 0.069^{d}	0.188 ± 0.068^{ef}
	50	0.422 ± 0.118^{c}	0.531 ± 0.237^{c}

$^{a-h}$ Different letters in the same column indicate significant differences ($P < 0.05$) between treatments.

* Sign indicates that the differences between the d4.3 at the top or at the bottom of emulsions are significant (Wilcoxon statistic test, $P < 0.05$) per level of pressure and oil concentration.

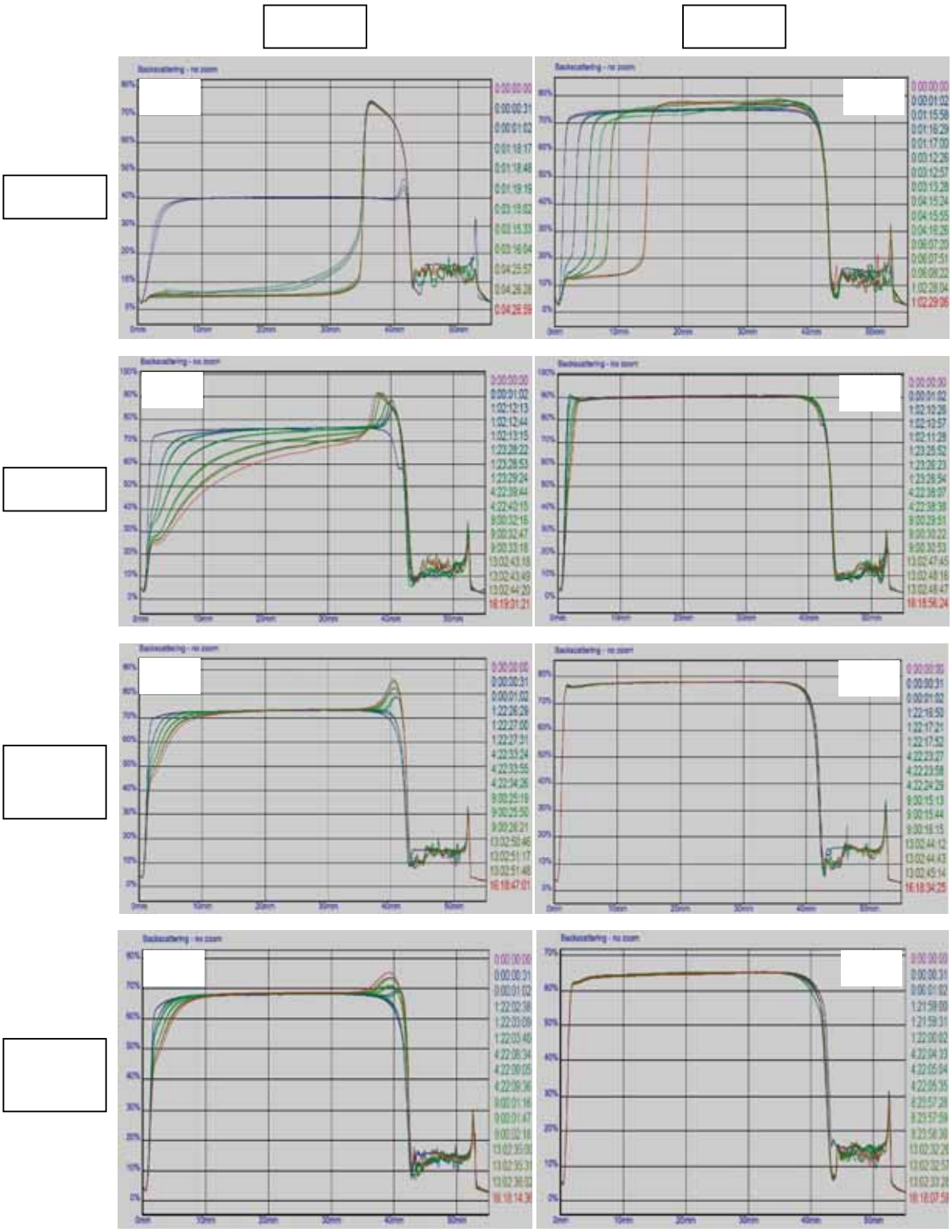

Figure 32. Changes in backscattering profiles of emulsions containing 4% of WPI and different oil contents, 10% (A, C, E, G) and 50% (B, D, F, H), prepared by (A, B) colloidal mill (CM), (C, D) conventional homogenization (CH), and ultra high pressure homogenization at 100 MPa (E, F) and 200 MPa (G, H), as a function of sample height with storage time (5 h for CM emulsions and 17 days for both CH and UHPH emulsions).

All UHPH emulsions remained fully turbid throughout the tube during the 17 days of storage without any visual changes of phase separation. Generally, UHPH led to excellent oil droplet stability vs. creaming and coalescence for all model emulsions, despite the probability of forming aggregates in emulsions containing 50% of oil. For instance, the migration velocity value calculated by Turbiscan for UHPH emulsions treated at 200 MPa and containing 10% of oil was much lower (15.2 μm/min) than that calculated for the CM and CH emulsions (273.6 and 120.4 μm/min, respectively), at the same concentration.

UHPH-treated emulsions at 200 MPa seem to be much more stable than those treated at 100 MPa. The d4.3 values at the top and at the bottom of UHPH emulsions indicated a slight creaming effect in emulsions with 10% oil treated at 100 MPa (Table 4, Fig. 7 C and Fig. 6 E), whereas emulsions treated at 200 MPa were more stable to creaming, as no significant differences could be proven between the top and the bottom (Table 4 and Fig. 7 E).

In structured emulsions the lipid droplets may be surrounded by dense biopolymer coatings (multilayer emulsions) or embedded in dense biopolymer particles (filled biopolymer particles), which may impact their tendency to cream or sediment. High-pressure homogenization improves the creaming stability of emulsions by decreasing the particle size according to Stoke's law, which in turn increases the density of particles and in turn the viscosity of the emulsion, slowing down the particle movement (Floury, Desrumaux, Axelos, & Legrand, 2002; Lee, Lefèvre, Subirade, & Paquin, 2009).

The stability results achieved for the UHPH emulsions are in agreement with the results found by Cortés-Muñoz et al. (2009). They observed a slight creaming effect in only a few cases, mainly after processing emulsions with 15% (w/w) oil treated at 100-150 MPa, while emulsions treated at 200 MPa led to excellent oil droplet stability vs. creaming and coalescence, especially when high oil concentration was used.

The oil concentration significantly affected the stability of emulsions. Increasing the oil concentration lowered the creaming phenomenon to a great extent. For the same droplet sizes, oil content plays an important role, as a large amount of oil in the emulsion results in a more stable system. The explanation for the lower tendency to creaming in emulsions containing higher amounts of oil, either in the conventional or in the UHPH emulsions, is an increase in the viscosity of the continuous phase that surrounds the oil

droplets, restricting the movement of droplets. It was reported that increasing the viscosity of the continuous water phase minimizes droplet mobility and decreases collision numbers. Cortés-Muñoz et al. (2009) reported an excellent stability in higher oil concentrations compared to lower oil concentrations.

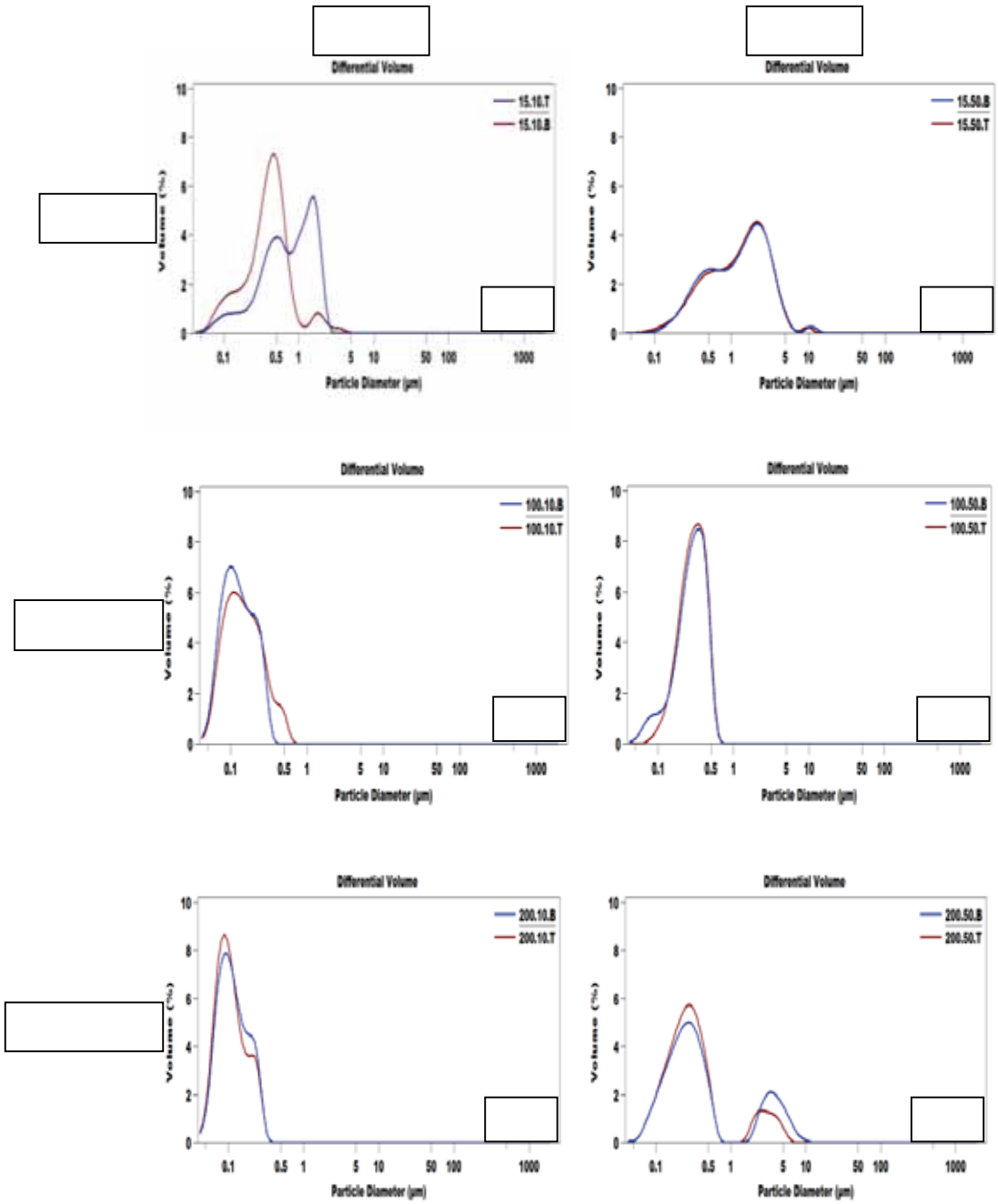

Figure 33. Droplet size distribution curves at the top (T) and the bottom (B) of O/W emulsions containing 10 and 50% oil plus 4% of WPI and processed by (A,B) conventional homogenization (CH) and ultra high pressure homogenization at 100 (C,D) and 200 (E,F) MPa after 9 days of storage at room temperature.

5.2.7. Oxidative stability

As lipids oxidize, hydroperoxides are formed and these are susceptible to further decomposition to secondary reaction products such as aldehydes, ketones, acids, hydrocarbons, and alcohol, which are responsible for the physicochemical characteristics and sensory properties of oxidized oils (McClements & Decker, 2000).

Hydroperoxide content and TBARS variations of emulsions treated by CM, CH and UHPH containing different oil concentrations plus 4% of WPI are shown in Table 5.

It is well known that, at a fixed oil concentration, total droplet surface increases as droplet diameter decreases, and therefore the rate of lipid oxidation is expected to increase (Nakaya, Ushio, Matsukawa, Shimizu, & Ohshima, 2005). However, this tendency can be modified, or even inverted, owing to the specific characteristics of the emulsion and the protective ability of the interface against oxidation, as was found in a number of studies (Azuma, Kimura, Hosokawa, & Miyashita, 2009; Nakaya et al., 2005; Lethuaut, Métro, & Genot, 2002; Hu, McClements, & Decker, 2003)

The results of the present study showed that the CM emulsions, which presented high particle size and low SSA, were more oxidized with a clearer discrimination than the corresponding CH and UHPH emulsions, with the lowest amounts of oxidation products being presented in UHPH emulsions, despite the low particle size and the high SSA. In this sense, Nakaya et al. (2005) measured higher lipid hydroperoxide and hexanal concentrations as well as lower residual oxygen concentrations in polyunsaturated triacylglycerol emulsions stabilized with sucrose lauryl ester or decaglycerol lauryl ester at larger, rather than smaller droplet size. Lethuaut et al. (2002) remarked that, although small droplets were more susceptible towards oxidation than large droplets in emulsions containing serum-albumin from bovine milk, the susceptibility was partly canceled out by the antioxidative activity of the protein. Atarés, Marshall, Akhtar, & Murray (2012) using a high-pressure jet homogenizer at 30 MPa evaluated the structure and oxidative stability of O/W emulsions formulated with whey protein and sunflower oil in the presence of flavonoid rutin. These authors found that high-pressure homogenization, through droplet size reduction, stabilized the emulsions against both creaming and oil oxidation. All these studies suggest that the surface area is not the only determining factor of the oxidative stability of emulsions.

Table 13. Mean ± SD of hydroperoxides (A_{510} nm) and TBA reactive substances (µg/ml) of O/W emulsions containing 4% (w/w) of whey proteins plus sunflower and olive oil (10, 30 and 50%) and prepared by colloidal mill (CM), conventional homogenization (15 MPa) and ultra high-pressure homogenization at 100 and 200 MPa.

Pressure (MPa)	Oil content (%)	Hydroperoxides (A_{510} nm)			TBARS (µg/ml)		
		Day 1	Day 10	Difference (day 10 - day 1)	Day 1	Day 10	Difference (day 10 - day 1)
CM	10	0.017 ± 0.004^{c}	0.326 ± 0.195^{ab}	0.309 ± 0.198^{a*}	0.097 ± 0.016^{a}	0.143 ± 0.017^{ab}	0.046 ± 0.031^{ab*}
	30	0.039 ± 0.005^{abc}	0.333 ± 0.026^{ab}	0.294 ± 0.022^{a*}	0.060 ± 0.003^{bcd}	0.079 ± 0.003^{cde}	0.020 ± 0.004^{abcd*}
	50	0.078 ± 0.003^{a}	0.433 ± 0.063^{a}	0.356 ± 0.063^{a*}	0.100 ± 0.007^{a}	0.160 ± 0.022^{a}	0.061 ± 0.017^{a*}
15	10	0.039 ± 0.015^{abc}	0.252 ± 0.032^{ab}	0.213 ± 0.047^{ab*}	0.054 ± 0.002^{bcd}	0.075 ± 0.007^{cde}	0.021 ± 0.007^{abcd*}
	30	0.041 ± 0.023^{abc}	0.178 ± 0.012^{c}	0.137 ± 0.034^{ab*}	0.058 ± 0.004^{bcd}	0.092 ± 0.009^{cd}	0.034 ± 0.006^{abcd*}
	50	0.057 ± 0.029^{ab}	0.245 ± 0.048^{ab}	0.187 ± 0.074^{ab*}	0.068 ± 0.005^{bc}	0.110 ± 0.035^{bc}	0.041 ± 0.034^{abc*}
100	10	0.022 ± 0.004^{bc}	0.027 ± 0.004^{cd}	0.005 ± 0.004$^{\mathbf{bc*}}$	0.052 ± 0.004^{cd}	0.044 ± 0.003^{e}	–0.080 ± 0.003^{e*}
	30	0.026 ± 0.006^{bc}	0.034 ± 0.0008^{cd}	0.009 ± 0.006^{bc*}	0.051 ± 0.004^{cd}	0.040 ± 0.008^{e}	–0.011 ± 0.008^{e*}
	50	0.028 ± 0.009^{bc}	0.033 ± 0.009^{cd}	0.0049 ± 0.004^{bc*}	0.041 ± 0.009^{d}	0.053 ± 0.008^{de}	0.012 ± 0.006^{bcd*}
200	10	0.025 ± 0.004^{bc}	0.024 ± 0.003^{d}	0.000 ± 0.0016^{c}	0.054 ± 0.008^{bcd}	0.088 ± 0.006^{cd}	0.034 ± 0.011^{abcd*}
	30	0.019 ± 0.006^{bc}	0.037 ± 0.003^{cd}	0.018 ± 0.004^{b*}	0.059 ± 0.008^{bcd}	0.066 ± 0.013^{de}	0.006 ± 0.017^{bcd}
	50	0.032 ± 0.006^{bc}	0.038 ± 0.004^{c}	0.006 ± 0.004^{bc*}	0.072 ± 0.007^{b}	0.072 ± 0.004^{cde}	0.000 ± 0.009^{cd}

$^{a\text{-}e}$ Different letters in the same column indicate significant differences *($P < 0.05$)* between treatments.

* sign indicates that the differences between day 10 and day 1 (oxidation evolution) is significant *($P < 0.05$)*.

Considerably high primary and secondary oxidation products were observed in CM emulsions, being CM emulsions containing 50% oil, the emulsions that presented the lowest oxidative stability at the first or at the last day of storage. The high hydroperoxide value in combination with the high levels of TBARS obtained in CM emulsions, especially those containing 10 or 50% oil, indicates the progression of oxidation from a primary to a secondary state, showing the high sensitivity of these emulsions to lipid oxidation. This high sensitivity of CM emulsions to oxidation may be attributed to the low protein coverage (Fig. 3 B) and the high coalescence rate in comparison to CH and UHPH emulsions, as described before.

CH emulsions presented oxidation level between CM and UHPH emulsions. At day 10 of storage, CH emulsions containing 10 and 50% oil presented significantly higher amounts of hydroperoxides in comparison to those containing 30% oil. Furthermore, the secondary oxidation products also increased as the oil content increased to 50% in CH emulsions, although the differences in TBARS were not significant between oil concentrations.

Our results are in agreement with those previously reported in safflower oil (Sims, Fioriti, & Trumbetas, 1979), canola oil (Osborn & Akoh, 2004), menhaden oil (Sun and Gunasekaran2009) and walnut oil (Gharibzahedi, Mousavi, Hamedi, Khodaiyan, & Razavi, 2012), in which the oil-phase volume fraction played a dominant role in determining the oxidative stability, affecting the oxidative stability of emulsions in a bad way when the oil content increased.

UHPH emulsions were the emulsions that presented the best oxidative stability. However, those treated at 100 MPa seem to be slightly less oxidized than those treated at 200 MPa, in which similar amounts of hydroperoxides were found after 10 days of storage, but higher amounts of TBARs were obtained in emulsions treated at 200 MPa, although this increase was only significant in emulsions containing 10% oil. The reasoning behind the higher oxidation rate observed in UHPH emulsions treated at 200 MPa, in respect to those treated at 100 MPa, could be the decrease in the efficiency of whey proteins to protect the oil droplets with increased pressure treatment, which may be related to the over processing phenomenon, because of the increase in the product

temperature at the outlet of the homogenization valve which affects the emulsifying properties of whey proteins.

5.3. References

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Chapter 6

Sodium caseinate oil-in-water emulsions stabilized by conventional and ultra-high pressure homogenization: Effects of protein concentration and pressure on structure and stability

6.1. Introduction

Nowadays the food industry has a growing interest in the replacement of synthetic emulsifiers by natural ones, such as polysaccharides and proteins. When proteins are used as emulsifiers, because of their amphiphatic nature, they adsorb at the O/W interface and then undergo unfolding and rearrangement to form a stabilizing layer at the droplet surface (Das & Kinsella, 1990; Dickinson, 1998). Casein is of particular importance as an emulsifier because of its ability for rapidly conferring a low interfacial tension during emulsification and because of the strongly amphiphilic characteristics of the major individual caseins. Moreover, sodium caseinate (SC) is a well-used ingredient because of its good solubility and emulsifying properties and its stability during heating.

One of most significant aspects of any food emulsion is its stability. The long-term stability of caseinate-based emulsions is attributed to a combination of both electrostatic and steric stabilization (Sun & Gunasekaran, 2009). In addition, caseins have been reported to be an efficient antioxidant protein in milk by absorbing to the surface of fat globule membrane and, in combination with other antioxidant compounds, caseins exhibited synergistic effect of antioxidant activity (Hegenauer, Saltman, Ludwig, Ripley, & Bajo, 1979). Caseins form a thicker interfacial layer (10 nm) in O/W emulsion droplets, which may explain why caseinate-stabilized O/W emulsions have been found to exhibit increased oxidative stability compared to whey protein isolate-stabilized emulsions (Hu, McClements, & Decker, 2003).

The high-pressure homogenization process of emulsions can produce emulsions with smaller droplet size, increasing the interaction between the interface (O/W) and the protein used as emulsifier.

Although a great deal of research has been focused on the physical stability and interfacial properties of protein-stabilized O/W submicron-emulsions (Desrumaux & Marcand, 2002; Floury, Desrumaux, Axelos & Legrand, 2003; Cortés-Muñoz, Chevalier-Lucia & Dumay, 2009; Kiokias, Reiffers-Magnani & Bot, 2004), very little research has focused on the use of sodium caseinate as an emulsifier and its effect on the physical and oxidative stability of these emulsions. Hence, the aim of the present work was to study the physical and oxidative stability of emulsions containing SC under various conditions of protein concentration and pressure using the UHPH technology in comparison with conventional homogenization. The methodology applied for this purpose is described in Chapter 3 and includes the study of physical and oxidative properties including: particle size distribution, rheological behavior, emulsifying activity, microstructure (CLSM and TEM microscopy), stability to creaming measured visually and by two light scattering techniques (particle size at the top and the bottom of emulsions and by Turbiscan lab), and the stability to oxidation, determining the hydroperoxides and thio-barbituric acid reactive substances (TBARS).

6.2. Results and discussion

6.2.1. Temperature rise during high-pressure homogenization

The temperature of the emulsions increased with increasing the pressure when passed through the homogenizer (Table 14), which is in agreement with the results of Floury, Desrumaux et al. (2003) working on emulsions treated by high-pressure homogenization using methylcelulose as emulsifier. The warming up of the emulsion is due to force-induced phenomena of shear, turbulence, and cavitation, which happen simultaneously, dissipating the mechanical energy as heat during emulsification. However, heating during homogenization occurs during a short period of time, depending on the cooling system of the UHPH equipment, and its contribution to modification of the macromolecules and droplet size is uncertain (Floury et al., 2003).

Temperature (T2) measured after the HP-valve increased by 47.7, 51 or 47.4°C per 100 MPa between 100 and 300 MPa for the three respective protein concentrations (1, 3 or 5%, respectively). The reason behind the temperature elevation decrease when the protein concentration increased to 5% may be the viscosity increase in these emulsions

emulsions could be explained by the viscous stress caused by the high velocity of the fluid flow, which then impinged on the valve (McClements, 2005). These results are similar to those of Desrumaux & Marcand (2002), Floury et al. (2003), and Bouaouina, Desrumaux, Loisel, & Legrand (2006) who reported a considerable temperature rise in the emulsions, despite using a cooling jacket at the exit of the valve of an ultra-high pressure system.

Table 14. Mean ± SD values of temperature measured before (T1) the high-pressure valve and at the outlet (T2) of the high-pressure valve for emulsions containing different concentrations of sodium caseinate (1, 3 and 5%) treated by ultra-high pressure homogenization at 100, 200 and 300 MPa (Tin = 25°C).

Protein content (%)	Pressure (MPa)	T1 (°C)[a]	T2 (°C)[b]
1	100	36.7 ± 1.53	59.3 ± 4.73
	200	42.0 ± 2.00	84.7 ± 1.53
	300	39.5 ± 3.5	107 ± 5.50
3	100	38.3 ± 1.15	59.0 ± 4.35
	200	43.0 ± 2.00	86.0 ± 4.36
	300	40.0 ± 6.00	110 ± 2.50
5	100	39.0 ± 1.00	60.6 ± 4.04
	200	42.6 ± 0.57	86.0 ± 3.00
	300	40.5 ± 5.50	108 ± 0.50

6.2.2. Particle size distribution

Emulsion droplet size plays a key role in many emulsion properties such as stability, color, appearance, texture, and rheology, and the aim of emulsification is usually to produce emulsion droplets as small as possible.

Droplet size indices (d3.2 and d4.3 μm) and specific surface area (SSA, m^2/ml) for emulsions containing 20% oil and different SC concentrations (1, 3 and 5%) are shown in Table 15 and Figure 34. CM emulsions had the largest particle size (d3.2 and d4.3) followed by CH emulsions and the minimum droplet size was found in emulsions stabilized by UHPH (Table 15). This decrease in the particle size was also confirmed by

CM emulsions with the highest particle size showed the lowest SSA; however, the decrease in the particle size when homogenization was applied (CH and UHPH-treated emulsions) increased the SSA.

The droplet size distributions of CM emulsions show a large peak (Fig. 34 A) at a size of around 10-20 µm (similar to the d4.3 values), probably because of the impotence of the homogenizer to create particles with small size and to the droplets coalescence as can be observed in Figure 37 (A-C). This may be a result of the high degree of surface tension in these emulsions, due to the low protein load at the interface, especially in CM emulsions containing 1% protein rather than those containing 3 and 5% protein. Indeed, the fine emulsions produced via conventional and UHPH homogenizers showed a much smaller size range of particle size distribution (Fig. 34). This occurred because the higher homogenization pressure led to an increase in impact forces that act on the droplets, causing disruption of the interfacial membranes (McClements, 2005) with a consequent increase in the interfacial area and interaction between oil and emulsifier (Floury, Desrumaux, & Lardières, 2000). Moreover, a temperature rise during homogenization reduces the viscosities of emulsion phases, and lowers the interfacial tension and Laplace pressure, thereby reducing the minimum thermodynamic energy necessary for emulsification which facilitates production of smaller droplets (Canselier et al., 2002; McClements, 2005). Similar results were found by Atarés, Marshall, Akhtar, & Murray (2012) evaluating the structure and oxidative stability of O/W emulsions formulated with whey protein and sunflower oil in the presence of flavonoid rutin using a high-pressure jet homogenizer at 30 MPa, where the particle size (d3.2) was reduced to the submicrom range (918 nm) in comparison to those measured in emulsions produced by ultraturrax rotor-stator (48.75 µm).

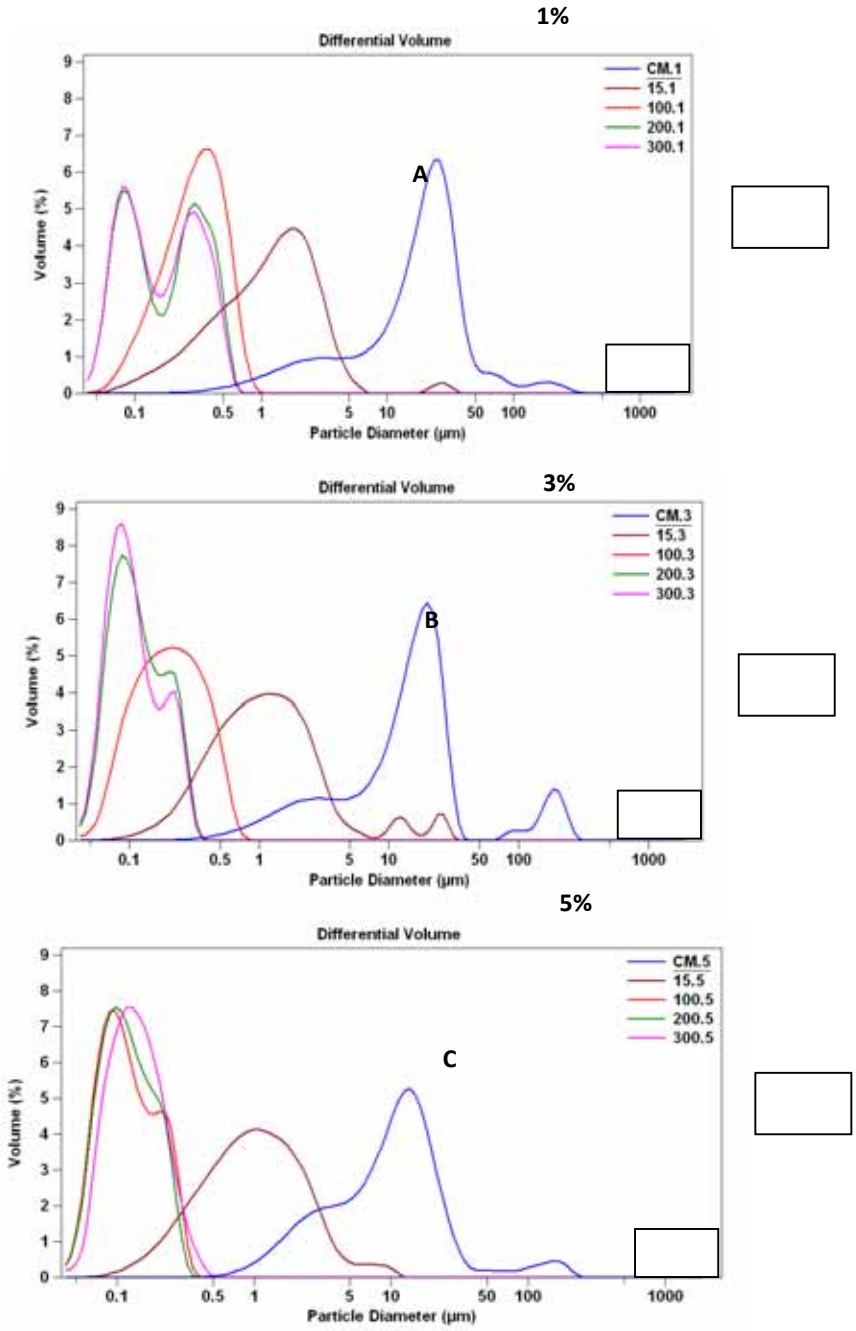

Figure 34. Droplet size distribution curves measured by light scattering of O/W emulsions containing 1 (A), 3 (B) and 5% (C) of SC plus 20% (w/w) of sunflower and olive oils and prepared by colloidal mill (CM), conventional homogenization (15 MPa) and ultra-high pressure homogenization (100 and 200 MPa).

Table 15. Mean ± SD of particle size distribution indices (d3.2 and d4.3) and specific surface area (SSA, m^2/ml) of O/W emulsions containing 20% (w/w) of sunflower and olive oils plus sodium caseinate (1, 3 and 5%) and prepared by colloidal mill (CM), conventional homogenization (15 MPa) and ultra-high pressure homogenization at 100, 200 and 300 MPa.

Pressure (MPa)	Protein content (%)	Particle size distribution		
		d3.2 (µm)	d4.3 (µm)	Specific surface area SSA (m^2/ml)
CM	1	6.828 ± 0.310^{a}	21.72 ± 4.325^{a}	0.894 ± 0.038^{g}
	3	5.641 ± 0.395^{b}	19.35 ± 6.026^{ab}	1.079 ± 0.066^{g}
	5	5.421 ± 0.362^{b}	13.29 ± 2.109^{b}	1.143 ± 0.053^{g}
15	1	0.578 ± 0.074^{c}	1.216 ± 0.103^{c}	10.57 ± 1.228^{f}
	3	0.597 ± 0.089^{c}	1.355 ± 0.189^{c}	10.00 ± 1.344^{f}
	5	0.572 ± 0.094^{c}	1.421 ± 0.216^{c}	9.677 ± 0.630^{f}
100	1	0.210 ± 0.046^{d}	0.294 ± 0.046^{d}	25.29 ± 0.364^{e}
	3	0.151 ± 0.014^{e}	0.219 ± 0.012^{e}	40.02 ± 4.433^{d}
	5	0.116 ± 0.009^{ef}	0.144 ± 0.006^{g}	54.63 ± 0.625^{ab}
200	1	0.141 ± 0.010^{ef}	0.223 ± 0.011^{e}	43.17 ± 1.781^{cd}
	3	0.120 ± 0.013^{ef}	0.157 ± 0.031f^{g}	53.58 ± 0.720^{abc}
	5	0.108 ± 0.008^{ef}	0.131 ± 0.011^{g}	59.21 ± 1.373^{a}
300	1	0.129 ± 0.002^{ef}	0.205 ± 0.005^{ef}	45.71 ± 1.721^{bcd}
	3	0.098 ± 0.001^{f}	0.121 ± 0.003^{g}	61.71 ± 0.892^{a}
	5	0.111 ± 0.009^{ef}	0.125 ± 0.006^{g}	53.86 ± 4.986^{abc}

$^{a-g}$ Different letters at the same column indicate significant differences *($P < 0.05$)* between treatments.

The d4.3 parameter allows coalescence and flocculation processes to be detected with more sensibility than the d3.2 value. However, even if the d4.3 values could be absolutely reliable, a large increase in d4.3 reflects the association of the emulsion droplets into large aggregates. CM emulsions presented higher d4.3 values than CH and UHPH emulsions, indicating the flocculation and high destabilization rate of these emulsions.

Protein concentration is known to influence emulsion droplet size, surface protein concentration, and storage stability (Dickinson, Murray, & Stainsby, 1988). Changes in protein concentration and pressure during homogenization affected both the average droplet diameter (d3.2 and d4.3, Table 15) and the droplet size distribution (Figure 34). The protein concentration affected the particle size of emulsions treated by CM, in which increasing the protein concentration from 1 to 3% resulted in a decrease in the d3.2 value, but further increase in the protein concentration to 5% had no additional effect on the particle size ($P<0.05$).

The d4.3 decreased significantly when the protein content increased to 5%, which is in accordance with the size distribution curves, being the CM emulsions containing 5% of SC, the emulsions that presented the lower d4.3 value. Furthermore, this decrease in the particle size could be visualized in the CLSM images (Fig. 35 A,B); the oil particles (red channel) are bigger in emulsions containing 1% protein and separated from the protein molecules (green channel), while a notable decrease in the particles size and a high protein coverage, refer to the yellowish color, which is a mix of the red channel (oil) and green channel (protein), could be clearly seen in the image of emulsion containing 5% protein.

The protein concentration had no effect on either the d3.2 or the d4.3 values in CH emulsions (Table 15). However, Figure 35 (C,D) shows that the emulsion droplets in emulsions containing 1% protein appeared to be homogeneous (Fig. 35 C), while emulsions containing 5% protein showed large numbers of small particles aggregated together and separated from the aqueous phase to form a network structure (Fig. 35 D).

Depending on the concentration of SC in the emulsions, protein-coated droplets may be destabilized by bridging or depletion flocculation (Gu, Decker, & McClements, 2004). Bridging flocculation occurs when the polymer concentration is not sufficient to completely saturate the droplet surfaces (as CH emulsions containing 1% of SC in our

study), leaving the polymer chains in a position to adsorb onto the surfaces of two droplets (Berli, Quemada, & Parker, 2002). The insufficiency of protein coverage could be observed by CLSM (Fig. 35 C) in the CH emulsion containing 1% protein, in which a lot of red particles without protein cover can be observed, a fact that is confirmed by the appearance, to some extent, of a double emulsion (O/W/O). The main destabilization mechanism for this emulsion was creaming of individual particles. On the other hand, in depletion flocculation, the presence of non-adsorbed molecules in the continuous phase of an emulsion (as CH emulsions containing 5% protein) causes an increase in the attractive forces between the droplets due to an osmotic effect associated with the exclusion of colloidal particles from a narrow region surrounding each droplet (McClements, 2005), producing an aggregated network structure that in our study was visible by CLSM and was confirmed by the shear thinning rheological behavior of these emulsions as will be explained later (Table 16). The aggregated network structure, with respect to the depletion flocculation formed with high concentrations of caseinate in emulsions, has been described in previous works (Dickinson & Golding, 1997; Srinivasan, Singh, & Munro, 2000). The first authors studied the effect of SC concentration on emulsion stability, observing that the emulsions formulated with 0.5 and 1% of SC destabilized mainly by creaming; however, for the emulsion containing 2% SC, both creaming and flocculation mechanisms were involved. The authors also found that concentrations below 0.5% SC seemed to be below that required for the saturation of monolayer coverage, since creaming rate was greater for 0.5% than for 1% SC. Further addition of protein led to high instability, producing flocculation and migration of flocculates.

Concerning UHPH emulsions, the droplet size decreased with increasing both the pressure and the concentration of SC. Concerning the effect of the homogenization pressure on d4.3 parameter, increasing the pressure from 100 to 200 MPa had a significant effect on the particle size (d4.3) and the SSA, especially in emulsions containing 1 and 3% SC; however, no further reduction was observed as the pressure increased to 300 MPa. Roach & Harte (2008) reported that the size of casein micelle decreases with increasing homogenization pressure up to 200 MPa, after which the size remains constant. Sandra & Dalgleish (2005) also reported that ultra-high pressure homogenization produced emulsions with smaller droplets up to a certain pressure; however, coalescence occurred above this pressure.

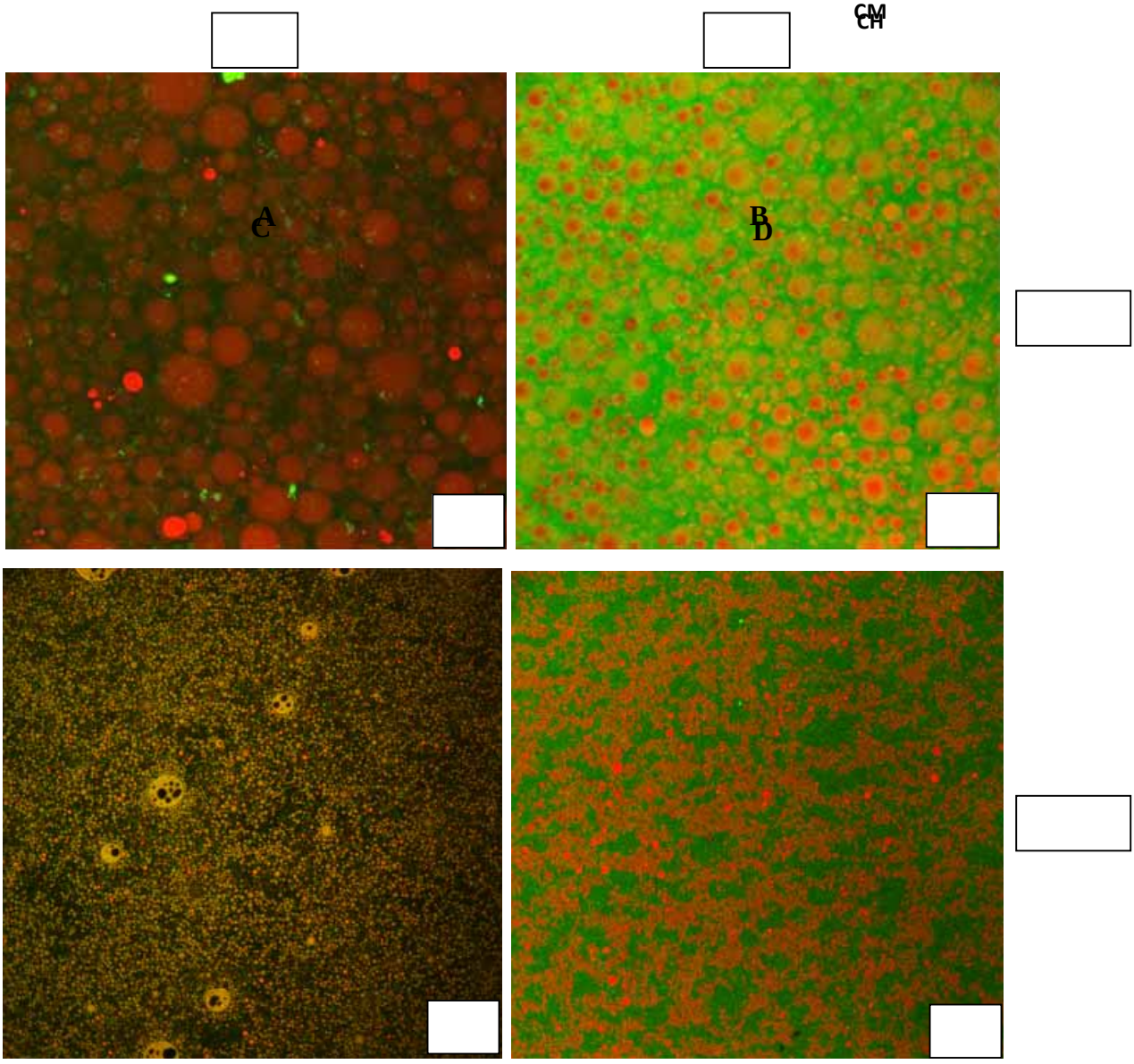

Figure 35. Confocal laser scanning microscope images of SC emulsions containing 1 and 5% SC and stabilized by (A,B) colloidal mill (CM) and (C,D) conventional homogenization (CH).

In UHPH emulsions treated at 100 MPa, the particle size (d3.2 and d4.3) decreased and the SSA increased as the protein concentration increased from 1 to 5%; however, the decrease in the d3.2 value was only significant when the protein content increased from 1 to 3%. This result was also confirmed by the TEM images (Fig. 37 G-I), in which an appreciable difference could be seen as the protein increased to 3%, but further increase to 5% had no effect. Furthermore, UHPH emulsions containing 1% SC and treated at100 MPa presented a mono modal distribution (Fig. 34 A) and the size distribution

curves were shifted towards small size as the protein concentration increased, with a slight bimodal distribution in emulsions containing 5% SC. According to Canselier et al. (2002), the droplet size, which determines emulsion formation and stability, is reduced when the surfactant concentration increases until a plateau is reached.

There are a number of possible reasons to account for the observed decrease in mean droplet size with increasing protein concentration: (i) the total droplet surface area that could be stabilized by the protein increased; (ii) the rate at which the droplet surfaces were covered with protein increased; (iii) the frequency of droplet collisions decreased due to the increase in aqueous phase viscosity. All of these factors should facilitate droplet disruption and prevent droplet coalescence within the homogenizer, thereby leading to the formation of smaller droplet sizes (McClements, 2005).

The d3.2 value was not significantly affected with the increase in the SC concentration in UHPH emulsions treated at 200 and 300 MPa. At low SC concentration (1%), UHPH emulsions treated at 200 and 300 MPa exhibited a significantly lower particle size in comparison to emulsions treated at 100 MPa, but they presented a bimodal droplet distribution (Fig. 34 A). The high-pressure homogenization was capable of producing smaller droplets, however, there were insufficient protein molecules to adsorb onto the newly formed surface producing the bimodal distribution. According to Euston & Hirst (1999), the bimodal nature at low protein contents is attributed to clustering of droplets through bridging flocculation, a fact that was confirmed in our study by the TEM images of UHPH emulsions treated at 200 MPa containing 1% protein (Fig. 37 J), in comparison to those of 3 and 5% protein (Fig. 37 K, L), where a visible decrease in the aggregation could be noticed. Increasing the SC concentration from 1 to 3% significantly ($P<0.05$) decreased the d4.3 value, however, there were no significant differences ($P>0.05$) in the d4.3 and SSA with further increase in SC concentration to 5%, a fact that was confirmed by the TEM images (Fig. 37 K, L). This may indicate that 3% of SC is sufficient to decrease the particle size and above this percent no effect on the particle size could be found. However, when protein was increased in emulsions treated at 200 and 300 MPa, droplet distribution changed from bimodal in emulsions containing 1% SC to monomodal distribution in emulsions containing 5% SC (Fig. 34 B,C).

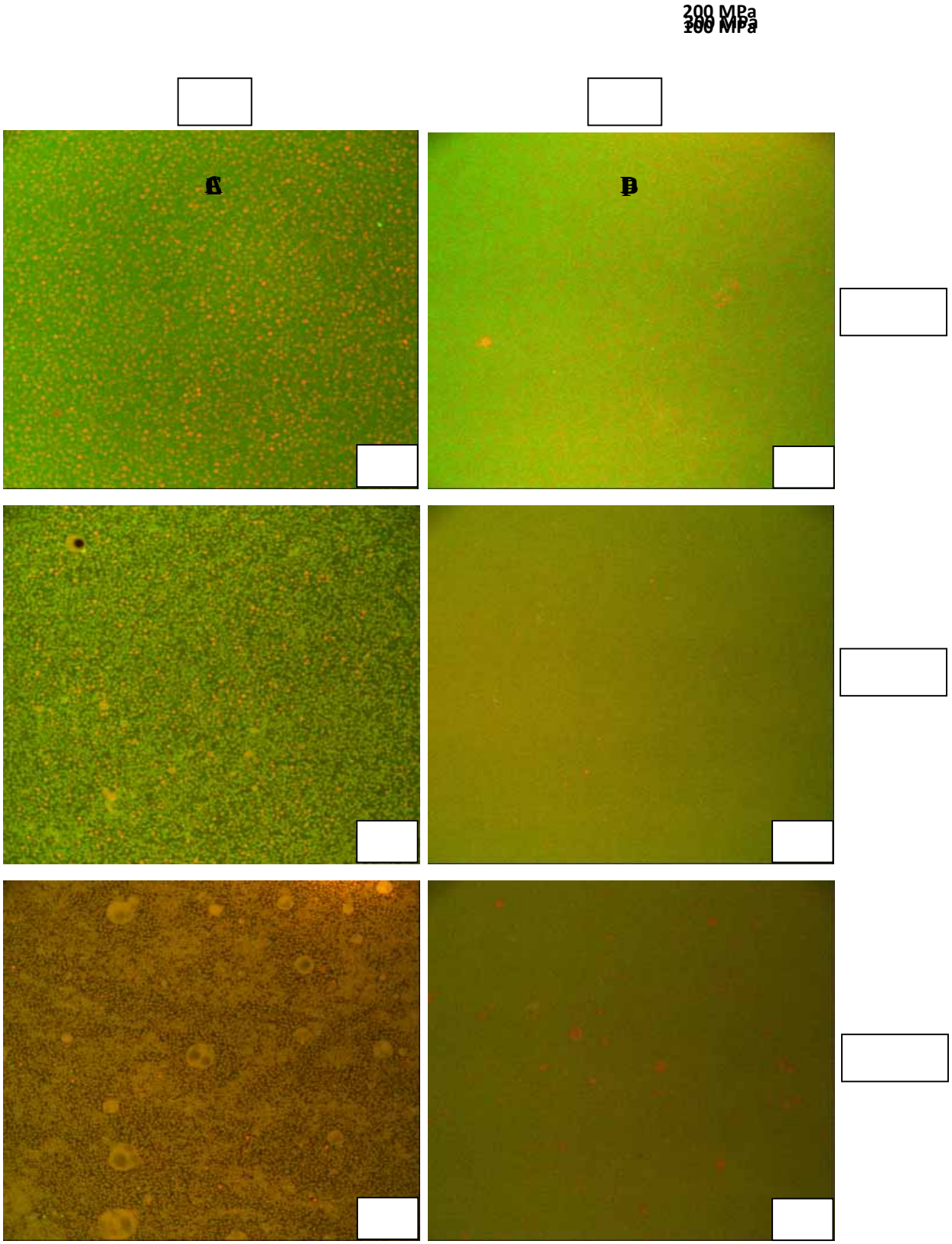

Figure 36. Confocal laser scanning microscope images of SC emulsions containing 1, 3 and 5% SC and stabilized by ultra-high pressure homogenization (UHPH) at 100 (A, B), 200 (C, D) and 300 MPa (E, F).

Table 16. Mean ± SD of rheological characteristics (flow and consistency indices) and emulsifying activity index EAI (m^2/g) of O/W emulsions containing 20% (w/w) of sunflower and olive oils plus sodium caseinate (1, 3 and 5%) and prepared by colloidal mill (CM), conventional homogenization (15 MPa) and ultra-high pressure homogenization (100, 200 and 300 MPa).

Pressure (MPa)	Protein content (%)	Rheological behavior			
		Consistency coefficient (K) mPa × s	Flow behavior index (n)	r^2	Emulsifying activity index EAI (m^2/g)
CM	1	0.0015 ± 0.0003^{e}	1.092 ± 0.017	0.998	6141 ± 272^{de}
	3	0.0047 ± 0.0017^{de}	1.041 ± 0.044	1.000	2495 ± 380^{f}
	5	0.0121 ± 0.0005^{cde}	1.006 ± 0.015	1.000	1959 ± 413^{f}
15	1	0.0018 ± 0.0002^{e}	0.994 ± 0.006	0.999	50232 ± 6018^{a}
	3	0.0201 ± 0.0094^{c}	0.776 ± 0.006	0.998	25427 ± 4041^{bc}
	5	0.0426 ± 0.0073^{ab}	0.739 ± 0.046	0.999	14490 ± 1298^{bcd}
100	1	0.0023 ± 0.0004^{e}	0.971 ± 0.020	0.999	31942 ± 3402^{b}
	3	0.0068 ± 0.0026^{de}	0.977 ± 0.029	1.000	9761 ± 1078^{d}
	5	0.0241 ± 0.0026^{cd}	0.911 ± 0.029	1.000	4359 ± 199^{e}
200	1	0.0033 ± 0.0020^{e}	0.930 ± 0.091	0.999	25702 ± 1727^{bc}
	3	0.0162 ± 0.0045^{cde}	0.850 ± 0.035	0.999	7514 ± 2277^{d}
	5	0.0307 ± 0.0077^{bc}	0.840 ± 0.042	0.999	3511 ± 1107^{e}
300	1	0.0028 ± 0.0005^{e}	0.966 ± 0.024	0.997	22077 ± 1670^{bc}
	3	0.0154 ± 0.0037^{cde}	0.863 ± 0.020	0.999	5406 ± 448^{de}
	5	0.0491 ± 0.0089^{a}	0.857 ± 0.032	0.997	2303 ± 102^{e}

[a-f] Different letters in the same column indicate significant differences *($P < 0.05$)* between treatments.

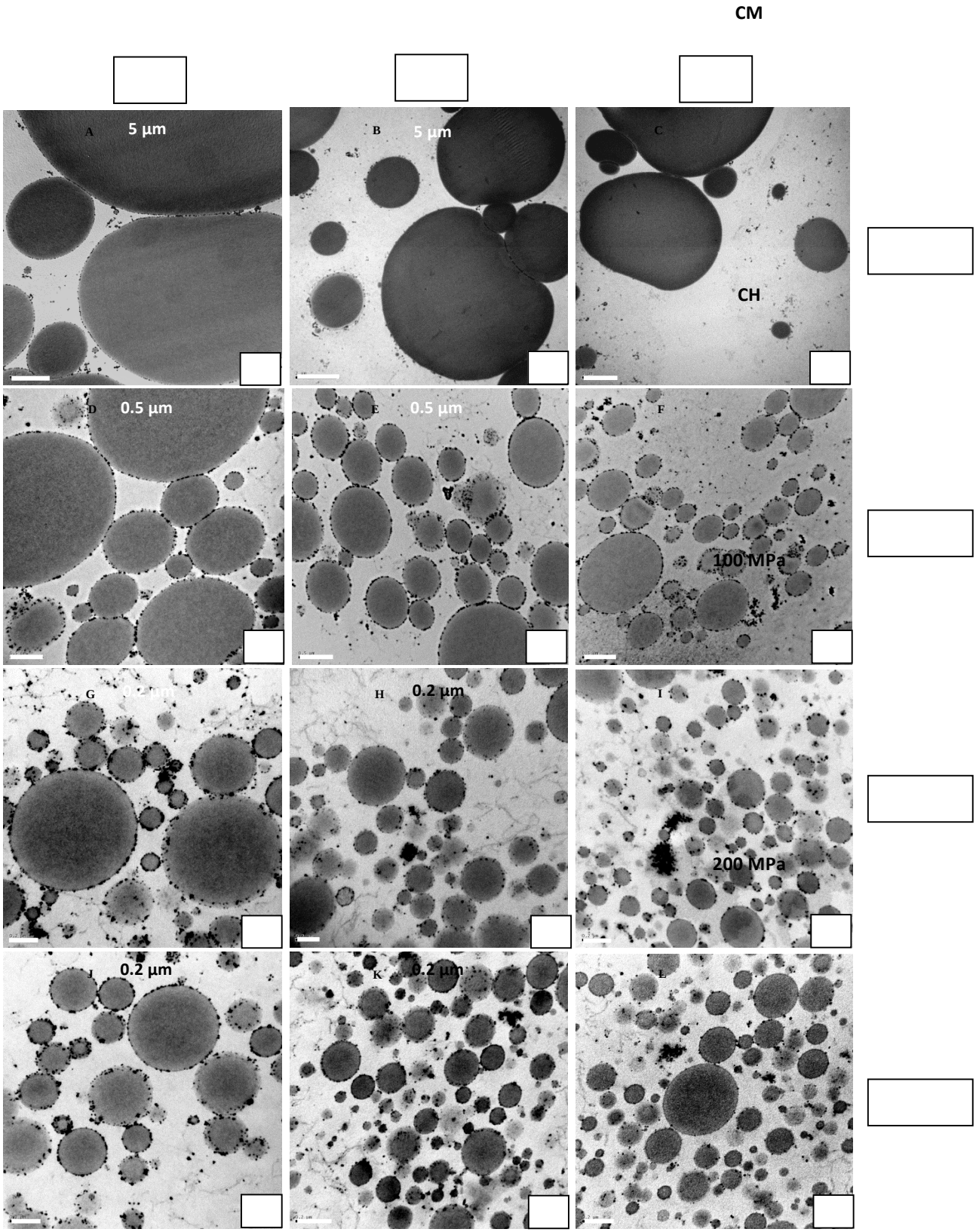

Figure 37. TEM images of emulsions containing1, 3 and 5% of SC and stabilized by (A-C) colloidal mill (CM) ×5000, (D-F) conventional homogenization (CH) ×25000 and by ultra-high pressure homogenization at 100 (G-I), and 200 MPa (J-L) ×50000.

6.2.4. Rheological behavior

Table 16 shows the consistency coefficient (K) value, which corresponds to the viscosity when the fluid is Newtonian, and the flow behavior index (n≈1 indicates Newtonian behaviour). CM emulsions showed a Newtonian flow behavior with low viscosity; possibly due to the small interaction between particles in these emulsions. Although, the protein concentration had no significant effect on CM emulsion viscosity, the viscosity increased with increasing the protein concentration.

The CH emulsions containing 1% SC showed a constant viscosity, independent of shear stress, i.e., a stable Newtonian liquid, while the emulsions prepared with 3 and 5% protein showed an increase in the consistency coefficient with a shear-thinning behavior; however, this increase was only significant in emulsions containing 5% protein. As mentioned previously, CH emulsions containing 5% SC presented an aggregated network structure (Fig. 35 D) from the depletion-flocculated emulsion droplets, due to the existence of a substantial excess amount of unadsorbed protein in the aqueous phase, which leads to an increase in the effective volume fraction of particle hydrodynamic interaction, thereby resulting in a much higher apparent viscosity. Berli et al. (2002) found that the rheological response of an emulsion containing SC was highly dependent on the concentration of SC. When the concentration of free proteins in the bulk solution was low, emulsions had a Newtonian behavior while, for higher SC concentration, emulsions became shear-thinning. Dickinson, Golding, & Povey (1997) reported that emulsions containing flocculated droplets have higher viscosities than those containing the same concentration of un-flocculated droplets.

The application of UHPH caused an increase in the emulsion viscosity at all the pressure treatments assayed but especially at 300 MPa when the protein level increased, although this increase was only significant between the emulsions containing 1 and 5% of protein. These results are consistent with the decrease of the droplet size as the pressure increased. The increase in viscosity with reduced droplet size could be attributed to an increase in hydrodynamic interactions between the droplets, since the mean separation distance between the droplets decreases when the droplet size is reduced (Pal, 2000). Moreover, a greater amount of absorbed protein or more tightly packed proteins at the O/W interface and the formation of viscoelastic layers around the

droplet can increase the emulsion viscosity (Innocente, Biasutti, Venir, Spaziani, & Marchesini, 2009).

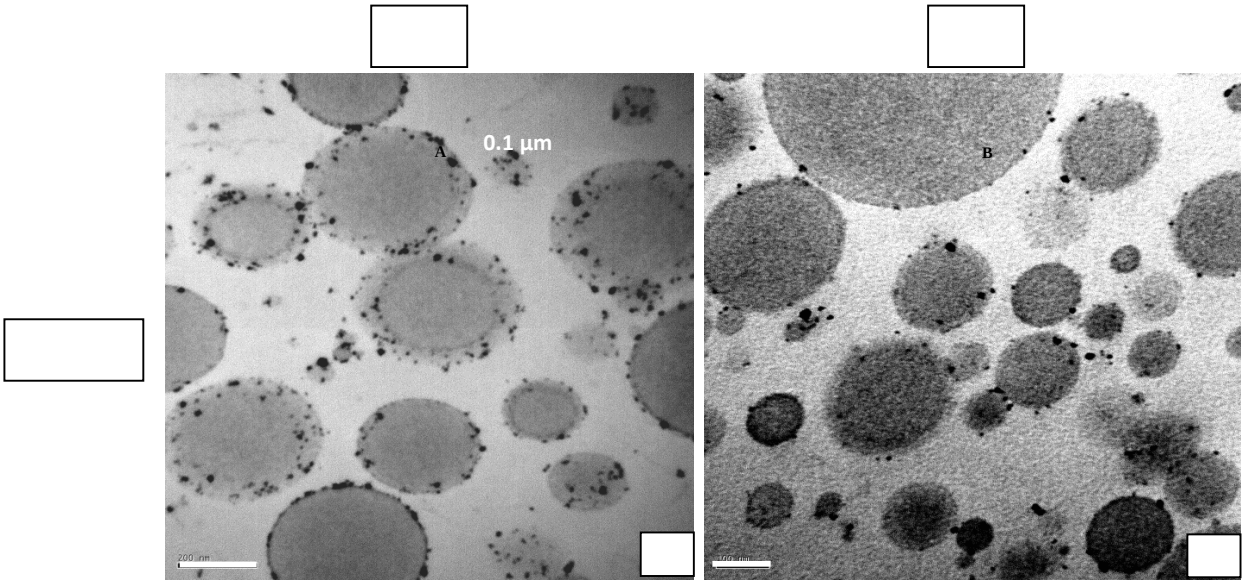

Figure 38. TEM images ×100000 of sodium caseinate O/W emulsions containing 1 (A) and 5% (B) and stabilized by ultra-high pressure homogenization at 200 MPa.

Extremely high-pressures (i.e. 300 MPa) and high protein concentrations (5%) may enhance the depletion flocculation due to the presence of excessive protein in the continuous phase, forming casein aggregates or protein gels, as can be seen in the CLSM image (Fig. 36 F, see the protein aggregates in the red channel), and increasing the viscosity of emulsions, a fact that was confirmed by the shear thinning behavior observed in these emulsions (n = 0.857). As known, the flocculation phenomenon increases the viscosity of emulsions and may eventually lead to the formation of a particle gel (McClements, 2005). Sodium caseinate at the concentrations generally used (above about 0.5% w/w protein) is not itself monomeric, but exists in the form of aggregates of the proteins containing about 30 molecules (Rollema, 1992), which are held together probably by hydrophobic forces. In the study of Pereda, Jaramillo, Quevedo, Ferragut, Guamis, & Trujillo (2007), higher viscosity was found after milk was subjected to high- pressure homogenization at 300 MPa, and this increase was attributed to the formation of fat aggregates that were not observed at relatively lower homogenization pressure (e.g., ≤ 200 MPa).

Dynamic high-pressure may induce gelation and is of growing interest as a texturing process in dairy and food emulsions. Floury, Desrumaux, & Legrand (2002) reported

that Newtonian liquid emulsions (20% w/w oil) stabilized by soy proteins (2%) were converted into shear-thinning emulsion gels after high-pressure homogenization (≥ 250 MPa).

With respect to the effect of protein concentration on the viscosity of UHPH-treated emulsions, emulsions treated at 100 MPa exhibited a flow Newtonian behavior whatever the protein content was. On the other hand, the Newtonian flow behavior was only observed in UHPH emulsions treated at 200 and 300 MPa containing low protein concentration (1%), whereas increasing the protein concentration to 3 and 5% tended to change the flow behavior towards the shear thinning behavior.

It seems that the rheology of the emulsion not only depends on the protein concentration but, also on the protein type. In the previous study, applying the same conditions of pressure (100-300 MPa) to treat O/W emulsions containing 20% oil plus different concentrations of whey protein isolate (1, 2 and 4%), all emulsions exhibited a Newtonian flow behavior regardless of the protein concentration. This may be due to the difference between the two proteins in the nature, and the ability of SC to form a gel-like structure at high-pressure when using high protein concentrations.

6.2.4. Emulsifying activity index (EAI)

The emulsifying activity index (EAI, m^2/g) is related to the surface area stabilized by a unit weight of proteins, which presents the ability of proteins to be adsorbed at the interface of fat globules and the aqueous phase. The EAI is a function of oil volume fraction, protein concentration, and the type of equipment used to produce the emulsion (Pearce and Kinsella, 1978).

CM emulsions exhibited low EAI (Table 16); however, applying low homogenization pressure (CH treatment) increased the EAI in comparison to CM emulsions, but further increase in the homogenization pressure (100, 200 and 300 MPa) decreased the emulsifying activity of SC.

The reasoning behind the low EAI values of the CM emulsions is that only a small part of SC is likely to be absorbed at the water/oil interface, because the kinetics of absorption in rotor-stator systems requires a much longer time than that for both CH and UHPH (Krešić, Lelas, Herceg, & Režek, 2006). It is evident that what is important in emulsion formation is not only the weight of oil, but also its interfacial area. Thus, if the

emulsion is made of large droplets, it will consume less surfactant than if small droplets are present. Corzo-Martínez et al. (2011) showed that the EAI of emulsion homogenized with Ultra-Turrax was much lower (77.61 ± 4.89 m^2/g) than that of the sonicated emulsions (>785 m^2/g) at 0.5 mg of SC/ml, due to the smaller particle size of emulsions obtained by high intensity ultra sound.

Since increasing homogenization pressure in the present study resulted in a decrease in mean diameter of fat droplets, one would expect that at ultra-high pressures there may not be sufficient protein present to completely cover the large interfacial area formed, resulting in a decrease in the EAI. However, as will be demonstrated later, the increase of the SC concentration did not improve the emulsions EAI, so other reasons may explain the EAI reduction in UHPH emulsions.

Another reason for the decrease in the EAI in UHPH-treated emulsions, in addition to the increase in the surface area, may be the surface hydrophobicity and flexibility of SC. Both surface hydrophobicity (which affects the affinity of the protein for the O/W interface) and molecular flexibility (which influences the ability to unfold and interact with other proteins) are important in determining emulsifying activity and for the film formation because rapid migration and adsorption on the O/W interface is critical (Monahan et al., 1993). Since the ultra-high pressures used in the present study are dissipated into heat, protein aggregation may occur by decreasing the availability of proteins to form films and emulsions (Phillips, Schulman, & Kinsella, 1990). The proteins could not adsorb at the interface when they were insolubilized and aggregated (Le Denmat, Anton, & Gandemer, 1999). Rodiles-López et al. (2008) observed that the decrease in emulsifying activity of egg yolk was related with that of protein solubility and reported that a high solubility is necessary for a good emulsifying activity.

Generally, increasing the protein concentration from 1 to 5% resulted in a decrease in the EAI. As can be seen in the TEM images (Fig. 38 A,B) for UHPH emulsions containing 1 (A) and 5% SC (B), higher protein coverage was observed when 1% SC was used than in emulsions containing 5% SC. Similarly, in CM and CH emulsions the differences were only significant when the protein content increased from 1 to 3% whereas, 5% of protein had no effect. Corzo-Martínez et al. (2011), working with high-intensity ultra sound and SC as emulsifier tried to select the optimal operating conditions to maximize EAI. Emulsions stabilized with 0.5 mg/ml SC solutions provided significantly higher EAI values (785 to 1287 m^2/g) than those obtained (128 to

312 m^2/g) at higher protein concentrations (2.5 and 4.5 mg of SC/ml, respectively). They reported that the droplet size, which determines emulsion formation and stability, is reduced when the surfactant concentration increases until a plateau is reached. Therefore, in the case of UHPH emulsions, it is likely that such a plateau was reached around 1% SC, with remaining unadsorbed caseinate at higher concentrations (3 and 5%). Other proteins, such as sweet potato proteins, almond protein, wheat gluten, denatured whey protein isolate (DWPI) and acidic subunits of soy 11S globulin also had similar characteristics (Agyarea, Addo, & Xiong, 2009; Liu, Lee, & Damodaran, 1999; Sze-Tao & Sathe, 2000; Britten, Giroux, & Gaudin, 1994; Guo and Mu, 2011). Britten et al. (1994) using DWPI at 5, 12, 19 and 26% found that the EAI was improved with increasing protein concentration to 19%. They attributed this improvement to the aggregated nature of DWPI which contributed to the formation of thicker membranes around fat droplets. However, further increase in DWPI to 26% resulted in decreased EAI.

Possible explanations for the decrease in the EAI with increasing SC concentrations have been suggested by Guo & Mu (2011) : (1) at lower protein concentrations protein adsorption at the O/W interface was diffusion controlled. The larger diffusion coefficients of protein at lower protein concentration facilitated formation of new droplets, resulting in greater EAI; (2) at higher protein concentrations the activation-energy barrier prevents protein migration in a diffusion-dependent manner. Further increases in protein concentration may decrease the effectiveness of protein adsorption, which leads to decreased EAI.

6.2.5. Physical Stability of Emulsions

The term stability for an emulsion refers to its ability to resist changes in its properties through time. The higher the emulsion stability, the lower the changes in its properties (McClements, 1999). Physical instability refers to the change in spatial arrangement or size distribution of emulsion droplets, such as creaming, flocculation or coalescence, whereas chemical instability includes change in the composition of the emulsion droplet itself, such as oxidation, hydrolysis, etc. (McClements & Decker, 2000; McClements, 2005).

Emulsion stability was visually examined (Fig. 39) and also assessed by measuring the d4.3 value at the top or at the bottom of the emulsion tubes stored at room temperature and under the same conditions for comparison (Table 17 and Fig. 40). Additionally, emulsion stability was also evaluated using the optical characterization method with a TurbiScan (Fig. 41).

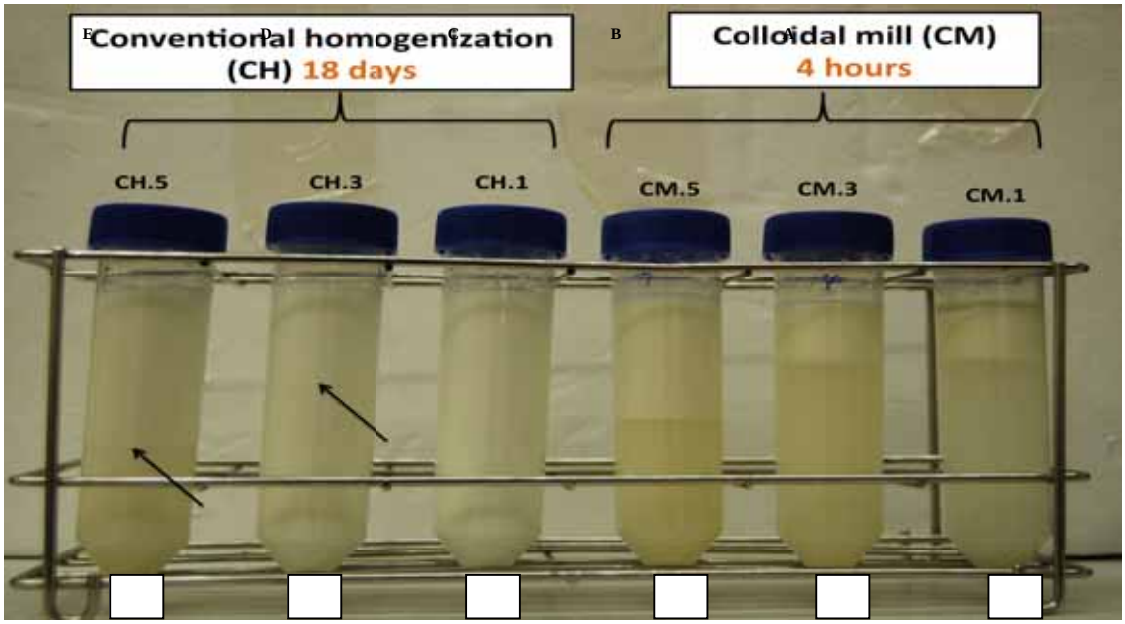

Figure 39. Visual creaming assessment of emulsions containing 1, 3 and 5% of SC prepared by colloidal mill (A-C) and conventional homogenization (D-F).

CM emulsions, at all protein concentrations, exhibited a high degree of creaming as a direct consequence of the large particle size, low viscosity and the high surface tension, which resulted in a high degree of coalescence as can be observed in the TEM images (Fig. 37 A-C); the emulsion was totally separated at the same day of preparation (Fig. 39 A-C and Fig. 41 A-C). CM emulsions containing 1% SC were the most instable emulsions, where the phase separation was completed in 30 min, however, increasing the protein concentration to 5% SC tended to slow down the creaming process, with a completed separation in approximately 4 h.

The CH emulsions were more stable against creaming than CM emulsions, although creaming could be detected in all CH emulsions by Turbiscan Lab (Fig. 41 D-F) and by the d4.3 values obtained at the top or the bottom of the CH emulsions tubes (Table 17 and Fig. 40 A-C). When the amount of protein used to stabilize the emulsion was 1%,

the optical characteristics of these emulsions showed slow changes in their backscattering patterns (Fig. 41 D), significant differences between the d4.3 values at the top or at the bottom of the emulsion (Table 17 and Fig. 40 A) with no visual separation during approximately 18 days of storage at room temperature (Fig. 39 D). The microscopic examination by TEM indicated the presence of bridging flocculation (Fig. 37), suggesting that bridging flocculation may have a stabilizing effect due to limited protein surface coverage (Dickinson et al., 1997) as red particles without protein coverage can be clearly seen in the CLSM image (Fig. 35 C). According to Day, Xu, Hoobin, Burgar, & Augustin (2007), bridging flocculation tends to progress much more slowly and not necessarily affect the creaming rate during the early part of emulsion storage.

Emulsions made with 3% SC showed extensive creaming, with the clarification front of the Turbiscan appearing after 3 days, indicating the limited shelf life of these emulsions. This result may be due to the excess of SC in the system, in agreement with the results of previous studies (Dickinson & Golding, 1997; Srinivasan et al., 2000). In emulsions containing high protein contents, the creaming rate of the system is greatly reduced due to depletion flocculation of protein-coated droplets by unadsorbed sub-micellar caseinate. Additional increase in the protein concentration in CH emulsions (from 3 to 5% SC) led to a reduction in the creaming rate (Fig. 41 F). This fact can be attributed to the formation of a network structure at higher SC concentrations, when the strength of the attractive depletion interaction was considerably stronger, resulting in a significant increase in the consistency coefficient (K value), which limits the droplets movement and decreases the migration velocity of particles (Table 17). These results were also confirmed by calculating the migration or creaming velocity V (t) in the clarification layer using the Turbiscan software. A lower creaming value was observed in emulsions containing 1% SC (207 μm/min), however, increasing the protein content from 1 to 3% increased the creaming rate (861 μm/min) while a further increase to 5% decreased the rate (272 μm/min).

Table 17. Mean ± SD of d4.3 values at the top or at the bottom of samples stored at room temperature for 9 days under the same conditions for comparison, of O/W emulsions containing 20% (w/w) of sunflower and olive oils plus sodium caseinate (1, 3 and 5%) and prepared by conventional homogenization (15 MPa) and ultra-high pressure homogenization (100, 200 and 300 MPa).

Pressure (MPa)	**Protein content (%)**	**Emulsion creaming stability after 9 days**		
		d4.3 (Top)	**d4.3 (Bottom)**	***P value***
15	**1**	2.428 ± 0.982^{ab}	0.961 ± 0.389^{a}	**0.0087***
	3	1.475 ± 0.046^{bc}	0.427 ± 0.090^{abc}	**0.0022***
	5	1.926 ± 1.220^{abc}	0.417 ± 0.128^{abc}	**0.0022***
100	**1**	3.643 ± 1.039^{a}	0.697 ± 0.335^{ab}	**0.0022***
	3	0.232 ± 0.014^{de}	0.203 ± 0.022^{c}	0.0627
	5	0.219 ± 0.047^{de}	0.145 ± 0.004^{c}	**0.0022***
200	**1**	0.971 ± 0.235^{bcd}	0.337 ± 0.168^{bc}	**0.0022***
	3	0.159 ± 0.021^{de}	0.169 ± 0.026^{c}	0.2207
	5	0.149 ± 0.007^{e}	0.146 ± 0.007^{c}	0.3636
300	**1**	0.671 ± 0.239^{cde}	0.354 ± 0.115^{bc}	**0.0259***
	3	0.144 ± 0.017^{e}	0.127 ± 0.015^{c}	0.1320
	5	0.134 ± 0.005^{e}	0.132 ± 0.007^{c}	0.5121

$^{a\text{-}e}$ Different letters in the same column indicate significant differences ($P < 0.05$) between treatments.* Sign indicates that the differences between the d4.3 at the top or at the bottom of emulsions are significant (Wilcoxon statistic test $P < 0.05$) per level of pressure and oil concentration.

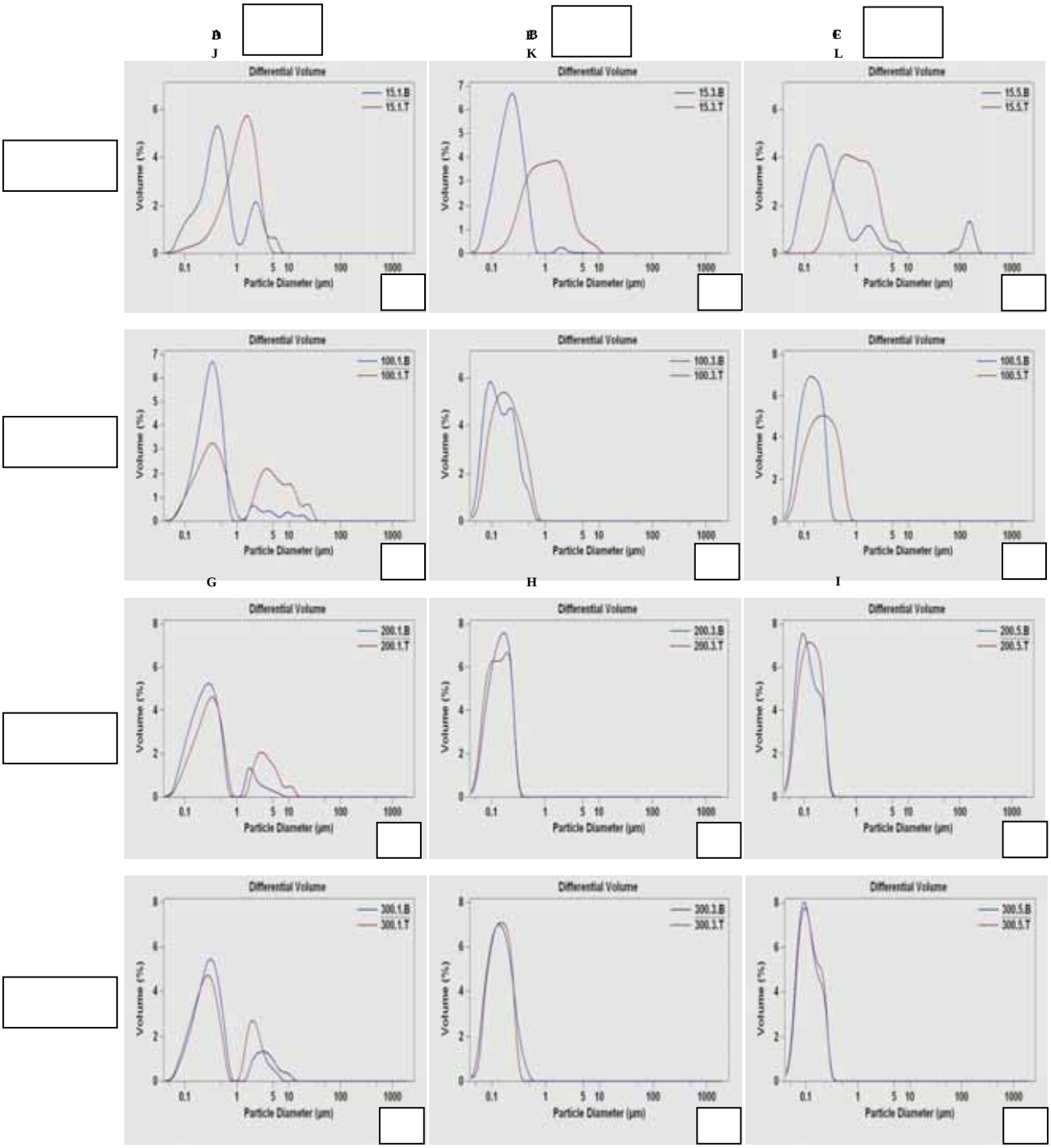

Figure 40. Droplet size distribution curves at the top and the bottom of O/W emulsions containing 1, 3 and 5% of SC plus 20% oil and processed by (A-C) conventional homogenization (CH) and ultra-high pressure homogenization at 100 (D-F), 200 (G-I) and 300 MPa (J-L) after 9 days of storage at room temperature.

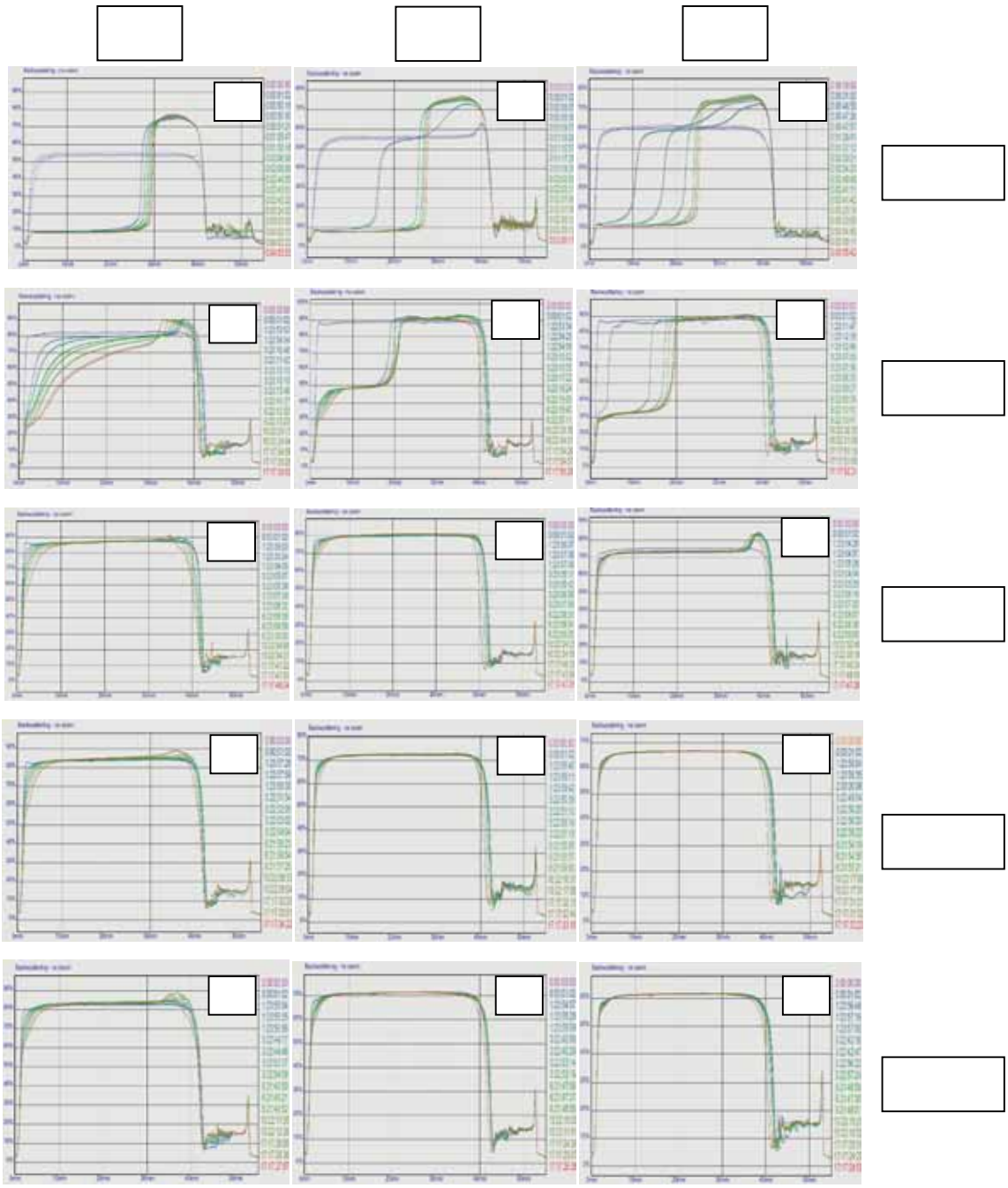

Figure 41. Changes in backscattering profiles of emulsions containing 20% oil and different SC contents, 1% (A, D, G, J, M), 3% (B, E, H, K, N) and 5% (C, F, I, L, O) and prepared by (A-C) colloidal mill (CM), (D-F) conventional homogenization (CH), and ultra-high pressure homogenization at 100 MPa (G-I), 200 (J-L) and 300 (M-O) MPa, as a function of sample height with storage time (5 h for CM emulsions and 18 days for both CH and UHPH emulsions).

Emulsions processed by UHPH were remarkably stable and remained fully turbid upon storage at room temperature for 18 days, with no creaming being visually noticed. Submicron/nano-emulsions typically have much better stability to gravitational separation than conventional emulsions because the relatively small particle size means that Brownian motion effects dominate gravitational forces (Tadros, Izquierdo, Esquena, & Solans, 2004). It has been shown that when the particle sizes are smaller than 100 nm (many particles in the present study fell into this range), creaming would be greatly reduced and aggregation become a dominant mechanism for emulsion instability (McClements, 2005). According to Lee, Lefèvre, Subirade, & Paquin (2009), the stabilization of emulsion may be partly attributed to the considerable increase of interaction between adsorbed proteins at the interface of the emulsion, because strong interactions between adsorbed proteins at the interface lead to the formation of a more rigid interfacial layer at higher pressures, so that it may effectively better protect emulsion droplets against destabilizing processes. The greater droplet size reduction and the rigid interfacial layers around oil droplets of UHPH emulsions resulted in the weightiness of fine emulsions as compared to the CM and CH emulsions. In addition, the low particle sizes in emulsions also increase the emulsion viscosity, which is the case of our emulsions treated at 200 and 300 MPa containing 3 and 5% SC (Table 16), limiting the movements of the oil particles and then lowering the creaming rate. For instance, in the case of UHPH emulsions, the migration velocity for those treated at 200 MPa and containing 3% of SC was much lower (13.5 μm/min) than that of CM and CH emulsions (106 and 861 μm/min, respectively) at the same SC concentration. According to Tadros et al. (2004), UHPH emulsions also tend to have better stability against droplet flocculation and coalescence because the range of the attractive forces acting between the droplets decreases with decreasing particle size, while the range of the steric repulsion is less dependent on particle size.

The protein concentration in combination with the homogenization pressure seemed to significantly affect the creaming stability of the UHPH emulsions. The d4.3 values at the top and at the bottom of UHPH emulsions (Table 17 and Fig. 40 D-L) and Turbiscan fingerprints (Fig. 41 G-O) indicated a slight creaming effect in emulsions containing 1 and 5% (w/w) SC treated at 100 MPa and in emulsions containing 1% treated at 200 and 300 MPa. Wang, Li, Wang, & Özkan (2010) reported that increasing protein concentration in the emulsion would facilitate producing relatively smaller droplets,

which may increase the protein amount adsorbed at the interface of oil droplet. High protein amounts at the interface may increase the density of droplets, and consequently decreasing the creaming rate. Even at high protein contents, there is a partial destabilization of the flocculated emulsion in the form of a strong particle gel network and particle movement is more limited at these concentrations (Dickinson, 2006). Roesch and Corredig (2003) working on emulsions containing higher concentrations of soy protein concentrate observed a decrease in the space between aggregates, lowering particle movement, and forming more stable emulsions to creaming.

6.2.6. Oxidative stability

It is well known that droplet disruption and shearing during homogenization result in a large intermediate surface area which may increase the oxidation rate. Therefore, lipid oxidation might be expected to be faster in emulsions with small droplets (CH and UHPH), owing to the larger total interfacial area compared to larger droplets (CM emulsions). Interestingly, CM emulsions were generally oxidized faster than those homogenized by CH and UHPH (Table 18). Some studies in emulsions support the hypothesis that an increase in total interfacial area has been shown to accelerate lipid oxidation (Gohtani, Sirendi, Yamamoto, Kajikawa, & Yamano, 1999). In contrast, other studies have shown no correlation between oil droplet size and lipid oxidation (Sun & Gunasekaran, 2009). In mayonnaise, lipid oxidation was observed to progress faster in smaller droplets than in larger ones in the initial part of the storage period, whereas no dependence of droplet size was observed on oxidative flavor deterioration in the later part of the storage period (Jacobsen et al., 2000). Nakaya, Ushio, Matsukawa, Shimizu, & Ohshima (2005) measured higher lipid hydroperoxide and hexanal concentrations as well as lower residual oxygen concentrations in polyunsaturated triacylglycerol emulsions stabilized with sucrose lauryl ester or decaglycerol lauryl ester at larger, rather than smaller droplet size. Similar results were obtained by Let, Jacobsen, & Meyer (2007) in emulsions created by homogenizing fish oil with milk; emulsions with smaller droplet sizes showed less production of lipid hydroperoxides and volatile lipid oxidation products.

Table 18. Mean ± SD of hydroperoxides (A_{510} nm) and TBA reactive substances (µg/ml) of O/W emulsions containing 20% (w/w) of sunflower and olive oils plus sodium caseinate (1, 3 and 5%) and prepared by colloidal mill (CM), conventional homogenization (15 MPa) and ultra-high pressure homogenization (100, 200 and 300 MPa).

Pressure (MPa)	Protein content (%)	Hydroperoxides (A_{510} nm)			TBARS (µg/ml)		
		Day 1	Day 10	Diference (Day 10 – Day 1)	Day 1	Day 10	Diference (Day 10 – Day 1)
CM	1	0.019 ± 0.005^{ab}	0.116 ± 0.050^{a}	$0.097 \pm 0.048^{a*}$	0.039 ± 0.018^{cd}	0.116 ± 0.033^{a}	$0.077 \pm 0.051^{a*}$
	3	0.022 ± 0.006^{ab}	0.097 ± 0.040^{ab}	$0.075 \pm 0.045^{a*}$	0.057 ± 0.019^{bc}	0.092 ± 0.009^{a}	$0.035 \pm 0.027^{ab*}$
	5	0.027 ± 0.002^{ab}	0.096 ± 0.024^{ab}	$0.070 \pm 0.023^{a*}$	0.079 ± 0.006^{a}	0.099 ± 0.016^{a}	$0.020 \pm 0.012^{ab*}$
15	1	0.018 ± 0.004^{ab}	0.091 ± 0.038^{ab}	$0.073 \pm 0.034^{a*}$	0.037 ± 0.017^{cd}	0.054 ± 0.019^{cd}	$0.016 \pm 0.003^{ab*}$
	3	0.025 ± 0.003^{ab}	0.107 ± 0.011^{a}	$0.082 \pm 0.008^{a*}$	0.042 ± 0.010^{cd}	0.059 ± 0.003^{cd}	$0.016 \pm 0.009^{ab*}$
	5	0.032 ± 0.010^{a}	0.114 ± 0.012^{a}	$0.082 \pm 0.003^{a*}$	0.047 ± 0.008^{cd}	0.057 ± 0.013^{cd}	0.010 ± 0.006^{b}
100	1	0.028 ± 0.003^{b}	0.057 ± 0.032^{cd}	$0.030 \pm 0.029^{ab*}$	0.066 ± 0.019^{ab}	0.072 ± 0.021^{bc}	0.006 ± 0.007^{b}
	3	0.036 ± 0.002^{a}	0.067 ± 0.016^{bc}	$0.031 \pm 0.015^{ab*}$	0.086 ± 0.005^{a}	0.063 ± 0.017^{bc}	$–0.042 \pm 0.055^{c*}$
	5	0.024 ± 0.007^{ab}	0.032 ± 0.010^{d}	0.008 ± 0.004^{b}	0.064 ± 0.005^{ab}	0.074 ± 0.005^{bc}	$0.010 \pm 0.009^{b*}$
200	1	0.034 ± 0.009^{a}	0.072 ± 0.035^{ab}	$0.038 \pm 0.026^{ab*}$	0.057 ± 0.014^{bc}	0.100 ± 0.014^{a}	$0.043 \pm 0.004^{ab*}$
	3	0.035 ± 0.011^{a}	0.096 ± 0.064^{ab}	$0.061 \pm 0.054^{a*}$	0.068 ± 0.023^{ab}	0.103 ± 0.019^{a}	$0.035 \pm 0.004^{ab*}$
	5	0.023 ± 0.006^{ab}	0.033 ± 0.010^{d}	0.010 ± 0.005^{b}	0.079 ± 0.015^{a}	0.067 ± 0.003^{bc}	$–0.012 \pm 0.015^{b}$
300	1	0.021 ± 0.002^{ab}	0.026 ± 0.009^{d}	0.005 ± 0.011^{b}	0.062 ± 0.011^{ab}	0.071 ± 0.013^{bc}	0.009 ± 0.004^{b}
	3	0.008 ± 0.001^{c}	0.006 ± 0.001^{e}	$–0.002 \pm 0.001^{b}$	0.056 ± 0.002^{bc}	0.094 ± 0.019^{a}	$0.038 \pm 0.018^{ab*}$
	5	0.005 ± 0.000^{c}	0.004 ± 0.001^{e}	$–0.001 \pm 0.000^{b}$	0.080 ± 0.010^{a}	0.085 ± 0.008^{ab}	0.004 ± 0.010^{b}

[a-e] Different letters in the same column indicate significant differences *(P < 0.05)* between treatments.

(*) sign indicates that the differences between day 10 and day 1 (oxidation evolution) is significant *(P < 0.05)*.

Considerable amounts of hydroperoxides and TBARS were observed in CM emulsions. This high concentration of oxidation products observed in CM emulsions indicates its high sensitivity toward oxidation, that it could be attributed to the high coalescence rate owing to the poor protein coverage at the emulsion interface.

A high level of primary oxidation products was formed in CH emulsions, but this was quite similar to the level found in CM emulsions. Although a significant evolution in the TBARS after 10 days was observed in CH emulsions, these amounts were lower than that of the corresponding CM emulsions, indicating that CH emulsions were more stable against oxidation.

UHPH-treated emulsions generally exhibited lower levels of hydroperoxides, in spite of the reduced particle size, in comparison to CM and CH emulsions. However, UHPH emulsions treated at 300 MPa seemed to be the most stable emulsions, with lower amounts of primary oxidation products being observed, especially when 5% of SC was used. UHPH-treated emulsions at 200 MPa were much more sensitive to oxidation than those treated at 100 MPa using low levels of SC (1 and 3%), however, the oxidative stability in UHPH emulsions treated at 200 MPa increased when 5% of SC was used.

Generally, increasing the protein concentration generally resulted in an increase in the oxidative stability of emulsions. When UHPH was applied to emulsions containing lower protein amounts (1%), smaller droplets were produced, creating a larger surface area, but SC was not sufficient to surround the oil droplets and cover such a large surface area (see Figure 36 (A,C) corresponding to emulsions treated at 100 and 200 MPa). However, increasing the pressure to 300 MPa tended to increase again the oxidative stability, although the particle size continued to decrease. This may be attributed to the good rearrangement of protein molecules to cover the interface.

Several studies with casein as emulsifier have shown that the rate of lipid oxidation decreases with increasing levels of casein (Faraji, McClements, & Decker, 2004; Hu et al., 2003; Kargar, Spyropoulos, & Norton, 2011; Ries, Ye, Haisman, & Singh, 2010). Ries et al. (2010), examining a wide range of casein concentrations (0.5-10%) to protect a linoleic acid emulsion from oxidation, found that the extent of lipid oxidation decreased as the concentration of protein in the system increased. In addition, the size of the emulsifier can affect the thickness of the interfacial layer of the emulsion droplet with larger hydrophilic head groups decreasing oxidation rates (Dalgleish, Srinivasan, &

Singh, 1995). Casein can form a thick interfacial layer around dispersed oil droplets of up to 10 nm, which is a high packing rate comparing with other emulsifiers i.e. whey proteins (1-2 nm) (Dickinson and McClements, 1995). The membrane acts as an efficient barrier to the diffusion of lipid oxidation initiators into the oil droplets (Coupland & McClements, 1996). Another reason behind the high oxidative stability in SC emulsions containing 5% of SC may be the presence of high amounts of protein in the aqueous phase, which in turn may act as antioxidants. SC in the continuous phase or as emulsifiers can enhance the oxidative stability of O/W emulsions (Faraji, McClements, & Decker, 2004; Hu et al., 2003). In the study of Sun et al. (2007), 0.2% whey protein isolate was sufficient to cover oil droplet surfaces in 5% O/W emulsions; the addition of 1 and 2% of whey protein provided more unadsorbed protein in the aqueous phase, which acted as an antioxidant to deactivate the iron. In addition, emulsions containing high protein amounts also present significant increases in emulsion viscosity (Table 16). It has been proposed that viscosity can affect oxidation by reducing the diffusion of potential pro-oxidative molecules, such as ferrous ions or lipid hydroperoxides (Sims, 1994; Imagi et al. 1992; Hsieh, and Harris, 1987).

In the case of secondary oxidation, UHPH emulsions presented high values of TBARS at day 1 after production. Droplet disruption by cavitation and subsequent rearrangement of oil droplets during homogenization promote distribution of oxygen, catalysts and lipid oxidation products between the newly arranged oil droplets, and may thus accelerate the lipid oxidation. Even if UHPH emulsions presented high values of TBARS at day 1, the evolution of secondary oxidation products during 10 days of storage was generally not significant at 100 and 300 MPa, except for some specific treatments. Emulsions treated at 200 MPa and containing 1-3% protein presented a significant increase of TBARS products during storage, but not when higher protein concentration (5%) was used.

6.3. References

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Chapter 7

Effects of oil-phase volume fraction and pressure on structure and stability of conventional and ultra-high pressure homogenized sodium caseinate oil-in-water emulsions

7.1. Introduction

Submicron/nano-emulsions with a narrow size distribution have been attracting considerable attention in recent years because of their certain inherent advantages. Due to the strong interfacial tension between the oil phase and water phase, nanoemulsions are thermodynamically unstable and must be stabilised by emulsifiers, which adsorb at the interfaces and reduce the interfacial tension.

Proteins emulsifiers, i.e., casein and caseinates, have the ability to form and stabilize emulsions by being absorbed to the oil-in-water interface during homogenization, reducing the interfacial tension between particles by an appreciable amount of proteins at the interface, thus preventing droplet coalescence (Dickinson, 2001). These proteins not only produce physically stable O/W emulsions, but also inhibit lipid oxidation (McClements & Decker, 2000).

Emulsions with low oil concentrations (i.e. 10-20%) are well described in the literature and are considered good systems for investigating a wide range of factors related to their production conditions. However, the knowledge on emulsions containing high oil concentrations (i.e. 30-50%) is scarce. When the aim is to use the emulsion as a delivery emulsion, a high oil concentration is preferable, particularly in food products where addition of water changes its texture in an unwanted way.

The choice of the oil concentration to be used to stabilize the emulsion without any sign of flocculation or coalescence is vital. At constant energy density (e.g. emulsification pressure), particle size rises with increasing oil content because the quantity of proteins available decreases. This limits the stabilizing benefits of the proteins, but increasing the

oil content may increase the emulsion viscosity and, as a result, slow down the creaming rate (Sun & Gunasekaran, 2009). Studies on protein stabilized O/W emulsions with varying volumes of the oil fraction have shown that a high oil fraction decreases lipid oxidation in safflower oil (Sims, Fioriti, & Trumbetas, 1979), canola oil (Osborn & Akoh, 2004), menhaden oil (Sun & Gunasekaran, 2009) and walnut oil (Gharibzahedi, Mousavi, Hamedi, Khodaiyan, & Razavi, 2012). The effect of using high oil volume fractions on physical stability in emulsions produced by UHPH using whey protein (Cortés-Muñoz, Chevalier-Lucia & Dumay, 2009; Floury, Desrumaux & Lardieres, 2000) and sodium caseinate (San Martín-González, Roach, & Harte, 2009) can be found in the literature. Besides providing kinetically stable emulsions, UHPH also enables the production of emulsions with a large range of flow behaviors (i.e., from highly fluid to highly thick samples) when combining the pressure level of homogenization and the oil volume fraction. In contrast, little research has been conducted on the oxidation behavior in simple O/W emulsions containing high oil contents.

As was concluded from the previous section, the best droplet breakdown, physical and oxidative stability were obtained when pressures in the range 200 and 300 MPa and 5% of sodium caseinate (SC) were used. However, using a high concentration of sodium caseinate (5%) in emulsions produced by conventional homogenization adversely affected the physical and oxidative stability, but on the other hand 1% was not sufficient to completely cover the surface area created. We thought that if an oil content of more than 20% were used, 1% of SC would not be sufficient to stabilize the emulsion. Therefore, the objective of this study was to evaluate the effect of homogenization pressures (200-300 MPa) and oil-phase volume fraction (10, 20, 30 and 50%) on the structure, rheological properties, physical and oxidative stability of emulsions containing 5% of SC. Emulsions treated by CH were prepared by adding both 1 and 5% SC.

As can be seen from Figure 42, increasing the oil content to 50% tended to produce an emulsion gel (mayonnaise-like product), so the results of the present study will focus only on emulsions containing 10, 20 and 30% oil. The methodology applied for this purpose is described in Chapter 3 and includes the study of physical properties such as particle size distribution, rheological behavior, emulsifying activity, microstructure (CLSM and TEM microscopy) and stability to creaming, measured visually and by two light scattering techniques (particle size at the top and the bottom of emulsions and

Turbiscan lab), and the stability to oxidation, determining the hydroperoxides and thio-barbituric acid reactive substances (TBARS).

Figure 42. Emulsions containing 50% oil and 5% sodium caseinate after treatment by ultra-high pressure homogenization.

7.2. Results and discussion

7.2.1. Temperature elevation during UHPH treatment

Temperatures of processed emulsions were measured before (T1) the high-pressure valve and at the outlet (T2) of the high-pressure valve. A mean temperature (T2) increase of 21.19, 21.5 and 23.7°C per 100 MPa for the three respective oil concentrations (10, 20 and 30%, respectively) in the pressure range 200-300 MPa was calculated from values presented in Table 19 when an Tin of 25°C was used. The temperature increase was proportional to the pressure applied and the oil concentration used. An increase in temperature of approximately 12-18°C per 100 MPa during high-pressure homogenization has been reported in similar studies (Sandra & Dalgleish, 2005; Thiebaud, Dumay, Picart, Guiraud, & Cheftel, 2003; Hayes & Kelly, 2003; Desrumaux & Marcand, 2002; Floury, Desrumaux, Axelos, and Legrand, 2003; Bouaouina, Desrumaux, Loisel, & Legrand, 2006). Floury et al. (2000) reported a temperature increase of 16.4°C per 100 MPa in a 10% O/W emulsion treated in the

range 20-300 MPa. The observed temperature increase of emulsions is partly due to adiabatic heating, while the majority may be due to the high velocity at which the fluid exits the primary homogenizing valve. The fluid will be exposed to high turbulence, shear and cavitation forces during UHPH, a large part of which may be transformed into thermal energy in the product.

A marked increase in the T2 was noticed when the oil concentration increased from 10-20 to 30% oil. An increase in the T2 of 0.459 and 0.585°C per 1% oil content (range 10 to 30%) in emulsions treated at 200 and 300 MPa, respectively, was calculated. This increase is in line with that observed in the study of Hayes & Kelly (2003), where milk outlet temperature increased in a linear manner (0.5°C per 1% fat) as milk fat content increased from 0-10% in a pressure range 50-200 MPa. A higher temperature increase should be expected for UHPH treatment of liquids with higher fat contents. The observed increase in heating during UHPH at higher oil contents may be a direct result of viscous dissipation or of the increased number of oil droplets. This larger population of oil droplets increases the probability of collisions between particles, which may, in turn, exert greater shear and other forces upon each other. In addition, different amounts of thermal energy could be absorbed by oil emulsions of varying fat contents due to adiabatic heating. Cortés-Muñoz et al. (2009) working on emulsions containing 4% of whey protein isolate and different oil contents (15, 30 and 45%) attributed this temperature increase to the fluid compression in the intensifier during the pressure build up. In addition, another possible explanation for the strong warming up of the fluid containing high oil content is the viscous stress caused by the high velocity of the fluid flow, which is then impinged on the ceramic valve.

The outlet temperature (T3), which was measured after the final cooling and exit from the homogenization valve, did not exceed 25ºC in all cases, even with varying the oil concentrations.

Table 19. Mean ± SD values of temperature measured before (T1) and at the outlet (T2) of the high-pressure valve for emulsions containing different oil concentrations (10, 20 and 30%) treated by ultra-high pressure homogenization at 200 and 300 MPa (Tin = 25°C).

Oil content (%)	Pressure (MPa)	T1 (°C)[a]	T2 (°C)[b]
10	200	41.00 ± 2.29	84.31 ± 3.01
	300	43.70 ± 2.52	105.5 ± 3.28
20	200	42.70 ± 0.58	86.00 ± 3.00
	300	40.50 ± 5.50	107.5 ± 0.50
30	200	44.00 ± 3.60	93.50 ± 3.77
	300	47.82 ± 3.82	117.2 ± 5.80

7.2.2. Particle size distribution

The particle size distribution of a material can be important in understanding its physical and chemical properties. During homogenization processes, there is usually a dynamic equilibrium between droplet break-up and coalescence which determines the final droplet size distribution.

Droplet size (d3.2 and d4.3 values) and specific surface area (SSA, m^2/ml) for emulsions containing 5% of sodium caseinate and different oil concentrations (10, 20 and 30%) are shown in Table 20, and the size distribution curves in Figure 43.

CM emulsions, at all oil concentrations, had the largest particle size (5.66 µm), showing a distribution curve with a large peak. However, the particle size was drastically decreased to ~ 0.552 µm in CH emulsions with a wide distribution curves, at all protein concentrations tested, and to ~ 0.104 µm in UHPH emulsions with a monomodal and narrow size distribution curves. According to Stang, Schuchmann, & Schubert (2001), droplet disruption in colloid mills is generally less efficient than in high-pressure devices because of having substantially larger volumes and longer residence times in their dispersion zones. Thus, at constant energy density, the mean power density in colloidal mills is lower than in CH and UHPH.

Table 20. Mean ± SD of particle size distribution indices (d3.2 and d4.3) and specific surface area (SSA, m^2/ml) of emulsions containing sunflower and olive oils (10, 20 and 30%) and prepared by colloidal mill (CM), ultra-high pressure homogenization at 200 and 300 MPa with 5% of sodium caseinate, and conventional homogenization (15 MPa) with 1 and 5% of sodium caseinate.

Pressure (MPa)	Oil content (%)	Particle size distribution		
		d3.2 (µm)	d4.3 (µm)	Specific surface area SSA (m^2/ml)
CM	10	6.358 ± 0.643[a]	18.06 ± 4.194[a]	0.915 ± 0.154[c]
	20	5.410 ± 0.303[b]	13.40 ± 2.776[a]	1.117 ± 0.068[c]
	30	5.232 ± 0.417[b]	12.73 ± 2.693[a]	1.152 ± 0.091[c]
15.1	10	0.562 ± 0.031[c]	1.086 ± 0.112[bc]	10.90 ± 0.530[b]
	20	0.588 ± 0.062[c]	1.382 ± 0.264[b]	10.57 ± 1.247[b]
	30	0.480 ± 0.006[c]	1.133 ± 0.160[bc]	12.93 ± 0.517[b]
15.5	10	0.521 ± 0.036[c]	0.961 ± 0.122[c]	11.56 ± 0.825[b]
	20	0.614 ± 0.042[c]	1.315 ± 0.234[bc]	9.841 ± 0.617[b]
	30	0.547 ± 0.106[c]	1.076 ± 0.104[bc]	11.40 ± 2.376[b]
200	10	0.110 ± 0.007[d]	0.131 ± 0.009[d]	54.91 ± 3.151[a]
	20	0.102 ± 0.004[d]	0.126 ± 0.005[d]	59.21 ± 1.801[a]
	30	0.108 ± 0.008[d]	0.130 ± 0.010[d]	55.70 ± 4.060[a]
300	10	0.093 ± 0.007[d]	0.111 ± 0.006[d]	65.16 ± 4.101[a]
	20	0.105 ± 0.014[d]	0.119 ± 0.007[d]	57.06 ± 6.991[a]
	30	0.103 ± 0.014[d]	0.121 ± 0.017[d]	59.48 ± 7.992[a]

[a-d] Different letters in the same column indicate significant differences *($P < 0.05$)* between treatments.

10%
20%
30%

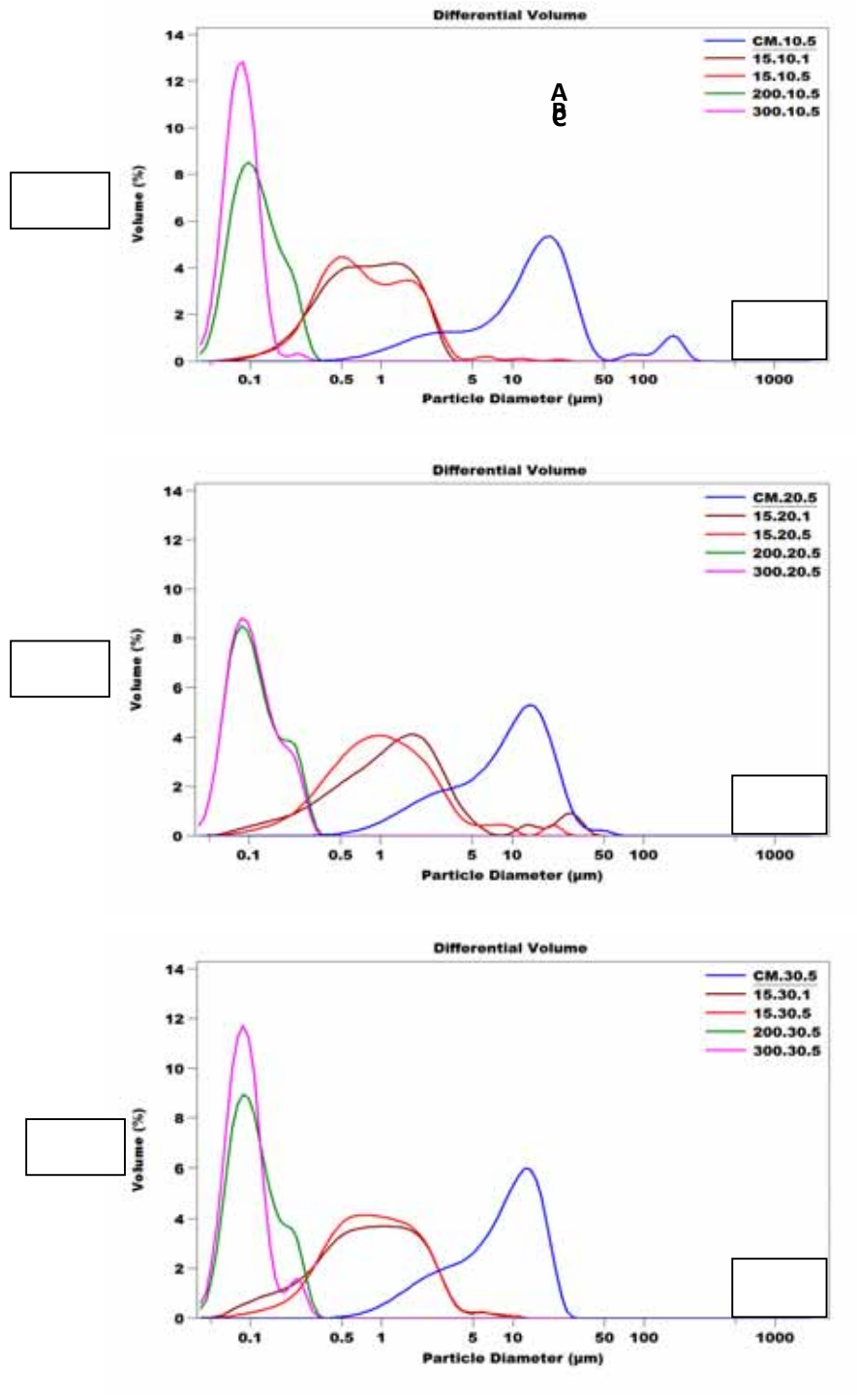

Figure 43. Droplet size distribution curves measured by light scattering of O/W emulsions containing sunflower and olive oils at 10 (A), 20 (B) and 30% (C), and prepared by colloidal mill (CM), ultra-high pressure homogenization at 200 and 300 MPa with 5% of sodium caseinate and conventional homogenization (15 MPa) with 1 and 5% of sodium caseinate.

Floury, Legrand, & Desrumaux (2004), and Schultz, Wagner, Urban, & Ulrich (2004) reported that the droplet disruption efficiency in turbulent flow increases in direct proportion to the power density and in inverse proportion to the length of the residence time in the dispersing zone of an emulsifying device. Therefore emulsions having size < 1 μm usually cannot be achieved using rotor-stator systems as occured in the present study. Furthermore, higher temperatures, as a direct result of high-pressure, reduce the emulsion viscosity, interfacial tension and Laplace pressure, facilitating droplet break-up (McClements, 2005). One other reason for the high particle size observed in CM emulsions may be the high coalescence rate between oil droplets observed in these emulsions (Fig. 44 A), due to the low surface protein coverage at the interface (Fig. 44 B), which may increase the surface tension of oil particles, owing to coalescing them together after homogenization.

In CM emulsions, the particle size (d3.2) was affected by the oil concentration. Increasing the oil concentration from 10 to 20% oil significantly reduced the particle size, but further increase in the oil content to 30% had no significant effect on the particle size. This result was confirmed by the CLSM images (Fig. 45 A-C), which showed similar size of oil particles for CM emulsions but a high degree of flocculation with big holes in emulsions containing 10% oil. On the other hand, no effect of the increase in the oil concentration on the d4.3 and SSA values was observed. This may indicate that 5% of sodium caseinate is sufficient to stabilize emulsions containing up to 20% of oil by decreasing the particle size, but the protein may be insufficient when using more than 20% oil, leaving oil particles without protein coverage and in turn, destabilize the emulsion.

As can be seen from the CLSM images (Fig. 45 D-I), high degree of flocculation could be observed in all CH emulsions, especially those containing 5% of SC, and it was generally higher in emulsions containing 10% oil. The high rate of flocculation observed in emulsions containing 5% SC may be attributed to the poor protein coverage, in comparison to those containing 1% SC, a fact that was confirmed by TEM images (Fig. 46 B, E).

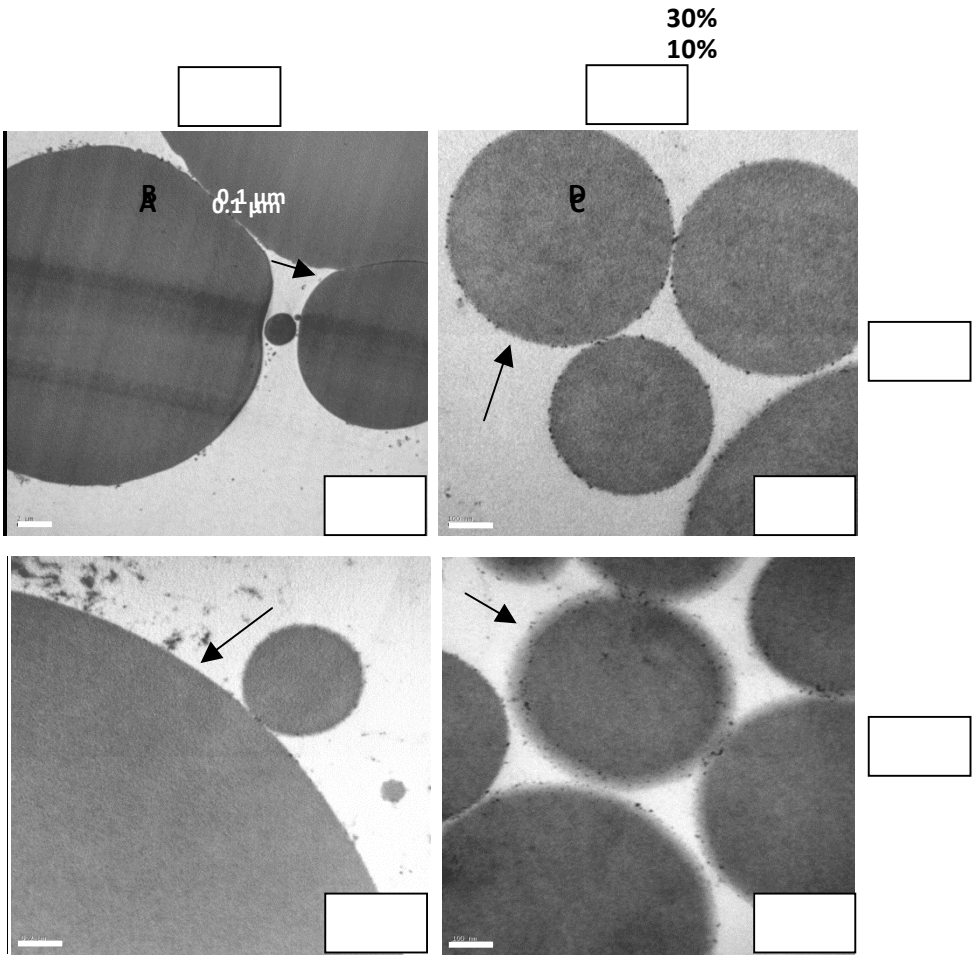

Figure 44. TEM images of O/W emulsions containing sunflower and olive oils at 10% (A,C) and 30% (B,D) and prepared by (A-B) colloidal mill (CM) ×4000 and ×50000, respectively, (C-D) conventional homogenization (15 MPa) with 5% of sodium caseinate ×100000.

The oil concentration had no effect on the particle size (d3.2 and d4.3 values) or on the SSA in CH and UHPH emulsions at all protein concentrations, (Table 20), a fact that was confirmed by the size distribution curves (Fig. 43 A-C), and also by the TEM images (Fig. 46 A-L).

Although UHPH emulsions showed no sign of flocculation (Fig. 46 G-L) between particles, some particles in the TEM images, in a few cases, showed particle coalescence, with a relatively high rate in emulsions containing 10% oil, which may be one of the results of the creaming observed in these emulsions, as will be explained in details later in the Physical Stability section (7.2.5). The coalescence observed in UHPH emulsions could be related to the high collision frequency (Tesch & Schubert, 2002).

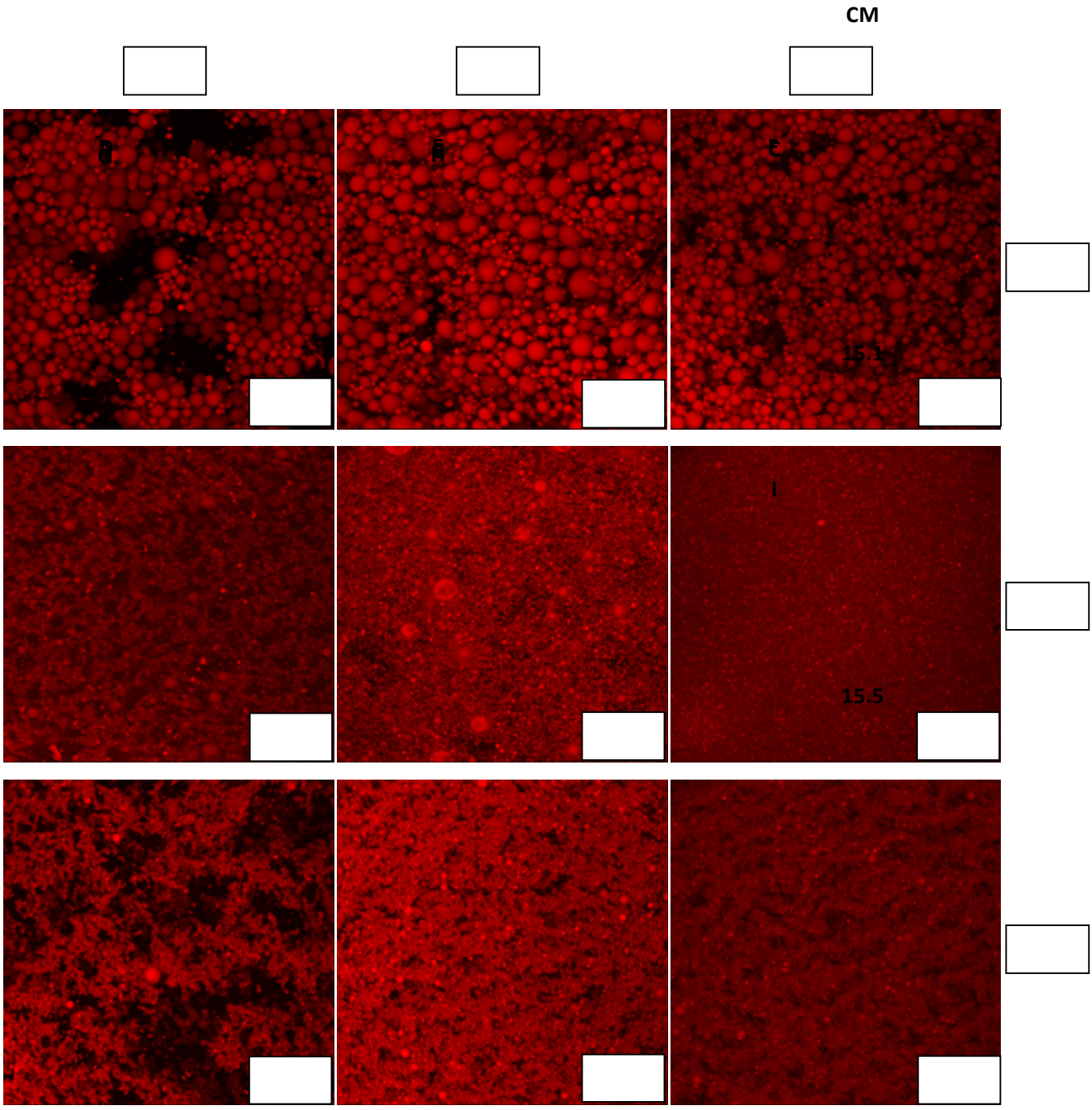

Figure 45. Confocal laser scanning microscope images of O/W emulsions containing sunflower and olive oils (10, 20 and 30%) and prepared by (A-C) colloidal mill (CM) with 5% of sodium caseinate, and by conventional homogenization (15 MPa) with 1 (D-F) and 5% (G-I) of sodium caseinate.

Normally, at constant energy density (e.g. emulsification pressure), particle size rises with increasing oil content. Some experiments by high-pressure valve homogenization (Phipps, 1985; Tesch, Gerhards, & Schubert, 2002) or ultrasound emulsification (Abismail, Canselier, Wilhelm, Delmas, & Gourdon, 1999) have confirmed this trend. At constant emulsifier concentration, and as the oil content increases, there may be an insufficient amount of protein present to completely cover the new droplets during high-pressure homogenization. An inadequate amount of protein in the aqueous phase could cause some aggregation of fat globules, as reported by Tomas, Paquet, Courthaudon, & Lorient, (1994). The results obtained in the present study indicate that 5% of SC is sufficient to stabilize the emulsions containing these oil concentrations, as no increase in the particle size was observed when the oil content increased.

7.2.3. Rheological behavior

According to McClements (2005), many factors influence rheology in emulsions such as dispersed phase volume fraction, rheology of component phases, droplet sizes, droplet charges, etc.

The influence of oil phase concentration on the apparent viscosity of the emulsions was determined by comparison of the consistency coefficient (K) value, which corresponds to the viscosity when the fluid is Newtonian, and the flow behavior index (n) obtained by fitting the flow curves for different concentration of oil phase.

Low viscosities and Newtonian behavior were observed in CM emulsions because of the low interaction between particles. However, a high consistency coefficient (K) and shear thinning behavior (or pseudo-plasticity) were observed in CH emulsions containing 5% of SC, decreasing their apparent viscosity with increasing shear stress, but not in those containing 1% of SC. Increasing the oil concentration from 10 to 30% significantly increased the viscosity of CM emulsions and CH emulsions containing 5% SC; however, this increase was only significant in CH emulsions containing 1% SC when the oil content increased from 10-20 to 30%. Dluzewska, Stobiecka, & Maszewska (2006), Wang, Li, Wang, & Özkan (2010) and Sun & Gunasekaran (2009) also reported that the increase of oil phase fraction led to an increase in emulsion viscosity.

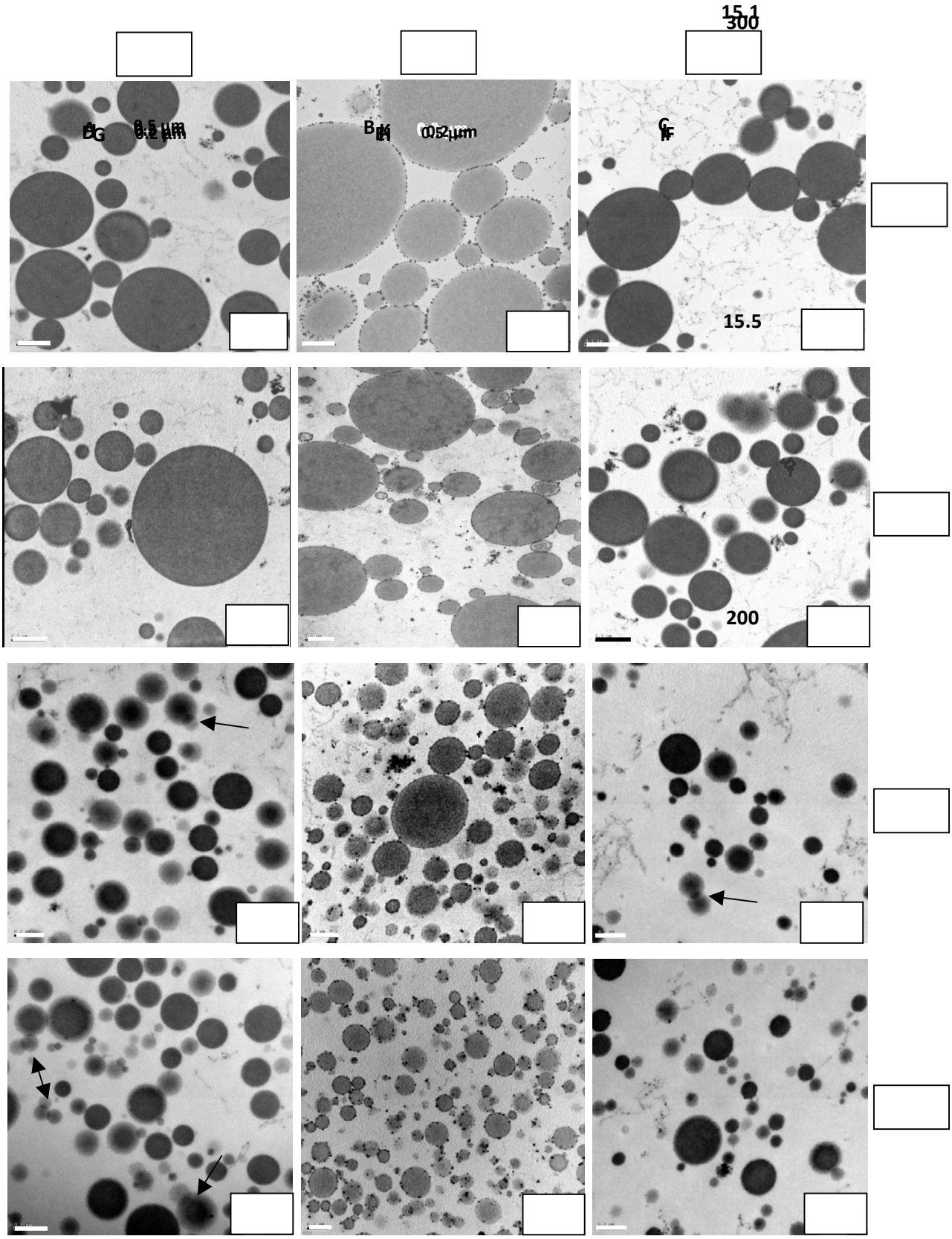

Figure 46. TEM images ×50000 of O/W emulsions containing sunflower and olive oils (10, 20 and 30%) and prepared by (A-F) conventional homogenization (15 MPa) with 1% (A-C) and 5% (D-F) of sodium caseinate, and by ultra-high pressure homogenization at 200 MPa (G-I) and 300 MPa (J-L).

As oil content increases, the particles become closer which leads to packing of the oil droplets and the strengthening of inter-particle interactions. The attractive forces between droplets drive the formation of flocs which could normally evolve into a space-filling particulate network (Mewis & Wagner, 2009). CM and CH emulsions, those containing 1%, exhibited a Newtonian flow behavior, whereas, the rheological behavior was changed toward shear thinning in CH emulsions containing 5% of SC.

In addition, the shear thinning behavior was more notable when the oil concentration increased from 10 to 30%, which indicates a high degree of aggregation by using high oil concentrations. Shear thinning behavior is observed in flocculated emulsions, as in the case of our CH emulsions containing 5% SC, as mentioned previously in the Particle Size Section (Fig. 45 G-I), because of deformation and breakdown of the aggregates as shear stresses increase. Dickinson & Golding (1997) observed that the low-shear viscosity of the 1% w/w SC emulsion, in which the depletion occurred, was much higher than that of both the 0.1% and 0.25% w/w SC emulsion, which destabilized by bridging flocculation. The aggregated structure from the depletion-flocculated emulsion droplets observed in CH emulsions containing high SC concentrations may be due to the existence of a substantial excess amount of unadsorbed protein in the aqueous phase, leading to an increase in the effective volume fraction of hydrodynamically interacting particles, thereby resulting in a much higher apparent viscosity than for the homogeneous emulsion, as in the case of CM and CH emulsions containing 1% SC.

UHPH application had a significant effect on the emulsions viscosity only when high oil content (30%) was used, which is in line with the decrease of the droplet size as the pressure increased. Pal (2000) attributed this increase in viscosity with reduced droplet size to an increase in hydrodynamic interactions between the droplets, since the mean separation distance between the droplets decreases when the droplet size is reduced.

Similar trends in the rheological characteristics of emulsions have been reported in some other studies using the UHPH in emulsion preparation. Floury, Desrumaux, & Legrand (2002) reported that Newtonian liquid emulsions (20% oil) stabilized by soy proteins (2%) were converted into emulsion gels with shear-thinning rheological behavior after high-pressure homogenization (> 250 MPa).

San Martin-González et al. (2009) showed that high-pressure homogenization is able to develop a gel-like structure in micellar casein stabilized emulsions containing 30% oil and 2 - 3.5% casein when homogenized between 20 and 100 MPa. The authors

hypothesized that homogenization results in the exposure of hydrophobic sites within the micelle core, which allows micelle-coated oil droplets to interact with neighboring particles, creating an elastic three-dimensional structure that becomes fairly strong at a threshold casein concentration. Pereda, Ferragut, Quevedo, Guamis & Trujillo (2007) observed higher viscosity after milk was subjected to high-pressure homogenization at 300MPa. They found that milks homogenized at pressure ~300 MPa were characterized by the formation of fat aggregates, due to an extensive flocculation process, that were not observed at relatively lower homogenization pressure (e.g., ≤ 200MPa).

The change in emulsion rheology caused by the high-pressure homogenization may not only be related to the change in the emulsion droplet size, as we initially thought, but might also be related to the protein adsorption at the droplets interface. The viscosity increase after homogenization has been related to the increased number of fat globules and the simultaneous adsorption of milk proteins on the increased fat globule surface (Hayes, Lefrancois, Waldron, Goff, & Kelly, 2003; Robins, 2000).

Concerning the effect of oil concentration on the viscosity of UHPH-treated emulsions, a significant increase in the viscosity was observed as the oil concentration increased from 10 to 30%. Floury et al. (2000) reported that UHPH allows the production of emulsions with a large range of flow behaviors (i.e., from highly fluid to highly thick samples) when combining the pressure level of homogenization and the oil volume fraction. The authors found that emulsions containing less than 20% of dispersed phase plus whey proteins (1.5%) followed Newtonian behavior (n = 1), whatever the homogenizing pressure applied. They attributed the Newtonian behavior of the fluids to the low particle-particle interactions in these emulsions, however, increasing the oil content to 50% tended to change completely the flow behavior to shear thinning. Cortés-Muñoz et al. (2009) using 4% whey protein isolate and 15-45% oil observed the Newtonian flow behavior only in emulsions containing 15 and 30%; however, emulsions containing 45% oil presented a shear thinning behavior. The explanation of the viscosity increase as oil-phase volume fraction increased was attributed by the authors to the increase in the packing fraction of oil droplets.

Table 21. Mean ± SD of rheological characteristics (flow and consistency indices) and emulsifying activity index (EAI, m^2/g) of emulsions containing sunflower and olive oils (10, 20 and 30%) and prepared by colloidal mill (CM), ultra high-pressure homogenization at 200 and 300 MPa with 5% of sodium caseinate, and conventional homogenization (15 MPa) with 1 and 5% of sodium caseinate.

Pressure (MPa)	Oil content (%)	Rheological behavior			
		Consistency coefficient (K) mPa × s	Flow behavior index (n)	r^2	Emulsifying activity index EAI (m^2/g)
CM	10	0.0052 ± 0.0007^{g}	0.988 ± 0.009	0.999	676 ± 37^{j}
	20	0.0121 ± 0.0006^{f}	0.986 ± 0.025	0.998	1552 ± 400^{hi}
	30	0.0235 ± 0.0021^{e}	1.003 ± 0.008	1.000	2817 ± 618^{fgh}
15.1	10	0.0018 ± 0.0006^{h}	1.027 ± 0.053	1.000	20116 ± 10886^{cd}
	20	0.0020 ± 0.0004^{h}	0.997 ± 0.010	0.998	52954 ± 9272^{b}
	30	0.0061 ± 0.0036^{fg}	0.972 ± 0.023	0.938	101916 ± 5828^{a}
15.5	10	0.0104 ± 0.0021^{f}	0.858 ± 0.019	0.999	5498 ± 436^{e}
	20	0.0444 ± 0.0117^{d}	0.754 ± 0.038	1.000	13312 ± 1776^{d}
	30	0.2092 ± 0.1043^{c}	0.608 ± 0.068	0.984	21743 ± 1002^{c}
200	10	0.0046 ± 0.0010^{g}	0.998 ± 0.017	0.999	893 ± 140^{ij}
	20	0.0384 ± 0.0086^{e}	0.885 ± 0.089	1.000	2692 ± 161^{fgh}
	30	2.8643 ± 1.2807^{b}	0.339 ± 0.052	0.988	3876 ± 441^{fg}
300	10	0.0046 ± 0.0010^{g}	1.011 ± 0.008	0.999	649 ± 98^{j}
	20	0.0490 ± 0.0091^{d}	0.850 ± 0.044	0.998	2055 ± 347^{gh}
	30	7.3833 ± 4.4120^{ab}	0.252 ± 0.039	0.987	2731 ± 542^{fgh}

[a-f] Different letters in the same column indicate significant differences *($P < 0.05$)* between treatments.

A number of protein solutions and O/W emulsions exhibit pseudoplastic and thixotropic behaviors. Thixotropy is a typical phenomenon observed in foods with significantly different dynamic and static yield stress data. Typically, emulsions that exhibit this behavior contain droplets that are aggregated by weak forces. The aggregated droplets are gradually disrupted and collapsed because of the shearing of the materials, hence the resistance to flow decreases and consequently cause the reduction of the apparent viscosity over time (Petrovic, Sovilj, Katona, & Milanovic, 2010).

In our results no thixotropic behavior or hysteresis loops were observed (Fig. 47), which may indicate that the aggregated structure is weak and could be ruptured with a small shearing and changing to separated particles, so no hysteresis loop could be seen. Similar results were observed by Srinivasan (1998) in 30% O/W emulsions made with sodium and calcium caseinate (0.5-5%), in which no hysteresis was evident in the flow curves, indicating that the emulsions did not exhibit thixotropic (time dependent) behavior.

7.2.4. Emulsifying activity index (EAI)

The EAI is related to the surface area stabilized by a unit weight of proteins, which presents the ability of proteins to be adsorbed at the interface of fat globules and the aqueous phase (Pearce & Kinsella, 1978).

Low EAI values were observed in CM emulsions, but applying low pressure homogenization (CH treatment) increased significantly the EAI; however, applying ultra-high pressures (200 and 300 MPa) decreased the emulsifying activity of sodium caseinate (Table 21).

It is evident that what is important in emulsion formation is not only the weight of oil, but also its interfacial area. CM emulsions presented the largest particle size with the lowest surface area, and thus, this surface area will consume less surfactant than if small droplets are present. Furthermore, the kinetics of absorption in rotor-stator systems requires a much longer time than that for homogenization by both CH and UHPH (Krešić, Lelas, Herceg, & Režek, 2006), which may be responsible for the low EAI in CM emulsions. Corzo-Martínez et al. (2011) showed that the EAI of emulsion homogenized with Ultra-Turrax, an example of rotor-stator systems, was much lower (77.61 ± 4.89 m^2/g) than that produced by sonication (> 785 m^2/g) at 0.5 mg of SC/ml.

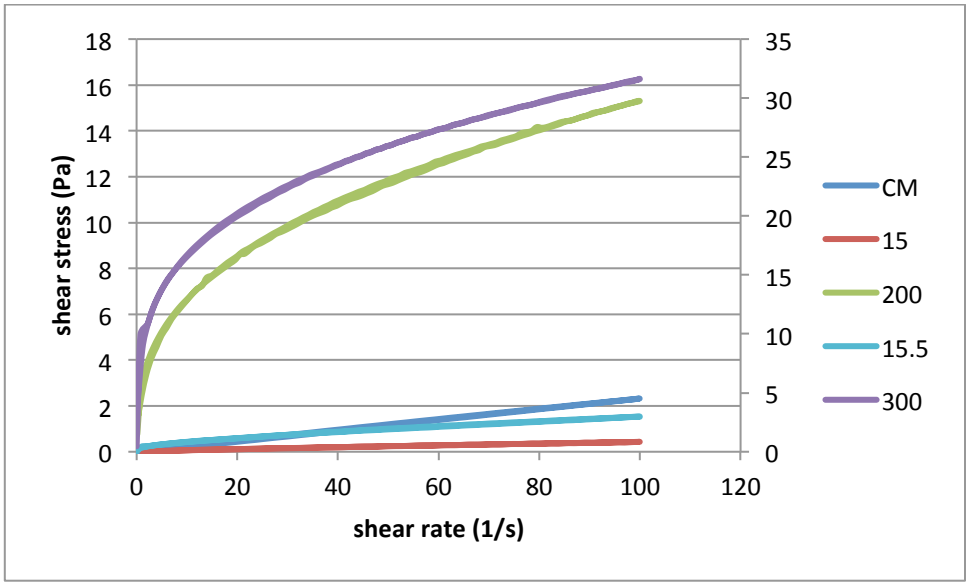

Figure 47. Shear stress curves of O/W emulsions containing 30% oil and 5% sodium caseinate stabilized by colloidal mill (—), conventional homogenization at 15 MPa with 1% (—) and 5% (—) sodium caseinate, and by ultra-high pressure homogenization at 200 MPa (—) and 300 MPa (—).

Applying the conventional homogenization significantly increased the EAI value, especially when low SC concentration was used. The EAI values of CH emulsions clearly decreased ($P < 0.05$) with increased protein concentration from 1 to 5% SC at all three respective oil concentrations, as can be seen in TEM images (Fig. 46 B, E) which show high protein amounts at the interface of CH emulsions containing 20% oil and stabilized by 1% SC (Fig. 46 B), in comparison to those containing 5% SC (Fig. 46 E). This result may be attributed to the high rate of aggregation or flocculation observed in CH emulsions containing 5% SC, which may result in a decreased efficiency of proteins to be adsorbed at the interface of oil droplets. One other possible explanation for the decrease in the EAI with increasing SC concentrations has been suggested by Guo & Mu (2011). They reported that at higher protein concentrations the activation-energy barrier prevents protein migration in a diffusion-dependent manner. Further increases in protein concentration may decrease the effectiveness of protein adsorption, which leads to a decrease in the EAI.

UHPH-treated emulsions exhibited low EAI values, similar to CM emulsions, as a consequence of the reduced particle size observed in these emulsions. One other reason which may explain the low EAI values of UHPH-treated emulsions rather than the

reduction in particle size produced by the treatment or the necessity of higher protein amounts to cover the newly created interface, maybe the decrease in the protein solubility and flexibility as a result of the protein aggregation caused by ultra-high pressures. Le Denmat, Anton, & Gandemer (1999) reported that the proteins could not adsorb at the interface when they are insolubilized and aggregated. The aggregated proteins caused by the heat dissipated from the high-pressure (117.2°C in UHPH emulsions treated at 300 MPa containing 30% oil) may make the proportion of proteins which could be adsorbed at the O/W interface insufficient to overcome the amounts of aggregated protein, which may decrease the availability of proteins to form films and emulsions (Phillips, Schulman, & Kinsella, 1990). High temperatures reduce the emulsion viscosity, interfacial tension and Laplace pressure, facilitating droplet break-up (McClements, 2005), but temperature rise has a complex effect, by adversely affecting the emulsifying properties of surface-active ingredients (Floury et al., 2003). It has been reported that when soy proteins were heat-treated at 90°C, proteins exhibited great surface hydrophobicity and formed disulphide bonds with neighboring proteins, which enhanced their emulsion stability, but conversely, excessive hydrophobic bonding among soy proteins treated at 120°C caused aggregates to form, which reduced their emulsifying capabilities (Wang et al., 2012). Whey protein adsorption in sunflower oil emulsions has been reported to increase with increasing pressure up to 100 MPa and to decrease at pressures of 200 or 300 MPa (Desrumaux & Marcand, 2002). This may be the reason for the coalescence of oil particles in some UHPH emulsions, as explained previously in the Particle Size section (7.2.2).

In respect to the effect of oil volume fraction on the EAI, it can be observed from Table 21 that, in CM and UHPH emulsions, increasing the oil concentration from 10 to 20% oil significantly increased the EAI; however, further increase in the oil concentration to 30% had no further effect on the EAI, indicating that the SC started to become limited in its ability to cover the O/W interface. A different trend was observed in CH emulsions, where a linear increase in the EAI was observed when the oil concentration increased from 10 to 30%, a fact that was confirmed by the TEM images (Fig. 44 C,D) in which a high protein coverage could be seen in emulsions containing 30% oil (Fig. 44 D) in comparison to those containing 10% oil (Fig. 44 C). Increasing the oil concentration while maintaining a constant protein level results in a reduction of protein at the interface, thus suggesting the spreading of protein at an interface to form a thinner

layer (Srinivasan et al., 1996). A similar trend was observed by Al-Malah, Azzam, & Omari (2000) in emulsions stabilized by 0.1% w/v bovine serum albumin in corn, soybean, sunflower and olive oils when the oil volume fraction increased from 25 to 56%, and by Gu, Decker, & McClements (2009), increasing the oil concentration from 10 to 20% in sunflower and soy oil emulsions. Britten, Giroux, & Gaudin, (1994), using the denatured whey protein isolate, found that the oil volume fraction of the emulsion was the main factor influencing the EAI. They reported that the surface area of the dispersed phase stabilized by the protein increased with the amount of oil to be homogenized.

7.2.5. Physical stability of emulsions

Emulsion stability is a complex issue and can be influenced by a number of factors including particle size, viscosity and environmental conditions such as temperature and shear force.

The light scattering fingerprints of emulsion samples obtained by Turbiscan lab, with oil concentrations of 10-30% and treated by CM, CH and UHPH, are shown in Figure 48. From these results, it can be observed that the emulsions prepared by the CM achieved the highest creaming rate at the first day of storage; a great variation with time could be observed in the backscattering profiles of Turbiscan. Furthermore, visual separation was observed in all CM emulsions at the same day of preparation. As explained before, the oil particles in CM emulsions are prone to coalesce together due to their low surface protein coverage, which leads to a tendency for a high interfacial tension between particles. One other reason for the high creaming rate in CM emulsions may be their low viscosities, which results from the high particle size and the low protein amount at the interface of the particles. The high creaming rate in CM emulsions was observed in those containing low oil concentration (10%) however, increasing the oil concentration from 10 to 30% significantly reduced the creaming rate to a great extent, a fact that was confirmed by the migration velocity value V (t) (μm/min) calculated by the Turbiscan, in which a high migration velocity of particles to the top of the Turbiscan tube was found in CM emulsions containing 10% oil (428.5 μm/min), in comparison to those containing 20 and 30% oil (85 and 58 μm/min), respectively. The low creaming stability of CM emulsions containing 10% oil could also be explained by the high particle size

(Table 20) and the high flocculation and coalescence rate (Fig. 45 A), in comparison to emulsions containing 20 and 30% oil (Fig. 45 B-C).

The CH emulsions exhibited higher creaming stabilities, in comparison to CM emulsions, especially when a low SC concentration (1%) was used. Table 22 and Figures 48 (D-I), 49 (A-F) and 50 (A-F) illustrate the physical stability against creaming in CH emulsions. The creaming was affected by both SC concentrations and the oil-phase volume fractions. Although significant differences in the d4.3 value at the top or at the bottom in all CH emulsions could be seen (Table 22 and Fig. 49 A-F), Turbiscan fingerprints (Fig. 48 D-I) and the visual stability test in emulsions stored at the room temperature for 20 days (Fig. 50 A-F) showed a higher creaming rate in CH emulsions containing 5% SC (Fig. 48 G-I and Fig. 50 D-F) than in those containing 1% SC (Fig. 48 D-F and Fig. 50 A-B). This result is in agreement with the confocal micrographs (Fig. 45 D-I), where a high flocculation rate was observed in emulsions containing 5%. Day, Xu, Hoobin, Burgar, & Augustin (2007) reported that, bridging flocculation at low SC concentrations leads to creaming progress much more slowly and does not necessarily affect the creaming rate during the early part of emulsion storage. Dickinson & Golding (1997) demonstrated that emulsions made with > 2% SC were more unstable towards creaming than emulsions made with lower caseinate concentrations. This destabilization was attributed to depletion flocculation caused by the presence of high concentrations of non-adsorbed caseinate.

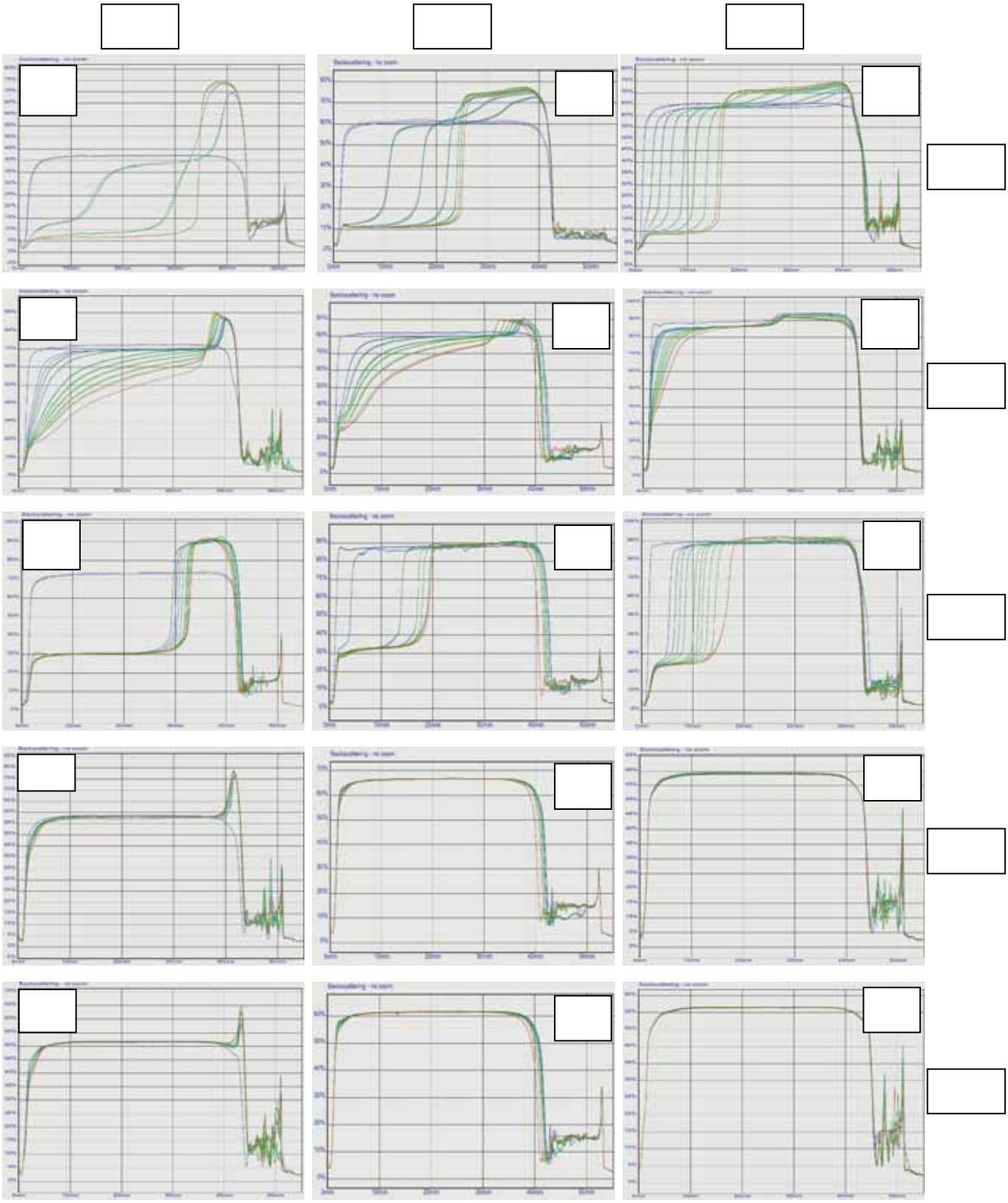

Figure 48. Changes in backscattering profiles of O/W emulsions containing sunflower and olive oils (10, 20 and 30%) and prepared by (A-C) colloidal mill (CM), conventional homogenization (15 MPa) with 1% (D-F) and 5% (G-I) of sodium caseinate, and by ultra-high pressure homogenization (UHPH) with 5% of sodium caseinate at 200 (J-L) and 300 MPa (M-O), as a function of sample height with storage time (5 h for CM emulsions and 18 days for both CH and UHPH emulsions).

Table 22. Mean ±SD of d4.3 values at the top or at the bottom in samples stored at room temperature for 9 days under the same conditions for comparison, of O/W emulsions containing sunflower and olive oils (10, 20 and 30%), and prepared by ultra high-pressure homogenization at 200 and 300 MPa with 5% of sodium caseinate, and conventional homogenization (15 MPa) with 1 and 5% of sodium caseinate.

Pressure (MPa)	**Oil content (%)**	**Emulsion creaming stability after 9 days**		
		d4.3 (Top)	**d4.3 (Bottom)**	***P value***
15.1	**10**	1.473 ± 0.452^{ab}	0.676 ± 0.075^{ab}	**0.002***
	20	2.428 ± 0.982^{a}	0.961 ± 0.389^{a}	**0.009***
	30	1.461 ± 0.368^{ab}	0.533 ± 0.050^{abc}	**0.002***
15.5	**10**	1.071 ± 0.102^{b}	0.409 ± 0.263^{bcd}	**0.002***
	20	1.113 ± 0.271^{b}	0.268 ± 0.118^{bcd}	**0.002***
	30	1.141 ± 0.225^{b}	0.382 ± 0.191^{bcd}	**0.002***
200	**10**	0.116 ± 0.013^{c}	0.117 ± 0.013^{d}	0.965
	20	0.135 ± 0.017^{c}	0.135 ± 0.016^{cd}	0.974
	30	0.125 ± 0.011^{c}	0.122 ± 0.012^{d}	0.844
300	**10**	0.103 ± 0.013^{c}	0.108 ± 0.007^{d}	0.374
	20	0.125 ± 0.017^{c}	0.122 ± 0.018^{d}	0.734
	30	0.114 ± 0.013^{c}	0.112 ± 0.008^{d}	0.859

$^{a-h}$ Different letters in the same column indicate significant differences ($P < 0.05$) between treatments.

* Sign indicates that the differences between the d4.3 at the top or at the bottom of emulsions are significant (Wilcoxon statistic test, $P < 0.05$) per level of pressure and oil concentration.

In this sense, Euston, Finnigan, & Hirst, (2002) reported that the removal of unadsorbed protein in the aqueous phase significantly reduced the aggregation rate. It was concluded that emulsions having full protein surface coverage, but relatively little excess unadsorbed protein in the continuous phase, were stable Newtonian liquids. In some cases, it has been found that stability decreases with increasing SC content up to a certain concentration (6% w/w).

Our results indicated that oil-phase volume fraction played an important role in the creaming stability of CH emulsions. As can be seen from Figure 48 (D-I) and 50 (A-F), the emulsion stability improved with the increased oil-phase volume fraction. The emulsion stability improved when the oil content increased because of the increase in packing fraction of oil droplets (Dickinson & Golding, 1997), which enhanced emulsion viscosity and lowered the creaming rate. According to Rezvani, Schleining, & Taherian (2012) this effect could also be explained by the fact that increasing the oil content leads to augmentation of the number of particles in the emulsion matrix. As a result, the presence of a large number of particles improved the resistance to the flow, and hence increased the apparent viscosity (Mirhosseini, Tan, Taherian, & Boo, 2008). Cortés-Muñoz et al. (2009) reported a much better stability at higher oil concentrations than at lower oil concentrations. At low oil-phase volume fraction (10%), the possible reason for the rapid creaming is that the smaller droplets were more sensitive to emulsion viscosity, than their size. For the emulsion with 20 and 30% oil-phase volume fraction, high viscosity (Table 21) made the creaming rates similar. The high viscosity could limit the motion of droplets and decrease the frequency of collisions between droplets and then the creaming rate. Sun & Gunasekaran (2009) reported that at low volume fraction of oil, creaming is rapid because the weakly flocculated network simply collapses under its own weight.

UHPH emulsions displayed better creaming stability than CM and CH emulsions at all oil concentrations, especially in emulsions containing 20 and 30% oil, with the emulsions remaining turbid with no visual separation during storage time (20 days). In addition, Turbiscan fingerprints (Fig. 48 J-O) and the particle size distribution curves at the top or at the bottom of emulsions (Fig. 49 G-L) showed no change in the light backscattering and the particle size, except for emulsions containing 10% oil, where a slight creaming was observed. However, the d4.3 value at the top or at the bottom presented in Table 22 did not show any change in the particle size in any UHPH

emulsions, even those containing 10% oil. The slight creaming observed in UHPH-treated emulsions containing 10% oil may be attributed to the slight flocculation or coalescence observed in these emulsions, as explained before in the Particle Size Distribution section (7.2.2).

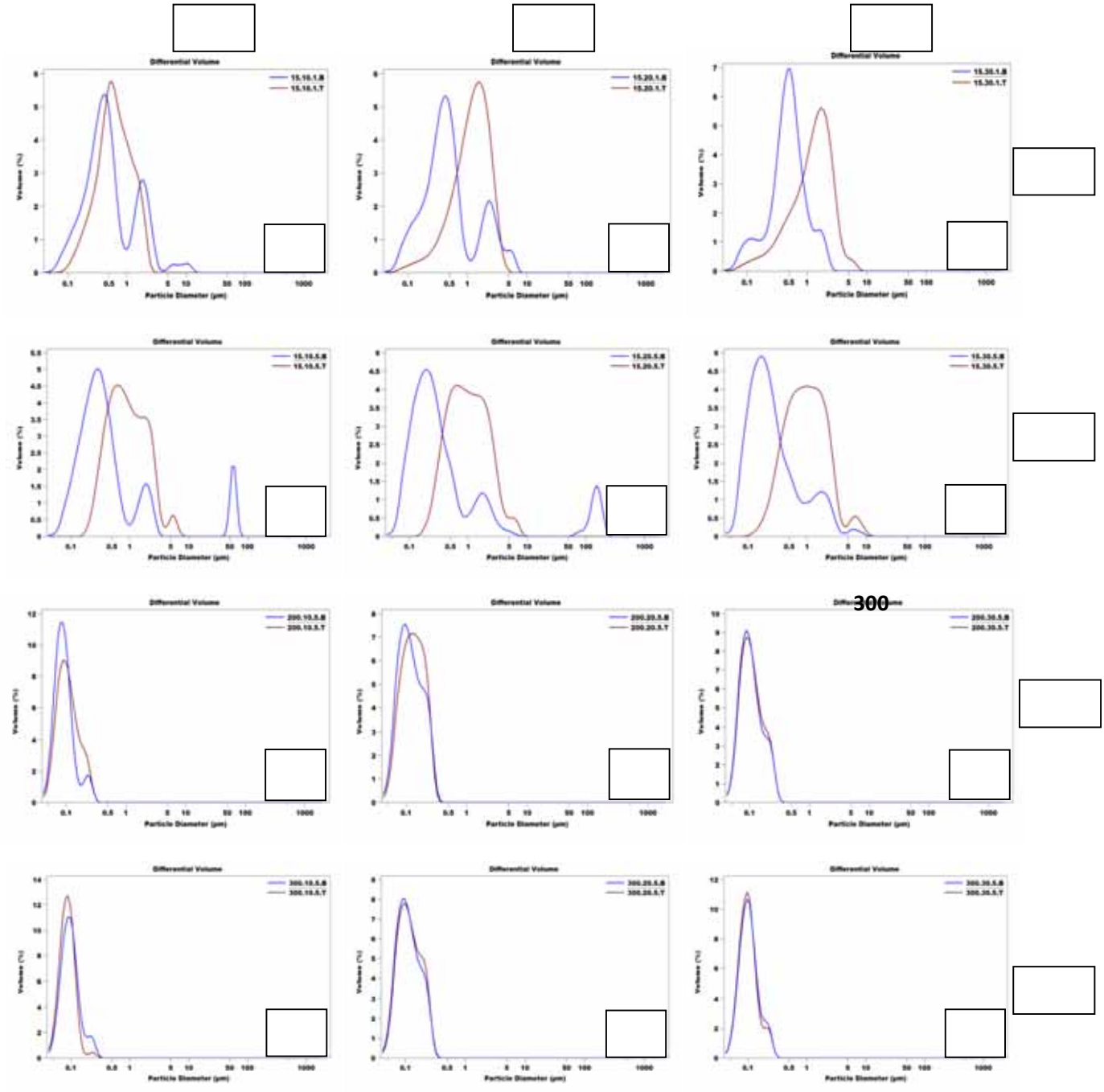

Figure 49. Droplet size distribution curves at the top (T) and the bottom (B) of O/W emulsions containing sunflower and olive oils (10, 20 and 30%) and prepared by (A-F) conventional homogenization (15 MPa) with 1% (A-C) and 5% (D-F) of sodium caseinate, and by ultra-high pressure homogenization at 200 (G-I) and 300 MPa (J-L) with 5% sodium caseinate.

The food industry is highly interested in submicron/nano-emulsions because of their certain inherent advantages. The very small droplet size results in low gravity forces such as Brownian motion, which may be sufficient to prevent creaming or sedimentation occurrence during storage (McClements, 2005).

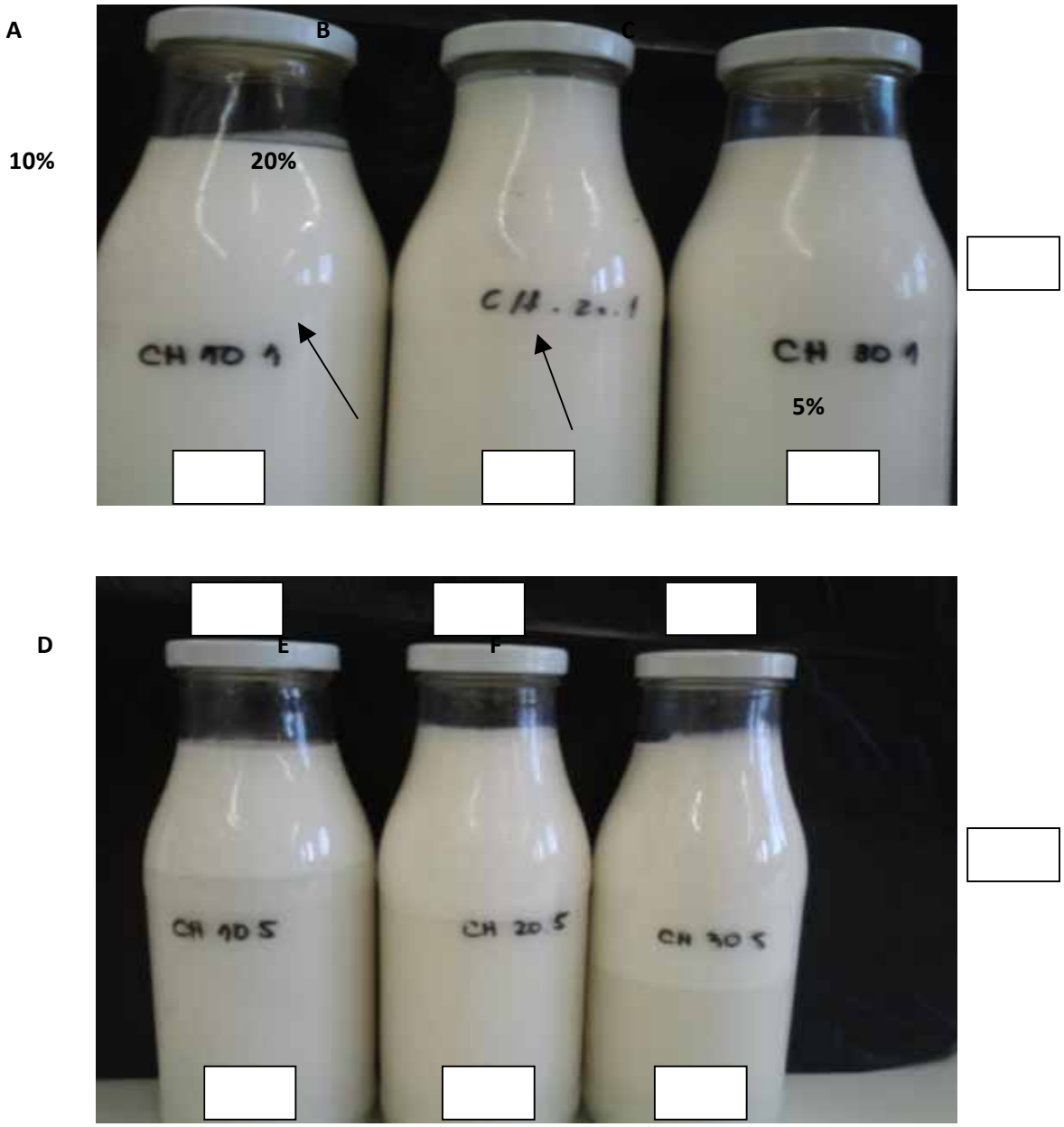

Figure 50. Visual creaming assessment of O/W emulsions containing 10, 20 and 30% of sunflower and olive oils and prepared by conventional homogenization (CH, 15 MPa) with 1% (A-C) and 5% (D-F) of sodium caseinate.

San Martín-González et al. (2009) observed that high-pressure (300 MPa), even at the low casein concentration (0.5 g/100 g, oil-free basis), resulted in a creaming index of 0 regardless of oil and casein concentration for up to 10 days. The authors attributed this high stability to the extensive disruption of the casein micelles which occurs at high-pressure, thus increasing the availability of emulsifying protein molecules. According to Lee, Lefèvre, Subirade, & Paquin (2009), high-pressure reduces droplet size and emulsions having smaller droplet size are more stable than large ones, which is consistent with Stokes law. However, high-pressure can change the amount of adsorbed proteins and the protein interactions at interface leading to the formation of a more rigid interfacial layer at higher pressure, so that it may effectively better protect emulsion droplets against destabilizing processes. The greater droplet size reduction and the rigid interfacial layers around oil droplets of emulsions treated at high pressure results in an increase in the emulsion density, which makes the migration of particles to the top very difficult. Additionally, the low particle size in emulsions also increases the emulsion viscosity (Table 21), limiting the movements of the oil particles and then lowering the creaming rate. For instance, in the case of our UHPH emulsions, the migration velocity for those treated at 200 MPa and containing 5% SC was much lower (7.7 μm/min) than that of CM and CH emulsions (85 and 272.9 μm/min, respectively) at the same oil concentration (20%).

The pressure of treatment and the oil concentration seem to have no effect on the emulsion stability, in which no significant differences could be observed when the pressure increased from 200 to 300 MPa and the oil concentration increased from 10 to 30%.

The stability results found for the UHPH emulsions are in agreement with the results found by Cortés-Muñoz et al. (2009). They observed a slight creaming effect in only a few cases, mainly after processing emulsions with 15% (w/w) oil treated at 100-150 MPa, while emulsions treated at 200 MPa led to excellent oil droplet stability vs. creaming and coalescence, especially when high oil concentration was used.

7.2.6. Oxidative stability

Hydroperoxide contents and TBARS of emulsions treated by CM, CH and UHPH containing different oil concentrations and SC are shown in Table 23.

CM emulsions showed the highest oxidation rates of all emulsions, where considerable high primary and secondary oxidation products were observed, being higher in emulsions containing 10 and 30% oil. The high hydroperoxide value in combination with the high levels of TBARS obtained in these emulsions, especially those containing 10% oil, indicates a well established oxidation from a primary to a secondary state, showing the high sensitivity of these emulsions to lipid oxidation.

This high sensitivity of CM emulsions to oxidation may be attributed to the high coalescence rate between oil droplets (Fig. 44 A), due to the low surface protein coverage at the interface (Fig. 44 B), making the particles sensible to the oxidation factors.

In CH emulsions containing 1% of SC, increasing the oil concentration from 10 to 30% resulted in a reduced oxidative stability. It can be observed that higher hydroperoxides with a significant evolution were formed in emulsions containing 10% after 10 day of storage; however, a decrease in hydroperoxides was observed when oil content increased to 30%. On the contrary, higher amounts of TBARS were observed in emulsions containing 20 and 30% oil. The low hydroperoxides accompanying with high TBARS in emulsions containing 20 and 30% oil may indicate the high oxidation rate in these emulsions and the change from the primary to secondary oxidation phase. It was concluded from the previous study (Chapter 6) with different SC concentrations (1, 3 and 5%) using 20% of oil, that 1% of SC produced a quite physically stable emulsion but was unable to completely cover all the oil particles formed, a fact that was confirmed in that study by the occurrence of oil red particles without protein coverage (Fig. 35). Based on this conclusion, it can be expected that 1% of SC may be sufficient to stabilize emulsions containing 10% oil, but a further increase in the oil content to 20 and 30% may affect the oxidative stability of emulsions in a bad way.

The higher hydroperoxide values of O/W emulsions containing lower oil fraction had been previously reported in safflower oil (Sims, Fioriti, & Trumbetas, 1979) and canola oil (Osborn & Akoh, 2004) emulsions. One explanation for the higher hydroperoxide values was that the number of radicals produced per oil droplet probably increased at

lower oil fraction concentration (Osborn & Akoh, 2004); at higher oil fraction more unsaturated fatty acids may have moved into the interior of the oil droplet, and thus these fatty acids became less accessible to direct interaction with the pro-oxidants at the interface (McClements & Decker, 2000).

CH emulsions containing 5% SC with 10 and 30% oil showed low hydroperoxide content, in comparison to emulsions containing 20% oil; however, lower amounts of TBARS were observed in emulsions containing 20% oil, indicating the high stability of these emulsions to oxidation.

When comparing emulsions containing 30% oil and stabilized by 1 and 5% SC, we can observe similar amounts of hydroperoxides with no significant differences, whereas significantly higher amounts of TBARS were formed in CH emulsions containing 1% SC which may confirm our previous suggestion that high protein amounts in the continuous phase is required to reduce the lipid oxidation in emulsions.

Several studies with casein as emulsifier have shown that the rate of lipid oxidation decreases with increasing levels of casein (Faraji, McClements, & Decker, 2004; Hu et al., 2003; Kargar, Spyropoulos, & Norton, 2011; Ries, Ye, Haisman, & Singh, 2010). Horn et al. (2011) reported that increasing the sodium caseinate concentration resulted in increased oxidative stability of O/W emulsions.

Studies using other emulsifiers have shown that when emulsifier (lecithin/Tween 20/whey protein isolate/monoglycerol/diacylglycerol/sucrose fatty acid esters) concentration was increased from 0.25% to 1%, lipid oxidation levels decreased (Fomuso, Corredig, & Akoh, 2002).

Emulsions containing 5% (w/w) SC would have more excess of protein in the continuous phase than in 1% SC emulsions, and then the interface of the emulsion droplets may become protein saturated in emulsions containing high protein concentrations. Since SC has antioxidative properties, it is possible that the SC excess in the continuous aqueous phase contributed to the better oxidative stability of the oil (Falch, Anthonsen, Axelson, & Aursand, 2004).

UHPH emulsions generally presented significantly lower amounts of hydroperoxides after 10 days of storage, in comparison to those prepared by CM and CH treatments. However, high TBARS content, similar or close to that corresponding to CM emulsions, was observed in some UHPH emulsions.

Table 23. Mean ± SD of hydroperoxides (A_{510} nm) and TBA reactive substances (µg/ml) of O/W emulsions containing sunflower and olive oils (10, 20 and 30%) and prepared by colloidal mill (CM), ultra-high pressure homogenization at 200 and 300 MPa with 5% of sodium caseinate, and conventional homogenization (15 MPa) with 1 and 5% of sodium caseinate.

Pressure (MPa)	Oil content (%)	Hydroperoxides (A_{510} nm)			TBARS (µg/ml)		
		Day 1	Day 10	Diference (Day 10 – Day 1)	Day 1	Day 10	Diference (Day 10 – Day 1)
CM	10	0.039 ± 0.024^{cd}	0.187 ± 0.018^{a}	0.148 ± 0.031^{a*}	0.217 ± 0.054^{a}	0.239 ± 0.055^{a}	0.022 ± 0.017^{cd}
	20	0.068 ± 0.049^{ab}	0.135 ± 0.067^{b}	0.067 ± 0.020^{bcd*}	0.107 ± 0.008^{e}	0.146 ± 0.008^{cd}	0.039 ± 0.014^{bc*}
	30	0.048 ± 0.021^{bc}	0.140 ± 0.027^{b}	0.092 ± 0.047^{abc*}	0.158 ± 0.063^{cd}	0.213 ± 0.032^{a}	0.055 ± 0.038^{ab*}
15.1	10	0.038 ± 0.030^{cd}	0.080 ± 0.040^{d}	0.043 ± 0.010^{cde*}	0.097 ± 0.028^{ef}	0.099 ± 0.029^{ef}	0.002 ± 0.004^{d}
	20	0.055 ± 0.009^{bc}	0.043 ± 0.002^{ef}	–0.012 ± 0.009^{ef*}	0.089 ± 0.015^{ef}	0.110 ± 0.013^{e}	0.022 ± 0.009^{cd*}
	30	0.072 ± 0.030^{ab}	0.062 ± 0.023^{de}	–0.010 ± 0.007^{ef}	0.141 ± 0.075^{def}	0.204 ± 0.018^{a}	0.063 ± 0.044^{ab*}
15.5	10	0.026 ± 0.003^{cd}	0.043 ± 0.015^{ef}	0.018 ± 0.017^{de*}	0.172 ± 0.029^{bc}	0.149 ± 0.035^{cd}	–0.022 ± 0.020^{d}
	20	0.032 ± 0.009^{cd}	0.114 ± 0.012^{c}	0.082 ± 0.003^{bcd*}	0.065 ± 0.011^{g}	0.077 ± 0.017^{g}	0.013 ± 0.008^{cd}
	30	0.096 ± 0.043^{a}	0.075 ± 0.030^{d}	–0.027 ± 0.024^{fg*}	0.154 ± 0.012^{cd}	0.171 ± 0.044^{bc}	0.017 ± 0.039^{cd}
200	10	0.053 ± 0.041^{bc}	0.020 ± 0.005^{g}	–0.032 ± 0.036^{fg*}	0.139 ± 0.009^{de}	0.183 ± 0.021^{b}	0.044 ± 0.023^{bc*}
	20	0.045 ± 0.034^{bc}	0.029 ± 0.006^{fg}	–0.016 ± 0.028^{ef}	0.107 ± 0.020^{e}	0.091 ± 0.004^{ef}	–0.015 ± 0.019^{d}
	30	0.057 ± 0.018^{bc}	0.026 ± 0.018^{g}	–0.032 ± 0.003^{fg*}	0.187 ± 0.008^{b}	0.200 ± 0.011^{ab}	0.013 ± 0.016^{cd}
300	10	0.049 ± 0.009^{bc}	0.018 ± 0.008^{g}	–0.030 ± 0.005^{fg*}	0.148 ± 0.041^{de}	0.239 ± 0.014^{a}	0.091 ± 0.052^{a*}
	20	0.005 ± 0.000^{e}	0.004 ± 0.001^{h}	–0.001 ± 0.000^{de}	0.108 ± 0.013^{e}	0.114 ± 0.011^{e}	0.005 ± 0.013^{d}
	30	0.026 ± 0.006^{cd}	0.023 ± 0.008^{g}	–0.003 ± 0.010^{de}	0.132 ± 0.022^{de}	0.155 ± 0.019^{cd}	0.023 ± 0.023^{cd}

$^{a-h}$ Different letters in the same column indicate significant differences *(P < 0.05)* between treatments.* Sign indicates that the differences between day 10 and day 1 (oxidation evolution) is significant *(P < 0.05).*

UHPH emulsions containing 10% oil, at all pressures tested, showed lower oxidative stability than that of their counterparts containing 20 and 30% oil. However, a decrease in the hydroperoxides was observed generally in all UHPH emulsions, significant TBARS evolution was only observed in emulsions containing 10% oil. The decrease in the hydroperoxides accompanied by the high amounts of TBARS observed in emulsions containing 10% oil may indicate a higher oxidation rate in these emulsions which would be attributed to the creaming observed, due to the flocculation or coalescence, and the decrease observed in the emulsifying activity (EAI), indicating low protection against oxidation in these emulsions as a result of the low protein coverage. The best oxidative stability in UHPH emulsions could be observed in those containing 20% oil, in which significantly lower TBARS were observed after 10 days of storage.

The lower oxidation levels in emulsions containing high oil contents may be due to the high viscosity of these emulsions. It has been proposed that viscosity can affect oxidation by reducing the diffusion of potential pro-oxidative molecules, such as ferrous ions or lipid hydroperoxides. Several research groups (Ponginebbi, Nawar, Chinachoti, 1999; Sims, 1994; Imagi et al. 1992; Hsieh, & Harris, 1987) have supported the view that elevated viscosities of the continuous aqueous phases of emulsions containing dissolved polyols inhibit oxygen diffusion and thereby cause a suppression of the oxidation of disperse phase lipids.

7.3. References

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Chapter 8

Final conclusions

1. The application of ultra high-pressure homogenization (UHPH) technology in emulsions containing whey protein isolate (WPI) and 20% oil (15% sunflower + 5% olive oils), leads to an improvement in droplet stability against creaming and coalescence when pressures between 100 and 200 MPa are applied, compared to emulsions produced by colloidal mill (CM, 5000 rpm for 5 min) and conventional homogenization (CH, 15 MPa); however, applying pressure of more than 200 MPa results in higher particle sizes and less stable emulsions. The best droplet breakdown, monomodal distribution and higher viscosities are achieved in UHPH-treated emulsions at 200 MPa when a high protein concentration (4%) is used. On the other hand, in CH emulsions the best droplet breakdown, high surface coverage and high stability against creaming are also observed when a high protein concentration (4%) is used.

2. The oxidative stability of emulsions containing WPI and 20% oil is improved using the UHPH technology in comparison to CM and CH emulsions, despite the high specific surface area (SSA) observed in UHPH emulsions showing that interfacial area is not the only determining factor of lipid oxidation. Increasing the pressure to moderate levels, especially at 100 MPa, increases the protein adsorption and protects the oil particles against oxidation, but further increase in the pressure to 200 MPa or more may make the proteins totally denatured and so unable to play their role.

3. The results of the present study reveal the potential of the UHPH technology for the production of submicron emulsions containing variable amounts of oil (10, 30 and 50%) and WPI. These emulsions show high physical and oxidative stability when pressures between 100 and 200 MPa are applied, compared to CM and CH emulsions. The oil concentration significantly decreases the particle size and surface coverage, and increases

the emulsifying activity index (EAI) in CM emulsions as the oil content increases, maintaining a Newtonian flow behavior. In general, an increase in the particle size and in the emulsifying activity of proteins are observed in CH and UHPH emulsions as the oil content increases to 30%, whereas an additional increase in the oil content to 50% in UHPH emulsions, affects the emulsifying activity of whey proteins in a bad way.

4. The increase of the oil fraction in CH and UHPH emulsions (50% oil in CH and 30-50% oil in UHPH emulsions) provokes a change in the rheological behaviour from Newtonian to shear thinning (with thixotropy) flow, these emulsions showing a great creaming stability. High oxidative stability is achieved in CH and UHPH emulsions, compared to CM emulsions, especially when the emulsion containing 30% oil is treated by UHPH at 100 MPa.

5. UHPH technology can produce emulsions containing 20% oil (15% sunflower + 5% olive oils) and sodium caseinate (SC) in the submicron/nano range of particle size, that are stable to physical destabilization mechanisms and oxidation in comparison to CM and CH emulsions, despite the high SSA observed in UHPH emulsions. The high physical and oxidative stabilities are achieved in these emulsions when pressures between 200 and 300 MPa are applied; however, applying pressure less than 200 MPa results in higher particle sizes and less stable emulsions.

6. In the case of UHPH emulsions containing 20% oil and SC, the addition of small amounts of SC (1%) results in emulsions with a bimodal distribution that are less stable to creaming and oxidation, possibly due to the limited protein which is unable to cover the new created interface. However, the best droplet breakdown, monomodal distribution and high oxidative stability are achieved in UHPH emulsions treated at 200 and 300 MPa when a high protein concentration (5%) is used. On the contrary, a low concentration of SC (1%) promotes the creaming and oxidative stability of the CH emulsions, however, higher protein amounts (5%), in general, increases the depletion flocculation and in turn, the creaming rate in these emulsions and decreases the oxidative stability.

7. The results of the study on the effect of different oil contents (10, 20 and 30%) in emulsions containing 5% of SC, reveals that the oil concentration has no effect on the particle size in CH and UHPH emulsions, but does have an effect in CM emulsions. CM and CH emulsions containing 1% SC and different oil contents (10, 20 and 30%), exhibit a Newtonian flow behavior with a slow creaming rate, whereas, a high degree of flocculation with a shear thinning behavior and higher creaming rates are observed in CH emulsions containing 5% of SC. UHPH application has a significant effect on the emulsion viscosity, showing shear thinning behavior only when high oil content (30%) is used. No thixotropic behavior or hysteresis loops are observed in emulsions containing SC.

8. The conventional homogenization treatment produces emulsions containing different oil contents (10, 20 and 30%) with higher EAI than emulsions treated by CM or UHPH, especially when low SC concentrations (1%) are used. The increase of the oil concentration, in general, increases the EAI and physical stability to creaming of emulsions.

9. UHPH technology produces emulsions, containing 5% SC with different oil contents (10, 20 and 30%), that are stable to oxidation, in comparison to CM emulsions. CH emulsions containing 5% SC have less oxidation products than those prepared with 1% SC, suggesting that high protein amounts in the continuous phase is required to reduce the lipid oxidation in emulsions.

10. The submicron emulsions obtained by UHPH, and containing 4% WPI or 5% SC with oil concentrations in the range of 20-30%, present high physical and oxidative stabilities suggesting potential applications for protecting food products containing bioactive lipophilic molecules such as polyunsaturated fatty acids (e.g. omega-3 fatty acids or conjugated linoleic acid), in their formulation.