Universitat Autònoma de Barcelona

STABILITY ASSESSMENT OF EMULSIONS TREATED BY ULTRA-HIGH PRESSURE HOMOGENIZATION AND THEIR INCORPORATION IN A UHT MILK-BASED PRODUCT FOR DELIVERY OF CONJUGATED LINOLEIC ACID

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Dr. ANTONIO JOSÉ TRUJILLO MESA, professor titular del Departament de Ciència Animal i dels Aliments de la Universitat Autònoma de Barcelona (UAB),

FA CONSTAR que CRISTINA FERNANDEZ AVILA ha realitzat, sota la seva direcció, en el àrea de Tecnologia dels Aliments de la Universitat Autonòma de Barcelona (UAB), el treball titulat **"Stability assessment of emulsions treated by Ultra-High Pressure Homogenization and their incorporation in a UHT milk-based product for delivery of conjugated linoleic acid"** que presenta per optar al grau de Doctor en Ciència dels Aliments.

I perquè així consti, signa el present document a:

Bellaterra, Cerdanyola del Vallès, 15 de Juliol del 2016.

Dr. Antonio José Trujillo Mesa

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"*Truth is always like oil-in-water, no matter how much water you add to depress it, it always floats on top*" **Anonymous**

"For most of history, Anonymous was a woman" **Virginia Woolf**

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ABSTRACT

Research on delivery of bioactive compounds by emulsion-based systems has increased over the last 10 years. Conventional oil-in-water emulsions (>1 μm) have poor physicochemical and oxidative stability; therefore, many efforts on novel technologies development have been employed to overcome this problem. Ultra-High Pressure Homogenization (UHPH) technology has been applied in different food emulsions in order to improve their physical stability. For these reasons, development of UHPH oil-in-water emulsions containing lipophilic bioactives, such as conjugated linoleic acid (CLA), to improve their physicochemical and nutritional characteristics and shelf-life is needed.

In this thesis dissertation, the influence of different technologies (UHPH and Conventional Homogenization; CH) and ingredients (soy protein isolate, SPI; pea protein isolate, PPI; soybean oil and CLA) in oil-in-water emulsions was evaluated. In addition, a final UHT milk-based product containing CLA-emulsions was developed.

This research has been divided into four parts. The first part studied the effect of UHPH (100-300 MPa, T_i=20 °C) compared to CH (15 MPa, T_i=20 °C) on physical and oxidative stability of soybean oil (10 and 20%) emulsions stabilized by SPI was assessed in order to determine the most stable formulations. Also, complete denaturation of SPI (95 °C, 15 min) before CH treatment was performed with the aim of analyzing possible improvement of emulsion characteristics. Particularly, UHPH emulsions treated at 100 and 200 MPa with 20% of oil were the most physically and oxidatively stable due to low particle size values, higher amount of oil, and greater viscosity and protein surface load at the interface. The heating of SPI did not improve physical stability of emulsions but enhanced oxidative stability when 10% oil was used.

The second study consisted in the assessment of different emulsions (20% oil) stabilized by SPI and PPI and homogenized by CH (55 MPa, T_i=20 °C) for 1 and 5 passes on the efficiency of 6% CLA encapsulation, its release and bioefficacy using *in vitro* digestion and a Caco-2 intestinal cell absorption model. All emulsions protected the encapsulated CLA better than the non-emulsified control in which CLA was oxidized during storage, as well as after *in vitro* digestion and delivery in Caco-2 cells. Similar percentages of CLA bioavailability were found for all emulsion treatments.

To evaluate the effect of UHPH (200 MPa, T_i=20 °C) in CLA-emulsions in comparison to CH (15 MPa, T_i=20 °C), a third study was performed. For another batch of CH emulsions, a heat treatment was also conducted applying a pasteurization treatment. UHPH produced a sterile CLA-emulsion and showed better physical stability during storage compared to other treatments, maintaining an optimal oxidative stability. Again, similar percentages of CLA bioavailability in Caco-2 cells were found for all emulsion treatments.

In the last study, the incorporation of CLA-emulsions obtained by UHPH (200 MPa, T_i=20 °C) and CH (15 MPa, T_i=20 °C) in a UHT milk-based product was evaluated. The final product containing the UHPH emulsions showed enhanced physicochemical stability and sensory properties during 4 months of storage.

RESUM

En els últims 10 anys s'ha incrementat la recerca en emulsions per a l'alliberament de compostos bioactius. Les emulsions convencionals d'oli-en-aigua (> 1 µm) presenten una estabilitat fisicoquímica i oxidativa insuficient; per tant, i per superar aquest problema, s'han dut a terme molts esforços en el desenvolupament de noves tecnologies. La tecnologia d'Homogeneïtzació a Ultra-Alta Pressió (UHPH) s'ha aplicat en diferents emulsions alimentàries per tal de millorar la seva estabilitat física. Per això, és necessari el desenvolupament d'emulsions UHPH d'oli-en-aigua que continguin bioactius lipòfils, com l'àcid linoleic conjugat (CLA), per tal de millorar les característiques nutricionals i fisicoquímiques de les emulsions, així com la seva vida útil.

En aquesta tesi doctoral, es va avaluar la influència de diferents tecnologies (UHPH i homogeneïtzació convencional; CH) i ingredients (aïllat proteic de soja, SPI; aïllat proteic de pèsol, PPI; oli de soja i CLA) en emulsions d'oli-en-aigua. A més, es va desenvolupar un producte final de base làctia UHT incorporant emulsions amb CLA.

La present recerca es va dividir en quatre parts. En un primer estudi, es va avaluar l'efecte de la UHPH (100-300 MPa, T_i=20 °C) en comparació de la CH (15 MPa, T_i=20 °C) sobre l'estabilitat física i oxidativa d'emulsions d'oli de soja (10 i 20%) i estabilitzades amb SPI per tal de determinar les formulacions més estables. A més, la desnaturalització completa de l'SPI (95 °C, 15 min) es va realitzar abans del tractament CH amb l'objectiu d'analitzar la possibilitat d'obtenir característiques millorades de les emulsions. En concret, les emulsions UHPH tractades a 100 i 200 MPa amb un 20% d'oli van ser les més estables a nivell físic i oxidatiu a causa de la disminució de la mida de partícula, la major quantitat d'oli, i una major viscositat i quantitat de proteïna a la interfase oli-en aigua. El tractament tèrmic de l'SPI no va millorar l'estabilitat física de les emulsions, però sí l'estabilitat oxidativa quan es va utilitzar un 10% d'oli.

El segon estudi consistí en la producció de diferents emulsions (20% d'oli) estabilitzades amb SPI i PPI i emprant 1 i 5 etapes de CH (55 MPa, T_i=20 °C) per avaluar l'eficiència d'encapsulació de CLA (6%), així com el seu alliberament i bioeficàcia utilitzant la digestió *in vitro* i un model cel·lular Caco-2 d'absorció intestinal. Totes les emulsions van protegir millor el CLA encapsulat a diferència del control (CLA en forma lliure), el qual es va oxidar durant l'emmagatzematge, així com després de la digestió *in vitro* i consegüent alliberament en les cèl·lules Caco-2. Es van trobar percentatges similars de biodisponibilitat del CLA en totes les emulsions.

Un tercer estudi va avaluar l'efecte de la UHPH (200 MPa, T_i=20 °C) en les emulsions amb CLA en comparació a la CH (15 MPa, T_i=20 °C). Per a un altre lot de emulsions CH, també es va dur a terme un tractament tèrmic de pasteurització. La UHPH va produir una emulsió estèril i durant l'emmagatzematge, va mostrar millor estabilitat física en comparació amb els altres tractaments, i a més, va mantenir una òptima estabilitat oxidativa. Un cop més, es varen trobar percentatges similars de biodisponibilitat del CLA en totes les emulsions.

En un l'últim estudi, es va avaluar la incorporació d'emulsions amb CLA tractades per UHPH (200 MPa, T_i=20 °C) i CH (15 MPa, T_i=20 °C) en un producte de base làctia UHT. El producte final que contenia les emulsions UHPH va mostrar una estabilitat fisicoquímica i propietats sensorials millorades durant 4 mesos d'emmagatzematge.

RESUMEN

En los últimos 10 años se ha incrementado la investigación en emulsiones para la liberación de compuestos bioactivos. Las emulsiones convencionales de aceite-en-agua (> 1 µm) presentan una estabilidad fisicoquímica y oxidativa insuficiente; por lo tanto, y para superar este problema, se han llevado a cabo muchos esfuerzos en el desarrollo de nuevas tecnologías. La tecnología de Homogeneización a Ultra-Alta Presión (UHPH) se ha aplicado en diferentes emulsiones alimentarias con el fin de mejorar su estabilidad física. Por estas razones, es necesario el desarrollo de emulsiones UHPH de aceite-en-agua que contengan bioactivos lipófilos, como el ácido linoleico conjugado (CLA), con el fin de mejorar las características nutricionales y físico-químicas de las emulsiones, así como su vida útil.

En esta tesis doctoral, se estudió la influencia de diferentes tecnologías (UHPH y homogeneización convencional; CH) e ingredientes (aislado proteico de soja, SPI; aislado proteico de guisante, PPI; aceite de soja y CLA) en emulsiones de aceite-en-agua. Además, se desarrolló un producto final de base láctea UHT conteniendo emulsiones con CLA.

La presente investigación se dividió en cuatro partes. En un primer estudio, se evaluó el efecto de la UHPH (100-300 MPa, T_i=20 °C) en comparación a la CH (15 MPa, T_i=20 °C) sobre la estabilidad física y oxidativa de emulsiones de aceite de soja (10 y 20%) y estabilizadas con SPI, con el fin de determinar las formulaciones más estables. Además, se desnaturalizó el SPI (95 ° C, 15 min) antes del tratamiento CH con el objetivo de analizar la posibilidad de obtener características de las emulsiones mejoradas. En concreto, las emulsiones UHPH tratadas a 100 y 200 MPa con un 20% de aceite fueron las más estables a nivel físico y oxidativo debido a la disminución del tamaño de partícula, la mayor cantidad de aceite, y una mayor viscosidad y cantidad de proteína en la interfase aceite-en-agua. El tratamiento térmico del SPI no mejoró la estabilidad física de las emulsiones, pero sí la estabilidad oxidativa cuando se utilizó un 10% de aceite.

El segundo estudio consistió en la producción de diferentes emulsiones (20% de aceite) estabilizadas con SPI y PPI, y se emplearon 1 y 5 pases de CH (55 MPa, T_i=20 °C) para determinar la eficiencia de encapsulación de CLA (6%), su liberación y bioeficacia utilizando la digestión *in vitro* y un modelo celular Caco-2 de absorción intestinal. Todas las emulsiones protegieron mejor el CLA encapsulado a diferencia del control (CLA en forma libre), el cual se oxidó durante el almacenamiento, así como después de la digestión *in vitro* y consiguiente liberación en las células Caco-2. Se encontraron porcentajes similares de biodisponibilidad del CLA en todas las emulsiones.

Para evaluar el efecto de la UHPH (200 MPa, T_i=20 °C) en las emulsiones con CLA en comparación a la CH (15 MPa, T_i=20 °C), se realizó un tercer estudio. Para otro lote de emulsiones CH, también se llevó a cabo un tratamiento térmico de pasteurización. La UHPH produjo una emulsión estéril y durante el almacenamiento, mostró mejor estabilidad física en comparación a los otros tratamientos, y además, mantuvo una óptima estabilidad oxidativa. Una vez más, se encontraron porcentajes similares de biodisponibilidad del CLA en todas las emulsiones.

En un el último estudio, se evaluó la incorporación de emulsiones con CLA tratadas por UHPH (200 MPa, T_i=20 °C) y CH (15 MPa, T_i=20 °C) en un producto de base láctea UHT. El producto final que contenía las emulsiones UHPH mostró una mayor estabilidad físico-química y propiedades sensoriales mejoradas durante 4 meses de almacenamiento.

Publications and presentations related to this thesis

Publications

Title: Ultra-high pressure homogenization enhances physicochemical properties of soy protein isolate-stabilized emulsions.
Authors: Fernández-Ávila, C., Escriu, R., & Trujillo, A. J.
Reference: *Food Research International* (2015), *75*, 357-366.

Title: Vegetable protein isolate-stabilized emulsions for enhanced delivery of conjugated linoleic acid in Caco-2 cells.
Authors: Fernandez-Avila, C., Arranz, E., Guri, A., Trujillo, A. J., & Corredig, M.
Reference: *Food Hydrocolloids* (2016), *55*, 144-154.

Title: Ultra-High Pressure Homogenization improves oxidative stability and interfacial properties of soy protein isolate-stabilized emulsions.
Authors: Fernandez-Avila, C., & Trujillo, A. J.
Reference: *Food Chemistry* (2016), *209*, 104-113.

Title: Enhanced stability of emulsions treated by Ultra-High Pressure Homogenization for delivering conjugated linoleic acid in Caco-2 cells.
Authors: Fernandez-Avila, C., & Trujillo, A. J.
Reference: *Food Hydrocolloids* (2016), submitted.

Title: Enhancement of physicochemical and sensory characteristics of a UHT milk-based product enriched with conjugated linoleic acid emulsified by Ultra-High Pressure Homogenization.
Authors: Fernandez-Avila, C., Gutierrez-Merida C., & Trujillo, A. J.
Reference: *Food Research International* (2016), submitted.

Scientific communications

Title**:** Efecto de la homogeneización a ultra alta presión (UHPH) en la estabilidad física de emulsiones submicrón de aceite de soja en agua.
Authors**:** Fernández-Ávila, C., Sánchez, J., Juan, B., Trujillo, A.J.
Meeting**:** Cibia9 - Congreso Iberoamericano de Ingeniería de Alimentos.
Place and date**:** Valencia (España), 13th – 16th January, 2013.
Type of communication**:** Oral communication.

Title: Effect of ultra-high-pressure homogenization on the oxidative stability and microstructure of submicron soy o/w emulsions.
Authors: Fernández-Ávila, C., Sánchez, J., Ferragut, V., Trujillo, A.J.
Meeting: 3rd International ISEKI Food Conference.

Place and date: Athens (Greece), 21th – 23th May, 2014.
Type of communication: Poster presentation.

Title: Physical and oxidative characteristics of soy protein isolate oil-in-water emulsions stabilized by conventional and ultra-high pressure Homogenization.
Authors: Fernández-Ávila, C., Trujillo, A.J.
Meeting: 28th EFFoST Conference and 7th International Conference on the Food Factory for the Future.
Place and date: Uppsala (Sweden), 26th – 29th November, 2014.
Type of communication: Oral communication.

Title: Effect of ultra-high pressure and conventional homogenization on the rheology and the adsorption of soy protein isolate in stabilized emulsions.
Authors: Fernández-Ávila, C., Trujillo, A.J.
Meeting: 28th EFFoST Conference and 7th International Conference on the Food Factory for the Future.
Place and date: Uppsala (Sweden), 26th – 29th November, 2014.
Type of communication: Poster presentation.

Title**:** Soy and pea protein isolate-stabilized emulsions for enhanced delivery of conjugated linoleic acid in Caco-2 cells.
Authors**:** Fernández-Ávila, C., Arranz, E., Guri, E., Trujillo, A.J., Corredig, M.
Meeting**:** 6th International Symposium on Delivery of Functionality in Complex Food Systems: Physically-Inspired Approaches from the Nanoscale to the Microscale.
Place and date**:** Paris (France), 14th – 17th July, 2015.
Type of communication**:** Oral communication.

Title**:** Ultra-High Pressure Homogenization enhances physical, microbiological and oxidative stability of emulsions containing conjugated linoleic acid.
Authors**:** Fernández-Ávila, C., Trujillo, A.J.
Meeting**:** 2nd UK Hydrocolloids Symposium.
Place and date**:** Birmingham (UK), 10th September, 2015.
Type of communication**:** Oral communication.

Table of contents

Abbreviation key

BS	backscattering
CH	conventional homogenization
CH-M	mixing of the emulsions treated by CH and the skim milk before UHT
CH-P	UHT milk-based product elaborated with emulsion treated by CH
CLA	conjugated linoleic acid
CLSM	confocal laser scanning microscopy
$d_{3.2}$	the surface-weighted mean diameter
$d_{4.3}$	the volume-weighted mean diameter
d_{50}	diameter for which 50% of the oil droplets are smaller than “d”
EAI	emulsifying activity index
FAA	free amino acids
FAME	fatty acid methyl esters
FFA	free fatty acids
GC-FID	gas chromatography-flame ionization detector
GC/MS	gas chromatography-mass spectrometry
GLM	general linear model
HTST	high-temperature-short-time
K	consistency index
MPa	megapascals
mPa	millipascals
n	flow behaviour index
O/W	oil-in-water
p	pressure

Pv	peroxide value
PPI	pea protein isolate
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIM	selected-ion monitoring
Span	dispersion index
SSA	specific surface area
SPC	surface protein concentration
SPI	soy protein isolate
T_i	the inlet temperature of the fluid
T_1	the temperature of the fluid after the high pressure valve
T_f	outlet temperature of the fluid
T	transmission
TEM	transmission electron microscope
TBARs	thiobarbituric acid-reactive substances
TSI	turbiscan stability index
UHT	ultra-high temperature
UHPH	ultra-high pressure homogenization
UHPH-M	mixing of the emulsions treated by UHPH and the skim milk before UHT
UHPH-P	UHT milk-based product elaborated with emulsion treated by UHPH
11S	glycinin
7S	β-conglycinin
ΔP	adsorbed protein percentage

Table Legends

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Chapter 7

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3. Sulforhodamine B assay (SRB) for cytotoxicity screening. A >80% cell viability is considered for further analysis of Caco-2 cells uptake at corresponding dilution and incubation time. Results are shown for both productions analysed.

Figure Legends

Chapter 1

1. Emulsions and final milk-based product procedure. The formulation ingredients of emulsions were as follows: 4% soy protein isolate (SPI) and 20% soybean oil (Experiments 1-2), 4% soy protein isolate (SPI) and pea protein isolate (PPI) and 14% soybean oil and 6% CLA (Experiment 3) and 4% SPI and 14% soybean oil and 6% CLA (Experiments 4-5).

2. Experimental analysis of Experiments 1 and 2.

3. Experimental analysis of Experiments 3 and 4.

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Chapter 2

1. Transmission Electron Microscopy (TEM) image (×25.000; the bar represent 0.5 μm) of emulsion treated by Ultra-High Pressure Homogenization at 200 MPa from this thesis dissertation (A). The soybean protein isolate concentration in continuous phase was 4%, and the volume soybean oil fraction 20% (A). A graphical scheme of the types of emulsion are also represented (B).

2. Overview of the main interrelationships between the formulation parameters of O/W emulsions and the associated emulsion physicochemical characteristics. Arrows connect the emulsion characteristics to their influencing formulation parameters. Orange "?'' tags point out emulsion parameters for which contradictory results have been obtained with respect to their effect on lipid oxidation, and that should be further investigated. Green ''✓'' tags point out emulsion properties that have been shown to affect lipid oxidation in a consistent and reproducible manner.

3. Surfactant classification according to the composition of their head: a) anionic (negatively charged); cationic (positively charged); and zwitterionic (two oppositely charged groups) (A). The structure of the interfacial membrane (B) depends on the molecular structure and interactions of the surface-active molecules: low-mass surfactants (d), flexible biopolymer (e) and globular biopolymer (f).

4. Hazard analysis and critical control points (HACCP) of the isolate process extraction of Pro-Fam 974 from soybean flakes (Lactotecnia, S.L.). CCP 1 is a critical control point in the procedure.

5. Overview of the main interrelationships between the interfacial characteristics of O/W emulsions: arrows connect the emulsion characteristics to their influencing interfacial characteristics (A). The conformational changes of protein at the interfacial layer depend on the ratio between the adsorption rate and the unfolding rate (B).

6. Emulsion instability pathways. Most emulsions are metastable and begin with (a) a homogeneous dispersion of oil droplets in water. The main instability processes are (b) flocculation, (c) coalescence, and (d) creaming (or sedimentation). Finally, the 2 phases may completely separate (e) and the system breaks into its component parts ("oiling off"). The arrows denote possible routes and indicate where reversibility is potentially achievable through gentle redispersion.

7. Ultra-High Pressure Homogenization system used in the present PhD thesis developed by Ypsicon SL, Barcelona, Spain.
8. Graphic scheme representing the complete UHPH system patented by UAB.
9. Homogenizing valves geometries from: Manton-Gaulin APV (a) and Stansted Fluid Power (b).
10. Scheme of the physical principles of Turbiscan^{TM} MA 2000.
11. Number of research articles and patens with the keywods "emulsion" and "functional food" published from 2005 to 2015 (Web of Science, June 2016).
12. Comparison of the structures of (A) linoleic acid (*cis*-9, *cis*-12 18:2), (B) *cis*-9, *trans*-11 conjugated linoleic acid or rumenic acid, and (C) *trans*-11 18:1 conjugated linoleic acid or vaccenic acid. Double bonds are denoted by arrows.

Chapter 3

1. Log $d_{3,2}$ vs. pressure (P) for emulsions treated by Ultra-High Pressure Homogenization at a fixed non-heated SPI concentration (4 %, w/v) with 10 % (v/v) (··♦··) and 20 % (v/v) (-■-) of soybean oil.
2. Size distribution profiles of o/w emulsions treated by conventional homogenization (CH) and stabilized by untreated or preheated SPIs (A-D), and o/w emulsions treated by Ultra-High Pressure Homogenization (UHPH) and stabilized by untreated SPI under different homogenizing pressures (100-300 MPa) (E-H). A, C, E, G: diluted in water; B, D, F, H; diluted in 2 % SDS. The protein concentration in continuous phase was 4 % (w/v), and the volume oil fraction 10 % (v/v) for A-B, E-F and 20 % (v/v) for C-D, G-H.
3. TEM images (×5000 and ×25000) of emulsions treated by conventional homogenization at 15 MPa and stabilized by non-heated (A and C) and heated SPIs (B and D). The protein concentration in continuous phase was 4 % (w/v), and the volume oil fraction 10 % (v/v) for A-B, and 20 % (v/v) for C-D. (pa) Protein aggregates, (cpa) cluster of protein aggregates, (fga) fat globule aggregate. Bars represent 2 and 0.5 µm, respectively.
4. TEM images (×5000 and ×50000) of emulsions treated by Ultra-High Pressure Homogenization at 100 MPa (A-B), 200 MPa (C-D) and 300 MPa (E-F). The protein concentration in continuous phase was 4 % (w/v), and the volume oil fraction 10 % (v/v) for A, C and E, and 20 % (v/v) for B, D and F. Bars represent 2 µm and 0.2 µm, respectively.
5. Flow behaviours (21 ºC) of emulsions treated by conventional homogenization stabilized by non-heated (15 MPa) and heated SPIs (15 MPa - D) and emulsions treated by Ultra-High Pressure Homogenization and stabilized by untreated SPI under different homogenizing pressures (100-300 MPa). The protein concentration in continuous phase was 4 % (w/v), and the volume oil fraction 20 % (v/v).
6. Variation of backscattering expressed as ΔBS (%) of emulsions stabilized by conventional homogenization (15 MPa) with 4 % (w/v) of non-heated SPI and heated SPI (15 MPa - D), and Ultra-High Pressure Homogenization at 100, 200 and 300 MPa during storage at the bottom (0-10 mm) for C-D and the top of the tube (35-40 mm) for A-B. The volume oil fraction was 10 % (v/v) for A and C, and 20 % (v/v) for B and D.
7. Variation of the particle mean diameter expressed as d (µm) in emulsions stabilized by conventional homogenization (15 MPa) with 4 % (w/v) of non-heated SPI and heated SPI (15 MPa - D) at the zone 15-30 mm of the tube. The volume oil fraction was 10 % (v/v).

Chapter 4

1. Scatterplot matrix for correlations between specific surface area (SSA, m^2/mL), emulsifying activity index (EAI, m^2/g) and surface protein concentration (SPC, mg/m^2) of emulsions treated by conventional homogenization stabilized by non-heated (15 MPa) and heated SPIs (15 MPa - d) and emulsions treated by Ultra-High Pressure Homogenization and stabilized by untreated SPI under different homogenizing pressures (100-300 MPa). The protein concentration in continuous phase was 4% (w/v), and the volume oil fraction 10% (v/v) for A, and 20% (v/v) for B.

2. TEM images (×50.000 and ×120.000) of emulsions treated by conventional homogenization at 15 MPa and stabilized by non-heated (A, B) and heated SPIs (C, D). The protein concentration in continuous phase was 4% (w/v), and the volume oil fraction 10% (v/v) for A and C, and 20% (v/v) for B and D. (pa) protein aggregates, (cpa) cluster of protein aggregates, (fga) fat globule aggregate. Bars represents 200 nm.

3. TEM images (×50.000 and ×120.000) of emulsions treated by Ultra-High Pressure Homogenization at 100 MPa (A-B), 200 MPa (C-D) and 300 MPa (E-F). The protein concentration in continuous phase was 4% (w/v), and the volume oil fraction 10% (v/v) for A, C and E, and 20% (v/v) for B, D and F. (pti) protein tails at the interface, (pa) protein aggregates, (fga) fat globule aggregate. Bars represent 100 (b, d, f) and 200 nm, respectively.

4. Lipid hydroperoxides (mmol/kg oil) and TBARs (µmol/kg oil) of emulsions treated by conventional homogenization stabilized by non-heated (15 MPa) and heated SPIs (15 MPa - d) and emulsions treated by Ultra-High Pressure Homogenization and stabilized by untreated SPI under different homogenizing pressures (100-300 MPa). The protein concentration in continuous phase was 4% (w/v), and the volume oil fraction 10% (v/v) for A, and 20% (v/v) for B. Bars represent the standard error of the mean.

5. Graphical scheme for correlations between lipid oxidation and interfacial properties of the emulsions.

Chapter 5

1. Size distribution profiles of oil in water emulsions stabilized with soy protein isolate (A,B) and pea protein isolate (C,D) homogenized with one (grey) or five (black) passes of homogenization. Emulsions were measured fresh (A,C) or after digestion (B,D). Dotted lines are the same emulsions after treatment with 2% SDS. Samples are representative of two independent experiments.

2. SDS-PAGE profiles under reducing conditions of SPI emulsions (A) and PPI emulsions (B) containing CLA and homogenized at one or five passes (E), their serum phases (S) and cream phases (C). MW: protein markers.

3. Amount of c9t11-CLA (A) and t10c12-CLA (B) recovered in the fresh oil-in-water emulsions. Emulsions were stabilized with soy protein isolate (white bars) or pea protein isolate (black bars), and homogenized with one (solid bars) or five passes (dotted bars). Data are shown as average ± SD of two independent experiments.

4. Chemical stability of CLA in cream phases. CLA isomer profile (%) of cream phases of fresh emulsions (A) and the quantitation of each CLA isomer (B). Data are represented as the average ± SD of two separate experiments. Means with different letters indicate significant differences (P<0.05).

5. Chemical stability of CLA in emulsions. CLA isomer profile (%) of freshly emulsions (A) and emulsions stored at 4˚C during 10 days (B), and the quantitation of each CLA isomer of freshly

emulsions (C) and emulsions stored at 4˚C during 10 days (D). Data are represented as the mean ± SD.

6. Peroxide value of emulsions and the control (CLA and soybean oil; ○) stored at 37˚C during 20 days (D). Emulsions were stabilized with soybean protein isolate (■□) or pea protein isolate (▲△), homogenized for one (■▲) or five passes (□△). Data are represented as the mean ± SD. * Indicates significant differences between treatments (P<0.05) at 10 and 20 days.

7. Chemical stability of CLA in digestates of the fresh emulsions. CLA isomer profile (%) of emulsions after being digested (A) and the quantitation of each CLA isomer (B). Data are represented as the mean ± SD. * It indicates significant differences between treatments (P<0.05).

8. Bioaccessibility (A, measured in the cell) and bioavailability (B, as measured in the basolateral phase after transport) of the main CLA isomers (c9t11 and t10c12) in digestates after absorption and transport through Caco-2 monolayers. Emulsions were stabilized with soy protein isolate (white bars) or pea protein isolate (black bars), and homogenized with one (solid bars) or five passes (dotted bars). Grey bars show control experiments (oil – no emulsion). Data are represented as the average ± SD of two separate experiments. Means with different letters indicate significant differences (P<0.05).

Chapter 6

1. Size distribution profiles of CLA emulsions stabilized with Ultra-High Pressure Homogenization (grey), conventional homogenization (dotted black) and conventional homogenization plus high-temperature short-time pasteurization (dashed black). Emulsions were measured fresh (A) or after digestion (B). Samples are representative of two independent experiments.

2. Confocal micrographs of fresh CLA emulsions stabilized with conventional homogenization (CH - 15 MPa), conventional homogenization plus high-temperature short-time pasteurization (CH-HTST - 15 MPa) and Ultra-High Pressure Homogenization (UHPH). The maximum intensity projection of 3D z-stacks (B) and their red channels in a surface plot (A) were shown. Red colour represents the oil phase of emulsions (A) and green colour represents the proteins. Samples are representative of two independent experiments.

3. Mean ± SD values of rheological properties of CLA emulsions stabilized with conventional homogenization (CH - 15 MPa), conventional homogenization plus high-temperature short-time pasteurization (CH-HTST - 15 MPa) and Ultra-High Pressure Homogenization (UHPH). The flow behaviour (n), the consistency index (K) and the initial shear stress (τ_o) of all emulsions were determined. The Area under the curve of emulsions which experimented thixotropic behaviour was also analysed. CH fresh emulsions (grey bars) are compared with their last day of shelf life (dotted bars, CH – 7 days and CH-HTST – 15 days) (A,C). Sterile UHPH emulsions were also analysed at 15 days. Within treatments, different letters indicate statistical significant differences (P<0.05). * Indicate statistically significantly differences between fresh and aged emulsions calculated using Student's t-test.

4. Physical stability of CLA emulsions stored at 4˚C during 165 days measured by Turbiscan Stability Index (TSI). Emulsions were stabilized with conventional homogenization (■), conventional homogenization plus high- temperature short-time pasteurization (▲) and Ultra-High Pressure Homogenization (●). Data are represented as the mean ± SD of two separate experiments.

5. Lipid hydroperoxides (mmol/kg oil) TBARs (µmol/kg oil) of CLA emulsions stabilized with conventional homogenization (CH - 15 MPa), conventional homogenization plus high-temperature short-time pasteurization (CH-HTST - 15 MPa) and Ultra-High Pressure Homogenization (UHPH). CH fresh emulsions (grey bars) are compared with their last day of shelf life (dotted bars, CH – 7 days and CH-HTST – 15 days) (A,C). Sterile UHPH emulsions were

also analysed at 15 days and further stored at 4˚C during 165 days (B,D). Data are represented as the average ± SD of two separate experiments. Within treatments, different letters indicate statistical significant differences (P<0.05). * Indicate statistically significantly differences between fresh and aged emulsions calculated using Student's t-test.

6. Non absorbed (A, measured in the apical phase), bioaccessibility (B, measured in the cell) and bioavailability (C, as measured in the basolateral phase after transport) of the CLA isomers (c9t11, t10c12 and *trans,trans*-CLA) in digestates after absorption and transport through Caco-2 monolayers. CLA emulsions were homogenized with Ultra-High Pressure Homogenization (white bars), conventional homogenization (grey bars) and conventional homogenization plus high- temperature short-time pasteurization (black bars). Dotted bars show control experiments (oil – no emulsion). Data are represented as the average ± SD of two separate experiments.

Chapter 7

1. Size distribution profiles of emulsions (CH-E and UHPH-E; dotted line), the mixing of the emulsions and the skim milk before UHT treatment (CH-M and UHPH-M; grey line) and the final milk-based product (CH-P and UHPH-P; black line) samples treated by conventional homogenization at 25 MPa (A) and Ultra-High Pressure homogenization at 200 MPa (B).

2. Pressure (MPa) (A) and temperature (B) reached in the high-pressure valve (T1) in emulsions treated by Ultra-High Pressure Homogenization (UHPH) at 200 MPa. Measures of emulsions passing through the UHPH-valve are represented with curly brackets.

3. Evolution in backscattering (BS) and transmission (T) of UHT milk-based products containing CLA-emulsions treated by conventional (A) or Ultra-high Pressure (B) homogenization during storage time.

4. Turbiscan Stability Index (TSI) in the bottom (▲), middle (■) and top (●) of UHT milk-based products containing CLA-emulsions treated by conventional (solid lines) or Ultra-High Pressure (dotted lines) Homogenization during storage time.

5. Sensorial attributes evaluated in the milk-based products containing CLA emulsified by Ultra-High Pressure Homogenization (UHPH-P) or by conventional homogenization (CH-P): odour (A), appearance (B) and flavour (C). Results have been represented as the UHPH-P in comparison to CH-P (standard). Preference test (D) was undertaken since the initial storage time (M0) until 4 months (M4).

Chapter 8

1. CLA peaks identification of a pure CLA standard (chromatogram from Experiment 3) and their isomers quantified.

2. Turbiscan glass vials filled with the final UHT milk-based products from Experiment 5 (CH-P and UHPH-P) after 5 months of storage at 21 °C.

CHAPTER 1: Literature review

1. Food emulsions

1.1. Definition and classification

An emulsion consists of two immiscible liquids (usually oil and water), with one of the liquids dispersed as small spherical droplets in the other (Fig. 1A) (McClements, 2005). Among the structures and structure-forming units within foods, emulsions play a major part. Indeed, they are key ingredients in the formation of structures in certain products, such as whipped toppings and ice creams, and more complex products, such as processed cheeses. The formulation of a food structure involving emulsions is a complex process, as it depends on the desired qualities of the food and the function of the emulsion into the product. Therefore, the understanding of the formation, structures, and properties of emulsions is essential to the creation and stabilization of structures in foods (Dickinson, 2003). For the efficient formulation and production of emulsions, it is necessary to take into account the following factors:

- ✓ Physical functionality of the ingredients.
- ✓ Restrictions imposed by certain religious groups or by public perceptions of health issues.
- ✓ Ingredients regulation by appropriate agencies.
- ✓ Economic costs of the process.
- ✓ Microbiological safety and the possibly need of heat treatment which may affect the stability of the emulsion during processing.

Emulsions are classified according to the distribution of the oil and aqueous phases (Dickinson, 2003; McClements, 2005). The substance that makes up the droplets in an emulsion is referred to as the dispersed phase, whereas the substance that makes up the surrounding liquid is called the continuous phase. There are three main types of emulsion which are important in foods (Fig. 1B):

- ✓ O/W or **oil-in-water emulsion**: oil droplets are dispersed in an aqueous phase (e.g., mayonnaise, milk, cream, soups, and sauces).
- ✓ W/O or **water-in-oil emulsion**: water droplets are dispersed in an oil phase (e.g., margarine, butter, and spreads)

- ✓ **Multiple emulsions** of the oil-in-water-in-oil (O/W/O) or water-in-oil-in-water (W/O/W) type. For example, a W/O/W emulsion consists of water droplets dispersed within larger oil droplets, which are themselves dispersed in an aqueous continuous phase. They can be used to control the release of certain ingredients, reduce the total fat content or isolate one ingredient from another (Clegg, Tavacoli, & Wilde, 2016; Kim, Cho, & Park, 2016).

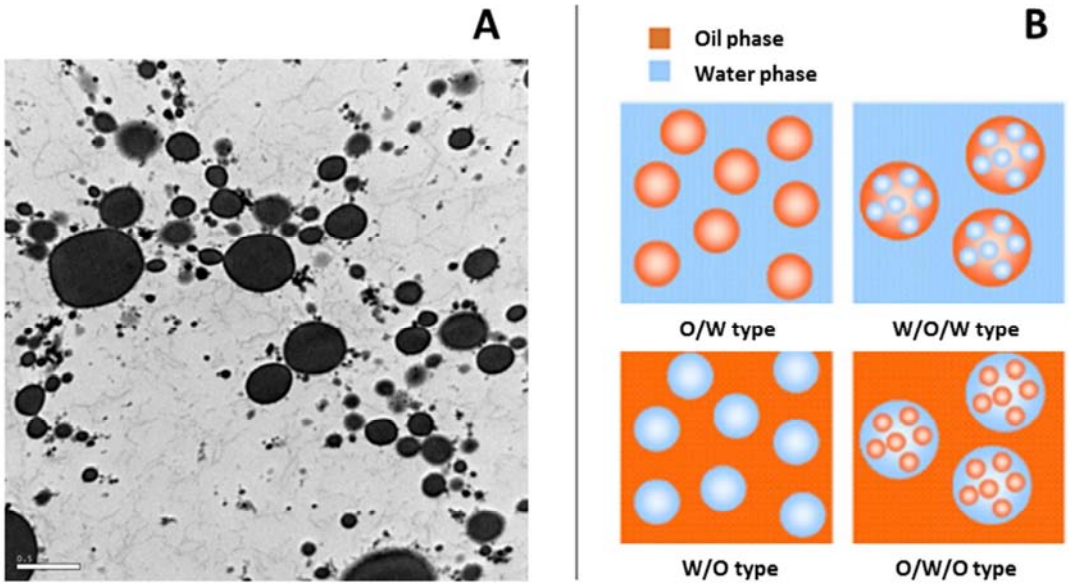

Figure 1. Transmission Electron Microscopy **(**TEM) image (×25.000; the bar represent 0.5 µm) of emulsion treated by Ultra-High Pressure Homogenization at 200 MPa from this thesis dissertation (A). The soybean protein isolate concentration in continuous phase was 4%, and the volume soybean oil fraction 20% (A). A graphical scheme of the types of emulsion are also represented (B).

In this introduction, concepts are focused on O/W emulsions as they have been studied through the following thesis dissertation. O/W emulsions fabricated from food-grade ingredients are commonly utilized in the food industry to encapsulate, protect, and deliver lipophilic functional components, such as biologically-active lipids (e.g., ω-3 fatty acids, conjugated linoleic acid) and oil-soluble flavors, vitamins, preservatives, and nutraceuticals.

1.2. Physically approaches from the microscale to the nanoscale

Many of the most important properties of emulsion-based food products (e.g., shelf life, physical and oxidative stability, appearance, texture, and flavor) are determined by the size of the droplets they contain (McClements, 2005). Consequently, it is important for food scientists to be able to reliably predict, control, measure, and report the droplet size in emulsions, from the nanoscale (nm) to the microscale (μm).

The droplets in **conventional emulsions** usually have diameters in the range of 100 nm to 100 μm, and are coated by a single layer of surface active components ("emulsifiers"; Fig 1A) that stabilize them against aggregation (McClements, 2012). Another definition is given for **submicron emulsions**, which are systems with diameter well below the micron (Cortés-Muñoz, Chevalier-Lucia, & Dumay, 2009). Then, the National Nanotechnology Initiative (NNI), a U.S. Government research and development initiative, defines nanotechnology as the "*science, engineering, and technology conducted at the nanoscale, which is about 1 to 100 nm*"[1]. Thus, production of **nanoemulsions** is achieved for particle size diameters below 100 nm. Establishing foundational knowledge at the nanoscale has been the main focus of the nanotechnology research community in the recent years, and it is already evolving toward becoming a general-purpose technology by 2020 (Roco, 2011). So far, the most relevant applications of nanotechnology to food products have been encapsulation of bioactive components to boost bioavailability, food safety, development of new products, stabilisation of bioactive ingredients, improvement of organoleptic characteristics, development of nanosensors and active/intelligent packaging and saving costs (Forum, 2009). Nanoemulsions have much better physical stability than conventional emulsions due to the smaller particle size (Wooster, Golding, & Sanguansri, 2008). However, there may also be some risks associated with the oral ingestion of nanoemulsions, such as their ability to change the biological fate of bioactive components within the gastrointestinal tract and the potential toxicity of some of the components used in their fabrication (McClements & Rao, 2011; Walker, Decker, & McClements, 2015).

[1] http://www.nano.gov/nanotech-101/what/definition

1.3. Dispersed oil-phase

Lipids (fats and oils) impact on the nutritional, organoleptic, and physicochemical properties of food emulsions in a variety of ways. Figure 2 shows the main interrelationships between the formulation parameters of O/W emulsions and the associated emulsion physicochemical characteristics (Berton-Carabin, Ropers, & Genot, 2014).

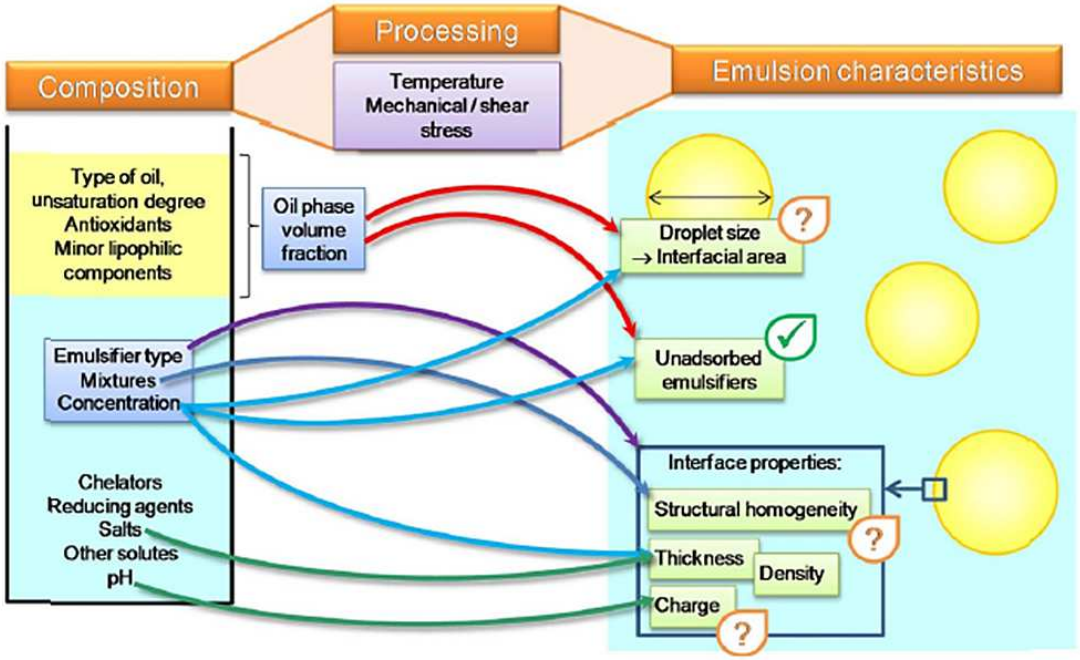

Figure 2. Overview of the main interrelationships between the formulation parameters of O/W emulsions and the associated emulsion physicochemical characteristics. Arrows connect the emulsion characteristics to their influencing formulation parameters. Orange "?'' tags point out emulsion parameters for which contradictory results have been obtained with respect to their effect on lipid oxidation, and that should be further investigated. Green ''✓'' tags point out emulsion properties that have been shown to affect lipid oxidation in a consistent and reproducible manner (Berton-Carabin et al., 2014).

Lipids are essential nutrients and a major source of energy in our diets; however, overconsumption of certain types (saturated fat, trans fatty acids) and underconsumption of polyunsaturated lipids has been link to various health concerns, such as obesity, cardiovascular disease, diabetes and cancer (Guasch-Ferré et al., 2015; Sala-Vila et al., 2016). Subsequently, in the past decade, there has been a trend in society and the food industry for consumption of low-fat food, which has turned into a decrease in the intake of lipophilic bioactives (Gao et al., 2014). However, one of the

most recent nutritional recommendations has been to increase amounts of polyunsaturated fats incorporated in foods (EFSA Panel on Dietetic Products, 2010). In this sense, the challenge to the food scientist is to create a product with similar characteristics than the original, enriched in polyunsaturated lipids and/or lacking lipophilic bioactives (McClements, 2005). The lipid phase can act as a solvent of these lipophilic bioactives, such as oil-soluble vitamins (A, D, E and K), antioxidants, preservatives and essential oils. As an example, dairy functional products are commonly fortified with oil-soluble vitamins and ω-3 fatty acids. In the recent literature, there has been growing interest in the development of natural antimicrobials, such as essential oils, to preserve foods. When essential oils are carried into nanoemulsions, their antibacterial activity is improved (Moghimi, Aliahmadi, McClements, & Rafati, 2016; Topuz et al., 2016).

All lipids are characterized by different physicochemical properties, which mainly depend on their fatty acid composition. These properties affect on the formation and stability of emulsion. In Table 1, a comparison of some bulk physical properties and fatty acid composition of liquid oils (soybean oil and Conjugated Linoleic Acid; CLA) used in this thesis dissertation and water at 20 °C is shown. For example, the contrast between the oil and water phase will have an important influence on long-term stability against creaming (see *Section 1.6*). Moreover, the ratio between the viscosity of the dispersed phase and the continuous phase will determine the minimum size of droplets that can be disrupted by the homogenizer (Floury, Legrand, & Desrumaux, 2004; Walstra, 1983; Walstra, Smulders, & Binks, 1998). However, the addition of an emulsifier such as proteins to the water phase increases its viscosity (Zayas, 2012), obtaining a favourable ratio between continuous and discontinuous phase for the droplets disruption.

Table 1. Comparison of some bulk physical properties and fatty acid composition of liquid oils (soybean oil and CLA) used in this thesis dissertation and water at 20°C.

Properties	Soybean oil	CLA (FFA form)[a]	Water
Density (kg·m^{-3})	922	890	998
Viscosity (mPa·s)	55	48	1.002
Fatty Acids (%)	Palmitic acid, C16:0 (7-13) Stearic acid, C18:0 (2-6) Oleic acid, C18:1 (17-35) Linoleic acid, C18:2 (45-60) Linolenic acid, C18:2 (4-11)	Palmitic acid, C16:0 (2.7) Stearic acid, C18:0 (2.3) Oleic acid, C18:1 (12.7) cis9,trans11-CLA (39.2) trans10,cis12-CLA (39)	NA[*]

[a] Free fatty acid (FFA) mixture high in isomers of CLA (Neobee®CLA80), Stepan Specialty Products LLC (Maywood, U.S.).
[*] NA= not applicable.

When the bulk physicochemical properties of many edible oils and fats are very similar, the choice of oil may not affect in a large extent the overall properties of an emulsion. Nevertheless, some oils do have different properties from the majority of other oils, such as CLA in a FFA form (Table 1), which may influence their functional characteristics as it is more prone to lipid oxidation compared to its triglyceride form. In the past, many researchers used model systems containing highly purified oils, with known chemical structures which are inert to oxidation, such as hydrocarbons, to establish the colloid basis of the emulsions (Kim, Decker, & McClements, 2002; Roozen, Frankel, & Kinsella, 1994). However, conclusions drawn from these studies should be carefully considered, as it is not a real food emulsion to be consumed.

The type and concentration of oil used will influence the droplet size of emulsions (Komaiko, Sastrosubroto, & McClements, 2015; Sun & Gunasekaran, 2009), as well as the homogenization technique (see *Section 1.7*). Both type/concentration of oil and particle size strongly impact on the rheology and flavour of food emulsions. Generally, viscosity increases when increasing droplet concentration (Sun & Gunasekaran, 2009). On the other hand, flavour is dependent on the lipid oxidation of the overall emulsion.

1.3.1. Lipid oxidation and additives

As stated in *Section 1.3*, health-promoting polyunsaturated lipids are currently added in the dispersed-oil phase of O/W emulsions (García-Moreno, Guadix, Guadix, & Jacobsen, 2016; Julio et al., 2015), although they are highly susceptible to lipid

oxidation. Lipid oxidation is a complex sequence of chemical reactions as a result of the interaction of lipids and oxygen active species (Genot, Meynier, Riaublanc, & Kamal-Eldin, 2003). It is commonly divided into three different stages: initiation, propagations and termination. The oxidation of lipids leads to the generation of volatile compounds that may cause undesirable rancid flavour and other "off-flavours", loss of polyunsaturated lipids and formation of potentially toxic products (McClements & Decker, 2000).

Therefore, manufacturers include additives to avoid lipid oxidation in food emulsions and enhance their stability, taste, texture and appearance. As an example, synthetic antioxidants such as BHT and BHA are highly effective at controlling lipid oxidation; however, nowadays consumers demand organic and natural/free additives food. Attempts to expand the number of natural antioxidants through screening of natural products are important, such as piceatannol which is an analogue and metabolite of resveratrol (Seyed, Jantan, Bukhari, & Vijayaraghavan, 2016); however, another alternative approach could be the use of the currently available "natural" antioxidants. An example of such an approach has been the design of O/W emulsions that promote synergistic interactions between various antioxidants (ascorbic acid, gallic acid, (−)-epicatechin, (−)-epigallocatechin-3-gallate), but effectiveness of such synergy has not successfully developed yet (Durand, Zhao, Coupland, & Elias, 2015). Thus, it is also important to develop other strategies or use antioxidant novel technologies to prevent/retard lipid oxidation during the shelf life of O/W emulsions which are prone to oxidation.

1.4. Emulsifiers

Emulsifiers are surface-active compounds that reduce the surface tension (or interfacial tension) between the dispersed and the continuous phase for the emulsion formation. During emulsification, emulsifiers are capable of adsorbing at the O/W interface and protect the emulsion droplets from aggregation and destabilization (see *Section 1.6*). The most commonly used emulsifiers in the food industry are: low-mass surfactants (Fig. 3A and B,d) and amphiphilic biopolymers (Fig. 3B,e-f).

In Figure 3A, low-mass surfactants are represented, which consist of a hydrophilic "head" group, which has a high affinity for water, and a lipophilic "tail" group, which has a high affinity for oil (McClements, 2005). Low-mass surfactants (such as monoglycerides, lecithins, glycolipids, fatty alcohols and fatty acids) are very mobile at the interface and they are particularly efficient reducing the interfacial tension. As a result, they rapidly coat the freshly created oil-water interface during emulsification (Kralova & Sjöblom, 2009). Water-soluble surfactants (i.e. Quillaja saponin) are often used to stabilize O/W emulsions, such as beverages and dressings (Yang, Leser, Sher, & McClements, 2013), as well as to displace proteins from interfaces during the production of ice-creams and whipped creams (Pelan, Watts, Campbell, & Lips, 1997). Oil-soluble surfactants (monoglycerides and lecithins) are habitually used to stabilize W/O emulsions, such as margarine and spreads (McClements, 2005). Another application could be the delivery of ω-3 fatty acids, as sunflower phospholipids have been shown to be viable natural emulsifiers to stabilize fish oil-in-water nanoemulsions (Komaiko, Sastrosubroto, & McClements, 2016).

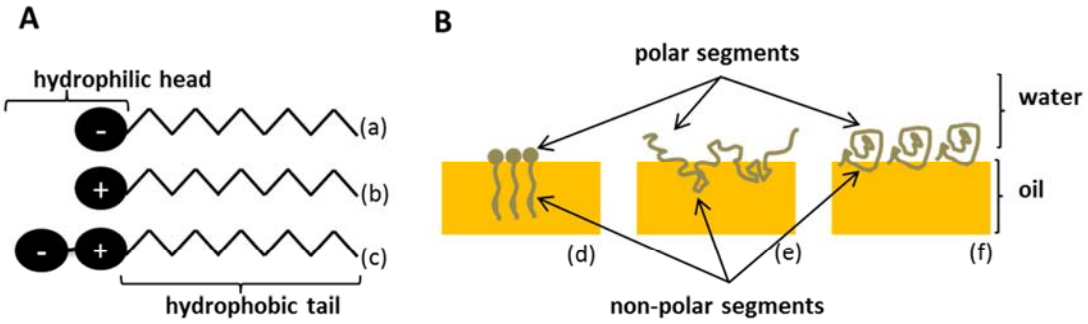

Figure 3. Surfactant classification according to the composition of their head: a) anionic (negatively charged); cationic (positively charged); and zwitterionic (two oppositely charged groups) (A). The structure of the interfacial membrane (B) depends on the molecular structure and interactions of the surface-active molecules: low-mass surfactants (d), flexible biopolymer (e) and globular biopolymer (f).

High-mass amphiphilic biopolymers cover protein and polysaccharide groups. Their functional properties (solubility, surface activity, thickening and gelation) are determined by their molecular characteristics (molecular weight, conformation, flexibility, polarity, hydrophobicity and colloid interactions). In the case of proteins, molecular characteristics are dependent on the type and the sequence of the amino

acids chain. Amino acids are classified according to its polarity into: ionic, polar, nonpolar or amphiphilic. Subsequently, protein contains a mixture of polar and non-polar segments which partially unfolds orientating their segments towards their affinity phase (Fig. 3B,e-f), and may interpenetrate in the lipid phase to various degrees (Kralova & Sjöblom, 2009). For example, proteins can adopt highly dynamic flexible conformation (Fig. 3B,e) or globular rigid structures (Fig. 3B,f). This conformation is determined by different physicochemical phenomena (see *Section 1.6*), including hydrophobic and electrostatic interactions, hydrogen bonding and van der Waals forces. It is important to note that high-mass amphiphilic biopolymers are slower at adsorbing the interfaces compared to small-mass surfactants (Pugnaloni, Dickinson, Ettelaie, Mackie, & Wilde, 2004). However, proteins cover the O/W interface with thicker layers than small-mass surfactants, which is more effective to prevent physical destabilization of O/W emulsions (see *Section 1.6*). The emulsifiers studied in the present thesis dissertation are vegetable proteins.

In the recent years, there is fast increasing interest in the development of plant protein-based emulsions, possibly due to the considerations of proteins being nutritional and functional, and even health-promoting (Aachary & Thiyam, 2012; Guang, Phillips, & Shang, 2012; Nishinari, Fang, Guo, & Phillips, 2014). The most extensively studied vegetable protein for its functional properties is soy protein. Soybeans have been cultivated for more than 3000 years in the Asian countries (China, Japan and Korea). Nowadays, the U.S. soy cultivation has the largest in the world[2]. The advantages of soybean proteins are listed below:

- ✓ A balanced nutritional composition, since all the essential amino acids are contained.
- ✓ They contain physiologically beneficial substances (isoflavones) to mantain the bone mineral density and decrease menopausal hot flushes (Toro, 2014).
- ✓ They have excellent technology properties such as gelling, emulsifying ability and water and oil-holding capacity (Nishinari et al., 2014).

[2] http://www.globalsoybeanproduction.com/

However, the use of other vegetable proteins in the food industry, such as pea protein, is also interesting because of their non-allergenic character compared to soybean proteins (Gharsallaoui, Cases, Chambin, & Saurel, 2009). Moreover, adsorption and rheological properties of globulins from both plant proteins were studied, and pea globulins exerted the highest surface active properties at the oil-water interface (Ducel, Richard, Popineau, & Boury, 2004).

The two major fractions of soy and pea proteins are the 11S legumin proteins and 7S vicilin proteins, which contain different molecular and functional characteristics (Gueguen, Chevalier, Barbot, & Schaeffer, 1988; Nishinari et al., 2014). Research to date has shown that it is possible to form stable O/W by using soy or pea proteins or their fractions as emulsifiers (Gharsallaoui et al., 2009; Roesch & Corredig, 2003). In the present, commercially protein isolates are available and commonly used by food manufacturers. However, most of the properties for those protein isolates are similar to heat-treated soy proteins. This might be caused by the steam heating marked as a critical control point in the procedure (Fig. 4), and the spray drying, as both processes cause protein aggregation and denaturation, which depends on the intensity of heat treatment applied. Consequently, protein isolate-stabilized O/W emulsions should contain relatively high droplet concentrations or high continuous phase viscosities (McClements, 2005).

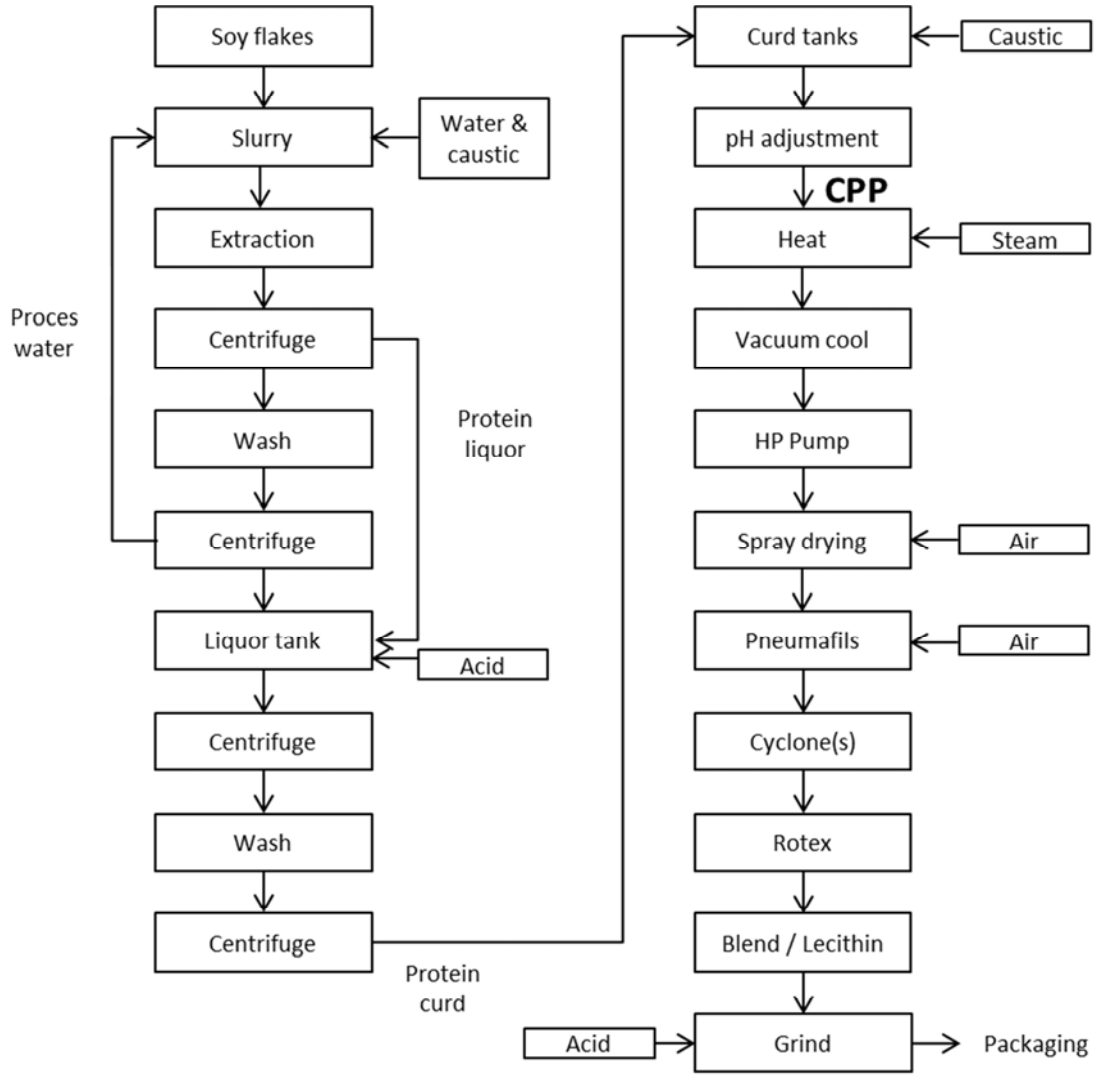

Figure 4. Hazard analysis and critical control points (HACCP) of the isolate process extraction of Pro-Fam 974 from soybean flakes (Lactotecnia, S.L.). CCP 1 is a critical control point in the procedure.

1.5. Interfacial properties

In O/W emulsions, the interface is the narrow region which separates the oil from the aqueous phase. Although it is a small fraction of the volume of an emulsion, it has an impact factor on the formation, rheology, physical/oxidative stability and flavour of the overall emulsion, as well as the fatty acid release and the delivery of nutrients (Berton-Carabin et al., 2014; Malaki, Wright, & Corredig, 2010). Nevertheless, an extensively review studied various parameters which affect lipid oxidation of O/W emulsions (Fig. 2), and contradictory results were obtained with respect to the interfacial region so it should be further investigated (Berton-Carabin et al., 2014). However, unadsorbed

emulsifiers have been shown to reduce lipid oxidation in a consistent manner (Fig. 2). Subsequently, high continuous phase viscosities for vegetable proteins (see *Section 1.4.1*), could play a positive role on lipid oxidation.

The interfacial characteristics of O/W emulsions are represented in Figure 5A, and how they impact on each other. It is important to note the major role of the interfacial composition in determining emulsion properties, such as type of emulsifier, concentration in the water-phase and degree of unfolding (e.g. native/denatured protein). The difference in behaviour between adsorbed protein lies in the degree of unfolding of the protein in the interfaces and the adsorption rate (McClements, 2005; Wierenga, Egmond, Voragen, & de Jongh, 2006). When the adsorption rate is lower than the unfolding rate of the protein, flexible biopolymers are obtained (Fig. 5B,a).

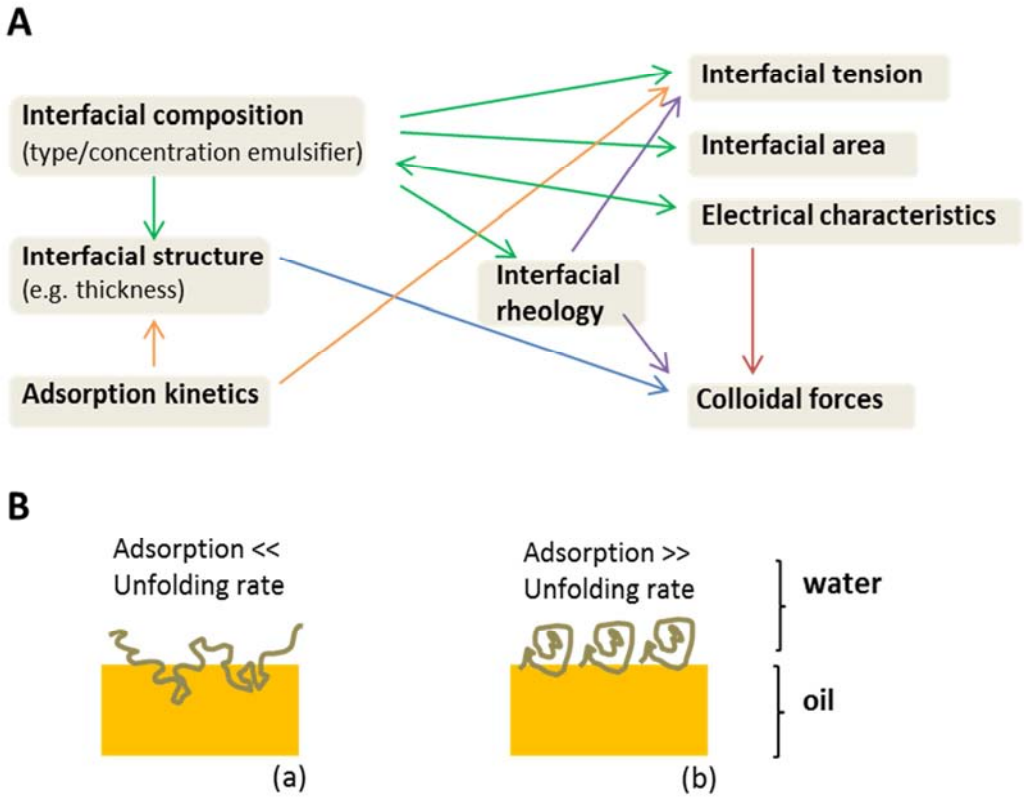

Figure 5. Overview of the main interrelationships between the interfacial characteristics of O/W emulsions: arrows connect the emulsion characteristics to their influencing interfacial characteristics (A). The conformational changes of protein at the interfacial layer depend on the ratio between the adsorption rate and the unfolding rate (B).

In contrast, more densely packed and compact interfaces are formed as a result of the faster adsorption rate compared to the unfolding rate of the protein (Fig. 5B,b).

The structural modification of protein caused by additional treatments, such as heating, also affects its interfacial properties (Wang et al., 2012). In particular, it has been reported that heating of soy protein-stabilized emulsions increases the surface load at the interface (Keerati-u-rai & Corredig, 2010), and the composition at the O/W interface depends not only on the temperature of heating (75 and 95 °C) but also on the order of heating and homogenization (Keerati-u-rai & Corredig, 2009). In this study, while heating the soy protein isolate before homogenization resulted in all the protein subunits to be present at the interface in an aggregated form, when heating was applied after homogenization, a portion of the α and the α' subunit of β-conglycinin as well as the acidic subunits of glycinin remained unadsorbed.

1.6. Destabilization phenomenon

All food emulsions are thermodynamically unstable systems which will eventually breakdown, and kinetics stability tell us the rate at which this will occur (McClements, 2005). "Emulsion stability" means the ability of an emulsion to resist physical and chemical changes over time. Physical changes are the modification on structural organization of molecules, whereas chemical changes result in the alteration of such molecules (e.g. lipid oxidation, lipolysis). The mechanisms by which these changes will take place depend on the emulsion composition (e.g. insufficient/excessive emulsifier concentration), processing operations (see *Section 1.7*), colloidal interactions among ingredients and environmental conditions during storage, such as temperature variations (Palazolo, Sobral, & Wagner, 2011).

There are five mechanisms that contribute to the physical instability of emulsions: creaming, sedimentation, aggregation (flocculation or coalescence), Ostwald ripening (molecules diffusing from small droplets to large ones) and phase inversion (Fig. 6). Flocculation (b), sedimentation and creaming (d) are reversible phenomena which result in migration of particles and can end resulting in the separation of the two phases (e). Coalescence (c), considered the ultimate degradation phenomenon is an

irreversible phenomenon which is characterized by a modification of the particle size (Fig. 6).

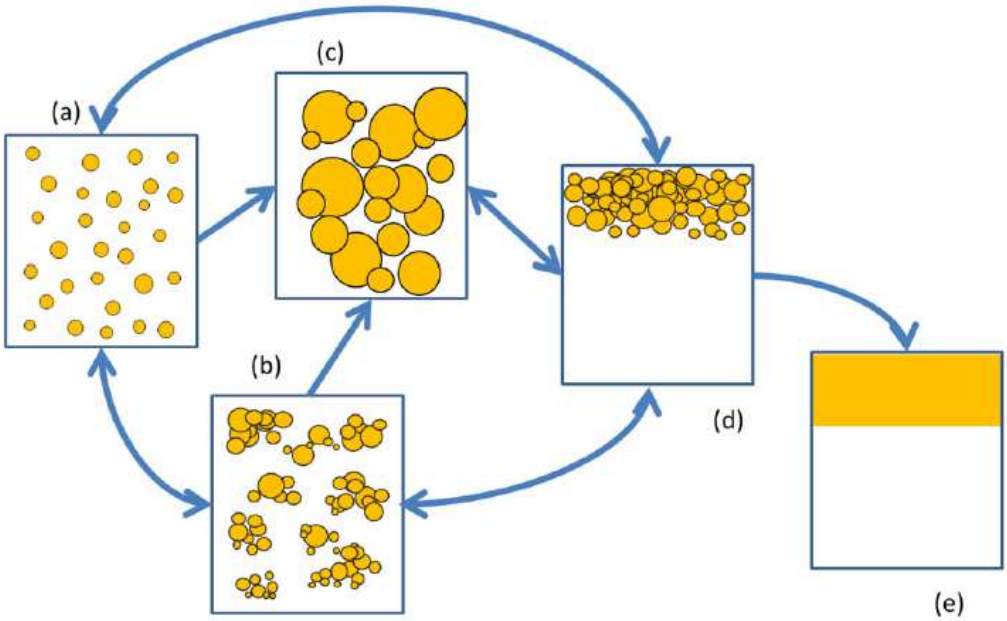

Figure 6. Emulsion instability pathways. Most emulsions are metastable and begin with (a) a homogeneous dispersion of oil droplets in water. The main instability processes are (b) flocculation, (c) coalescence, and (d) creaming (or sedimentation). Finally, the 2 phases may completely separate (e) and the system breaks into its component parts ("oiling off"). The arrows denote possible routes and indicate where reversibility is potentially achievable through gentle redispersion (Degner, Chung, Schlegel, Hutkins, & McClements, 2014).

The droplets in O/W emulsions tend to move upward and cream, as the densities of liquid oils are lower than water (Table 1). The kinetics of the creaming phenomenon can be explained by the general law of sedimentation or the Stokes Law extended to concentrated dispersions (Mills & Snabre, 1994):

$$V(\varphi, d) = \frac{|p_p - p_c| \times g \times d^2}{18 \times \nu \times p_c} \cdot \frac{[1-\varphi]}{1 + \left(\frac{4{,}6\varphi}{(1-\varphi)^3}\right)}$$

where: V = particle migration velocity ($m.s^{-1}$), p_c = continuous phase density ($kg.m^{-3}$), p_p = particle density ($kg.m^{-3}$), g = gravity constant ($9.81 m.s^{-2}$), d = particle mean diameter (µm), ν = continuous phase dynamic viscosity (cP), φ = volume oil fraction (without unit).

According to this equation, the creaming rate could be reduced increasing continuous phase density and volume oil fraction, as well as reducing the particle size of oil droplets. Both measures could be achieved by increasing the protein concentration in the continuous phase and applying higher energy input during emulsification, respectively (Hebishy, 2013; Juliano et al., 2011).

Physical properties and physical stability of emulsions have been studied through numerous techniques such as particle sizing, microscopy and rheology. However, most of these techniques involve some form of dilution which might not reveal the real causes that contribute to destabilization (Herrera, 2012). The Turbiscan^{TM} method scans the turbidity profile over time and allows us to obtain quantitative data and make comparisons among different formulations (Mengual, 1999).

1.7. Homogenization technique

Mechanical homogenization refers to the capability of producing a homogeneous size distribution of particles suspended in a liquid, by forcing the liquid under the effect of high pressure trough a disruption valve. The first invention for “intimately mixing milk” and other liquids was patented in 1899 by Auguste Gaulin using pressures up to 30 MPa (U.S. Patent no.756953). Since then, conventional homogenization extended the pressure range until 60 MPa, by forcing the fluid trough a gap of approximatively 100-300 µm. High-pressure Homogenization (HPH) or dynamic HPH enables pressures 10-15 times higher than traditional homogenizers and covers the range 100-400 MPa, within which the pressure range of 200-400 MPa has been referred to Ultra-High-Pressure Homogenization (UHPH), despite the controversy that exists in the literature as it is just referred for 300 and 400 MPa (Dumay et al., 2013). HPH technology must not be mistaken with High Hydrostatic Pressure (Trujillo, Capellas, Saldo, Gervilla, & Guamis, 2002), as HPH is a continuous process which combines both homogenization and pressure leading to shear forces, turbulence and cavitation, produced due to the pass of the fluid throughout the HPH valve. Currently, there are numerous pilot or lab-scale equipments that use the HPH processing, although the maximum pressure achieved depends on the engineering design. In Table 2, high-pressure processors that

combine superior (flow rate and pressure) and scalable results for pilot and production environments had been chosen.

Table 2. Current high-pressure homogenizers that combine superior (flow rate and pressure) and scalable results for pilot and production environments.

Corporation	Model	Pressure (MPa)	Flow (L/h)
Microfluidics (U.S.)[3]	Microfluidizer M-7250	275	816
BEE International (U.S.)[4]	DeBEE 2000P-250/45	310	1.500
Avestin (Canada)[5]	EmulsiFlex-C1000	207	1.000
Gea Niro Soavi (Italy)[6]	Ariete NS5355	150	60.000
APV (U.K.)[7]	Rannie 75	150	800
Stansted Fluid Power (U.K.)[8]	SPF-nG11300	400	240
Ypsicon (Spain)[9]	UHPH-A1	350	150

To date, a number of papers have been published on the effect of homogenization pressure on stability of food emulsions (Dumay et al., 2013; Georget, Miller, Callanan, Heinz, & Mathys, 2014). Many studies have confirmed that homogenization pressure, especially above 100 MPa, significantly influences emulsion stability (Hebishy, Buffa, Guamis, Blasco-Moreno, & Trujillo, 2015; Poliseli-Scopel, Hernández-Herrero, Guamis, & Ferragut, 2012). Appropriate pressure is believed to be beneficial to the stabilization and emulsifying properties of protein emulsion, and an excessive pressure may cause detrimental effect, such as particle size distribution and flocculation (Floury, Desrumaux, & Legrand, 2002).

2. Ultra-High Pressure homogenization (UHPH)

2.1. UHPH equipment and applications

The continuous UHPH system is a revolutionary product on the market, as it enables the simultaneous stabilization and sanitization (pasteurization or sterilization depending on the pressure and the inlet temperature of the product applied) of liquid

[3] http://www.microfluidicscorp.com/microfluidizer-processors/m-700-series
[4] http://www.beei.com/products/industrial-homogenizers
[5] http://www.avestin.com/English/cxpage.html
[6] http://www.gea.com/es/es/products/homogenizers-ariete-series.jsp
[7] http://www.spxflow.com/en/apv/
[8] http://www.homogenizersystems.com/production_high_pressure_homogenizers.html
[9] http://www.ypsicon.com/main.asp?pagina=pdetall&pdetall=923

products. The line is fully automated and all the parameters can be controlled by one operator through a touch panel (Figure 7). The operation can be divided in three basic steps:

- ✓ Preheating of the product to choose the inlet temperature of the product (T_{in}).
- ✓ Application of the continuous ultra-high pressure homogenization.
- ✓ Instant cooling.

Figure 7. Ultra-High Pressure Homogenization system used in the present PhD thesis developed by Ypsicon SL, Barcelona, Spain.

Figure 8 represents the complete UHPH system, which is made up of a product tank (1), filters (2), heat exchangers (3), an UHPH system including pumps (4), an UHPH valve gap of few µm in width (5), heat exchangers using cooled water (6) and an aseptic tank (7). The UHPH system can be sterilized previously to processing, using steam or water reheated at 140 °C for 45 min in a closed circuit, allowing the stabilization of sterilized products, according the UHPH conditions applied. This UHPH system, which is capable of sterilizing pumpable liquids, has been patented by a group of researchers from Universitat Autònoma de Barcelona (UAB) (Guamis, Trujillo, Ferragut, Quevedo, Lopez, & Buffa, 2015).

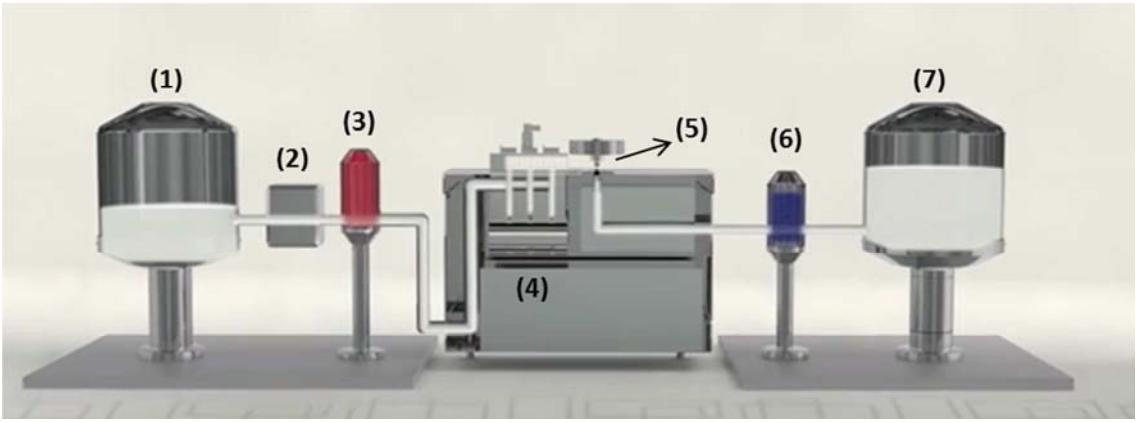

Figure 8. Graphic scheme representing the complete UHPH system patented by UAB.

Production starts by filling the tank with the liquid product (1). The product is them immediately pumped to the heat exchanger (3), where depending on its characteristics, it can be kept at cold/room temperature or pre-heated up to a specific temperature to reduce its viscosity and/or guarantee the aimed temperature peak reached at the **UHPH-valve** (5) according to the objective of the treatment applied. Once the right temperature has been reached, the product is pumped (4) to the homogenizer, where the fluid is forced to pass through the UHPH-valve (5) at a specific pressure (until 400 MPa maximum). At the UHPH-valve, such an increase in pressure modifies the temperature of the product, about 2-2.5 °C per 10 MPa, in less than a fraction of second. This short time exposure at the temperature peak clarifies that there is an insignificant damage of the product caused by temperature. For this reason, and although the temperature reached at the UHPH-valve seems to have a crucial effect on sanitization conditions of the product, the UHPH technology could be considered as a non-thermal treatment for the processing of pumpable fluids (Diels & Michiels, 2006; Zamora & Guamis, 2014). Right after the UHPH valve, the homogenized product enters to a cooling system (6) to guarantee its instant cooling, in order to reduce its thermal damage. After that, if sterilization conditions are achieved, the fluid can be sent to an aseptic tank (7) for subsequent aseptic packaging, such as cardboard, polyethylene or glass.

The UHPH process is an **emerging technique**, since the innovative design of the homogenization valve, originally developed by Stansted Fluid Power Ltd., modified the geometry and material of the valve to ceramic, which is known to withstand to ultra-

high-pressure levels (Fig 9b). This type of equipment can attain pressures up to 150-200 MPa considered HPH and up to 300-400 MPa as UHPH (Zamora & Guamis, 2014).

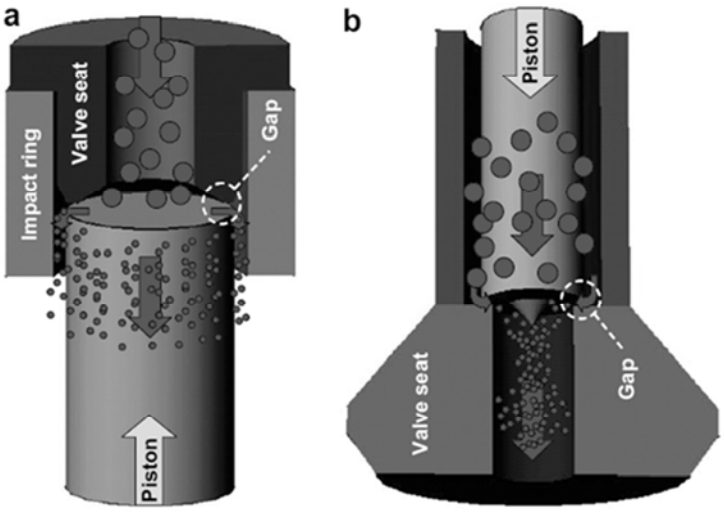

Figure 9. Homogenizing valves geometries from: Manton-Gaulin APV (a) and Stansted Fluid Power (b) (Donsì, Ferrari, & Maresca, 2009).

In the UHPH valve, as it is shown in Figure 9b, the fluid is first fed axially along the mobile part of the valve and then accelerates radially through the narrow gap between the valve and valve seat. On contrary, in the classical valve design from APV-Gaulin, the flow direction of fluid is fed in the opposite direction (Fig 9a). The geometry of the valve is a critical design element with reference to the quality of the finished product, as well as the physical processes that takes place (Donsì, Annunziata, & Ferrari, 2013; Donsì, Sessa, & Ferrari, 2012). Different **physical processes** have been proposed as the causes of disruption of droplets and cells in UHPH, and each mechanism shows up at different extent as a result of various factors such as the viscosity of the product and the operating parameters (**pressure**, **temperature**, flow rate):

- ✓ **Shear stress**: is defined as the inertial forces within particles through a material cross section. It is the only mechanism that contributes to droplets disruption in a laminar flow (Floury et al., 2004); however, both laminar and turbulence regimes coexist in this type of homogenizer (Fig 9b).
- ✓ **Turbulence**: is a mechanism caused by the inertial forces generated in the highly turbulent region, located just at the exit of the valve (Donsì et al., 2009).

- ✓ **Cavitation**: is a phenomenon when liquid passes from low-pressure, in which cavities/air bubbles are formed, to high-pressure region, when they will be collapsing between each other. Intensities of cavitation strongly increase with homogenising pressure (Floury et al., 2004), and decrease with higher fluid viscosity (Diels, Callewaert, Wuytack, Masschalck, & Michiels, 2005).
- ✓ **Impingement**: is the impact of the droplets or cells against the walls of the UHPH valve.
- ✓ **Extensional stress**: stretches droplets/cells which are disrupted by elongational stresses (Floury et al., 2004).

The continuous UHPH system can be applied in a wide variety of sectors, such as the food, pharmaceutical, biotechnology, chemical and cosmetics industries. Generally, it can also be used in any pumpable product, chemically compatible with the system, obtaining a product susceptible to be aseptically packaged (Guamis, Trujillo, Ferragut, Quevedo, Lopez, & Buffa, 2015).

In **cosmetics**, UHPH could be successfully applied by means of microencapsulation. For example, perfumes show a strong initial odour which easily vaporizes, due to high volatility of compounds. In this sense, Rexona® developed an innovative deodorant that combines fine fragrances with patented encapsulated odour-fighting technology, to offer long lasting fragrance. After that, marketing of the product could use the slogan "*Rexona won't let you down*"[10]. So far, UHPH-processed O/W submicron emulsions stabilised with a lipid-based surfactant and containing retinyl acetate were tested in ex vivo pig's ear skin (Benzaria et al., 2014). Those researchers indicated that the UHPH-induced decrease in droplet size facilitated skin permeation compared to coarse emulsions.

In the case of **pharmaceutical** applications, nano/submicron O/W emulsions are targeted to be used as delivery vehicles. The objective is to reduce droplet size in the nano/submicron range to improve solubility of bioactive components and its further bioavaibility (Dumay et al., 2013). These compounds could be protected against external agents, such as light and oxygen, for further release into target tissues

[10] https://www.unilever.com/brands/our-brands/rexona.html

without previous degradation. Recently, a milk-based powder formulation has been succesfully developed for pediatric delivery of ritonavir, which was integrated to the protein, especially casein micelles, during UHPH treatment (Corzo-Martínez, Mohan, Dunlap, & Harte, 2015).

2.2. UHPH effects on physico-chemical properties

The physical properties of O/W emulsions can be characterized by a the combination of various techniques, such as visual observation, rheological methods, ultrasound profiling, electroacoustic spectroscopy (zeta potential), surface protein concentration, microscopic analysis, nuclear magnetic resonance and optical methods (dynamic light scattering, diffusing wave spectroscopy and Turbiscan^{TM}) (Herrera, 2012). In practice, dynamic light scattering has become of vital importance to characterize particle sizing of emulsions, along with the other available techniques.

2.2.1. Particle size and viscosity

The particle size of dairy and other food emulsions is an important factor in defining structural, oxidative and sensory characteristics. Numerous studies have reported the effect on reducing particle size by increasing the pressure of the UHPH treatment (Cortés-Muñoz et al., 2009; Dumay et al., 2013; Floury et al., 2004). The particle size/shape of emulsion has a strong influence on the bulk or macro properties, such as rheology, which gives an indication of emulsion's colloidal state and interactions between the different constituents. In general, at constant emulsifier concentration, viscosity increases with reducing particle size by means of UHPH (Floury et al., 2002; Hebishy et al., 2015). As an example, milk UHPH-treated achieves the narrowest particle size distribution with increasing pressures up to 200 MPa (Pereda, Ferragut, Quevedo, Guamis, & Trujillo, 2007; Roach & Harte, 2008). However, in those studies, when 300 MPa was applied, an increase in the particle size was observed, probably due to the fact that there is insufficient protein for the larger surface area created and then, flocculation occurs. By contrast, when there is enough protein at constant volume oil fraction, viscosity increases with reducing the polydispersity of size

distribution profiles (Cortés-Muñoz et al., 2009). Moreover, at constant number of particles, the flow behaviour will generally migrates from Newtonian to "shear thinning" (pseudoplastic) with increasing the volume oil fraction (Chevalier-Lucia, Cortés-Muñoz, Picart-Palmade, & Dumay, 2009). Nevertheless, other factors (e.g. protein denaturation) influence on viscosity of the emulsions and they should be carefully taken into account.

2.2.2. Protein denaturation

Proteins respond differently to heat and pressure treatments (Floury et al., 2002). The addition of protein ligands which improves physical protein stability, could delay the formation of higher molecular weight aggregates against heat or pressure treatments (Chi, Krishnan, Randolph, & Carpenter, 2003). In this sense, some authors employed heat (between 40 and 93 °C) and high-hydrostatic pressure treatment (between 50 and 800 MPa for 30 min) on β-lactoglobulin (β-LG) B to determine the extent of unfolding and aggregation in the presence of different ligands: CLA and myristic acid (Considine, Patel, Singh, & Creamer, 2007). It was found that pressurizing β-LG B dispersions at higher pressures (from 50 to 800 MPa), only CLA had the ability to inhibit the protein transition from; a) mainly non-native monomers and dimers, to; b) larger polymers. In contrast, UHPH technology exhibit **different physical processes** (see *Section 2.1*) along with **pressure** and **temperature** contribute to protein denaturation. UHPH processing can be used as a tool to modify the functional aspects of proteins in:

- ✓ Liquid products, such as dairy-based foods.
- ✓ The development of new ingredients to change rheological/textural properties of other foodstuff.

In **liquid products**, UHPH treatment at 300 MPa has shown to produce the same extent of denaturation in soymilk proteins (Cruz et al., 2007) and an increase in the surface hydrophobicity of almond UHPH-beverages (200 and 300 MPa) (Valencia-Flores, Hernández-Herrero, Guamis, & Ferragut, 2013), in comparison to an UHT treatment. Surprisingly, cow milk UHPH-treated (200 and 300 MPa), regardless of the increase of

temperature experienced in the UHPH valve (<85 °C and 100 °C, respectively), suffered less whey protein denaturation compared to a commercial pasteurized milk (90 °C,15 s) which leads to a better nutritional value of the milk (Pereda, Ferragut, Quevedo, Guamis, & Trujillo, 2009). On a pilot-scale study, some researchers showed that the amount of denatured β-LG was much greater (17%) for UHPH-treated cow milk at 200 MPa, which reached approximately 75°C for a very short time, than for light-pasteurized milk at 72°C for 15 s (Zamora, Ferragut, Jaramillo, Guamis, & Trujillo, 2007). Such results corroborated the idea that not only heat but also physical processes and pressure during UHPH treatment induce the denaturation of whey protein.

Moreover, UHPH has been used as a tool to modify the **functional aspects of proteins** in skim milk (Roach & Harte, 2008), especially, casein micelles are potentially suitable and efficient carrier systems of hydrophobic drugs (Corzo-Martínez et al., 2015), as it has been demonstrated with α-tocopherol acetate (Dominique Chevalier-Lucia, Blayo, Gràcia-Julià, Picart-Palmade, & Dumay, 2011). In combination with polysaccharides, UHPH processing at 300 MPa modified casein-hydroxypropyl cellulose interactions and improved the stability of the aqueous system (Ye & Harte, 2014). Also, the partial denaturation of whey proteins from cow milk treated by UHPH (200 and 300 MPa; single-stage) improved the cheese-making properties of milk, in comparison to conventional homogenized-pasteurized milk (Zamora et al., 2007).

2.2.3. Physical stability

Among optical methods (see *Section 2.2*), Turbiscan™ overcomes the major drawback of light scattering techniques which is the need of sample dilution, as it modifies the structure and composition of the real emulsion (Huang, Yu, & Ru, 2010). The Turbiscan™ can be used for easy and fast comparison and ranking of the samples, and in-depth characterisation of destabilization phenomena (see *Section 1.6*). Figure 10 shows a scheme of the physical principles of Turbiscan™ MA 2000, which is composed by a reading head (pulsed near-IR light source; λ = 850 nm, and two synchronous detectors of transmission and backscattering) which scans the sample glass tube.

Changes in the profile curves during time allow the quantification of any destabilization phenomena.

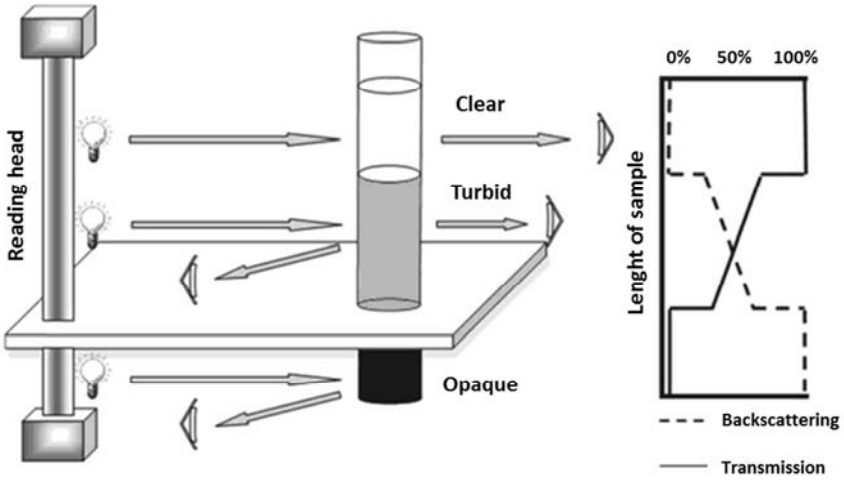

Figure 10. Scheme of the physical principles of Turbiscan^{TM} MA 2000 (Mengual, 1999).

In Table 3, an overview of the current UHPH food emulsions studied and their **stabilization phenomena** have been summarized. The best physical stability of those food emulsions reviewed depends not only on smaller particle size, but also by the absence of an increase of particle aggregates during storage that sediment (Poliseli-Scopel, Hernández-Herrero, Guamis, & Ferragut, 2014), the possibly denaturation of milk proteins by the intense temperatures reached at the exit of the UHPH-valve (Addo & Ferragut, 2015), and particularly on the increase on protein content and viscosity against creaming phenomenon (Hebishy et al., 2015), which will determine the acceptability by consumers.

Table 3. Overview of the stabilization phenomena that contributes to the best physical stability in different UHPH food emulsions.

Food emulsion	Pressure (MPa)	T_{in} (°C)	Best physical stability	Stabilization phenomena	Reference
UHPH O/W emulsion (1, 2 and 4% whey protein isolate, WPI; and 15% sunflower oil and 5% olive oil)	15 100 and 200	60 25	Emulsions with 4% WPI treated at 100 and 200 MPa against **creaming**	↑protein concentration ↑aqueous phase viscosity ↓droplet diameter	(Hebishy et al., 2015)
UHPH soymilk	200 Control (Pasteurized: 90 °C, 30 s)	55 and 75	Both UHPH samples against **sedimentation**	↓droplet diameter	(Poliseli-Scopel, Hernández-Herrero, Guamis, & Ferragut, 2013)
UHPH soymilk	300 Control (UHT: 142 °C, 6 s)	80	Soymilk treated at 300 MPa against **sedimentation**	↓droplet diameter, and same $d_{4,3}$ values during storage indicated no increase in particle aggregates	(Poliseli-Scopel et al., 2014)
Tiger Nut beverage (*orxata de xufla*)	200 300 Control (Homogenized-pasteurized: 18+4 MPa and 80 °C, 15 s)	40 40	Soymilk treated at 300 MPa against **creaming**	↓droplet diameter	(Codina-Torrella, 2014)
Donkey milk	100 200 300 Control (Pasteurized: 70 and 85 °C, 60 s)	40 40 40	Donkey milk treated at 100 MPa against **creaming** and **sedimentation**	↓droplet diameter ↓T(°C) at the exit of the UHPH-valve (high T causes denaturation and aggregation)	(Addo & Ferragut, 2015)
Cow milk	200 200 300 300 Control (High-pasteurized: 90 °C, 15 s)	30 40 30 40	All UHPH samples against **creaming**	↓droplet diameter	(Pereda et al., 2007)

2.3. UHPH effects on microbial stability

UHPH has been suggested as an alternative to thermal pasteurization to preserve liquid food products (Pereda et al., 2007; Saldo, Suárez-Jacobo, Gervilla, Guamis, & Roig-Sagués, 2009; Tahiri, Makhlouf, Paquin, & Fliss, 2006). The best efficiency of UHPH against most microorganisms depends on the pressure applied but also by applying several cycles (Diels & Michiels, 2006; Tahiri et al., 2006). All the physical processes explained above (see *Section 2.1*) along with higher T_{in} of samples will increase temperature at the exit of the UHPH-valve, provoking a very short time thermal effect which could also induce lethality of microorganisms (Roig-Sagués et al., 2009). In fact, this ultra-short time thermal effect, along with the pressure applied, might be the reason of reaching sterilization conditions (Georget et al., 2014a). In Table 4, the effect of UHPH inactivation of naturally present bacterial spores on various food emulsions have been reported. UHPH treatment at 200-300 MPa has been shown to be as effective as thermal pasteurization (Poliseli-Scopel et al., 2012; Valencia-Flores et al., 2013) and sterilization (Amador-Espejo, Suàrez-Berencia, Juan, Bárcenas, & Trujillo, 2014; Poliseli-Scopel et al., 2014) to ensure the microbiological stability (Table 2). The literature reviewed has showed that the pressure and T_{in} combination had a significant impact on the lethal effect of UHPH treatment (Table 4). In cow milk and soymilk, sterilization has been achieved when maximum temperature of the fluid passing through the UHPH-valve was closer to 140 °C (Amador-Espejo et al., 2014; Poliseli-Scopel et al., 2014). In fact, the first attempt on the validation of the UHPH for bacterial spores inactivation has been conducted with thermostable strains such as the wet heat sterilization indicator *G. stearothermophilus* ATCC7953 spores in a model buffer system, and up to 2 $\log_{10}$ were inactivated at 300 MPa and >145 ºC at the exit of the UHPH-valve (Georget et al., 2014b). Moreover, it is important to note that characteristics of food matrix, such as fat content and viscosity, had also been reported in the literature to influence microbial inactivation (Diels et al., 2005; Roig-Sagués et al., 2009). In this sense, higher viscosity and fat content should contribute to the development of UHPH emulsions for the purpose of bacterial inactivation.

Table 4. Overview of literature on successful pasteurization and sterilization of beverage emulsions by UHPH inactivation of naturally present bacterial spores.

Food emulsion	Pressure (MPa)	T_{in} (°C)	Max T (°C) UHPH-valve	Initial count (log cfu/mL)	Maximal microbial stability	Reference
Almond beverage	200 300	55 65 75	129.3±12.6	4.00	ND* after 20 days at 30 °C (300 MPa/65-75 °C)	(Valencia-Flores et al., 2013)
Soymilk	200 300	55 65 75	135.7±1.5	1.54	ND after 20 days at 30 °C (300 MPa/75 °C)	(Poliseli-Scopel et al., 2012)
Milk 3.5 % fat	200 300	55 65 75 85	139.0±2.7	1.00	ND after 15 days at 30 °C and 7 days at 45° (300 MPa/75-85 °C)	(Amador-Espejo et al., 2014)
Soymilk	300	80	144	2.18	ND after 20 days at 30 °C and 10 days at 55 °C	(Poliseli-Scopel et al., 2014)
Almond and soymilk beverage	200 300	55 65 75	135.7±1.5	3.18 (soymilk) - 4.21 (almond)	ND (200MPa/75°C and 300MPa/65-75°C)	(Ferragut, Hernández-Herrero, Poliseli, Valencia, & Guamis, 2011)

* ND= not detected.

2.4. UHPH effects on oxidative stability

Lipid oxidation in emulsions has become a renewed concern and it is important to develop new technologies to replace synthetic antioxidants (Waraho, Cardenia, Decker, McClements, & Elias, 2010). When oil oxidises it produces a series of breakdown products in stages, starting with primary oxidation products (peroxides, dienes, free fatty acids), then secondary products (carbonyls, aldehydes, trienes), particularly aldehydes, responsible for undesirable odours and flavours, and finally tertiary products (Gunstone & Martini, 2010). Many factors influence the rate of oxidation such as temperature, light exposure, presence of metals, oxygen, oil composition and higher specific area on emulsions (Waraho et al., 2010).

So far, however, there has been little discussion about the effect of UHPH on oxidative stability of oil-in-water emulsions. In particular, Hebishy et al. (2015) examined the effect of UHPH (100 and 200 MPa) on oxidative stability in emulsions with similar oil content (20% sunflower oil) than the actual thesis dissertation. It was found that UHPH emulsions were more oxidatively stable than their CH counterparts.

Nonetheless, there is reliable evidence that UHPH could act as an antioxidant technology on emulsion beverages as the following studies have shown, especially at 200 MPa. UHPH-milk treated at 300 MPa showed lower aldehyde content compared with commercial milk samples (Pereda et al., 2008c). In the later samples, heat treatment induced the formation of volatile compounds related to heat and oxidized off-flavours, such as aldehydes and methyl ketones. Similarly, other researchers have successfully applied the UHPH technology (200 and 300 MPa) on “tiger nut beverage” (*orxata de xufla*) as an alternative method of production and preservation, compared to conventional homogenized-pasteurized beverages and conventional homogenized-pasteurized beverages plus the addition of preservatives (Codina-Torrella, 2014). After production, all UHPH-tiger nut beverages resulted in less aldehyde content compared to conventional treatments. Within UHPH-tiger nut beverages, which were kept in refrigeration until their shelf life, the treatment performed at 300 MPa drastically increased total aldehyde content at day 14, and then kept constant. Comparing with the treatment at 200 MPa, the authors attributed this increase to the temperature

reached at the exit of the UHPH-valve in the treatment at 300 MPa (~116 °C). Similarly, cow milk UHPH-samples treated at 300 MPa presented higher malondialdehyde and hexanal content compared to those treated at 200 MPa, possibly due to the fact that higher temperature values (until 100 °C) were reached after the exit of the second UHPH-valve (Pereda, Ferragut, Quevedo, Guamis, & Trujillo, 2008). In another study, UHPH-soymilk treated at 300 MPa, and reaching 144 °C at the exit of the UHPH-valve (Poliseli-Scopel et al., 2014), showed decrease values of hydroperoxide index and hexanal levels in UHPH soymilk comparing to the counterpart soymilk treated by UHT. Interestingly, some authors observed that UHPH (0-350 MPa) did not significantly affect the fatty acid content and the possibly loss by oxidation/isomerization of CLA isomers profile in cow, ewe and goat milk (Rodríguez-Alcalá, Harte, & Fontecha, 2009). In contrast, UHT sterilization resulted in a isomerization of *cis*9,*trans*11-CLA to *trans*9,*trans*11-CLA, and this extent was greater in those milks which were naturally enriched in PUFA.

2.5. UHPH effect on sensory attributes

Sensory evaluation is a discipline that analyses and measures human responses to the composition of food and drink, e.g. appearance, touch, odour, texture, temperature and taste. Sensory evaluation panels (group of trained testers) are increasingly being used to help make vital decisions about food product improvement, quality control, new product development, production process change and competitor benchmarking. In a thesis dissertation in which UHPH-treated milk (100-300 MPa) and conventionally pasteurized milk were studied (Pereda, 2009), a trained sensory panel considered cow milk UHPH treated at 200 MPa as ‘‘rancid’’, which could have been related to their increase in free fatty acids as that treatment could not inactivate the native milk lipoprotein lipase (Pereda et al., 2008b). However, when 300 MPa was performed, almost no defects were noticed as no significant differences were observed on the increase in proteolysis and lipolysis between UHPH at 300 MPa and pasteurized milk (Pereda, Ferragut, Buffa, Guamis, & Trujillo, 2008; Pereda et al., 2008b). Nevertheless, UHPH-milk treated at 300 MPa showed higher aldehyde content compared to pasteurized milk (Pereda et al., 2008), albeit just 25% of people from the sensory panel

characterized the UHPH-milk treated at 300 MPa as 'oxidized' (Pereda, 2009). Nonetheless, UHPH-milk at 300 MPa was preferred to conventionally pasteurized milk by the sensory panel (Pereda, 2009). A possible explanation for this might be the retention of hexanal with increasing homogenisation pressure, as it has been previously observed on HPH emulsions (0, 18, 100 and 150 MPa) of sodium caseinate-whey protein (2% + 2%) emulsions, in which interactions between hexanal and caseinate were suggested (Innocente, Marchesini, & Biasutti, 2014). In a similar way, Codina-Torrella et al. (2014) concluded that UHPH-tiger nut beverages (200 an 300 MPa) showed similar sensory characteristics and acceptability by the sensory panel during their shelf life, even though the treatment at 300 MPa showed higher aldehyde content. Similar results were obtained with soymilk, where Poliseli-Scopel et al. (2014) concluded that chemical changes that occurred during storage did not impact on the organoleptic characteristics of soymilk treated at 300 MPa when comparing to UHT (sterile beverages). Contrary when non-sterile soymilk (treated at 200 MPa) was compared to a pasteurized sample, better acceptance of UHPH-treated soymilk was achieved (67%) (Poliseli Scopel, 2012).

3. Delivery of lipophilic bioactive substances

3.1. Background

Changes in agricultural practice over the past 50 years have increased the world's capacity to provide food, which has resulted in considerable changes in food consumption on developed countries (Kearney, 2010). Consequently, agricultural sector have promoted unhealthy diets over the past years, which in turn, it has increased the development of non-communicable diseases and health risks such as obesity and hypertension (Hawkes, 2007). In this sense, the food industry is focused on the development of **functional food** products, as they offer great potential to improve health and/or help to prevent certain diseases when taken as part of a balanced diet and healthy lifestyle (Alasalvar & Bolling, 2015; Sikand, Kris-Etherton, & Boulos, 2015). Nonetheless, the oral bioavailability of lipophilic health-promoting dietary components is relatively low, as they are limited by various physicochemical and physiological

phenomena (McClements, Li, & Xiao, 2015). For this reason, there has been a great interest in the past years for the design of **food-based delivery systems** to boost the oral bioavailability of lipophilic bioactives. In the literature, quite a few research articles has focused on numerous nano or micro delivery-systems such as emulsions, liposomes, micelles, hydrogel particles, solid lipid particles or colloidosomes (McClements & Li, 2010).

Figure 11 summarizes the **growth in research** articles published in the last decade (2005-2015) containing "**emulsion**" and "**functional food**" as keywords (Web of Science, Thomson Scientific, June 2016). However, the publication of patents drastically decreased in 2010 (Fig. 11).

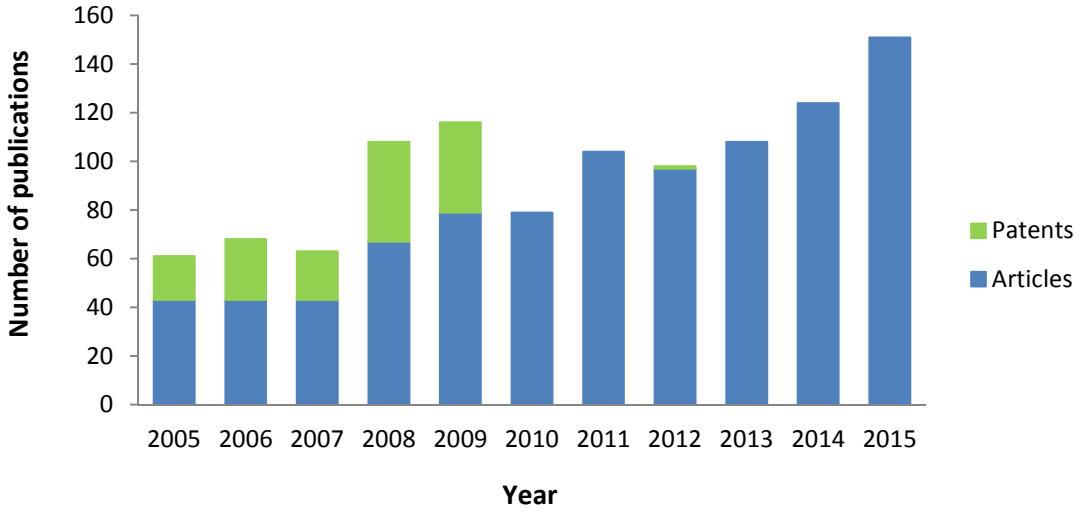

Figure 11. Number of research articles and patens with the keywods "emulsion" and "functional food" published from 2005 to 2015 (Web of Science, June 2016).

In contrast, when "delivery systems" and "food" are selected as keywords, a gradually increase up to 650 research articles are found and a constant number of patents over the same years reviewed (Arranz, Corredig, & Guri, 2016). Similar trend than these authors was observed when "emulsion" and "food" were chosen as keywords (data not shown). The decrease in the number of patents since 2010 when the terms "emulsion" and "functional food" are employed (Fig. 11) could be related to the lack of nanoemulsions in the market. Among all emulsions, nanoemulsions play an important role due to their potential to boost oral bioavailability (McClements & Li, 2010).

However, much of the debate about the safety aspects of nanotechnology in the food sector has focused on the uncertainties and the lack of toxicological data (Martirosyan & Schneider, 2014), as nanoparticles show greater reactivity owing their higher specific surface area and could cross the blood-brain barrier. As this sector is under fast development, EFSA have developed a guidance document for the development of nanoemulsions (EFSA Panel, 2011), which follows European Commission's regulations, and those uncertainties are clearly stated:

"*There are currently uncertainties related to the identification, characterisation and detection of engineered nanomaterials (ENM) that are related to the lack of suitable and validated test methods to cover all possible applications, aspects and properties of ENM. Similarly, there are a number of uncertainties related to the applicability of current standard biological and toxicological testing methods to ENM.*"

Nevertheless, UE regulation (EFSA) on nanofood is not universal and has discrepancies with other institutions, such as Food and Drug Administrations in the U.S. (FDA).

3.2. Bioavailability and food matrix effect

When eating food, which contain macromolecules, our organism digests many of them into nanoparticles in order to obtain enough energy for physiological processes. In Table 5, an overview of the complex processes occurring during **digestion**, which induce physical and chemical changes on the food matrix consumed, is summarized (Arranz et al., 2016). These processes involve the solubilisation of the food components, their transformation (chemical, enzymatic), metabolism and matrix interactions, which will affect on the **release of bioactives** of interest.

Fortifying food matrices with bioactives compounds is commonly used in the food industry for the development of functional foods. However, this practice does not mean that bioactive compounds are delivered to the consumer in the intended state and concentration to accomplish the claimed health effect (González-Ferrero & Sáiz-Abajo, 2015).

Table 5. The four main steps of the gastrointestinal tract and their general digestive function.

Mouth	✓ Macrostructure breakdown ✓ Bolus formation ✓ Initiation of starch and lipid digestion
Stomach	✓ Mixing ✓ Structure breakdown ✓ Protein hydrolysis ✓ Lipid digestion
Small intestine	✓ Enzymatic digestion ✓ Nutrient absorption (amino acids, fatty acids, monosaccharides)
Colon	✓ Microbial fermentation ✓ Micronutrient reabsorption ✓ Water removal

To ensure the release and bioefficacy of bioactives in the body, encapsulation and controlled-release of bioactive constituents can be attained by new engineered food systems (McClements, Decker, Park, & Weiss, 2009). In order to be efficient delivery systems, the matrices should preserve the bioactive molecule through their food matrix, as well as maintain its bioactivity during digestion and get it release in the appropriate site of the organism to exert its bioefficacy. Typically, lipophilic bioactives are trapped within lipid-based colloidal particles in food matrices, and their liberation depends on the digestion of triacylglycerols and the formation of mixed micelles for their solubilisation (McClements, 2013). When using viscous or gel-like matrices delivery systems, the extent of lipid digestion is reduced (Li, Hu, Du, Xiao, & McClements, 2011; Li & McClements, 2011), with the concomitant reduction of the extent of micelles available to solubilize lipophilic bioactives. Considering all complex processes during the digestive tract (Table 5), compositional and structural changes of delivery systems, such as emulsions, have been studied before, during, and after digestion (Malaki, Corredig, & Wright, 2011). Recently, an **international consensus** on *in vitro* digestion has been published in order to standardize the protocol, as a consequence of the impossibility to relate or compare the results from different research groups (Minekus et al., 2014). During *in vitro* digestion, interfacial properties of protein-stabilized emulsions play a major role in fatty acid release and the potential bioavailability of lipophilic molecules (Malaki et al., 2010). Thus, interfacial characteristics can be modified, by changing their composition and processing (Fig. 2), to enhance the bioactives release. After that, **bioavailability** takes place, which is defined as the portion of the nutrient of interest that reaches the blood circulation and

has a metabolic endpoint. A recent review has developed a "nutraceutical bioavailability classification scheme" (NuBACS), which classifies the nutraceuticals according to factors limiting their oral bioavailability (McClements et al., 2015). Classifying nutraceuticals according to their bioavailability and transformation limiting factors during gastrointestinal tract, could be a useful tool to develop strategies to boost bioavailability of each molecule. For example, lipophilic bioactives have **poor solubility** within the aqueous intestinal fluids, which is a limiting factor on oral bioavailability (McClements, 2013).

In vitro digestion simulating the gastric and intestinal phases (Minekus et al., 2014) coupled to absorption studies using epithelial cells, such as Caco-2, is a great tool to evaluate the uptake but also the transformation of the bioactive during cells absorption (Guri, Gülseren, & Corredig, 2013). In fact, among bioavailability assays, ***in vitro*** assays are often utilized to design improved food delivery systems in first instance (Arranz et al., 2016). As an example of UHPH emulsions subjected to *in vitro* digestion coupled to Caco-2 cells, the binding of curcumin (0-20 μM) to UHPH-treated (200 and 300 MPa) native-like phosphocaseins (PC, 2.5%) at pH 6.6 was assessed (Benzaria, Maresca, Taieb, & Dumay, 2013). Surprisingly, at the highest curcumin concentrations (10 and 20 μM), curcumin cells uptake was significantly lower for UHPH-processed than native-like PC micelles, providing the possibility of controlled release for UHPH. Nevertheless, the TC7-cells model was incubated for 15 min, and 2 h of incubation should be carrying out in order to mimic the human body. However, the quantitation of bioactives during the process could be limited, as it depends on the level of cytotoxicity of emulsion digestates. Thus, further dilutions of the digestates are needed in uptake experiments, until desired cytotoxicity levels are achieved to not harm the cells. In contrast, ***in vivo*** studies consist on the ingestion of a molecule and the biomarker of bioactivity tracking (Fernández-Ávila et al., 2015). In both cases (*in vitro/vivo* assays), challenges are focused on development of **highly sensitive analytical techniques**, such as Mass Spectrometry and Orbitrap, which are necessary to quantify the bioactives and their metabolites.

4. Conjugated linoleic acid (CLA)

4.1. Natural food sources and biological effects

Among lipophilic bioactive substances, conjugated linoleic acid (CLA) has created widespread interest. Since 1940, there have been published more than 16.000 research papers containing "conjugated linoleic acid" as a keyword (Web of Science, Thomson Scientific, June 2016). Polyunsaturated fatty acids (PUFAs) typically contain *cis* double bonds that are separated by an interceding carbon (methylene group), as linoleic acid (cis9,cis12 18:2) is represented in Fig 12A. However, CLAs are a mixture of positional and geometric **isomers of linoleic acid** and the double bonds are adjacent with no interceding methylene group (Fig 12B,C). Furthermore, the geometric orientation of the conjugated double bonds may be *cis*-*trans*, *cis*-*cis*, *trans*-*cis*, or *trans*-*trans*, and the position at 7, 8, 9, 10, 11, 12, or 13, as found in natural products. These double bonds reduce the oxidative stability of CLA, which may result in a decrease in the nutritional quality and the development of strange flavors (Campbell, Drake, & Larick, 2003).

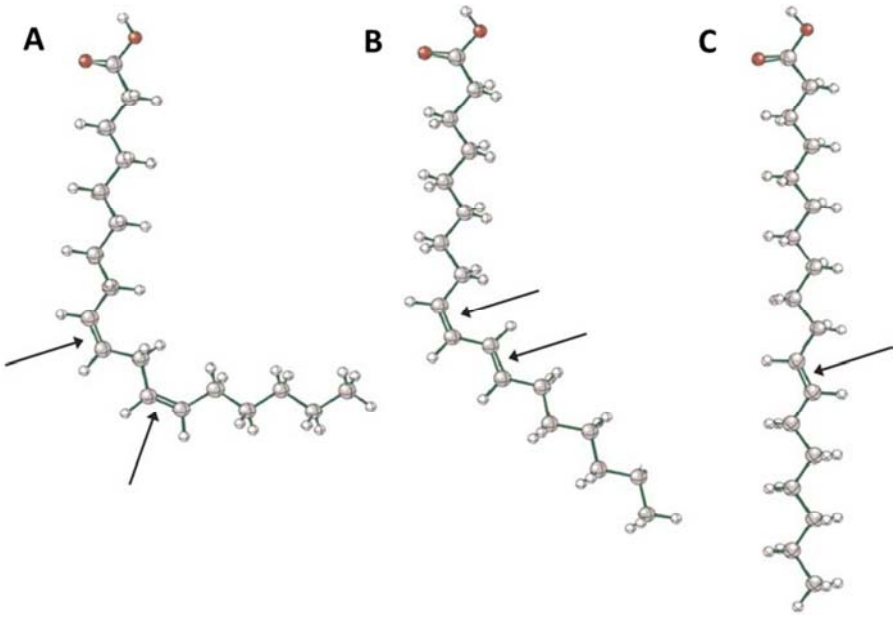

Figure 12. Comparison of the structures of (A) linoleic acid (*cis*-9, *cis*-12 18:2), (B) *cis*-9, *trans*-11 conjugated linoleic acid or rumenic acid, and (C) *trans*-11 18:1 conjugated linoleic acid or vaccenic acid. Double bonds are denoted by arrows (Bauman & Lock, 2006).

The **origin** of CLA is related to rumen fermentation of dietary PUFAs. Thus, ruminant-derived foods are the major dietary source of CLA, with dairy products and ruminant meats representing over 90% of human intake (Kim, Kim, Kim, & Park, 2016). The c9,t11-CLA isomer is the more present in foodstuff; however, when CLA is prepared from linoleic acid using chemical processes, a significant amount of t10,c12-CLA is formed along with the other main isomer, c9,t11-CLA, owing to chemical stability. Chemically prepared CLA formulations typically contain a proportion of 1:1 of both isomers, and different predominant health effects have been attributed for each isomer (Bhattacharya, Banu, Rahman, Causey, & Fernandes, 2006). Along with its original discovery as an anticancer component (Ha, Grimm, & Pariza, 1987), CLA has been shown to prevent the development of atherosclerosis, reduce body fat while improving lean body mass, and modulate immune and/or inflammatory responses (Koba & Yanagita, 2013).

In the U.S., CLA status was approved as GRAS (generally recognized as safe) in 2008. However, EFSA regulation has discrepancies with FDA (U.S.), as it addressed an unfavourable opinion on **health claims** in relation to CLA isomers and contribution to the maintenance or achievement of a normal body weight, increase in lean body mass, increase in insulin sensitivity, protection of DNA, proteins and lipids from oxidative damage, and contribution to immune defences by stimulation of production of protective antibodies in response to vaccination (EFSA Panel, 2010). To date, last negative opinion has been given to Clarinol® and Tonalin® (both are an equimolar mixture of the CLA isomers c9,t11 and t10,c12), as they tried to get the following claim effect accepted: “contributes to a reduction in body fat mass” (EFSA Panel, 2015).

4.2. Designing CLA delivery systems

Milk has a great potential for naturally fortification of CLA, as fatty acid composition depend on cow’s diet. In fact, it has been observed that milk from cows grazed or fed fresh forage, especially from species-rich grasslands or forage legumes, has a considerably higher content of vaccenic acid (t11 18:1-CLA) than milk from cows fed silage or hay (Kalač & Samková, 2010). As an example of naturally enriched milks in the

market, Feiraco Unicla® (0.04 g CLA/100 mL) was the result of a research project conducted in collaboration with Galician universities[11]. Various milk processing showed a differential effect on CLA preservation, which was mainly affected by high temperature (121 °C, 15 min), leading to higher concentrations of t9,t11-CLA (Rodríguez-Alcalá, Alonso, & Fontecha, 2014). In this study, this effect was more evident when cow milk was naturally enriched with PUFA. Other researchers applied UHPH (0-350 MPa) in different milks (cow, goat and ewe) and the absent of significant modifications on the naturally CLA isomers profile were reported (Rodríguez-Alcalá et al., 2009), which showed the potential of UHPH on preserving CLA (<1 g CLA/100 mL), as well as for emulsion stabilization and milk sanitization (pasteurization or sterilization). In addition, enriched CLA milk was treated with pressure-assisted thermal sterilization that simultaneously applied high pressure (100, 350 and 600 MPa) and high temperature (60, 90 and 120 °C) until different treatment times (0-14 min), and it was found that after 14 min at 100 MPa, at least 80% of CLA was retained, regardless of temperature used (Martínez-Monteagudo, Saldaña, Torres, & Kennelly, 2012). Similar results were also observed after 14 min at 350 MPa when 60 or 90 °C were applied.

However, CLA concentrations naturally present or fortified by animal's diet are not in the range of what is necessary to deliver a sufficient amount to be nutritionally significant: ~3 g/day (Kim et al., 2016). In this sense, commercially products are available that provide similar daily doses. As an example of CLA evaluation during refrigerated storage, various commercial skim milk dairy products fortified with CLA-Tonalin® (milk, milk powder, fermented milk, yogurt, fresh cheese, and milk-juice blend), containing 1.2 g/serving, were tested (Rodríguez-Alcalá & Fontecha, 2007). In this study, the major isomers (c9,t11-CLA and t10,c12-CLA) were not significantly affected by processing but a decrease in total CLA in fresh cheese samples was detected after 10 weeks. Nevertheless, refrigerated storage and, particularly, thermal treatment resulted in significant decreases of some of the minor CLA isomers and a significant increase of *trans*,*trans*-CLA isomers. However, Campbell et al. (2003) found a significant loss of c9,t11-CLA after high-temperature short-time (HTST) pasteurization

[11] http://feiraco.es/en/productos/leche-feiraco-unicla/

of 2% CLA-fortified skim milk, along with a significant decrease after 3 weeks of refrigerated storage. In this work, such a loss in CLA was attributed to the heat processing and to excessive microbial growth during the storage of the milk samples. Thus, there has been an increasing interest to investigate a variety of approaches to deliver CLA, as an alternative of fortification of products.

CLA has very **low water solubility** and therefore should not be incorporated into aqueous based food and beverage products. Instead, it has to be encapsulated within colloidal delivery systems, such as O/W emulsions. However, different aqueous formulations containing CLA are found in the market. As an example, Stepan Specialty Products LLC comercializes a skim UHT-milk chocolate drink with Clarinol® G-80 (triglyceride form), corresponding to 3 g CLA isomers per serving[12]. Interestingly, it also provides Clarinol Emulsion® for food and beverage applications, which is a micro-encapsulated form of Clarinol G-80 oil (almost 9%), although the processing and the type of delivery system remain unknown. Most of the literature reviewed has focused on CLA preservation by spray-dried microcapsules (Choi, Ryu, Kwak, & Ko, 2010; Costa et al., 2015; He et al., 2016; Jimenez, Garcia, & Beristain, 2008); however, CLA shows very poor chemical stability during thermal processing and can decompose to furan fatty acids in the presence of air (Buhrke, Merkel, Lengler, & Lampen, 2012). These compounds may exert toxicological properties, but also antioxidant (Spiteller, 2005), so that spray-drying may not be a suitable processing (Gao et al., 2014). In this sense, Gao et al. (2014) successfully developed soy lipophilic protein nanoparticles as a novel delivery vehicle for CLA. O/W beverage CLA-emulsion containing acacia gum and xanthan gum was also investigated, showing the onset of oxidation after 28 days of storage at 50 °C (TBA = 3.75) (Nasrabadi, Goli, & nasirpour, 2015).

Additionally, CLA is used in its triglyceride form (CLA-TG) owing the better taste than CLA in a free fatty acid from (CLA-FFA), which shows more rancidity and its commonly supplied in capsules. However, one might think that bioavailability of CLA-FFA might be higher than CLA-TG. Recently, lecithin-nanoemulsification of CLA-FFA enhanced the antiobesity effecs in rats (Kim, Park, Kweon, & Han, 2013), and recent evidence confirms the role of improved *in vivo* bioavailability of those nanoemulsions (Heo, Kim,

[12] http://www.acclaimdrink.com/

Pan, & Kim, 2016). Heo et al. (2016) also compared both CLA forms (CLA-TG and CLA-FFA) and no differences in bioavailability were observed between nanoemulsions, although it is important to note that they exhibit different particle size: 70 and 230 nm, respectively. With all the scientific and technological progress in the design of O/W emulsions, it is imperative that these food structures can be sustainable and produced by economically viable processing operations that can assure their stability during storage, such as UHPH.

5. References

Aachary, A. A., & Thiyam, U. (2012). A pursuit of the functional nutritional and bioactive properties of canola proteins and peptides. *Critical Reviews in Food Science and Nutrition*, *52*(11), 965–79.

Addo, C. N. A., & Ferragut, V. (2015). Evaluating the Ultra-High Pressure Homogenization (UHPH) and Pasteurization effects on the quality and shelf life of donkey milk. *International Journal of Food Studies, 4*(1).

Alasalvar, C., & Bolling, B. W. (2015). Review of nut phytochemicals, fat-soluble bioactives, antioxidant components and health effects. *The British Journal of Nutrition*, *113 Suppl* , S68–78.

Amador-Espejo, G. G., Suàrez-Berencia, A., Juan, B., Bárcenas, M. E., & Trujillo, A. J. (2014). Effect of moderate inlet temperatures in ultra-high-pressure homogenization treatments on physicochemical and sensory characteristics of milk. *Journal of Dairy Science*, *97*(2), 659–71.

Arranz, E., Corredig, M., & Guri, A. (2016). Designing food delivery systems: challenges related to the in vitro methods employed to determine the fate of bioactives in the gut. *Food Funct (In press).*

Bauman, D. E., & Lock, A. L. (2006). Conjugated Linoleic Acid: Biosynthesis and Nutritional Significance. In *Advanced Dairy Chemistry Volume 2 Lipids* (pp. 93–136). Boston, MA: Springer US.

Benzaria, A., Gràcia-Julià, A., Picart-Palmade, L., Hue, P., Chevalier-Lucia, D., Marti-Mestres, G., … Dumay, E. (2014). UHPH-processed O/W submicron emulsions stabilised with a lipid-based surfactant: physicochemical characteristics and behaviour on in vitro TC7-cell monolayers and ex vivo pig's ear skin. *Colloids and Surfaces. B, Biointerfaces*, *116*, 237–46.

Benzaria, A., Maresca, M., Taieb, N., & Dumay, E. (2013). Interaction of curcumin with phosphocasein micelles processed or not by dynamic high-pressure. *Food Chemistry*, *138*(4), 2327–2337.

Berton-Carabin, C. C., Ropers, M.-H., & Genot, C. (2014). Lipid Oxidation in Oil-in-Water Emulsions: Involvement of the Interfacial Layer. *Comprehensive Reviews in Food Science and Food Safety*, *13*(5), 945–977.

Bhattacharya, A., Banu, J., Rahman, M., Causey, J., & Fernandes, G. (2006). Biological effects of conjugated linoleic acids in health and disease. *The Journal of Nutritional Biochemistry*, *17*(12), 789–810.

Buhrke, T., Merkel, R., Lengler, I., & Lampen, A. (2012). Absorption and metabolism of cis-9, trans-11-CLA and of its oxidation product 9,11-furan fatty acid by Caco-2 cells. *Lipids*, *47*(4), 435–442.

Campbell, W., Drake, M. A., & Larick, D. K. (2003). The impact of fortification with conjugated linoleic acid (CLA) on the quality of fluid milk. *Journal of Dairy Science*, *86*(1), 43–51.

Chevalier-Lucia, D., Blayo, C., Gràcia-Julià, A., Picart-Palmade, L., & Dumay, E. (2011). Processing of phosphocasein dispersions by dynamic high pressure: Effects on the dispersion physico-chemical characteristics and the binding of α-tocopherol acetate to casein micelles. *Innovative Food Science & Emerging Technologies*, *12*(4), 416–425.

Chevalier-Lucia, D., Cortés-Muñoz, M., Picart-Palmade, L., & Dumay, E. (2009). Submicron emulsions processed by ultra-high pressure homogenization. *High Pressure Research*, *29*(4), 732–738.

Chi, E. Y., Krishnan, S., Randolph, T. W., & Carpenter, J. F. (2003). Physical Stability of Proteins in Aqueous Solution: Mechanism and Driving Forces in Nonnative Protein Aggregation. *Pharmaceutical Research*, *20*(9), 1325–1336.

Choi, K.-O., Ryu, J., Kwak, H.-S., & Ko, S. (2010). Spray-dried conjugated linoleic acid encapsulated with Maillard reaction products of whey proteins and maltodextrin. *Food Science and Biotechnology*, *19*(4), 957–965.

Clegg, P. S., Tavacoli, J. W., & Wilde, P. J. (2016). One-step production of multiple emulsions: microfluidic, polymer-stabilized and particle-stabilized approaches. *Soft Matter*, *12*(4), 998–1008.

Codina-Torrella, I. (2014). Optimización del proceso de elaboración y aplicación de la homogenización a ultra alta presión como tecnología de conservación de licuado de chufa. Thesis (Bellaterra): Universitat Autònoma de Barcelona.

Considine, T., Patel, H. A., Singh, H., & Creamer, L. K. (2007). Influence of binding conjugated linoleic acid and myristic acid on the heat- and high-pressure-induced unfolding and aggregation of β-lactoglobulin B. *Food Chemistry*, *102*(4), 1270–1280.

Cortés-Muñoz, M., Chevalier-Lucia, D., & Dumay, E. (2009). Characteristics of submicron emulsions prepared by ultra-high pressure homogenisation: Effect of chilled or frozen storage. *Food Hydrocolloids*, *23*(3), 640–654.

Corzo-Martínez, M., Mohan, M., Dunlap, J., & Harte, F. (2015). Effect of ultra-high pressure homogenization on the interaction between bovine casein micelles and ritonavir. *Pharmaceutical Research*, *32*(3), 1055–71.

Costa, A. M. M., Nunes, J. C., Lima, B. N. B., Pedrosa, C., Calado, V., Torres, A. G., &

Pierucci, A. P. T. R. (2015). Effective stabilization of CLA by microencapsulation in pea protein. *Food Chemistry*, *168*, 157–66.

Cruz, N., Capellas, M., Hernández, M., Trujillo, A. J., Guamis, B., & Ferragut, V. (2007). Ultra high pressure homogenization of soymilk: Microbiological, physicochemical and microstructural characteristics. *Food Research International*, *40*(6), 725–732.

Degner, B. M., Chung, C., Schlegel, V., Hutkins, R., & McClements, D. J. (2014). Factors Influencing the Freeze-Thaw Stability of Emulsion-Based Foods. *Comprehensive Reviews in Food Science and Food Safety*, *13*(2), 98–113.

Dickinson, E. (2004). Food emulsions. Stig E. Friberg, Kare Larsson and Johan Sjoblom (Eds.). New York: Marcel Dekker Inc.

Diels, A. M. J., Callewaert, L., Wuytack, E. Y., Masschalck, B., & Michiels, C. W. (2005). Inactivation of Escherichia coli by high-pressure homogenisation is influenced by fluid viscosity but not by water activity and product composition. *International Journal of Food Microbiology*, *101*(3), 281–91.

Diels, A. M. J., & Michiels, C. W. (2006). High-pressure homogenization as a non-thermal technique for the inactivation of microorganisms. *Critical Reviews in Microbiology*, *32*(4), 201–16.

Donsì, F., Annunziata, M., & Ferrari, G. (2013). Microbial inactivation by high pressure homogenization: Effect of the disruption valve geometry. *Journal of Food Engineering*, *115*(3), 362–370.

Donsì, F., Ferrari, G., & Maresca, P. (2009). CHAPTER 19 – High-Pressure Homogenization for Food Sanitization. In *Global Issues in Food Science and Technology* (pp. 309–352).

Donsì, F., Sessa, M., & Ferrari, G. (2012). Effect of Emulsifier Type and Disruption Chamber Geometry on the Fabrication of Food Nanoemulsions by High Pressure Homogenization. *Industrial & Engineering Chemistry Research*, *51*(22), 7606–7618.

Ducel, V., Richard, J., Popineau, Y., & Boury, F. (2004). Adsorption kinetics and rheological interfacial properties of plant proteins at the oil-water interface. *Biomacromolecules*, *5*(6), 2088–2093.

Dumay, E., Chevalier-Lucia, D., Picart-Palmade, L., Benzaria, A., Gràcia-Julià, A., & Blayo, C. (2013). Technological aspects and potential applications of (ultra) high-pressure homogenisation. *Trends in Food Science & Technology*, *31*(1), 13–26.

Durand, E., Zhao, Y., Coupland, J. N., & Elias, R. J. (2015). Assessing Interactions between Lipophilic and Hydrophilic Antioxidants in Food Emulsions. *Journal of Agricultural and Food Chemistry*, *63*(49), 10655–61.

EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies) (2010). Scientific Opinion on the substantiation of health claims related to conjugated linoleic acid (CLA) isomers and contribution to the maintenance or achievement of a normal body weight, increase in lean body mass… *EFSA Journal*, 8(10):1794.

EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies) (2011). Guidance on the risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain. *EFSA Journal*, 9(5):2140.

EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies) (2015). Scientific Opinion on the substantiation of a health claim related to an equimolar mixture of the CLA isomers c9,t11 and t10,c12 (marketed as Clarinol® and Tonalin®) and "contributes to a reduction in body fat mass" pursuant to Article 13(5) of Regulation. *EFSA Journal*, *13*(1):3953.

Hebishy, E. H. (2013). 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. Universitat Autònoma de Barcelona. Thesis (Bellaterra): Universitat Autonòma de Barcelona.

Ferragut V, Hernández-Herrero M, Poliseli F, Valencia D, Guamis B. (2011). Ultra high pressure homogenization (UHPH) treatment of vegetable milks: improving hygienic and colloidal stability. In Yanniotis S, Taoukis P, Stoforos NG, Karathanos VT, (Eds). *Proceedings of the 11th International Congress on Engineering and Food (ICEF11) - Food Process Engineering in a Changing World*, Vol. II. Athens: Cosmosware. p.1193–4.

Fernández-Ávila, C., Montes, R., Castellote, A. I., Chisaguano, A. M., Fitó, M., Covas, M. I., … López-Sabater, M. C. (2015). Fast determination of virgin olive oil phenolic metabolites in human high-density lipoproteins. *Biomedical Chromatography : BMC*, *29*(7), 1035–41.

Floury, J., Desrumaux, A., & Legrand, J. (2002). Effect of Ultra-high-pressure Homogenization on Structure and on Rheological Properties of Soy Protein-stabilized Emulsions. *Journal of Food Science*, *67*(9), 3388–3395.

Floury, J., Legrand, J., & Desrumaux, A. (2004). Analysis of a new type of high pressure homogeniser. Part B. study of droplet break-up and recoalescence phenomena. *Chemical Engineering Science*, *59*(6), 1285–1294.

Forum, Institute of Medicine (US) Food Forum (2009). Application of Nanotechnology to Food Products. USA: National Academies Press.

Gao, Z.-M., Zhu, L.-P., Yang, X.-Q., He, X.-T., Wang, J.-M., Guo, J., … Yin, S.-W. (2014). Soy lipophilic protein nanoparticles as a novel delivery vehicle for conjugated linoleic acid. *Food & Function*, *5*(6), 1286–93.

Gaulin A (1899). System for intimately mixing milk. USA Patent Application 125440.

García-Moreno, P. J., Guadix, A., Guadix, E. M., & Jacobsen, C. (2016). Physical and oxidative stability of fish oil-in-water emulsions stabilized with fish protein hydrolysates. *Food Chemistry*, *203*, 124–35.

Genot, C., Meynier, A., & Riaublanc, A. (2003). Lipid oxidation in emulsions. In A. Kamal-Eldin (Ed.), Lipid oxidation pathways (pp. 190–244). Illinois: AOCS Press.

Georget, E., Miller, B., Aganovic, K., Callanan, M., Heinz, V., & Mathys, A. (2014). Bacterial spore inactivation by ultra-high pressure homogenization. *Innovative Food Science & Emerging Technologies*, *26*, 116–123.

Georget, E., Miller, B., Callanan, M., Heinz, V., & Mathys, A. (2014). (Ultra) high pressure homogenization for continuous high pressure sterilization of pumpable foods - a review. *Frontiers in Nutrition*, *1*, 15.

Gharsallaoui, A., Cases, E., Chambin, O., & Saurel, R. (2009). Interfacial and emulsifying characteristics of acid-treated pea protein. *Food Biophysics*, *4*(4), 273–280.

González-Ferrero, C., & Sáiz-Abajo, M.-J. (2015). Characterization and stability studies of bioactive compounds and food matrices as evidence in support of health claims. *International Journal of Food Sciences and Nutrition*, *66 Suppl 1*, S4–S12.

Guang, C., Phillips, R. D., & Shang, J. (2012). Functional and nutritional properties of peanut and cowpea proteins. *Journal of Food, Agriculture and Environment, 10, 19*–25.

Guasch-Ferré, M., Babio, N., Martínez-González, M. A., Corella, D., Ros, E., Martín-Peláez, S., … Salas-Salvadó, J. (2015). Dietary fat intake and risk of cardiovascular disease and all-cause mortality in a population at high risk of cardiovascular disease. *The American Journal of Clinical Nutrition*, *102*(6), 1563–73.

Gueguen, J., Chevalier, M., And, J. B., & Schaeffer, F. (1988). Dissociation and aggregation of pea legumin induced by pH and ionic strength. *Journal of the Science of Food and Agriculture*, *44*(2), 167–182.

Gunstone, F. D., & Martini, S. (2010). Chemical and physical deterioration of bulk oils and shortenings, spreads and frying oils. In L. H. Skibsted, J. Risbo, & M. L. Andersen (Eds.), Chemical deterioration and physical instability of food and beverages (pp. 413–438). UK: Woodhead Publishing Limited.

Guri, A., Gülseren, I., & Corredig, M. (2013). Utilization of solid lipid nanoparticles for enhanced delivery of curcumin in cocultures of HT29-MTX and Caco-2 cells. *Food & Function*, *4*(9), 1410–9.

Ha, Y. L., Grimm, N. K., & Pariza, M. W. (1987). Anticarcinogens from fried ground

beef: heat-altered derivatives of linoleic acid. *Carcinogenesis*, *8*(12), 1881–7.

Hawkes, C. (2007). Promoting healthy diets and tackling obesity and diet-related chronic diseases: what are the agricultural policy levers? *Food and Nutrition Bulletin*, *28*(2 Suppl), S312–22.

He, H., Hong, Y., Gu, Z., Liu, G., Cheng, L., & Li, Z. (2016). Improved stability and controlled release of CLA with spray-dried microcapsules of OSA-modified starch and xanthan gum. *Carbohydrate Polymers*, *147*, 243–250.

Hebishy, E., Buffa, M., Guamis, B., Blasco-Moreno, A., & Trujillo, A.-J. (2015). Physical and oxidative stability of whey protein oil-in-water emulsions produced by conventional and ultra high-pressure homogenization: Effects of pressure and protein concentration on emulsion characteristics. *Innovative Food Science & Emerging Technologies 32, 79–90*.

Heo, W., Kim, J. H., Pan, J. H., & Kim, Y. J. (2016). Lecithin-Based Nano-emulsification Improves the Bioavailability of Conjugated Linoleic Acid. *Journal of Agricultural and Food Chemistry*, *64*(6), 1355–1360.

Herrera, M. (2012). *Analytical Techniques for Studying the Physical Properties of Lipid Emulsions*. New York: Springer.

Huang, Q., Yu, H., & Ru, Q. (2010). Bioavailability and delivery of nutraceuticals using nanotechnology. *Journal of Food Science*, *75*(1), R50–7.

Innocente, N., Marchesini, G., & Biasutti, M. (2014). Effect of high-pressure homogenisation on the retention of selected aroma compounds in model dairy emulsions. *International Journal of Food Science & Technology*, *49*(9), 1992–2000.

Jimenez, M., Garcia, H. S., & Beristain, C. I. (2008). Sensory evaluation of dairy products supplemented with microencapsulated conjugated linoleic acid (CLA). *LWT - Food Science and Technology*, *41*(6), 1047–1052.

Juliano, P., Kutter, A., Cheng, L. J., Swiergon, P., Mawson, R., & Augustin, M. A. (2011). Enhanced creaming of milk fat globules in milk emulsions by the application of ultrasound and detection by means of optical methods. *Ultrasonics Sonochemistry*, *18*(5), 963–73.

Julio, L. M., Ixtaina, V. Y., Fernández, M. A., Sánchez, R. M. T., Wagner, J. R., Nolasco, S. M., & Tomás, M. C. (2015). Chia seed oil-in-water emulsions as potential delivery systems of ω-3 fatty acids. *Journal of Food Engineering*, *162*, 48–55.

Kalač, P., & Samková, E. (2010). The effects of feeding various forages on fatty acid composition of bovine milk fat: A review. *Czech Journal of Animal Science, 55(12), 521-537*.

Kearney, J. (2010). Food consumption trends and drivers. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *365*(1554), 2793–807.

Keerati-u-rai, M., & Corredig, M. (2009). Heat-induced changes in oil-in-water emulsions stabilized with soy protein isolate. *Food Hydrocolloids*, *23*(8), 2141–2148.

Keerati-u-rai, M., & Corredig, M. (2010). Heat-Induced changes occurring in oil/water emulsions stabilized by soy glycinin and β-conglycinin. *Journal of Agricultural and Food Chemistry*, *58*(16), 9171–80.

Kim, B.-K., Cho, A.-R., & Park, D.-J. (2016). Enhancing oral bioavailability using preparations of apigenin-loaded w/o/w emulsions: in vitro and in vivo evaluations. *Food Chemistry*, *206*, 85–91.

Kim, D., Park, J.-H., Kweon, D.-J., & Han, G. D. (2013). Bioavailability of nanoemulsified conjugated linoleic acid for an antiobesity effect. *International Journal of Nanomedicine*, *8*, 451–9.

Kim, H.-J., Decker, E. A., & McClements, D. J. (2002). Impact of Protein Surface Denaturation on Droplet Flocculation in Hexadecane Oil-in-Water Emulsions Stabilized by β-Lactoglobulin. *Journal of Agricultural and Food Chemistry*, *50*(24), 7131–7137.

Kim, J. H., Kim, Y., Kim, Y. J., & Park, Y. (2016). Conjugated Linoleic Acid: Potential Health Benefits as a Functional Food Ingredient. *Annual Review of Food Science and Technology*, *7*, 221–44.

Koba, K., & Yanagita, T. (2013). Health benefits of conjugated linoleic acid (CLA). *Obesity Research & Clinical Practice, 8(6),* 525–532.

Komaiko, J., Sastrosubroto, A., & McClements, D. J. (2015). Formation of oil-in-water emulsions from natural emulsifiers using spontaneous emulsification: sunflower phospholipids. *Journal of Agricultural and Food Chemistry*, *63*(45), 10078–88.

Komaiko, J., Sastrosubroto, A., & McClements, D. J. (2016). Encapsulation of ω-3 fatty acids in nanoemulsion-based delivery systems fabricated from natural emulsifiers: Sunflower phospholipids. *Food Chemistry*, *203*, 331–9.

Kralova, I., & Sjöblom, J. (2009). Surfactants Used in Food Industry: A Review. *Journal of Dispersion Science and Technology*, *30*(9), 1363–1383.

Li, Y., Hu, M., Du, Y., Xiao, H., & McClements, D. J. (2011). Control of lipase digestibility of emulsified lipids by encapsulation within calcium alginate beads. *Food Hydrocolloids*, *25*(1), 122–130.

Li, Y., & McClements, D. J. (2011). Controlling lipid digestion by encapsulation of protein-stabilized lipid droplets within alginate–chitosan complex coacervates. *Food*

Hydrocolloids, *25*(5), 1025–1033.

Lopez, B. G., Mesa, A. J. T., Perez, V. F., Terre, J. M. Q., Pedemonte, T. J. L., & Dunat, M. N. B. (2015). Continuous system and procedure of sterilization and physical stabilization of pumpable fluids by means of an ultra-high pressure homogenization. UE Patent Application WO2012010284 A2.

Malaki, N. A., Corredig, M., & Wright, A. J. (2011). Release of lipophilic molecules during in vitro digestion of soy protein-stabilized emulsions. *Molecular Nutrition & Food Research*, *55 Suppl 2*, S278–89.

Malaki N., A., Wright, A. J., & Corredig, M. (2010). Interfacial design of protein-stabilized emulsions for optimal delivery of nutrients. *Food & Function*, *1*(2), 141–8.

Martínez-Monteagudo, S. I., Saldaña, M. D. a., Torres, J. A., & Kennelly, J. J. (2012). Effect of pressure-assisted thermal sterilization on conjugated linoleic acid (CLA) content in CLA-enriched milk. *Innovative Food Science & Emerging Technologies*, *16*, 291–297.

Martirosyan, A., & Schneider, Y.-J. (2014). Engineered nanomaterials in food: implications for food safety and consumer health. *International Journal of Environmental Research and Public Health*, *11*(6), 5720–50.

McClements, D. J. (2005). *Food Emulsions: Principles, Practice, And Techniques*. (2nd ed.). Boca Raton, FL: CRC Press.

McClements, D. J. (2012). Advances in fabrication of emulsions with enhanced functionality using structural design principles. *Current Opinion in Colloid & Interface Science*, *17*(5), 235–245.

McClements, D. J. (2013). Utilizing food effects to overcome challenges in delivery of lipophilic bioactives: structural design of medical and functional foods. *Expert Opinion on Drug Delivery*, *10*(12), 1621–32.

McClements, D. J., & Decker, E. A. (2000). Lipid Oxidation in Oil-in-Water Emulsions: Impact of Molecular Environment on Chemical Reactions in Heterogeneous Food Systems. *Journal of Food Science*, *65*(8), 1270–1282.

McClements, D. J., Decker, E. A., Park, Y., & Weiss, J. (2009). Structural design principles for delivery of bioactive components in nutraceuticals and functional foods. *Critical Reviews in Food Science and Nutrition*, *49*(6), 577–606.

McClements, D. J., Li, F., & Xiao, H. (2015). The Nutraceutical Bioavailability Classification Scheme: Classifying Nutraceuticals According to Factors Limiting their Oral Bioavailability. *Annual Review of Food Science and Technology*, *6*, 299–327.

McClements, D. J., & Li, Y. (2010). Structured emulsion-based delivery systems: controlling the digestion and release of lipophilic food components. *Advances in Colloid and Interface Science*, *159*(2), 213–28.

McClements, D. J., & Rao, J. (2011). Food-Grade Nanoemulsions: Formulation, Fabrication, Properties, Performance, Biological Fate, and Potential Toxicity. *Critical Reviews in Food Science and Nutrition 51(4),* 285–330.

Mengual, O. (1999). Turbiscan MA 2000: multiple light scattering measurement for concentrated emulsion and suspension instability analysis. *Talanta*, *50*(2), 445–456.

Mills, P., & Snabre, P. (1994). Settling of a Suspension of Hard Spheres. *Europhysics Letters (EPL)*, *25*(9), 651–656.

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., … Brodkorb, A. (2014). A standardised static in vitro digestion method suitable for food - an international consensus. *Food & Function*, *5*(6), 1113–24.

Moghimi, R., Aliahmadi, A., McClements, D. J., & Rafati, H. (2016). Investigations of the effectiveness of nanoemulsions from sage oil as antibacterial agents on some food borne pathogens. *LWT - Food Science and Technology*, *71*, 69–76.

Nasrabadi, M. N., Goli, S. A. H., & nasirpour, A. (2015). Stability assessment of conjugated linoleic acid (CLA) oil-in-water beverage emulsion formulated with Acacia and xanthan gums. *Food Chemistry*, *199*, 258–264.

Nishinari, K., Fang, Y., Guo, S., & Phillips, G. O. (2014). Soy Proteins: A review on composition, aggregation and emulsification. *Food Hydrocolloids 39,* 301–318.

Palazolo, G. G., Sobral, P. A., & Wagner, J. R. (2011). Freeze-thaw stability of oil-in-water emulsions prepared with native and thermally-denatured soybean isolates. *Food Hydrocolloids*, *25*(3), 398–409.

Pelan, B. M. C., Watts, K. M., Campbell, I. J., & Lips, A. (1997). The Stability of Aerated Milk Protein Emulsions in the Presence of Small Molecule Surfactants. *Journal of Dairy Science*, *80*(10), 2631–2638.

Pereda, J. (2009). Utilización de la Ultra Alta Presión por Homogenización como alternativa al tratamiento de pasteurización para la obtención de leche de consumo. (Thesis) Bellaterra: Universitat Autònoma de Barcelona.

Pereda, J., Ferragut, V., Buffa, M., Guamis, B., & Trujillo, A. J. (2008a). Proteolysis of ultra-high pressure homogenised treated milk during refrigerated storage. *Food Chemistry*, *111*(3), 696–702.

Pereda, J., Ferragut, V., Quevedo, J. M., Guamis, B., & Trujillo, A. J. (2007). Effects of

ultra-high pressure homogenization on microbial and physicochemical shelf life of milk. *Journal of Dairy Science*, *90*(3), 1081–93.

Pereda, J., Ferragut, V., Quevedo, J. M., Guamis, B., & Trujillo, A. J. (2008b). Effects of ultra-high-pressure homogenization treatment on the lipolysis and lipid oxidation of milk during refrigerated storage. *Journal of Agricultural and Food Chemistry*, *56*(16), 7125–30.

Pereda, J., Ferragut, V., Quevedo, J. M., Guamis, B., & Trujillo, A. J. (2009). Heat damage evaluation in ultra-high pressure homogenized milk. *Food Hydrocolloids*, *23*(7), 1974–1979.

Pereda, J., Jaramillo, D. P., Quevedo, J. M., Ferragut, V., Guamis, B., & Trujillo, A. J. (2008c). Characterization of volatile compounds in ultra-high-pressure homogenized milk. *International Dairy Journal*, *18*(8), 826–834.

Poliseli-Scopel, F. H. (2012). Study of the Ultra High Pressure Homogenization (UHPH) technology for producing high quality soymilk. (Thesis) Bellaterra: Universitat Autònoma de Barcelona.

Poliseli-Scopel, F. H., Hernández-Herrero, M., Guamis, B., & Ferragut, V. (2012). Comparison of ultra high pressure homogenization and conventional thermal treatments on the microbiological, physical and chemical quality of soymilk. *LWT - Food Science and Technology*, *46*(1), 42–48.

Poliseli-Scopel, F. H., Hernández-Herrero, M., Guamis, B., & Ferragut, V. (2013). Characteristics of soymilk pasteurized by ultra high pressure homogenization (UHPH). *Innovative Food Science & Emerging Technologies*, *20*, 73–80.

Poliseli-Scopel, F. H., Hernández-Herrero, M., Guamis, B., & Ferragut, V. (2014). Sterilization and aseptic packaging of soymilk treated by ultra high pressure homogenization. *Innovative Food Science & Emerging Technologies 22,* 81–88.

Pugnaloni, L. A., Dickinson, E., Ettelaie, R., Mackie, A. R., & Wilde, P. J. (2004). Competitive adsorption of proteins and low-molecular-weight surfactants: computer simulation and microscopic imaging. *Advances in Colloid and Interface Science*, *107*(1), 27–49.

Roach, A., & Harte, F. (2008). Disruption and sedimentation of casein micelles and casein micelle isolates under high-pressure homogenization. *Innovative Food Science & Emerging Technologies*, *9*(1), 1–8.

Roco, M. C. (2011). The long view of nanotechnology development: the National Nanotechnology Initiative at 10 years. *Journal of Nanoparticle Research*, *13*(2), 427–445.

Rodríguez-Alcalá, L. M., Alonso, L., & Fontecha, J. (2014). Stability of fatty acid composition after thermal, high pressure, and microwave processing of cow milk as affected by polyunsaturated fatty acid concentration. *Journal of Dairy Science*, *97*(12), 7307–7315.

Rodríguez-Alcalá, L. M., & Fontecha, J. (2007). Hot topic: Fatty acid and conjugated linoleic acid (CLA) isomer composition of commercial CLA-fortified dairy products: evaluation after processing and storage. *Journal of Dairy Science*, *90*(5), 2083–90.

Rodríguez-Alcalá, L. M., Harte, F., & Fontecha, J. (2009). Fatty acid profile and CLA isomers content of cow, ewe and goat milks processed by high pressure homogenization. *Innovative Food Science & Emerging Technologies*, *10*(1), 32–36.

Roesch, R. R., & Corredig, M. (2003). Texture and microstructure of emulsions prepared with soy protein concentrate by high-pressure homogenization. *LWT - Food Science and Technology*, *36*(1), 113–124.

Roig-Sagués, A. X., Velázquez, R. M., Montealegre-Agramont, P., López-Pedemonte, T. J., Briñez-Zambrano, W. J., Guamis-López, B., & Hernandez-Herrero, M. M. (2009). Fat content increases the lethality of ultra-high-pressure homogenization on Listeria monocytogenes in milk. *Journal of Dairy Science*, *92*(11), 5396–402.

Roozen, J. P., Frankel, E. N., & Kinsella, J. E. (1994). Enzymic and autoxidation of lipids in low fat foods: model of linoleic acid in emulsified hexadecane. *Food Chemistry*, *50*(1), 33–38.

Sala-Vila, A., Guasch-Ferré, M., Hu, F. B., Sánchez-Tainta, A., Bulló, M., Serra-Mir, M., … Tur, J. (2016). Dietary α-Linolenic Acid, Marine ω-3 Fatty Acids, and Mortality in a Population With High Fish Consumption: Findings From the PREvención con DIeta MEDiterránea (PREDIMED) Study. *Journal of the American Heart Association*, *5*(1).

Saldo, J., Suárez-Jacobo, Á., Gervilla, R., Guamis, B., & Roig-Sagués, A. X. (2009). Use of ultra-high-pressure homogenization to preserve apple juice without heat damage. *High Pressure Research*, *29*(1), 52–56.

Seyed, M. A., Jantan, I., Bukhari, S. N. A., & Vijayaraghavan, K. (2016). A Comprehensive Review on the Chemotherapeutic Potential of Piceatannol for Cancer Treatment, with Mechanistic Insights. *Journal of Agricultural and Food Chemistry*, *64*(4), 725–37.

Sikand, G., Kris-Etherton, P., & Boulos, N. M. (2015). Impact of functional foods on prevention of cardiovascular disease and diabetes. *Current Cardiology Reports*, *17*(6), 39.

Spiteller, G. (2005). Furan fatty acids: Occurrence, synthesis, and reactions. Are furan

fatty acids responsible for the cardioprotective effects of a fish diet? *Lipids*, *40*(8), 755–771.

Sun, C., & Gunasekaran, S. (2009). Effects of protein concentration and oil-phase volume fraction on the stability and rheology of menhaden oil-in-water emulsions stabilized by whey protein isolate with xanthan gum. *Food Hydrocolloids*, *23*(1), 165–174.

Tahiri, I., Makhlouf, J., Paquin, P., & Fliss, I. (2006). Inactivation of food spoilage bacteria and Escherichia coli O157:H7 in phosphate buffer and orange juice using dynamic high pressure. *Food Research International*, *39*(1), 98–105.

Topuz, O. K., Özvural, E. B., Zhao, Q., Huang, Q., Chikindas, M., & Gölükçü, M. (2016). Physical and antimicrobial properties of anise oil loaded nanoemulsions on the survival of foodborne pathogens. *Food Chemistry*, *203*, 117–23.

Toro Funes, N. (2014). Valor nutritivo y biofuncional de las bebidas de soja y de almendra estabilizadas por ultra alta presión de homogeneización. (Thesis) Barcelona: Universitat de Barcelona.

Trujillo, A. J., Capellas, M., Saldo, J., Gervilla, R., & Guamis, B. (2002). Applications of high-hydrostatic pressure on milk and dairy products: a review. *Innovative Food Science & Emerging Technologies*, *3*(4), 295–307.

Valencia-Flores, D. C., Hernández-Herrero, M., Guamis, B., & Ferragut, V. (2013). Comparing the effects of ultra-high-pressure homogenization and conventional thermal treatments on the microbiological, physical, and chemical quality of almond beverages. *Journal of Food Science*, *78*(2), E199–205.

Walker, R., Decker, E. A., & McClements, D. J. (2015). Development of food-grade nanoemulsions and emulsions for delivery of omega-3 fatty acids: opportunities and obstacles in the food industry. *Food & Function*, *6*(1), 42–55.

Walstra, P. (1983). Formation of emulsions. In P Becher (Ed.), *Encyclopedia of Emulsion Technology.* New York: Marcel Dekker.

Walstra, P., Smulders, P. E. A., & Binks, B. P. (1998). Emulsion Formation. In B. P. Binks (Ed.), *Modern aspects of emulsion science.* Cambridge: Royal Society of Chemistry.

Wang, J.-M.-M., Xia, N., Yang, X.-Q.-Q., Yin, S.-W.-W., Qi, J.-R.-R., He, X.-T.-T., … Wang, L.-J.-J. (2012). Adsorption and dilatational rheology of heat-treated soy protein at the oil-water interface: Relationship to structural properties. *Journal of Agricultural and Food Chemistry*, *60*(12), 3302–3310.

Waraho, T., Cardenia, V., Decker, E. A., McClements, D. J., & Elias, R. J. (2010). Lipid oxidation in emulsified food products. In E. A. Decker, R. J. Elias, & D. J. McClements

(Eds.), Oxidation in foods and beverages and antioxidant applications. Management in different industry sectors (Vol. 2, pp. 306–343). UK: Woodhead Publishing Limited.

Wierenga, P. A., Egmond, M. R., Voragen, A. G. J., & de Jongh, H. H. J. (2006). The adsorption and unfolding kinetics determines the folding state of proteins at the air-water interface and thereby the equation of state. *Journal of Colloid and Interface Science*, *299*(2), 850–7.

Wooster, T. J., Golding, M., & Sanguansri, P. (2008). Impact of oil type on nanoemulsion formation and Ostwald ripening stability. *Langmuir : The ACS Journal of Surfaces and Colloids*, *24*(22), 12758–65.

Yang, Y., Leser, M. E., Sher, A. A., & McClements, D. J. (2013). Formation and stability of emulsions using a natural small molecule surfactant: Quillaja saponin (Q-Naturale®). *Food Hydrocolloids*, *30*(2), 589–596.

Ye, R., & Harte, F. (2014). High pressure homogenization to improve the stability of casein - hydroxypropyl cellulose aqueous systems. *Food Hydrocolloids*, *35*, 670–677.

Zamora, A., Ferragut, V., Jaramillo, P. D., Guamis, B., & Trujillo, A. J. (2007). Effects of ultra-high pressure homogenization on the cheese-making properties of milk. *Journal of Dairy Science*, *90*(1), 13–23.

Zamora, A., & Guamis, B. (2014). Opportunities for Ultra-High-Pressure Homogenisation (UHPH) for the Food Industry. *Food Engineering Reviews*, *7*(2), 130–142.

Zayas, J. F. (2012). *Functionality of Proteins in Food*. New York: Springer Science & Business Media.

CHAPTER 2: Statement of the problem, objectives and working plan

1. Statement of the problem

Nowadays, conventional or low-homogenized O/W emulsions (>1 µm) have poor physicochemical and oxidative stability; therefore, many efforts on novel technologies development have been employed to overcome this problem. UHPH technology has been applied in different food emulsions such as milk and vegetable beverages in order to improve physical stability of such emulsions, and has been suggested as an alternative to thermal pasteurization, due to its effect on inactivating pathogenic and other food spoilage microorganisms.

Research on delivery of bioactive compounds by emulsion-based systems has increased over the last 10 years and different formulations and technologies have been studied. For those emulsion-based delivery systems, *in vitro* methods employed to determine the fate of bioactives in the gut have been widely applied, in comparison to *in vivo* methods. Moreover, considerably more work will need to be done to determine the potential toxicology of nanoemulsions (<100 nm). Nonetheless, it would be interesting to assess the effects of UHPH-submicron emulsions (<1 µm) on more functional ingredients and their possibly interactions with the other constituents to further improve their nutritional, physicochemical and sensory characteristics.

2. Objectives

2.1 Main objective

To assess the physico-chemical stability of O/W emulsions treated by ultra-high pressure homogenization (UHPH) and conventional homogenization (CH) for their incorporation in a UHT milk-based product and delivery of conjugated linoleic acid.

2.2 Specific objectives

- ✓ To investigate the effect of UHPH (100-300 MPa) compared to CH (15 MPa) on physical stability of soybean oil (10 and 20%) emulsions stabilized by soy protein isolate (SPI, 4%). *(Experiment 1).*

- ✓ To evaluate the effect of UHPH (100-300 MPa) compared to CH (15 MPa) on oxidative stability and interfacial properties of soybean oil (10 and 20%) emulsions stabilized by SPI (4%). *(Experiment 2).*
- ✓ To determine whether complete denaturation of SPI before CH treatment would similarly affect the emulsions characteristics or improve the physical and oxidative stability. *(Experiment 1-2).*
- ✓ To study the effect of different conventional O/W emulsions (14% soybean oil) stabilized by soy and pea protein isolates (SPI and PPI, 4%) and homogenized for 1 and 5 passes on the efficiency of CLA (6%) encapsulation, its release and bioefficacy using in vitro digestion and a Caco-2 intestinal cell absorption model. *(Experiment 3).*
- ✓ To investigate the microbial and physicochemical stability of the UHPH emulsion (200 MPa) with 20% of oil which exhibited the best physical and oxidative stability from Experiments 1-2 and containing CLA (6%), compared to CH (15 MPa), for delivering conjugated linoleic acid in Caco-2 cells. *(Experiment 4).*
- ✓ To assess the effect of emulsions from *Experiment 4*, incorporated in a liquid milk-based product for further UHT treatment and aseptic packaging, on physicochemical stability and sensory properties of the final product during storage (4 months). *(Experiment 5).*

3. Working plan

According to the objectives, Figure 1 schematically represents the experimental design and Figures 2-4 the analysis of all the experiments conducted in this thesis.

The first stage of the present work was to experiment with the effect of different pressure treatments for UHPH (100, 200 and 300 MPa) and volume oil fraction (10 and 20%) on physical and oxidative stability, along with a fixed protein concentration (SPI, 4%). Additionally, CH was tested as a control.

Once the best pressure (200 MPa) and oil content (20%) were determined, the second stage of the thesis consisted in studying the delivery of a lipophilic compound (CLA)

after *in vitro* digestion and further uptake in Caco-2 cells, with different emulsion-based systems (UHPH and CH).

Finally, in the third stage of the present study, 2 of those emulsion-based systems (UHPH and CH) were chosen to be incorporated in a UHT milk-based product, and study its stability and consumer acceptance during storage.

Dispersed at 250 rpm for 1 h at 25 °C (**Experiments 1-5**), and another batch heated at 95 °C for 5 min (**Experiments 1-2**)	Stored overnight at 4 °C (**Experiments 1-5**)	Dispersed using a rotor-stator emulsifying unit at 15.000 rpm (**Experiments 1-5**)	100, 200, and 300 MPa (single-stage) and 15 MPa (**Experiments 1-2**) 55 MPa for 1 and 5 passes using (**Experiment 3**) 200 MPa (single-stage) and 15 MPa (**Experiments 4-5**)	Applied high-temperature-short-time conditions (72 °C for 20 s; CH-HTST emulsion) (**Experiment 4**)	1:10 (emulsion: skim milk) to obtain fat content of 2% in the final product (**Experiment 5**)	140 °C for 4 s (**Experiment 5**)	Aseptically packed in 200 mL Tetra Brick aseptic slim containers (**Experiment 5**)

Figure 1. Emulsions and final milk-based **product procedure**. The formulation ingredients of emulsions were as follows: 4% soy protein isolate (SPI) and 20% soybean oil (**Experiments 1-2**), 4% soy protein isolate (SPI) and pea protein isolate (PPI) and 14% soybean oil and 6% CLA (**Experiment 3**) and 4% SPI and 14% soybean oil and 6% CLA (**Experiments 4-5**).

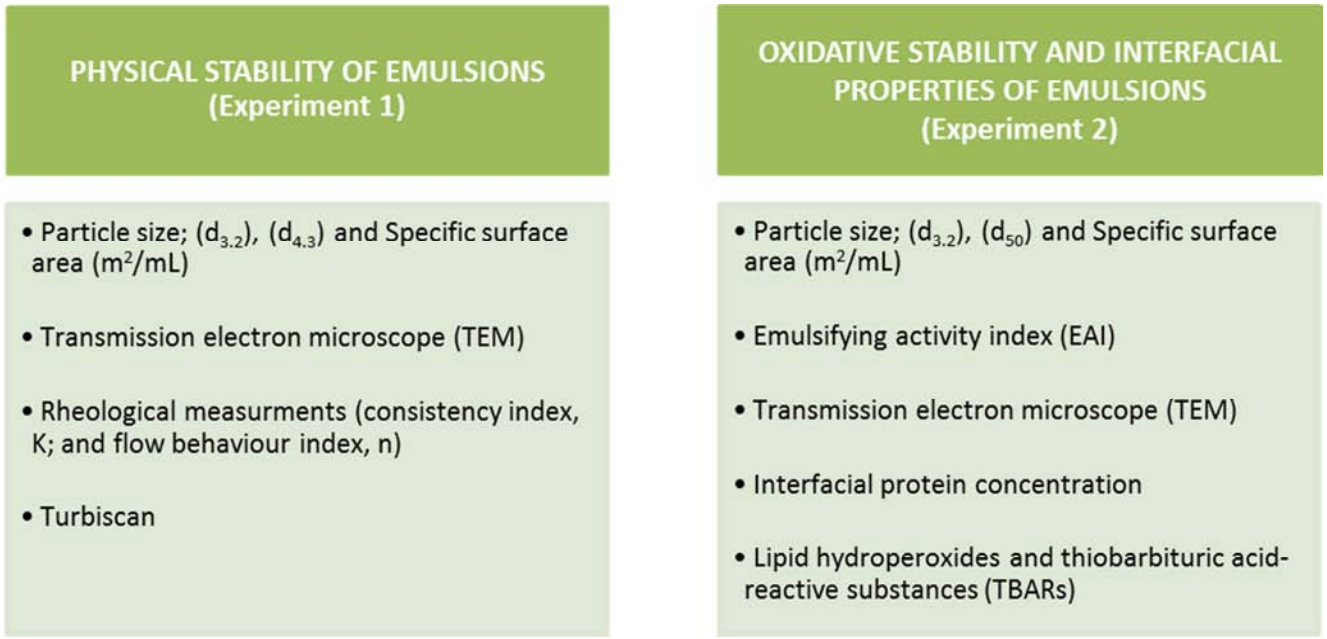

Figure 2. Experimental analysis of **Experiments 1 and 2**.

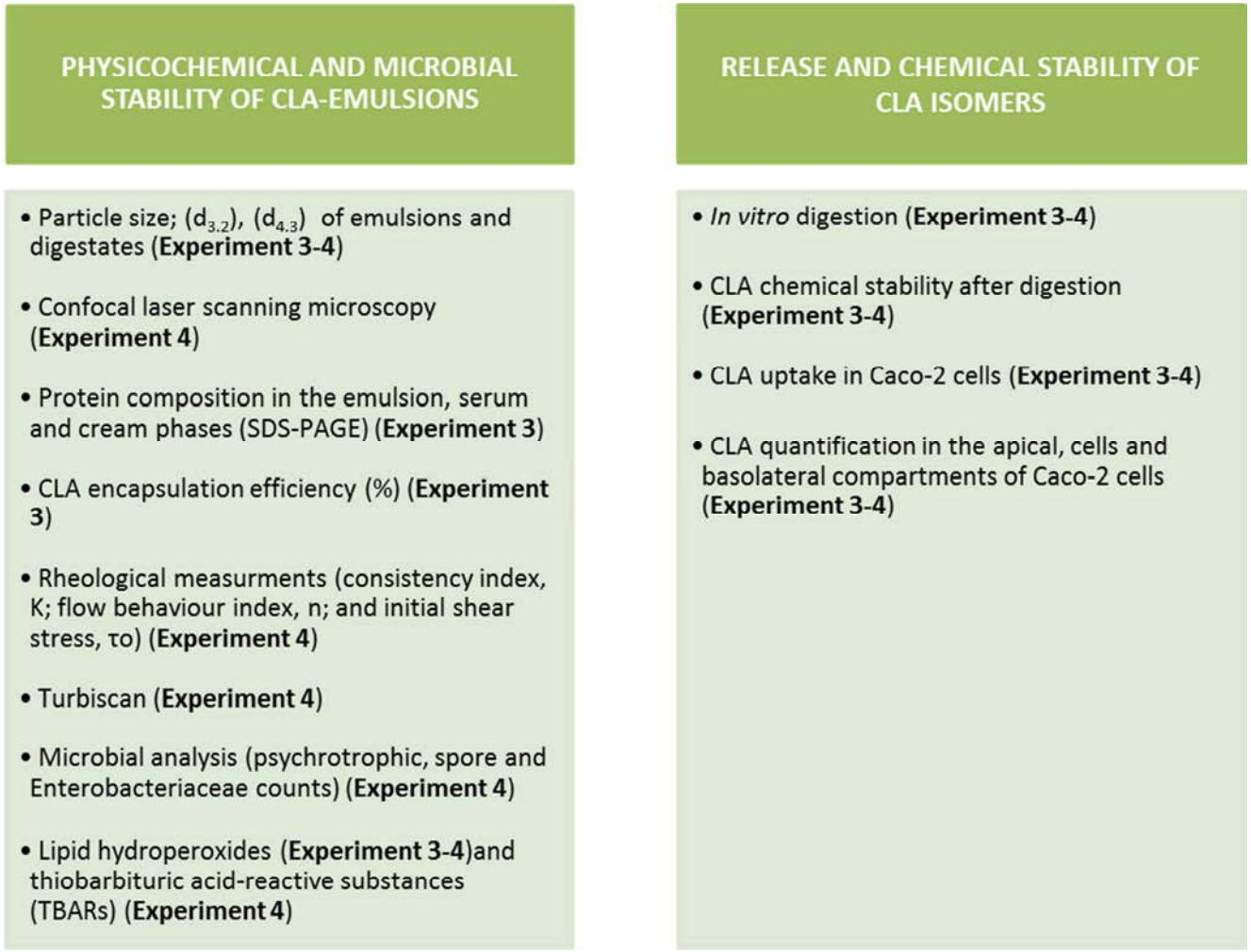

Figure 3. Experimental analysis of **Experiments 3 and 4**.

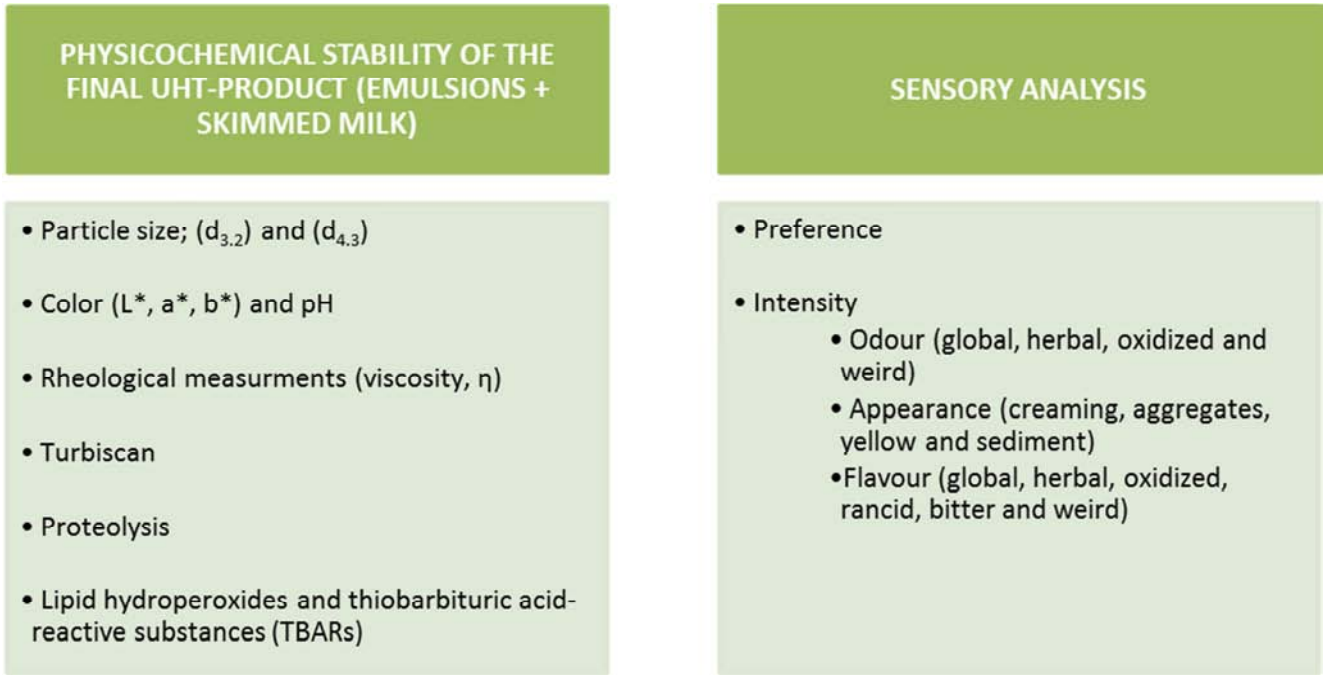

Figure 4. Experimental analysis of **Experiment 5**.

CHAPTER 3: Ultra-High Pressure Homogenization enhances physicochemical properties of soy protein isolate-stabilized emulsions

[Experiment 1]

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Ultra-High Pressure Homogenization enhances physicochemical properties of soy protein isolate-stabilized emulsions

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ABSTRACT

The effect of Ultra-High Pressure Homogenization (UHPH, 100–300 MPa) on the physicochemical properties of oil-in-water emulsions prepared with 4.0% (w/v) of soy protein isolate (SPI) and soybean oil (10 and 20%, v/v) was studied and compared to emulsions treated by conventional homogenization (CH, 15 MPa). CH emulsions were prepared with non-heated and heated (95 °C for 15 min) SPI dispersions. Emulsions were characterized by particle size determination with laser diffraction, rheological properties using a rotational rheometer by applying measurements of flow curve and by transmission electron microscopy. The variation on particle size and creaming was assessed by Turbiscan® analysis, and visual observation of the emulsions was also carried out. UHPH emulsions showed much smaller $d_{3,2}$ values and greater physical stability than CH emulsions. The thermal treatment of SPI prior CH process did not improve physical stability properties. In addition, emulsions containing 20% of oil exhibited greater physical stability compared to emulsions containing 10% of oil. Particularly, UHPH emulsions treated at 100 and 200 MPa with 20% of oil were the most stable due to low particle size values ($d_{3,2}$ and Span), greater viscosity and partial protein denaturation. These results address the physical stability improvement of protein isolate-stabilized emulsions by using the emerging UHPH technology.

1. Introduction

An emulsion consists of two immiscible liquids (usually oil and water), with one of the liquids dispersed as small spherical droplets in the other. An oil-in-water (o/w) emulsion consists of oil droplets dispersed in an aqueous phase (McClements, 2005). Emulsifiers, such as proteins, can act to form viscoelastic films around dispersed oil droplets to improve physical stability. By physical stability, the ability of an emulsion to resist destabilization phenomena caused by particle migration or modification of particle size is meant (Herrera, 2012). An example of emulsifier is protein obtained from soybean. Soybean is the dominant oilseed in world markets, which comes from a variety of factors, including favorable agronomic and economic characteristics and high-quality of protein and edible oil products (Wilson, 2008). Soy protein isolate (SPI), which contains glycinin (11S) and β-conglycinin (7S) as major globulin fractions (>80%), has been widely applied in a wide range of food formulations due to their functional properties such as solubility, emulsification, foaming properties, gelation, water-binding capacity, and water-holding capacity (Nishinari, Fang, Guo, & Phillips, 2014). Manufacturers normally use commercial protein isolates. However, they exhibit poor solubility compared to other protein fractions or high-soluble isolates obtained in the laboratory due to the isolation processes (Keerati-u-rai & Corredig, 2010; Wang et al., 2012). It has been previously reported that a thermal treatment of high-soluble soy protein isolate promotes greater surface hydrophobicity due to protein unfolding, which improves surface activity (Wang et al., 2012).

In the food industry, to improve the stability of emulsions, a homogenization process is carried out using mechanical devices, which usually subject the liquids to intense mechanical agitation (e.g., high speed blenders, high-pressure valve homogenizers and colloid mills) (Perrier-Cornet, Marie, & Gervais, 2005). An emergent technology to produce fine and stable submicron emulsions (<1 μm) is Ultra-High Pressure Homogenization (UHPH) (Chevalier-Lucia, Cortés-Muñoz, Picart-Palmade, & Dumay, 2009; Dumay et al., 2013). High-homogenized emulsions are more stable physically than conventional or low-homogenized emulsions (>1 μm), mainly by the decrease in the particle size (Lee, Lefèvre, Subirade, & Paquin, 2009). Furthermore, UHPH modifies emulsion properties due to conformational changes of proteins, which results in changes of the physical stability of emulsions (Dumay et al., 2013). Besides the homogenization pressure applied, the interfacial layers formed are also affected by different conditions, such as the volume oil fraction, the nature and concentration of protein, and the temperature reached at the exit of the homogenization valve (Hebishy, 2013.; Floury, Desrumaux, & Legrand, 2002; Roesch & Corredig, 2003).

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With respect to other homogenization techniques, soy protein solutions heated (75 and 95 °C, 15 min) before emulsification by a Microfluidizer® and emulsions heated after homogenization cause protein–protein interactions, leading to changes in the supramolecular structure of the aggregates at the oil-in-water interface, which increase the viscosity (Keerati-u-rai & Corredig, 2009). Generally, the more the viscosity of emulsions, the more the physical stability in emulsions (Floury et al., 2002). Viscosity of emulsions is influenced by colloidal interaction between oil droplets, which depend on the structural organization of molecules. At molecular level, the dominant interaction energies that act can be classified into covalent, electrostatic, van der Waals, and steric overlap (Piorkowski & McClements, 2013). These energies along with some entropy effects contribute to form compound interactions, as hydrogen bonding and hydrophobic interactions (McClements, 2005). Compared to these forces, gravitational ones act over large distances at the macroscopic level, leading to reversible phenomena of sedimentation, creaming or flocculation of droplets. Another common phenomenon after flocculation is coalescence, which consists in the fusion of the oil droplets with an increase of the particle size (Petsev, 2004).

Ultra-High Pressure Homogenization, as a novel trend in food technology, could produce stable emulsions to act as carriers of biological compounds with the advantage in a reduction of the microbial load of processed products (Dumay et al., 2013). The relevance of UHPH treatment compared to conventional processes, by applying a single stage of homogenization, is clearly supported by the improvement to emulsion physical and microbiology stabilities in stock before being sold. Actually, a recent research at our laboratory has allowed to develop aseptically packaged beverages by UHPH-processing at 300 MPa (Amador-Espejo, Suàrez-Berencia, Juan, Bárcenas, & Trujillo, 2014; Poliseli-Scopel, Hernández-Herrero, Guamis, & Ferragut, 2014).

To our knowledge, o/w emulsions stabilized with SPI and treated by UHPH have not been studied yet. For the first time, the studied composition envisaged the combination of soybean oil with soy protein isolate (SPI) as a whole (i.e. without previous extraction of any protein fraction) in emulsions treated by UHPH. The aim of this study was to investigate the effect of UHPH (100–300 MPa) compared to conventional homogenization (CH) at 15 MPa on soybean oil emulsions stabilized by SPI. The relevance of using a commercial SPI implies industrial benefits to manufacturers because it is not economical to use each extracted/separated protein fractions in food industry (Nishinari et al., 2014). Additionally, a thermal treatment was carried out on the protein solutions before CH treatment, to determine whether denaturation of proteins would affect similarly the emulsions characteristics compared to UHPH emulsions or would improve the physical stability. Droplet size distribution, flow behavior, microstructure and stability against creaming and coalescence of prepared emulsions were evaluated for various soybean oil concentrations (10 and 20%, v/v) emulsified with SPI.

2. Materials and methods

2.1. Materials

A commercial SPI (PRO-FAM 974) was purchased from Lactotecnia (Barcelona, Spain). The composition of this commercial SPI according to manufacturer was: 90% protein, 4% fat, 6% moisture, and less than 5% ash (dry basis, w/w). SPI PRO-FAM 974 has acid character and the isoelectric point is 4.6 (Kinsella, 1979) due to the high content of glutamic acid (19.2%) and aspartic acid (11.5%). Solubility of PRO-FAM 974 at pH = 7 is 39.5% (Bissegger, 2007). Soybean oil was purchased from Gustav Heess (Barcelona, Spain). Peroxide value of the soybean oil was below 10 meq O_2/kg and the acidity index was below 0.5 mg KOH/g.

All other chemicals used were of analytical or better grade.

2.2. Preparation of oil-in-water emulsions

Oil-in-water emulsions were prepared with a fixed content of SPI (4.0%, w/v) and different contents of soybean oil (10 and 20%, v/v). Firstly, the stock protein dispersion (4.0%, w/v) was prepared by dispersing SPI in deionized water by using a high-speed dispersing unit at a rate of about 250 rpm for 1 h at 25 °C. The heated SPI dispersions were prepared by heating the stock dispersion at 95 °C for 15 min to allow complete denaturation of soy protein (Keerati-u-rai & Corredig, 2009), and then cooled immediately in an ice bath to room temperature. Protein dispersions were stored overnight at 4 °C to allow complete hydration. Protein dispersions and oil were equilibrated at 20 °C (inlet temperature) before mixing. Pre-emulsions (or coarse emulsions) were prepared by mixing the non-heated or heated protein dispersions with the soy oil using a rotor-stator emulsifying unit (model Diax 900, Heidolph, Kehlheim, Germany) at 15,000 rpm for 4 min. The coarse emulsions were further homogenized through a high-pressure homogenizer or by conventional homogenization (Stansted Benchtop Homogenizator nG12500, Stansted Fluid Power Ltd., Essex, UK). The emulsions were treated at 100, 200, and 300 MPa (single-stage) by the high-pressure homogenizer (flow rate of 8–14 L/h) provided with a high-pressure ceramic needle-seat valve. The prehomogenized non-heated and heated dispersions were further homogenized at 15 MPa with a ceramic ball-seat valve (conventional homogenization treatments, single-stage). Pre-emulsions were passed through both devices with an inlet temperature (T_{in}) of 20 °C. The outlet temperature of emulsions were controlled by a heat exchanger (Inmasa, Reus, Spain) located immediately after the high-pressure valve or ball-seat valve. During treatments, the T_{in}, the temperature after the high pressure valve (T_1), and outlet temperature (T_f) were monitored.

2.3. Determination of oil droplet size distribution

The particle size distribution in the emulsion samples was determined immediately after homogenization process 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 2 g/L sodium dodecyl sulfate (SDS) at least 30 min before light scattering analyses to check for the presence of aggregated or coalesced droplets that could be dissociated by SDS (Pearce & Kinsella, 1978). The optical parameters used were: a refractive index of 1.475 for the soybean oil and a refractive index of 1.332 for the water. The Span or dispersion index defined as [d(90)–d(10) ÷ d(50)] where "d[x]" is the diameter for which x% of the oil droplets are smaller than "d", the surface-weighted mean diameter ($d_{3,2}$, μm), the volume-weighted mean diameter ($d_{4,3}$, μm), and the specific surface area (SSA, m^2/mL) were determined.

2.4. Transmission electron microscope (TEM)

In order to assess the microstructure of emulsions, transmission electron microscope was used. 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% (w/v) low-temperature gelling agarose at a 1:1 ratio in the same day of homogenization process. The mixture was allowed to gel and was chopped into 1 mm^3 cubes. The cubes were fixed using a solution of 2 % (w/v) paraformaldehyde and 2.5 % (v/v) glutaraldehyde (EM grade, Merck, Darmstadt, Germany) in phosphate buffer (PB; 0.1 M, pH 7.4; Sigma-Aldrich, Steinheim, Germany) and placed on a rocking platform for 2 h. Following fixation, the samples were washed four times with PB. Then, samples were post-fixed for 2 h with 1% (w/v) osmium tetroxide (TAAB Lab., UK) containing 0.8 % (w/v) potassium hexocyanoferrate (Sigma-Aldrich, Steinheim, Germany) prepared in PB, followed by four washes with deionized

water and sequential dehydration in acetone. Samples were embedded in Eponate 12™ resin (Ted Pella Inc., Redding, California), polymerized at 60 °C and cut with an ultramicrotome. Finally, ultrathin sections placed in copper grids were contrasted with conventional uranyl acetate and Reynolds lead citrate solutions and observed using a Jeol 1400 transmission electron microscope (Jeol Ltd, Tokyo, Japan) equipped with a GATAN ES1000W Erlangshen camera.

2.5. Rheological measurements

Rheological measurements were performed using a controlled stress rheometer (Haake Rheo Stress 1, Thermo Electron Corporation, Karlsruhe, Germany) using a cone (1°, 60 mm diameter) and plate geometry probe at 21 °C. Flow curves (shear stress compared to shear rate) were determined at increasing and decreasing shear rates between 0.001 s^{-1} and 100.0 s^{-1} in 1 min (up and down flow curves). Flow curves were fitted to the Ostwald–de Waele rheological model: $\tau = K(\gamma^{\cdot})^n$ and the consistency coefficient (K, mPa × s^n) and flow behavior index (n) were obtained. Experiments were carried out after 48 h of the homogenization process maintaining the samples in refrigeration at 4 °C.

2.6. Turbiscan measurements

The stability of emulsions was also determined through the use of the optical analyser Turbiscan MA 2000 (Formulaction, Toulouse, France). This equipment allows the optical characterization of any type of dispersion. The Turbiscan measures backscattered and transmitted light as a function of the axial tube coordinate and time by means of two synchronous optical sensors that respectively receive light transmitted through the sample and light backscattered by the sample. The light source is an electro-luminescent diode in the near infrared (λ_{air} = 850 nm). The propagation of light through a concentrated dispersion can be used to characterize the emulsion physico-chemical stability (Juliano et al., 2011). 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 Turbiscan MA 2000. In the present study, fresh emulsions treated by UHPH and CH and containing 0.02% (w/v) of sodium azide were filled into the Turbiscan tube and stored in quiescent conditions in a chamber at constant temperature at 4 °C. For CH emulsions, scans every 15 min were performed for further 2 h due to their faster destabilization's phenomenon expected. For all emulsions, scans were performed at preset intervals over a predetermined period of time (5 months). The creaming destabilization kinetics was evaluated by measuring the (Backscattering) BS profiles measured at 0 h (BS_0) from those measured at time t (BS_t); $\Delta BS = BS_t - BS_0$ (bottom and top zones) (Herrera, 2012). For emulsions that also destabilized by flocculation, the mean droplet diameter variation (d) was also performed over 5 months in the zone 15–30 mm of the tube to avoid the area affected by creaming. Visual determination of the serum layer thickness of the emulsions was also carried out.

2.7. Statistical analyses

Descriptive statistics, mean and standard deviation, were listed for each variable in this study. In order to evaluate the physical stability of emulsions among type of emulsion (CH or UHPH) and oil concentration (10 and 20%), a General Linear Model (GLM) was performed. Variables of interest related to physical stability needed to be transformed using log-transformation in order to stabilize the variance. The statistical analysis was performed using the SPSS® v17.0 package to a 95% level of significance and Tukey adjustment was performed for multiple comparisons of the means ($P < 0.05$). Experiments were repeated and performed in duplicate as separate, independent runs.

3. Results and discussion

3.1. Temperature at the exit of the valve and droplet disruption mechanisms

The temperature increase of the UHPH emulsions at the exit of the homogenization valve was proportional to the pressure applied and the oil concentration used (Table 1). A mean temperature increase of 21.0 and 22.5 °C per 100 MPa for the two oil concentrations (10 and 20% v/v, respectively) from the pressure 100 to 200 MPa with T_{in} = 20 °C was calculated, and analyzing the mean temperature increase from the pressure 200 to 300 MPa, values of 27.5 and 27.0 °C per 100 MPa were found. In similar studies (Chevalier-Lucia et al., 2009; Cortés-Muñoz, Chevalier-Lucia, & Dumay, 2009; Desrumaux & Marcand, 2002), an increase in T_1 up to 22.8 °C per 100 MPa for emulsions was found. However, CH treatments showed a similar temperature increase in the valve (T_1 = 31.6 °C) for all the emulsions treated at 15 MPa. The outlet temperature (T_f) did not exceed 25 °C in all treatments. The increase of temperature during UHPH treatment is the consequence of the short-life heating of the fluid that passes the valve gap and the high turbulence, shear, and cavitation forces were transformed into thermal energy (Cortés-Muñoz et al., 2009; Floury, Bellettre, Legrand, & Desrumaux, 2004). When the emulsion passes through the homogenizing valve it undergoes deformation, break-up of droplets, adsorption of protein at the formed interface between phases, collision, and possible recoalescence of oil droplets (Floury et al., 2002). These phenomena contribute differently to the disruption of oil droplets, in addition to the pressure to which the emulsions are exposed and to their formulation. However, depending on the flow regime (laminar or turbulent), some models to predict the extent of oil droplets disruption mechanisms have been described in the literature ($d_{3,2} \propto p^{3/2}$ or $p^{-3/4}$ or $p^{-0.9}$) (Floury, Legrand, & Desrumaux, 2004). Fig. 1 shows the results of mean droplet diameter versus homogenizing pressure obtained after UHPH treatments to adjust prediction models for oil droplets disruption mechanisms. Using this small Ultra-High Pressure Homogenizer device, none of the models described in the literature was able to be validated by our data due to smaller values of both exponents: $d_{3,2} \propto p^{-0.24}$ for 10% (v/v) and $d_{3,2} \propto p^{-0.19}$ for 20% (v/v) o/w emulsions. These results are in line with those of Floury et al. (2002), in which submicron o/w emulsions of 20% (v/v) sunflower oil stabilized with 1 or 2% (w/w) 11S soy globulins were produced by UHPH. However, as both laminar and turbulence regimes coexist in this type of homogenizer, it is complicated how to predict experimentally the particle disruption of submicron emulsions due to different mechanisms that emulsions experience (Floury et al., 2004).

Table 1
Mean ± SD values of temperature reached in the high-pressure valve (T_1) for emulsions treated by conventional homogenization at 15 MPa with native and denatured soy protein isolate, and by ultra-high pressure homogenization at 100–300 MPa with native soy protein isolate (T_{in} = 20 °C).

Emulsion treatment		T_1 (°C)
MPa	Oil (%)	
15	10	32.00 ± 1.41
15.D*		31.50 ± 3.54
100		51.50 ± 2.12
200		72.50 ± 2.12
300		100.00 ± 0.00
15	20	31.50 ± 3.54
15.D		31.50 ± 3.54
100		54.00 ± 1.41
200		76.50 ± 0.71
300		103.50 ± 2.12

* D = emulsions containing heated SPI (95 °C, 15 min) and stabilized by conventional homogenization.

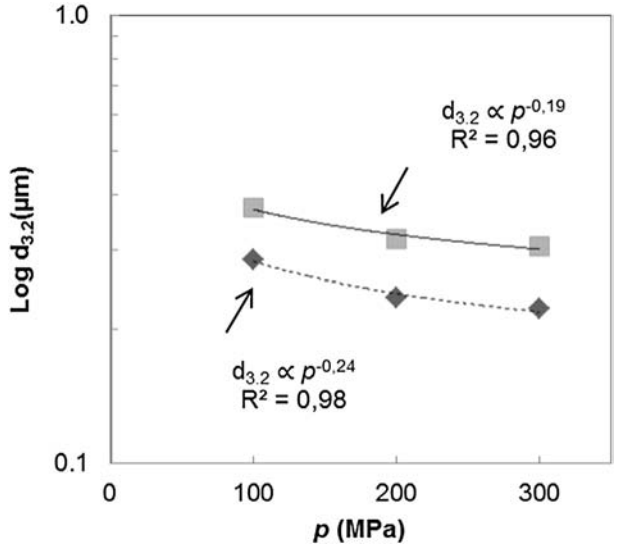

Fig. 1. Log $d_{3,2}$ vs. pressure (P) for emulsions treated by Ultra-High Pressure Homogenization at a fixed non-heated SPI concentration (4%, w/v) with 10% (v/v) (· · ♦ · ·) and 20% (v/v) (-■-) of soybean oil.

3.2. Influence of homogenization conditions on oil droplet size distribution

3.2.1. Increase of mean droplet size with oil-phase volume fraction

At constant emulsification pressure, $d_{3,2}$ rose with increasing oil content (Table 2). This could be explained because higher oil contents increase the emulsion viscosity (Tang & Liu, 2013), and as a result, droplet disruption in the valve becomes more difficult. Another factor that might affect the rise in $d_{3,2}$, due to the increase of oil fraction, is the increase of the high shear stress and temperature in the homogenizing valve, and consequently the rate of flocculation/coalescence among oil droplets could increase (Floury, Desrumaux, & Lardières, 2000; Floury et al., 2002).

At constant emulsifier concentration, there might be insufficient coverage of the droplet interface by proteins at higher oil-phase volume fraction (McClements, 2005). According to different authors (Floury et al., 2002; Keerati-u-rai & Corredig, 2009) the protein concentration in the range of 0.5–2.0% of high-soluble soy protein fractions can stabilize emulsions containing 10–20% oil. However, our commercial SPI is a low-soluble isolate compared to those high-soluble SPI which are commonly used and studied elsewhere (Guo et al., 2012; Keerati-u-rai & Corredig, 2010). For that reason, and in order to stabilize our emulsions at pH = 7.14, we used higher SPI content (4% w/v) to compensate the elimination of the purification step of SPI for manufacturers. Preliminary data of UHPH emulsions (100–300 MPa) made in our lab with 2% (w/v) of SPI showed greater particle size (data not shown) than their homologous emulsions containing 4% (w/v). This clearly support the fact that more soluble protein can migrate to the interfaces when 4% (w/v) is used to stabilize the great SSA generated in UHPH emulsions.

There were no significant differences ($P > 0.05$) for Span and SSA values between CH-treated emulsions containing different oil contents (10 and 20% v/v) (Table 2). By contrast, significant differences as a result of the statistic GLM applied ($P < 0.05$) of these parameters were found within UHPH-treated emulsions containing different oil contents (10 and 20% v/v) since the increase of the oil content caused the increase of the Span and decrease of SSA values.

3.2.2. Effect of pressure level on droplet size distribution

CH emulsions, at both oil concentrations, had the largest particle size ($d_{3,2}$) followed by emulsions stabilized by UHPH at 100, 200 and 300 MPa, in this order (Table 2). A significant increase in the Span value of emulsions was observed when the pressure increased from 100–200 to 300 MPa at both oil concentrations (Table 2). The Span value increase in the emulsion treated at 300 MPa could be explained by the coalescence within oil droplets after crossing the homogenizing valve (Floury et al., 2000, 2002), due to formation of protein aggregates produced by partial or total denaturation of SPI owing to the increase of the temperature (≥100 °C) at the exit of the UHPH valve (Table 1). These facts were confirmed by the presence of large tails of flocs in 300 MPa-treated emulsions with 10% (v/v) soybean oil and by the absence of these tails after changing the environmental conditions with 2% SDS (Fig. 2E). In the case of emulsions containing 20% (v/v) oil, although these tails decreased, 2% of SDS was not enough to break up the flocs. In the CH and UHPH emulsions (100 and 200 MPa), extensive droplet flocculation did not occur, as it was confirmed by SDS environment in emulsions, where bimodal distributions of particle size did not change to monomodal (Fig. 2). All emulsions exhibited bimodal distribution, although increasing the homogenization pressure in UHPH emulsions almost shifted the bimodal curve to monomodal with a narrower size distribution, compared to CH emulsions. Another device widely used as emulsion homogenization treatment is the Microfluidizer® (Keerati-u-rai & Corredig, 2009; Shao & Tang, 2014; Tang & Li, 2013; Tang & Liu, 2013). Oil-in-water emulsions of 20% (v/v) soybean oil stabilized by 4% (w/v) SPI and treated by microfluidization (one pass at 40 MPa) (Shao & Tang, 2014) presented higher $d_{4,3}$ values and were more polydisperse (0.76 vs. 0.42 and 0.75 μm) in comparison with our UHPH-treated emulsions

Table 2
Mean ± SD of particle size distribution indices ($d_{3,2}$ and Span), specific surface area (SSA, m^2/mL) and rheological characteristics (flow and consistency indices) of emulsions containing soybean oil (10 and 20%, v/v) and stabilized by conventional homogenization (15 MPa) and ultra-high pressure homogenization at 100, 200, and 300 MPa with 4% (w/v) of SPI in continuous phase.

Emulsion treatment		Particle size			Rheological parameters	
MPa	Oil (%)	$d_{3,2}$ (μm)	Span	SSA (m^2/mL)	K (mPa × s^n)	n
15		0.51 ± 0.01^{bc}	3.49 ± 0.17^{cd}	11.69 ± 0.29^{d}	8.66 ± 0.68^{d}	0.98 ± 0.00
15.D*		0.50 ± 0.02^{c}	3.47 ± 0.39^{cd}	12.15 ± 0.57^{d}	4.55 ± 0.09^{e}	1.01 ± 0.00
100	10	0.28 ± 0.00^{ef}	3.73 ± 0.05^{cd}	21.32 ± 0.19^{b}	8.22 ± 2.10^{d}	0.96 ± 0.02
200		0.23 ± 0.01^{f}	2.31 ± 0.87^{d}	25.63 ± 0.54^{a}	8.45 ± 0.67^{d}	0.96 ± 0.00
300		0.23 ± 0.01^{f}	8.30 ± 2.88^{b}	26.68 ± 1.72^{a}	7.05 ± 0.39^{d}	0.97 ± 0.00
15		0.60 ± 0.05^{a}	3.29 ± 0.21^{cd}	10.08 ± 0.89^{d}	58.67 ± 6.57^{b}	0.85 ± 0.02
15.D		0.56 ± 0.01^{ab}	2.95 ± 0.15^{cd}	10.81 ± 0.26^{d}	29.97 ± 1.74^{c}	0.87 ± 0.02
100	20	0.37 ± 0.02^{d}	4.83 ± 0.36^{c}	16.10 ± 0.75^{c}	114.19 ± 38.69^{a}	0.74 ± 0.03
200		0.32 ± 0.04^{de}	4.81 ± 0.31^{c}	19.21 ± 2.34^{b}	83.53 ± 13.03^{ab}	0.76 ± 0.01
300		0.30 ± 0.01^{e}	26.23 ± 15.04^{a}	19.80 ± 0.92^{b}	34.38 ± 3.97^{c}	0.84 ± 0.01

Different letters from $^{a-f}$ in the same column indicate significant differences ($P < 0.05$) between treatments.
* D = emulsions containing heated SPI (95 °C, 15 min) and stabilized by conventional homogenization.

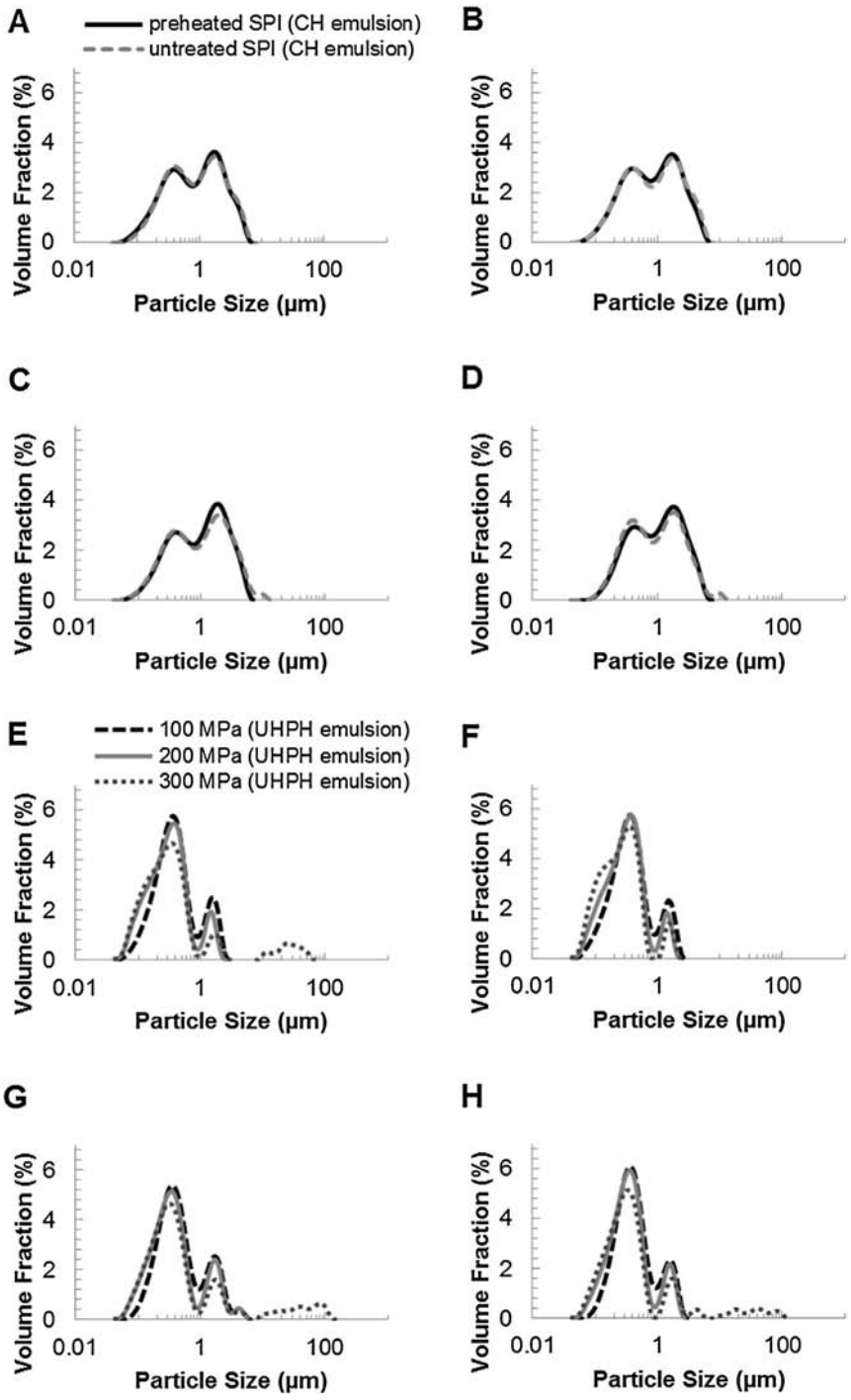

Fig. 2. Size distribution profiles of o/w emulsions treated by conventional homogenization (CH) and stabilized by untreated or preheated SPIs (A–D), and o/w emulsions treated by Ultra-High Pressure Homogenization (UHPH) and stabilized by untreated SPI under different homogenizing pressures (100–300 MPa) (E–H). A, C, E, G: diluted in water; B, D, F, H; diluted in 2% SDS. The protein concentration in continuous phase was 4% (w/v), and the volume oil fraction was 10% (v/v) for A–B, E–F and 20% (v/v) for C–D, G–H.

of 20% (v/v) soybean oil stabilized by 4% (w/v) SPI and treated by UHPH at 100 and 200 MPa.

3.3. Characteristics of emulsion microstructure

Fig. 3 shows the microstructure of the emulsions studied by TEM at magnifications of ×5000 and ×25,000 and Fig. 4 shows it at magnifications of ×5000 and ×50,000. Results indicate that all emulsions contained spherically shaped droplets. As it was expected, UHPH reduced the mean particle size in emulsions compared to CH emulsions. In addition, the greater SSA created in UHPH emulsions (Table 1) could promote more adsorption of the protein to the interfaces, as it is shown in the micrographs (Fig. 4). On the other hand, CH emulsions contained larger protein aggregates or cluster of protein aggregates (Fig. 3), leading to less coverage of the emulsion interface (McClements, 2005). In UHPH emulsions containing 20% (v/v) of oil, some larger droplets were produced by the build-up and break-up processes occurring during emulsion formation, as a result of increasing oil content in formulations (Fig. 4B, D and F).

Electrostatic repulsion and attractive interactions, especially hydrophobic interactions between the adsorbed soy proteins on individual oil droplets, contribute the stability of the emulsions (Tang

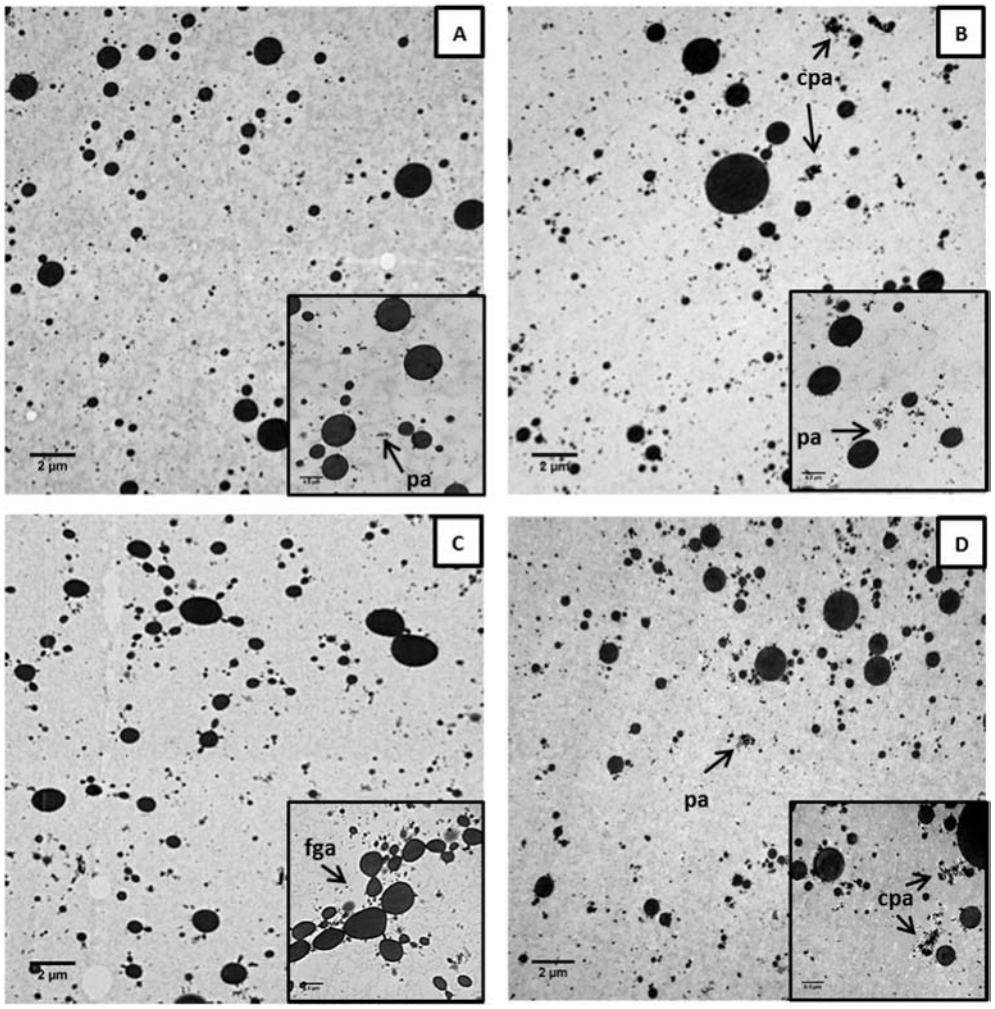

Fig. 3. TEM images (×5000 and ×25,000) of emulsions treated by conventional homogenization at 15 MPa and stabilized by non-heated (A and C) and heated SPIs (B and D). The protein concentration in continuous phase was 4% (w/v), and the volume oil fraction was 10% (v/v) for A–B, and 20% (v/v) for C–D. (pa) Protein aggregates, (cpa) cluster of protein aggregates, (fga) fat globule aggregate. Bars represent 2 and 0.5 μm, respectively.

& Liu, 2013). However, comparing CH and UHPH emulsions, there were fewer groups of oil droplets in CH emulsions that remained within close proximity of each other (Fig. 3), so the UHPH emulsions presented greater flocculation phenomena. Particularly, the most groups of oil droplets were clearly observed in the TEM images of UHPH emulsions with 20% (v/v) homogenized at 200 MPa (Fig. 4D). According to other authors (Cruz et al., 2007; Floury et al., 2002), the homogenization treatments applied at high pressures induce conformational protein changes in the non-heated SPI emulsions that affect the structural organization of the droplets. Thus, UHPH treatment could increase the number of bonds per droplet in comparison with CH-treated emulsions (Figs. 3 and 4). Even so, the information on the adsorbed soy protein layers within emulsion droplets are characterized by their complexity due to different types of structure and composition of proteins (Keerati-u-rai & Corredig, 2009, 2010). In that manner, it is difficult to understand the relationship between colloidal interactions at molecular and macroscopic level (McClements, 2005; Petsev, 2004; Piorkowski & McClements, 2013).

3.4. Rheological behavior

Measurements of viscosity vs. shear rate can be used to provide information about the strength of protein–protein interaction. During the rheological measurements, the shape of the upward and downward flow curves were not different, so that emulsions did not present thixotropic behavior (data not shown), and for this reason droplets were not aggregated by weak forces (McClements, 2005). The flow curves of emulsions containing 10% (v/v) oil showed a Newtonian fluid behavior ($n \approx 1$) (Table 2). However, emulsions containing 20% (v/v) presented a shear-thinning behavior (Table 2; Fig. 5) being the UHPH emulsions treated at 100 and 200 MPa those that showed the highest consistency indices, as the statistic GLM showed ($P < 0.05$). However, lower consistency index was observed in the emulsions treated at 300 MPa due to denaturation of SPI globulins (Floury et al., 2002), by the high temperature increase at the exit of the valve (Table 1). Thus, it is possible to hypothesize that the partial protein denaturation produced by the UHPH treatment at 100 and 200 MPa caused much greater effective volume fraction than their actual volume fraction (McClements, 2005; Quemada, 2002), according to the greatest viscosity data exhibited in these emulsions (Table 2). However, the CH emulsion with 20% (v/v) of soybean oil, which is not expected to experience denaturation of SPI, showed less viscosity. Otherwise, CH emulsions containing heated SPI and 20% (v/v) of soybean oil were less viscous than their counterparts made with non-heated SPI (Table 2; Fig. 5). This result might be related to less flocculation and protein coverage of protein in the interfaces of this emulsion as confirmed by TEM images (Fig. 3C, D). Additionally, total heat denaturation of SPI before CH treatment leads to similar consistency index values (K) that UHPH treatment at 300 MPa in o/w emulsions with 20% (v/v) of soybean oil (Table 2). This result also sustains the denaturation of SPI produced at 300 MPa by UHPH.

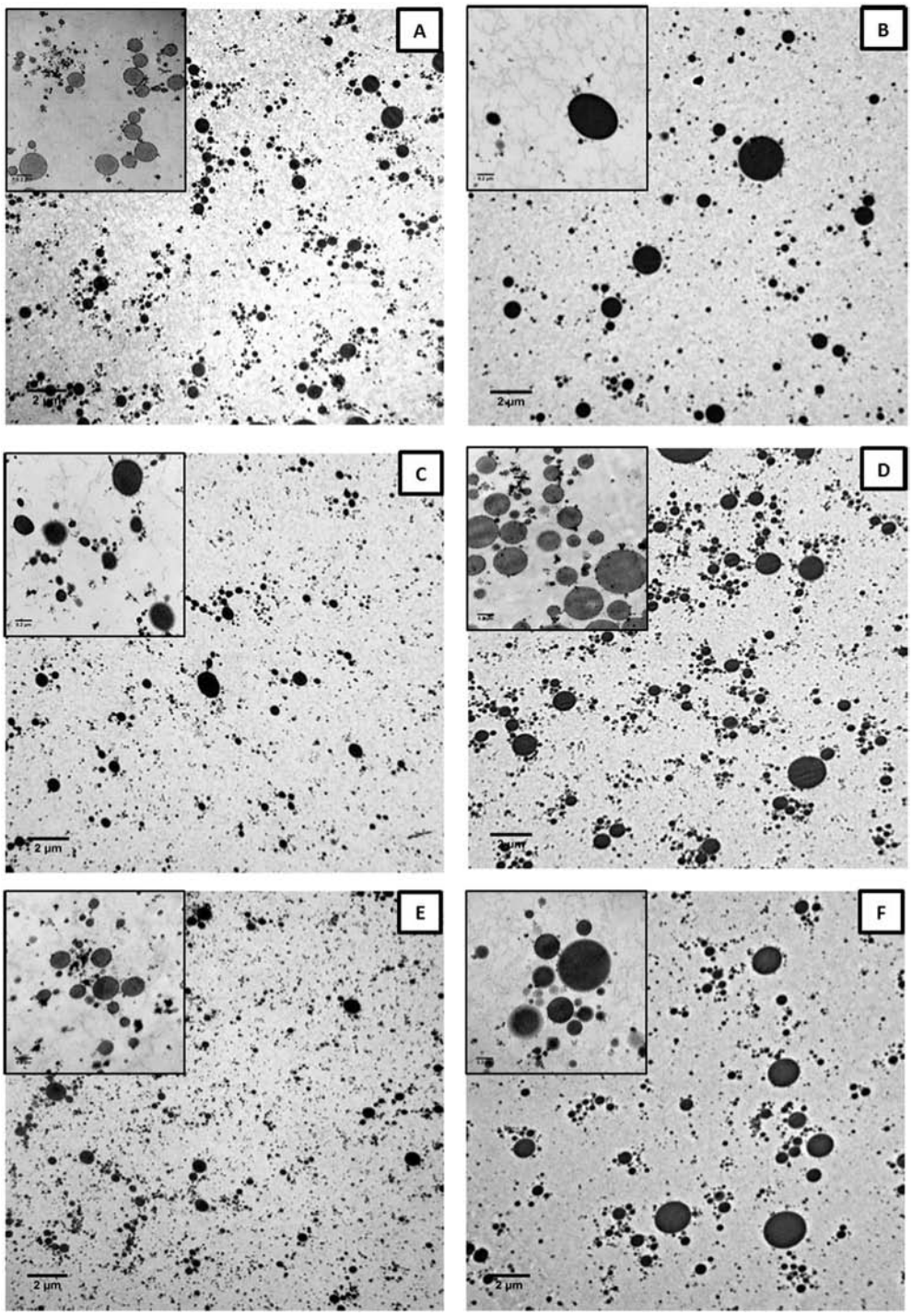

Fig. 4. TEM images (×5000 and ×50,000) of emulsions treated by Ultra-High Pressure Homogenization at 100 MPa (A–B), 200 MPa (C–D), and 300 MPa (E–F). The protein concentration in continuous phase was 4% (w/v), and the volume oil fraction was 10% (v/v) for A, C, and E, and 20% (v/v) for B, D, and F. Bars represent 2 µm and 0.2 µm, respectively.

It is known that shear-thinning behavior is caused by flocculation, the increase in the disperse phase volume fraction and the balance between build-up and breakdown of oil droplets that move together (Quemada, 2002). Flocculation phenomena was evident in emulsions containing the higher oil content (20%, v/v), as Figs. 3 (C, D) and 4 (B, D, F) show. Furthermore, when more electrostatic forces exist between the droplets of emulsions at a fixed oil volume fraction, an increase in viscosity is obtained (McClements, 2005; Quemada, 2002).

Some authors have confirmed the presence of protein aggregates in the interfaces of 10% o/w emulsions containing 1% SPI by heating (75 and 95 °C) after homogenization by Microfluidizer®, which increased the shear-thinning behavior compared to unheated emulsions (Keeratiu-rai & Corredig, 2009). Nevertheless, they reported that all the SPI subunits were present at the interface in an aggregated form when heating of protein dispersions was conducted prior to homogenization compared to heating after emulsification.

By contrast, clusters of protein aggregates were found in our CH emulsions with heated SPI before homogenization without adhesion at the interface (Fig. 3B, D). This result was expected, since aggregations of proteins are caused by low surface protein load and larger particle

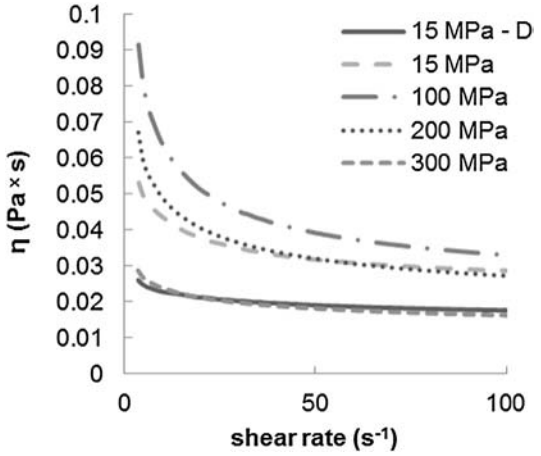

Fig. 5. Flow behaviors (21 °C) of emulsions treated by conventional homogenization stabilized by non-heated (15 MPa) and heated μSPIs (15 MPa — D) and emulsions treated by Ultra-High Pressure Homogenization and stabilized by untreated SPI under different homogenizing pressures (100–300 MPa). The protein concentration in continuous phase was 4% (w/v), and the volume oil fraction was 20% (v/v).

size (Williams, Phillips, & Walstra, 2002). Consequently, our results showed that CH emulsions with heated SPI were less viscous than their counterparts with the non-heated protein (Table 2), so that the heating prior emulsification process was not the best option in our formulations by the lack of protein aggregates formation at the interface (Keerati-u-rai & Corredig, 2009).

Floury et al. (2002) working on o/w emulsions stabilized by the 11S protein fraction and treated by UHPH (20–350 MPa) observed that viscosity values increased proportionally to pressure. According to the authors, above 200 MPa, UHPH treatment produces a modification of the conformation of 11S globulins, which tends to unfolding and aggregation, increasing hydrophobic effects leading to protein–protein interactions. However, our results of SPI emulsions treated by UHPH presented the highest consistency coefficient value at 100 MPa and were gradually reduced up to 300 MPa (Table 2; Fig. 5), possibly due to over-processing of emulsions after passing through the valve (Table 1). The differences on viscosity in UHPH emulsions stabilized with SPI compared to those from Floury et al. (2002) could be attributed to the fact that SPI contains more protein fractions, mostly 7S and 11S, and the thermal behavior varies. The thermal aggregation behavior of each protein fraction alone (7S or 11S) or in a complex 7S/11S at pH 7.0 is different (Guo et al., 2012). According to these authors, after heat treatment, the insoluble aggregates of the 11S protein fraction alone show more active sites available for aggregation. Nevertheless, when the 11S/7S protein fractions are combined, soluble aggregates are formed due to the end of the assembly by N-linked glycans on 11S protein fraction.

3.5. Physical stability of emulsions

3.5.1. Stability against creaming by Turbiscan measurements

When o/w emulsions are prepared with proteins, the rate of creaming strongly increases with the droplet diameter and with aggregation of the droplets (Williams et al., 2002).

It is known that the size variation phenomena could be computed by Turbiscan with the mean value for each ΔBS profile in between two defined limits as a function of time. Fig. 6 shows the creaming destabilization kinetics of emulsions measured by ΔBS (%) as a function of time during more than 5 months of storage. Fig. 6C, D shows the clarification in the bottom of the tube, while parts A and B of Fig. 6 represent the creaming in the top of the tube. From this figure, it is clearly visible that CH emulsions were more unstable than UHPH emulsions as a result of having larger particle size compared to those treated by UHPH (Table 2). Most of CH emulsions exhibited clarification in the bottom and creaming in the top of the tube (Fig. 6), except for CH emulsion containing 20% (v/v) of soybean oil with non-heated SPI, which only presented clarification in the bottom. CH emulsions formulated with non-heated SPI presented greater extent of destabilization phenomena. These differences could be attributed to the lesser values of the consistency coefficients (Table 2). Consequently, CH emulsions stability was greatly dependent on structure and composition of proteins at the interface (non-heated or heated) and the state of aggregation in the continuous phase. Additionally, CH emulsion with 20% (v/v) oil and stabilized by non-heated SPI were more stable than emulsion with 10% (v/v) of soybean oil owing to more consistency by increasing oil content (Fig. 6; Table 2). Indeed, this emulsion also exhibited greater thickness of SPI around the interface and the absence of clusters of protein aggregates (Fig. 3C).

All UHPH emulsions containing 10% (v/v) of soybean oil showed clarification and creaming of droplets, being the emulsion treated at 100 MPa the least stable. This variability could be attributed to the greater particle size of this emulsion (Table 2) and the excess of protein used, leading to faster clarification (Fig. 6C). With respect to the emulsion prepared with 20% (v/v) of soybean oil, only the emulsions homogenized at 300 MPa showed some clarifications and creaming phenomena (Fig. 6B, D), as a result of the formation of protein aggregates confirmed by the Span values and the size distribution profiles (Table 2; Fig. 2G, H). By contrast, after more than 5 months of cold storage, emulsions containing 20% (v/v) and treated at 100 and 200 MPa did not show any sign of variation in the creaming extent (Fig. 6B, D). This could be explained by the lack of larger aggregates in fresh emulsions (Fig. 2G, H), as well as the depletion flocculation of protein-coated droplets by unadsorbed proteins in the aqueous phase (Herrera, 2012). Particularly, TEM images of UHPH emulsions containing 20% of soybean oil and treated at 200 MPa showed a network of flocculated droplets, which is also evident by the high consistency coefficient level of the emulsion (Fig. 4D; Table 2).

Proteins generally provide good protection against the irreversible phenomenon of coalescence, due to strong colloidal repulsion provided and the high interfacial tension oil–water (Williams et al., 2002). By contrast, Fig. 7 shows that emulsions containing 10% (v/v) of soybean oil and stabilized with CH increased the mean particle size ought to flocculation/coalescence in the medium zone of the tube (15–30 mm). Although the backscattering method is able to detect changes with a less sensibility than laser diffraction measurements, it allows the evaluation of changes in particle size in non-diluted emulsions stored at long-term (Palazolo, Sorgentini, & Wagner, 2005). Additionally, Fig. 3A–B shows that emulsions did not contain the surface load as a densely packed monolayer, which could cause coalescence of droplets along with the higher particle size (Table 2) presented in these emulsions (Williams et al., 2002).

3.5.2. Visual observation

Emulsion separation was visually observed in CH emulsions, being clarification and creaming clearly evident in the emulsions containing 10% (v/v) of soybean oil. Moreover, only clarification was observed in CH emulsions with 20% (v/v) of oil. Furthermore, UHPH emulsions containing 20% (v/v) of oil did not showed any clear destabilization phenomena to the human eye.

4. Conclusions

UHPH technology is suitable to produce SPI-stabilized submicron emulsions containing soybean oil with a high physical stability when compared to emulsions obtained by CH. The creaming of oil droplets was strongly dependent on the viscosity, since the more stable emulsions exhibited the highest shear-thinning behavior which were the UHPH emulsions containing 20% (v/v) of soybean oil and homogenized at 100 and 200 MPa. Otherwise, when UHPH emulsions did not contain the effective volume oil fraction, flocculation led to

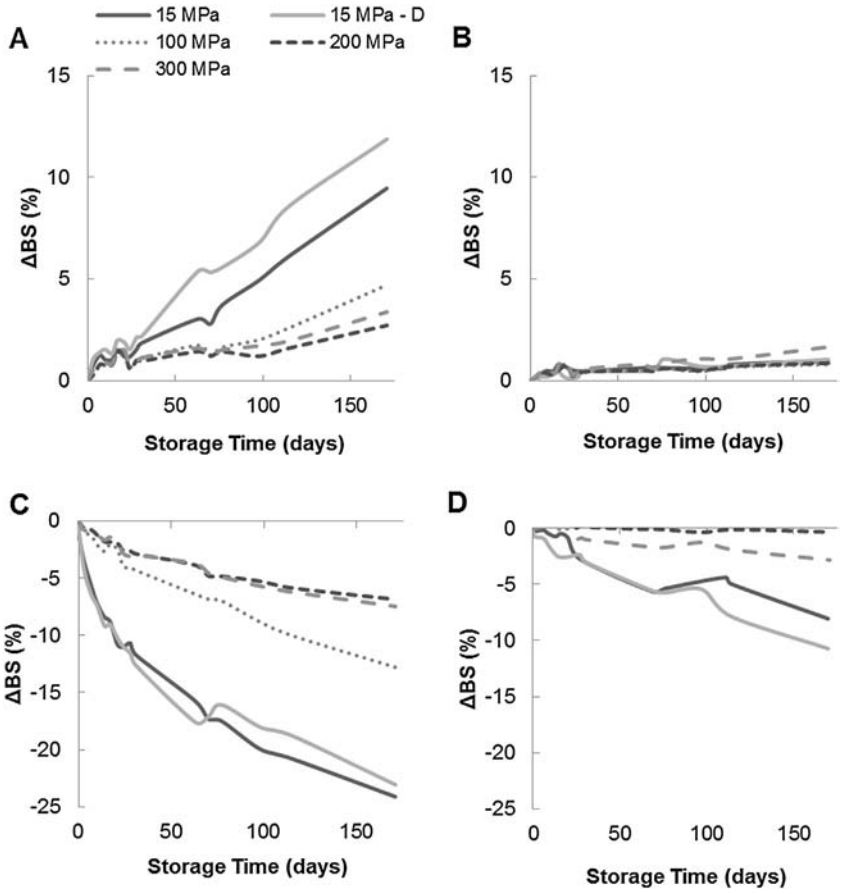

Fig. 6. Variation of backscattering expressed as ΔBS (%) of emulsions stabilized by conventional homogenization (15 MPa) with 4% (w/v) of non-heated SPI and heated SPI (15 MPa — D), and Ultra-High Pressure Homogenization at 100, 200, and 300 MPa during storage at the bottom (0–10 mm) for C–D and the top of the tube (35–40 mm) for A–B. The volume oil fraction was 10% (v/v) for A and C, and 20% (v/v) for B and D.

accelerated creaming with lower pressures applied, as we can observe in the emulsion with 10% (v/v) of soybean oil and treated at 100 MPa. In UHPH emulsions (100 and 200 MPa) containing 20% (v/v) of soybean oil, physical stability was also enhanced by low particle size ($d_{3,2}$) and polydispersity (Span). In addition, the beneficial effects of high-pressure homogenization (100 and 200 MPa) on physical stability of emulsions have been attributed to the partial unfolding of soy proteins. Moreover, physical stability was negatively affected in the emulsion with 20% (v/v) of soybean oil and treated at 300 MPa due to higher temperatures reached at the exit of the valve. Our results show that heating of soy protein isolate dispersions before CH treatment did not improve physical stability of emulsions. This information could be used by food industry to produce physically stable submicron emulsions. Taken together, these findings suggest the advantages for the elimination of an expensive protein extraction step to obtain the emulsifier in promoting UHPH technology. However, studies on the oxidative stability of emulsions should be conducted in order to explore the role of pressure treatment when enriched with biological compounds, and their relationship to preservation.

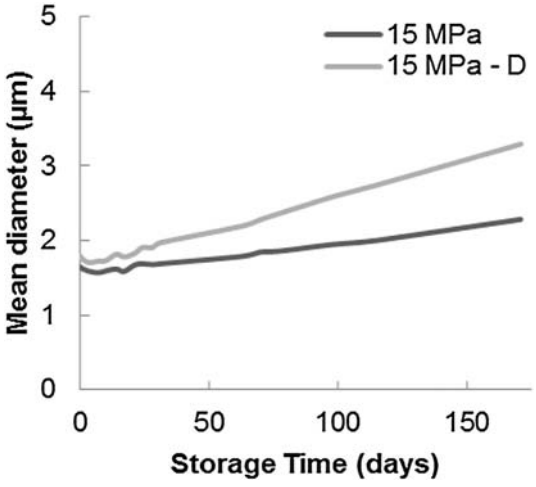

Fig. 7. Variation of the particle mean diameter expressed as d (μm) in emulsions stabilized by conventional homogenization (15 MPa) with 4% (w/v) of non-heated SPI and heated SPI (15 MPa — D) at the zone 15–30 mm of the tube. The volume oil fraction was 10% (v/v).

Acknowledgments

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References

Amador-Espejo, G. G., Suàrez-Berencia, A., Juan, B., Bárcenas, M. E., & Trujillo, A. J. (2014). Effect of moderate inlet temperatures in ultra-high-pressure homogenization treatments on physicochemical and sensory characteristics of milk. *Journal of Dairy Science*, *97*(2), 659–671.

Bissegger, S. (2007). *Production of bioactive soy peptides.* (Thesis) HES-SO Valais.

Chevalier-Lucia, D., Cortés-Muñoz, M., Picart-Palmade, L., & Dumay, E. (2009). Submicron emulsions processed by ultra-high pressure homogenization. *High Pressure Research*, *29*(4), 732–738.

Cortés-Muñoz, M., Chevalier-Lucia, D., & Dumay, E. (2009). Characteristics of submicron emulsions prepared by ultra-high pressure homogenisation: Effect of chilled or frozen storage. *Food Hydrocolloids*, *23*(3), 640–654.

Cruz, N., Capellas, M., Hernández, M., Trujillo, A. J., Guamis, B., & Ferragut, V. (2007). Ultra high pressure homogenization of soymilk: Microbiological, physicochemical and microstructural characteristics. *Food Research International*, *40*(6), 725–732.

Desrumaux, A., & Marcand, J. (2002). Formation of sunflower oil emulsions stabilized by whey proteins with high-pressure homogenization (up to 350 MPa): Effect of pressure on emulsion characteristics. *International Journal of Food Science and Technology*, *37*(3), 263–269.

Dumay, E., Chevalier-Lucia, D., Picart-Palmade, L., Benzaria, A., Gràcia-Julià, A., & Blayo, C. (2013). Technological aspects and potential applications of (ultra) high-pressure homogenisation. *Trends in Food Science & Technology*, *31*(1), 13–26.

Floury, J., Bellettre, J., Legrand, J., & Desrumaux, A. (2004a). Analysis of a new type of high pressure homogeniser. A study of the flow pattern. *Chemical Engineering Science*, *59*(4), 843–853.

Floury, J., Desrumaux, A., & Lardières, J. (2000). Effect of high-pressure homogenization on droplet size distributions and rheological properties of model oil-in-water emulsions. *Innovative Food Science & Emerging Technologies*, *1*(2), 127–134.

Floury, J., Desrumaux, A., & Legrand, J. (2002). Effect of ultra-high-pressure homogenization on structure and on rheological properties of soy protein-stabilized emulsions. *Journal of Food Science*, *67*(9), 3388–3395.

Floury, J., Legrand, J., & Desrumaux, A. (2004b). Analysis of a new type of high pressure homogeniser. Part B. study of droplet break-up and recoalescence phenomena. *Chemical Engineering Science*, *59*(6), 1285–1294.

Guo, J., Yang, X. -Q., He, X. -T., Wu, N. -N., Wang, J. -M., Gu, W., et al. (2012). Limited aggregation behavior of β-conglycinin and its terminating effect on glycinin aggregation during heating at pH 7.0. *Journal of Agricultural and Food Chemistry*, *60*(14), 3782–3791.

Hebishy, E. H. (2013). *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.* (Thesis) Bellatera: Autonomous University of Barcelona.

Herrera, M. (2012). *Analytical techniques for studying the physical properties of lipid emulsions.* New York: Springer.

Juliano, P., Kutter, A., Cheng, L. J., Swiergon, P., Mawson, R., & Augustin, M. A. (2011). Enhanced creaming of milk fat globules in milk emulsions by the application of ultrasound and detection by means of optical methods. *Ultrasonics Sonochemistry*, *18*(5), 963–973.

Keerati-u-rai, M., & Corredig, M. (2009). Heat-induced changes in oil-in-water emulsions stabilized with soy protein isolate. *Food Hydrocolloids*, *23*(8), 2141–2148.

Keerati-u-rai, M., & Corredig, M. (2010). Heat-induced changes occurring in oil/water emulsions stabilized by soy glycinin and β-conglycinin. *Journal of Agricultural and Food Chemistry*, *58*(16), 9171–9180.

Kinsella, J. E. (1979). Functional properties of soy proteins. *Journal of the American Oil Chemists Society*, *56*(3), 242–258.

Lee, S. -H., Lefèvre, T., Subirade, M., & Paquin, P. (2009). Effects of ultra-high pressure homogenization on the properties and structure of interfacial protein layer in whey protein-stabilized emulsion. *Food Chemistry*, *113*(1), 191–195.

McClements, D. J. (2005). *Food emulsions: Principles, practice, and techniques* (2nd ed.). Boca Raton, FL: CRC Press.

Nishinari, K., Fang, Y., Guo, S., & Phillips, G. O. (2014). Soy proteins: A review on composition, aggregation and emulsification. *Food Hydrocolloids*, *39*, 301–318.

Palazolo, G. G., Sorgentini, D. A., & Wagner, J. R. (2005). Coalescence and flocculation in o/w emulsions of native and denatured whey soy proteins in comparison with soy protein isolates. *Food Colloids 2004 (Harrogate)*, *19*(3), 595–604.

Pearce, K. N., & Kinsella, J. E. (1978). Emulsifying properties of proteins: Evaluation of a turbidimetric technique. *Journal of Agricultural and Food Chemistry*, *26*(3), 716–723.

Perrier-Cornet, J. M., Marie, P., & Gervais, P. (2005). Comparison of emulsification efficiency of protein-stabilized oil-in-water emulsions using jet, high pressure and colloid mill homogenization. *Journal of Food Engineering*, *66*(2), 211–217.

Petsev, D. N. (2004). Theory of emulsion flocculation. In D. N. Petsev (Ed.), *Emulsions: Structure, stability and interactions* (pp. 313–350). Elsevier.

Piorkowski, D. T., & McClements, D. J. (2013). Beverage emulsions: Recent developments in formulation, production, and applications. *Food Hydrocolloids*, *44*, 5–41.

Poliseli-Scopel, F. H., Hernández-Herrero, M., Guamis, B., & Ferragut, V. (2014). Sterilization and aseptic packaging of soymilk treated by ultra high pressure homogenization. *Innovative Food Science & Emerging Technologies*, *22*, 81–88.

Quemada, D. (2002). Energy of interaction in colloids and its implications in rheological modeling. *Advances in Colloid and Interface Science*, *98*(1), 51–85.

Roesch, R. R., & Corredig, M. (2003). Texture and microstructure of emulsions prepared with soy protein concentrate by high-pressure homogenization. *LWT — Food Science and Technology*, *36*(1), 113–124.

Shao, Y., & Tang, C. -H. (2014). Characteristics and oxidative stability of soy protein-stabilized oil-in-water emulsions: Influence of ionic strength and heat pretreatment. *Food Hydrocolloids*, *37*, 149–158.

Tang, C. -H., & Li, X. -R. (2013). Microencapsulation properties of soy protein isolate and storage stability of the correspondingly spray-dried emulsions. *Food Research International*, *52*(1), 419–428.

Tang, C. -H., & Liu, F. (2013). Cold, gel-like soy protein emulsions by microfluidization: Emulsion characteristics, rheological and microstructural properties, and gelling mechanism. *Food Hydrocolloids*, *30*(1), 61–72.

Wang, J. -M., Xia, N., Yang, X. -Q., Yin, S. -W., Qi, J. -R., He, X. -T., et al. (2012). Adsorption and dilatational rheology of heat-treated soy protein at the oil-water interface: Relationship to structural properties. *Journal of Agricultural and Food Chemistry*, *60*(12), 3302–3310.

Williams, P. A., Phillips, G. O., & Walstra, P. (2002). In P. A. Williams, & G. O. Phillips (Eds.), *Gums and stabilisers for the food industry 11*, *Vol. 11*, Cambridge: Royal Society of Chemistry.

Wilson, R. F. (2008). Soybean: Market driven research needs. In G. Stacey (Ed.), *Genetics and genomics of soybean* (pp. 3–15). New York, NY: Springer New York.

CHAPTER 4: Ultra-High Pressure Homogenization improves oxidative stability and interfacial properties of soy protein isolate-stabilized emulsions

[Experiment 2]

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Ultra-High Pressure Homogenization improves oxidative stability and interfacial properties of soy protein isolate-stabilized emulsions

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ABSTRACT

Ultra-High Pressure Homogenization (100–300 MPa) has great potential for technological, microbiological and nutritional aspects of fluid processing. Its effect on the oxidative stability and interfacial properties of oil-in-water emulsions prepared with 4% (w/v) of soy protein isolate and soybean oil (10 and 20%, v/v) were studied and compared to emulsions treated by conventional homogenization (15 MPa). Emulsions were characterized by particle size, emulsifying activity index, surface protein concentration at the interface and by transmission electron microscopy. Primary and secondary lipid oxidation products were evaluated in emulsions upon storage. Emulsions with 20% oil treated at 100 and 200 MPa exhibited the most oxidative stability due to higher amount of oil and protein surface load at the interface. This manuscript addresses the improvement in oxidative stability in emulsions treated by UHPH when compared to conventional emulsions.

1. Introduction

A current major health issue at the heart of our understanding is to follow the most recent nutritional recommendations (EFSA Panel on Dietetic Product, 2010), one being, increasing amounts of polyunsaturated fats incorporated in foods (Walker, Decker, & McClements, 2015). At the same time, consumers look for foods containing fewer additives, especially synthetic antioxidants. However, synthetic antioxidants are added to food containing chemically unstable compounds, such as polyunsaturated fats. This is why lipid oxidation in formulated foods has become a renewed concern for the food industry and it is important to develop new technologies to replace synthetic antioxidants. Lipid oxidation consists of the reaction of molecular oxygen with unsaturated fatty acid, resulting in unsaturated hydroperoxides (Gunstone & Martini, 2010). Lipid hydroperoxides are unstable molecules that break down into other products, particularly aldehydes, responsible for undesirable odours and flavours. Thus, lipid oxidation is the main reason for the deterioration of fats and oils (Matthäus, 2010). Many food products contain oil and water. These phases are immiscible, but can coexist in a stable form in food products through emulsification, with one of the liquids dispersed as small spherical droplets in the other. In particular, oil-in-water (ow) emulsions consist of oil droplets dispersed in an aqueous phase (McClements, 2005).

Since the late 1980s, substantial work has been performed with respect to lipid oxidation in simplified model emulsions (Berton-Carabin, Ropers, & Genot, 2014; Waraho, Cardenia, Decker, & McClements, 2010). The rates and pathways of oxidation reactions are influenced by the physical environment of the molecules involved, for example, whether they are located in the oil, water or interfacial region (Berton-Carabin et al., 2014; Faraji, McClements, & Decker, 2004; Fernandez-Avila, Arranz, Guri, Trujillo, & Corredig, 2015). The interfacial region, which is the contact region between the oil phase and the aqueous phase, represents a particularly critical area in the system with regard to the development of lipid oxidation. For these reasons, it is important to have a better understanding of the mechanisms of lipid oxidation in food dispersions so that novel antioxidant technologies can be developed (Waraho et al., 2010).

Ultra-High Pressure Homogenization (UHPH) is an emergent technology to produce fine and stable submicron emulsions (<1 μm) (Dumay et al., 2013; Fernández-Ávila, Escriu, & Trujillo, 2015; Hebishy, Buffa, Guamis, Blasco-Moreno, & Trujillo, 2015). UHPH modifies emulsion properties due to conformational changes of proteins, which results in changes of the physical stability of emulsions. These changes in particle size and proteins structure are caused by different phenomena occurring at the exit of the UHPH valve, such as high turbulence, cavitation and shear

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stress (Desrumaux & Marcand, 2002; Floury, Desrumaux, & Legrand, 2002). Over the past decade, the changes experienced by UHPH treatment compared to conventional processes (high speed blenders, high-pressure valve homogenizers and colloid mills) remain unprecedented. The benefits obtained by applying a single stage of homogenization with UHPH technology are supported by time and cost savings in the food industry. The most significant improvements of these emulsions are their physical and microbiological stabilities, which allow maintaining emulsions in stock (Dumay et al., 2013). In fact, recent research at our laboratory has allowed to develop aseptically packaged beverages by UHPH-processing at 300 MPa (Amador-Espejo, Suàrez-Berencia, Juan, Bárcenas, & Trujillo, 2014; Poliseli-Scopel, Hernández-Herrero, Guamis, & Ferragut, 2014). However, a major problem with this kind of technology is the lower particle size associated with UHPH in products when compared to conventional treatments. It is widely known that when emulsions show higher specific surface area, it is likely for lipid oxidation to easily occur (McClements & Li, 2010). Nonetheless, the debate continues about the best strategies for management of lipid oxidation, and many other factors that affect it, such as the physical structure of emulsions, amount and type of emulsifiers or bulk oil-phase (Berton-Carabin et al., 2014). Most lipid oxidation studies in emulsions have been carried out using only antioxidants or other emulsification techniques (Cui, Kong, Chen, Zhang, & Hua, 2014; Shao & Tang, 2014; Wan, Wang, Wang, Yuan, & Yang, 2014). Hebishy et al. (2015) has been the only research which has examined the effect of UHPH (100 and 200 MPa) on oxidative stability thus far. UHPH emulsions were found to be more oxidatively stable than their CH counterparts. In particular, emulsions stabilized with 1 and 2% whey protein isolate (WPI) and treated with 100 MPa and emulsions stabilized with 4% WPI and treated with 200 MPa showed the most promising results against lipid oxidation. However, further research on this topic needs to be accomplished before the association between UHPH technology and lipid oxidation is more clearly understood. The aim of this study was to investigate the effect of UHPH (100–300 MPa) compared to conventional homogenization (CH) at 15 MPa on oxidative stability of soybean oil emulsions stabilized by SPI. Our previous research showed that o/w emulsions stabilized with SPI and treated by UHPH were more physically stable than CH emulsions (Fernández-Ávila et al., 2015). Particularly, UHPH emulsions treated at 100 and 200 MPa with 20% oil were the most stable due to lower particle size, greater viscosity and partial protein denaturation (Fernández-Ávila et al., 2015). It is important to note that the studied composition of the emulsions in this manuscript proposed the combination of soybean oil as a source of polyunsaturated fat with high propensity to be oxidized and soy protein isolate (SPI) as an emulsifier. Additionally, a thermal treatment was carried out on the protein solutions before CH treatment, to determine whether complete denaturation of proteins would similarly affect the emulsions characteristics or improve the oxidative stability. Droplet size distribution, emulsifying activity index, microstructure, surface protein concentration and lipid oxidation of prepared emulsions were evaluated for various soybean oil concentrations (10 and 20%, v/v) emulsified with SPI.

2. Material and methods

2.1. Materials

A commercial SPI (PRO-FAM 974) was purchased from Lactotecnia (Barcelona, Spain). The composition of this commercial SPI according to the manufacturer was: ⩾90% protein, <4% fat, <6% moisture, and less than 5% ash (dry basis, w/w). SPI PRO-FAM 974 has acid character and an isoelectric point of 4.6 (Kinsella, 1979) due to the high content of glutamic acid (Glu, 19.2%) and aspartic acid (Asp, 11.5%). Solubility of PRO-FAM 974 at pH = 7 is 39.5% (Bissegger, 2007). Soybean oil was purchased from Gustav Heess (Barcelona, Spain). Peroxide value of the soybean oil was below 5 mmol O_2/kg and the acidity index was below 8.9×10^{-3} mmol KOH/g. All other chemicals used were of analytical or better grade.

2.2. Preparation of oil-in-water emulsions

Oil-in-water emulsions were prepared with a fixed content of SPI (4%, w/v) and different contents of soybean oil (10 and 20%, v/v). Firstly, the stock protein dispersion (4%, w/v) was prepared by dispersing SPI in deionized water using a high-speed dispersing unit at a rate of about 250 rpm for 1 h at 25 °C. The heated SPI dispersions were prepared by heating the stock dispersion at 95 °C for 15 min to allow complete denaturation of soy protein (Keerati-u-rai & Corredig, 2009), and then cooled immediately in an ice bath to room temperature. Protein dispersions were stored overnight at 4 °C to allow complete hydration. Protein dispersions and oil were equilibrated at 20 °C (inlet temperature) before mixing. Pre-emulsions (or coarse emulsions) were prepared by mixing the native or denatured protein dispersions with the soybean oil using a rotor-stator emulsifying unit (model Diax 900, Heidolph, Kehlheim, Germany) at 15000 rpm for 4 min. The coarse emulsions were further homogenized through a high-pressure homogenizer or by conventional homogenization (Stansted Benchtop Homogenizator nG12500, Stansted Fluid Power Ltd., Essex, UK). The emulsions were treated at 100, 200 and 300 MPa (single-stage) by the high-pressure homogenizer (flow rate of 8 L/h) provided with a high-pressure ceramic needle-seat valve. The pre-homogenized native and denatured dispersions were further homogenized at 15 MPa with a ceramic ball-seat valve (conventional homogenization treatments, single-stage). Pre-emulsions were passed through both devices with an inlet temperature (T_{in}) of 20 °C. The outlet temperature of emulsions were controlled by a heat exchanger (Inmasa, Reus, Spain) located immediately after the high-pressure valve or ball-seat valve. During treatments, the T_{in}, the temperature after the high pressure valve (T_1), and outlet temperature were monitored. The pH of all the resulting emulsions was 7.14.

2.3. Determination of oil droplet size distribution

The particle size distribution in the fresh emulsion samples (UHPH and CH emulsions) 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. The optical parameters used were: a refractive index of 1.475 for the soybean oil and a refractive index of 1.332 for water. The volume-weighted mean diameter ($d_{4.3}$, μm), the median particle size by volume (d_{50}) and the specific surface area (SSA, m^2/mL) were determined. The specific surface area (SSA) was calculated according to the following Eq. (1):

$$SSA\,(m^2/mL) = \left(\frac{6\varnothing}{d_{3.2}}\right) \quad (1)$$

where $\varnothing$ is the oil volume fraction and the $d_{3.2}$ the volume-surface average diameter of the particles.

2.4. Emulsifying activity index

Emulsifying activity index (EAI) was determined according to the method of Pearce and Kinsella (1978) with some modifications. Aliquots (10 μL) were taken from the emulsions and diluted in 10 mL of 0.1% (w/v) SDS solution. After vortex mixing, the absorbance of the diluted emulsions was determined in a 1-cm path length cuvette at a wavelength of 500 nm in the UV-visible spectrophotometer (CECIL model 9000 series, Cambridge, UK). EAI value was calculated using the following Eq. (2):

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

where (DF) is the dilution factor which could be changed from emulsion to another depending on the particle size and particles concentration, (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 (∅) is the optical path (0.01 m). Emulsions were analyzed immediately after production expressing the emulsifying activity index (EAI).

2.5. Interfacial protein concentration

Surface protein concentration of oil droplets was determined according to the method described by Desrumaux and Marcand (2002). According to the authors, the cream layer was isolated by centrifugation, clarified, and then the protein content was determined. The protein content in the isolated purified protein layers was determined by the Kjeldahl procedure on a Kjeltec Auto 1030 Analyzer (Tecator, Höganäs, Sweden), calculating crude protein content as N × 6.38. Surface protein concentration (SPC) was calculated using the following Eq. (3):

$$SPC\,(mg/m^2) = \frac{mg\ adsorbed\ protein/mL\ emulsion}{SSA\,(m^2/mL)} \quad (3)$$

Adsorbed protein percentage (ΔP, %) in the cream phase was calculated with respect to initial protein concentration.

2.6. Analysis of lipid oxidation

2.6.1. Lipid hydroperoxides content

CH and UHPH emulsions with sodium azide (0.02% w/v) were oxidized at 37 °C for 14 days. The formation of lipid peroxides was evaluated according to the method of Hu, Julian McClements, and Decker (2004). Lipid hydroperoxide concentrations (mmol/L of emulsion) were determined using a standard curve made from cumene hydroperoxide. Afterwards, data was expressed in mmol/kg of oil in order to differentiate between emulsions with low and high volume oil-phase fractions regarding the formation of hydroperoxides.

2.6.2. Thiobarbituric acid-reactive substances (TBARs)

To determine the formation of secondary oxidation products, TBARs technique was performed. It detects the formation of malondialdehyde, which reacts with thiobarbituric acid (TBA). Malondialdehyde or TBARs content of the emulsions upon storage at 37 °C for 14 days was determined using a process as described by Sørensen and Jørgensen (1996), with a few modifications. In brief, emulsions (3 mL) were mixed with 6 mL of trichloracetic acid (TCA) reagent (7.5%, w/v) and were vortexed. The resultant mixtures were filtered with a 1 μm microporous membrane after 20 min of the TCA addition. The filtered (2 mL) were placed in test tubes containing TBA reagent (0.8%, w/v) and they were mixed by vortexing. The resultant mixtures were heated in a water bath (95 °C) for 15 min, and then cooled immediately in an ice-bath to slow down the reaction. The absorbance of the final extracts was recorded at 532 nm. The TBARs concentration (μmol/L of emulsion) was determined according to a standard curve with 1,1,3,3-tetraethoxypropane. Again, data was expressed in μmol/kg of oil to consider the effect of oil content on lipid oxidation.

2.7. Transmission electron microscopy (TEM)

To examine the changes in emulsion microstructure, emulsion samples were observed by transmission electron microscopy, preparing samples as described by Fernández-Ávila et al. (2015). Emulsions were mixed with warm 2% low-temperature gelling agarose (type VII, Sigma Aldrich) at a 1:1 ratio the same day of homogenization process. The mixture was allowed to gel and was cut 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 60 °C for 48 h. Semithin sections (0.03–0.05 μm thick) were cut with a Reichert ultracut microtome, 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. The software ImageJ was used for the analysis of the micrographs, containing steps such as contrasting, and numerical evaluation of the thickness (nm) of the droplet cross sectional areas.

2.8. Statistical analyses

Descriptive statistics, mean and standard deviation, were listed for each variable in this study. In order to evaluate the physical stability of emulsions among type of emulsion (CH or UHPH) and oil concentration (10 and 20%), one-way ANOVA was performed. The statistical analysis was performed using the SPSS® v17.0 package to a 95% level of significance and Tukey adjustment was performed ($P < 0.05$). Experiments were repeated in duplicate and on two independent occasions.

3. Results and discussion

3.1. Emulsifying activity index (EAI)

The kinetics aspects of protein adsorption are very important for the practical performance of proteins as emulsifying agents, such as the so-called EAI (Pearce & Kinsella, 1978).

The EAI increased for CH and UHPH emulsions with 20% (v/v) of soybean oil in comparison with their counterparts made with 10% (v/v) (Table 1). This fact indicates that during homogenization, the generated area was enough to compensate more protein to cover oil droplets at 20% (v/v) (Hebishy et al., 2015).

Nevertheless, the amount of emulsifier required to stabilize an emulsion depends on the oil-water interfacial area, rather than the oil concentration, and so the emulsifying capacity depends on the size of the droplets during agitation (McClements, 2005). In this study, d_{50} and $d_{4,3}$ of pre-emulsions were around 120 and 150 μm, respectively. Such a large particle size is due to the fact that commercial SPI contains aggregates as a result of the partial denaturation of the protein. Subsequently, particle size values of pre-emulsions were about 20-fold larger than the UHPH and CH

Table 1
Mean ± SD of temperature reached in the high-pressure valve (T_1), particle size distribution indices (d_{50} and $d_{4,3}$), specific surface area (SSA, m^2/mL), emulsifying activity index (EAI, m^2/g), surface protein concentration (SPC, mg/m^2) and adsorbed protein percentage (ΔP%) of emulsions containing soybean oil (10 and 20%, v/v) and stabilized by conventional homogenization (15 MPa) and Ultra-High Pressure Homogenization at 100, 200 and 300 MPa with 4% (w/v) of SPI in continuous phase (T_{in} = 20 °C).

Emulsion treatment			Particle size			Emulsifying properties	Interfacial protein layer	
MPa	Oil (%)	T_1 (°C)	d_{50} (μm)	$d_{4,3}$ (μm)	SSA (m^2/mL)	EAI (m^2/g)	SPC (mg/m^2)	ΔP (%)
15	10	32.0 ± 1.4	1.0 ± 0.0^{bc}	1.5 ± 0.2^{bc}	11.7 ± 0.0^{ef}	5.9 ± 1.1^{ef}	2.4 ± 0.0^{bc}	2.6 ± 0.3
15.D*		31.5 ± 3.5	0.8 ± 0.2^{b}	1.3 ± 0.1^{bc}	12.1 ± 0.6^{ef}	6.3 ± 0.1^{de}	2.2 ± 0.3^{bc}	2.4 ± 0.1
100		51.5 ± 2.1	0.4 ± 0.0^{d}	0.6 ± 0.0^{c}	21.3 ± 0.2^{bc}	4.9 ± 0.2^{ef}	1.1 ± 0.2^{de}	2.3 ± 0.5
200		72.5 ± 2.1	0.3 ± 0.0^{d}	0.7 ± 0.4^{bc}	25.6 ± 0.1^{ab}	3.9 ± 0.2^{ef}	0.9 ± 0.0^{e}	2.4 ± 0.1
300		100.0 ± 0.0	0.4 ± 0.1^{d}	3.7 ± 0.7^{a}	26.7 ± 2.1^{a}	3.4 ± 0.1^{f}	0.8 ± 0.1^{e}	2.2 ± 0.3
15	20	31.5 ± 3.5	1.2 ± 0.1^{a}	2.2 ± 0.5^{ab}	10.1 ± 0.9^{f}	14.1 ± 1.3^{a}	3.4 ± 0.2^{a}	3.4 ± 0.6
15.D		31.5 ± 3.5	1.1 ± 0.0^{ab}	1.5 ± 0.1^{bc}	10.8 ± 0.1^{f}	11.9 ± 0.6^{ab}	2.8 ± 0.1^{ab}	3.3 ± 0.5
100		54.0 ± 1.4	0.5 ± 0.0^{d}	0.6 ± 0.3^{c}	16.1 ± 0.9^{de}	10.9 ± 0.4^{bc}	2.2 ± 0.3^{bc}	3.6 ± 0.3
200		76.5 ± 0.7	0.4 ± 0.0^{d}	0.8 ± 0.1^{bc}	19.2 ± 2.8^{cd}	9.6 ± 0.1^{bc}	2.0 ± 0.3^{c}	3.8 ± 0.0
300		103.5 ± 2.1	0.4 ± 0.0^{d}	3.1 ± 0.6^{a}	19.8 ± 1.1^{cd}	9.0 ± 1.0^{cd}	1.9 ± 0.2^{cd}	3.7 ± 0.1

$^{a–f}$ Different letters in the same column indicate significant differences ($P < 0.05$) between treatments.
* D = O/w emulsions containing preheated SPI (95 °C, 15 min) and stabilized by conventional homogenization.

emulsions, indicating an efficient reduction in particle size by the homogenization procedure.

In general, CH emulsions presented larger EAI values than UHPH emulsions owing to an increase in area created per unit mass in the later emulsions during emulsification (Table 1). In fact, the EAI of the emulsions containing 10 and 20% (v/v) of soybean oil treated from 15 to 300 MPa was inversely correlated with SSA when it was expressed per unit volume (Fig. 1). Significantly ($P < 0.05$) different EAI levels between CH emulsions stabilized with the non-heated SPI and UHPH emulsions containing 20% (v/v) of soybean oil were evident, and were correlated with their SSA (m^2/mL) values. Emulsifying characteristics are related to denaturation of proteins and their decreased solubility. Floury et al. (2002) showed total denaturation of glycinin in UHPH emulsions homogenized at 300 MPa. We can assume that changes in the emulsifying properties were affected due to structural changes by partial/total denaturation of proteins after UHPH treatments, being that the UHPH treatment at higher pressure (300 MPa) is the most intense (Table 1). This is supported by the largest $d_{4,3}$ values of emulsions treated by 300 MPa ($P < 0.05$, Table 1), as $d_{4,3}$ is a parameter more sensitive to aggregation than d_{50}, which showed no significant difference between UHPH emulsions ($P > 0.05$, Table 1). Indeed, it has been previously reported that UHPH destroys the original aggregates of pre-emulsions but can also produce new aggregates (Fernández-Ávila et al., 2015).

3.2. Interfacial properties of protein-stabilized emulsions

3.2.1. Adsorbed protein at the interfacial layer

Surface protein concentration (SPC) of emulsions, which corresponds to the mass of emulsifier required to cover a unit area of droplet surface, was directly correlated with EAI (Fig. 1). Thus, emulsions found to exhibit more interfacial packing, further showed an increased emulsifying capacity. Moreover, surface loads encountered in the emulsions of this study are consistent with those of other studies that presented a range from 0.8 to 4.5 mg/m^2 (Bos & van Vliet, 2001; Cui et al., 2014; Hebishy et al., 2015).

UHPH emulsions, which showed higher SSA, exhibited significantly ($P < 0.05$) lower protein concentration at the interface compared to CH emulsions, as SPC shows (Table 1). This fact could be attributed to the fastest spreading during UHPH and rearrangement of adsorbed protein molecules at the interface. However, this data must be interpreted with caution, because the mass of emulsifier adsorbed onto the droplet surface per unit volume appears to be the same in emulsions containing 10 or 20% (v/v) ($P > 0.05$), and UHPH emulsions homogenized at 100, 200 and 300 MPa containing 20% (v/v) oil were the most packed

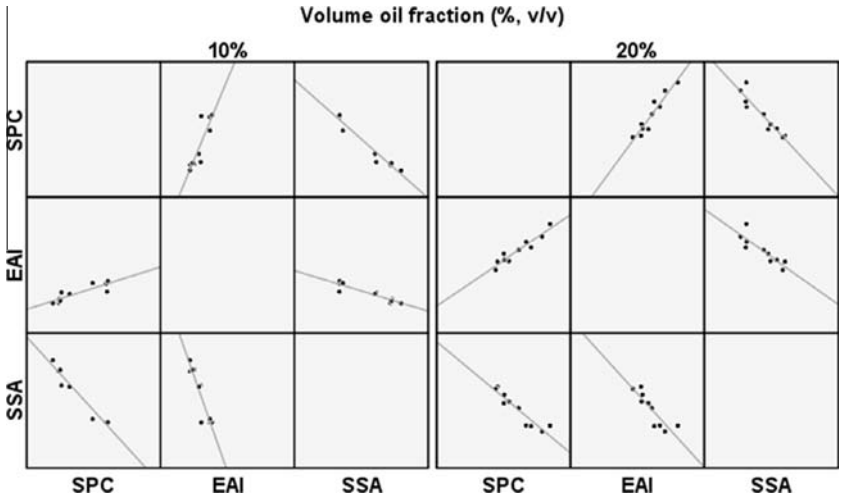

Fig. 1. Scatterplot matrix for correlations between specific surface area (SSA, m^2/mL), emulsifying activity index (EAI, m^2/g) and surface protein concentration (SPC, mg/m^2) of emulsions treated by conventional homogenization stabilized by non-heated (15 MPa) and heated SPIs (15 MPa – d) and emulsions treated by Ultra-High Pressure Homogenization and stabilized by untreated SPI under different homogenizing pressures (100–300 MPa). The protein concentration in continuous phase was 4% (w/v), and the volume oil fraction 10% (v/v) for A, and 20% (v/v) for B.

(35.9 ± 3.0 mg/mL, 37.1 ± 0.6 mg/mL and 36.9 ± 1.1 mg/mL, respectively). These values are also supported by the same ΔP (%) in all treatments, in which those homogenized at 100 and 200 MPa express higher protein content at the interfacial region (Table 1). In all emulsions, the mass was not equal to the initial concentration (40 mg/mL or 4% v/v).

In the case of CH emulsion containing 20% (v/v) stabilized with non-heated SPI, a thicker monolayer of proteins is present at the interfaces, as TEM image (Fig. 2B) and the highest SPC value (Table 1) showed. The adsorbed layer increases the effective size of droplets, and therefore the creaming rate is increased by a factor of $(1 + \delta/r)^2$, where δ is the thickness of the adsorbed layer (McClements, 2005). Hence, it is possible that this effect on creaming was significant as the thickness of the adsorbed layer was around 17 nm. In effect, preliminary work on physical stability of the same emulsions reported that CH emulsions experienced more creaming compared to their UHPH emulsions counterparts (100, 200 and 300 MPa) (Fernández-Ávila et al., 2015). Additionally, CH emulsions containing 20% (v/v) stabilized with non-heated SPI presented higher SPC than the homologous CH emulsions stabilized with heated SPI, because the heating of SPI increases their surface hydrophobicity and decreases its solubility. In this sense, more protein aggregates and protein aggregate clusters were observed in the continuous phase rather than at the interfacial area (Fig. 2C and D). When more aggregates are present, more protein is required to obtain full coverage of the interface, and this could be the reason for an observed reduction in surface load of CH emulsions containing 20% (v/v) stabilized with heated SPI (Table 1). Another possible explanation for this is that the surface protein excess concentration depends on the rate of the conformational changes compared to the adsorption rate (McClements, 2005). If adsorption onto the surface is faster than unfolding, the interfacial membrane will tend to be thicker. Then, as proteins were already denatured, resultant adsorption was slower than unfolding. The membrane appeared to be thinner because significant spreading of SPI molecules occurred at the continuous phase (Fig. 2C and D).

3.2.2. Microstructure of emulsions

Emulsions stabilized by soy proteins are particularly sensitive to pressure and thermal treatments, because these proteins unfold exposing reactive nonpolar and sulfhydryl groups (Cruz et al., 2009; Faraji et al., 2004). Nevertheless, it has been previously

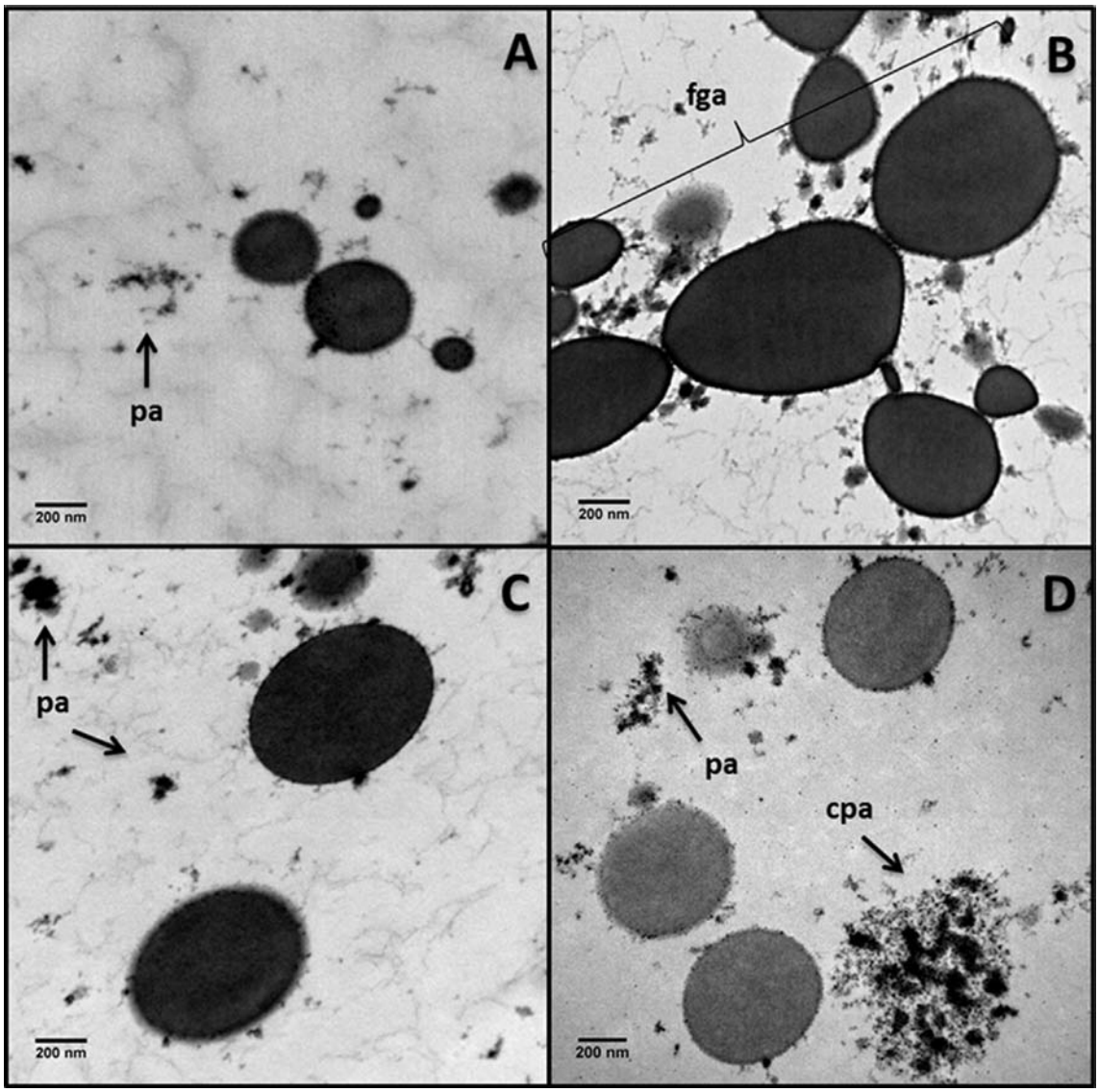

Fig. 2. TEM images (×50.000 and ×120.000) of emulsions treated by conventional homogenization at 15 MPa and stabilized by non-heated (A, B) and heated SPIs (C, D). The protein concentration in continuous phase was 4% (w/v), and the volume oil fraction 10% (v/v) for A and C, and 20% (v/v) for B and D. (pa) protein aggregates, (cpa) cluster of protein aggregates, (fga) fat globule aggregate. Bars represents 200 nm.

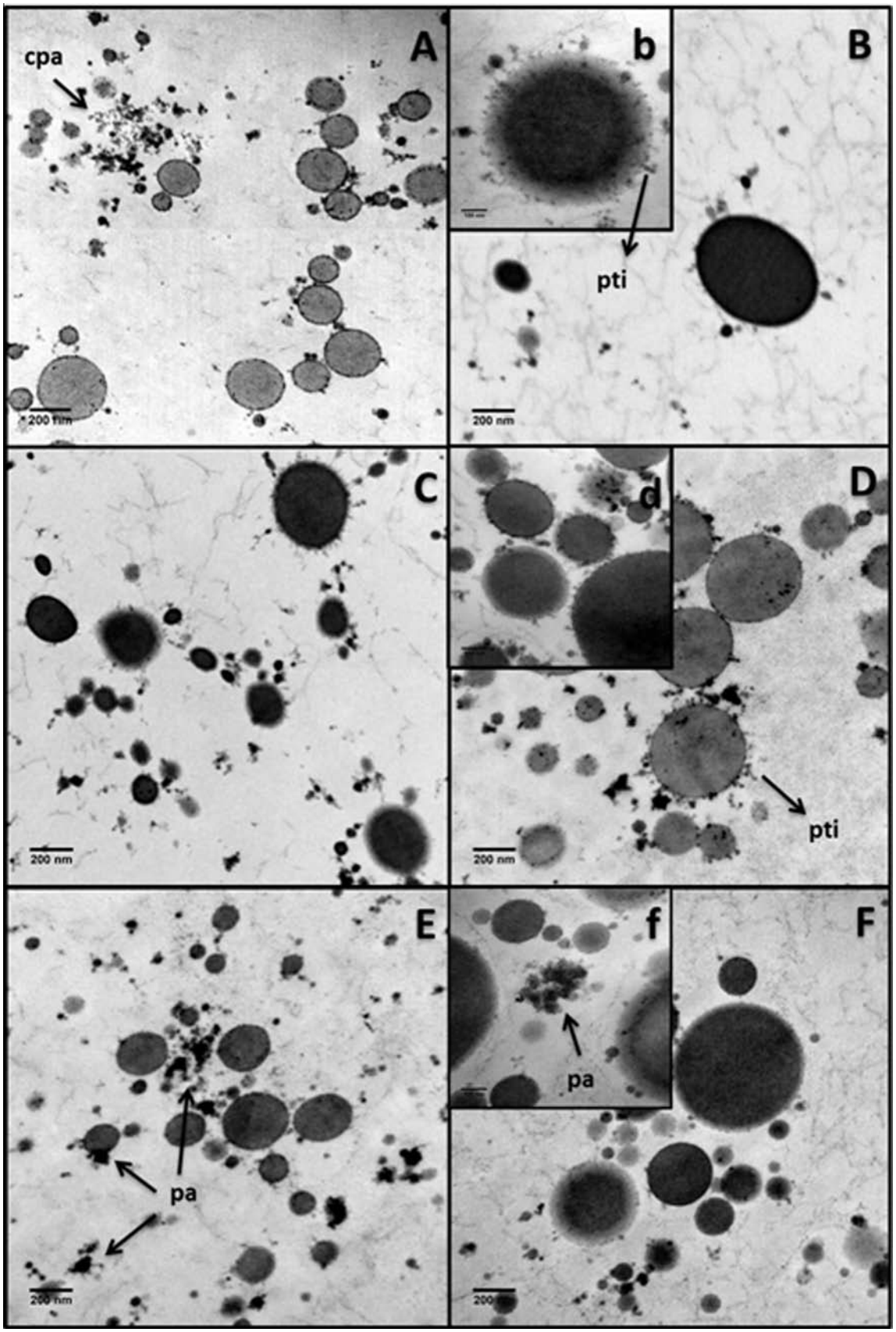

Fig. 3. TEM images (×50.000 and ×120.000) of emulsions treated by Ultra-High Pressure Homogenization at 100 MPa (A, B), 200 MPa (C, D) and 300 MPa (E, F). The protein concentration in continuous phase was 4% (w/v), and the volume oil fraction 10% (v/v) for A, C and E, and 20% (v/v) for B, D and F. (pti) protein tails at the interface, (pa) protein aggregates, (fga) fat globule aggregate. Bars represent 100 (b, d, f) and 200 nm, respectively.

reported that the processing of the protein during pilot plant isolation (most likely heat treatment and spray drying) causes changes in the supramolecular structure of the soy proteins, but still shows quaternary structure (Keerati-u-rai, Wang, & Corredig, 2011). These changes will also depend on their molecular structure; more flexible structures can better adjust to the environmental changes and decrease their free energy during adsorption. In fact, low-pressure emulsions (CH, 15 MPa) stabilized with non-heated SPI, the treatment less affected by pressure or the absence of heating, presented the greater surface load and a more compact monolayer leading to flocculation (Table 1, Fig. 2B). The greater particle size of this emulsion in all treatments (Table 1) could have resulted in droplet flocculation, as fat globule aggregates are observed in Fig. 2B. This is supported as the maximum strength of the depletion interaction increases as the size of emulsion droplets increases (McClements, 2005). Then, the initial flocculation of this emulsion might be the cause of faster creaming and clarification than their UHPH emulsions counterparts (200 and 300 MPa), as previously characterized in our lab (Fernández-Ávila et al., 2015).

UHPH and CH emulsions differed in the configuration that proteins adopted at the interface. The reasons for this are the different phenomena of cavitation, shear and turbulence produced by UHPH technology on the macromolecular conformation of proteins (Dumay et al., 2013). UHPH emulsions showed more random-coil proteins attached at the interfaces as Fig. 3 shows. According to McClements (2005), they are relatively flexible molecules and can therefore rearrange their structures fairly rapidly. In particular, protein tails at the interfaces were observed in UHPH emulsion homogenized at 100 and 200 MPa containing 20% (v/v) oil (Fig. 3B and D).

3.3. Determination of lipid oxidation

3.3.1. Decrease of lipid oxidation with oil-phase volume fraction

At constant emulsification pressure, lipid oxidation decreased with increasing oil content (Fig. 4). This finding is in agreement with other research which found a decrease in lipid oxidation when oil volume fraction increased from 5 to 40% (Sun & Gunasekaran, 2009), or from 5 to 30% (Kargar, Spyropoulos, & Norton, 2011). A possible explanation for the better oxidative stability of emulsions containing 20% (v/v) oil might be their improved creaming stability, as previously reported (Fernández-Ávila et al., 2015). Poor creaming stability leads to the cream phase formation, which is closer to the ambient, so lipid oxidation is favoured (Sun & Gunasekaran, 2009). Also, the propagation of the oxidation proceeds easily in the cream phase, where the distance between droplets is reduced (Berton-Carabin et al., 2014). Another possible explanation for the decrease of lipid oxidation with oil-phase volume fraction is that the aqueous phase fraction decreases proportionally, as well as the water soluble prooxidants, and consequently decrease the amount of free radicals (Kargar et al., 2011). However, as hydroperoxide content from all emulsions were in the

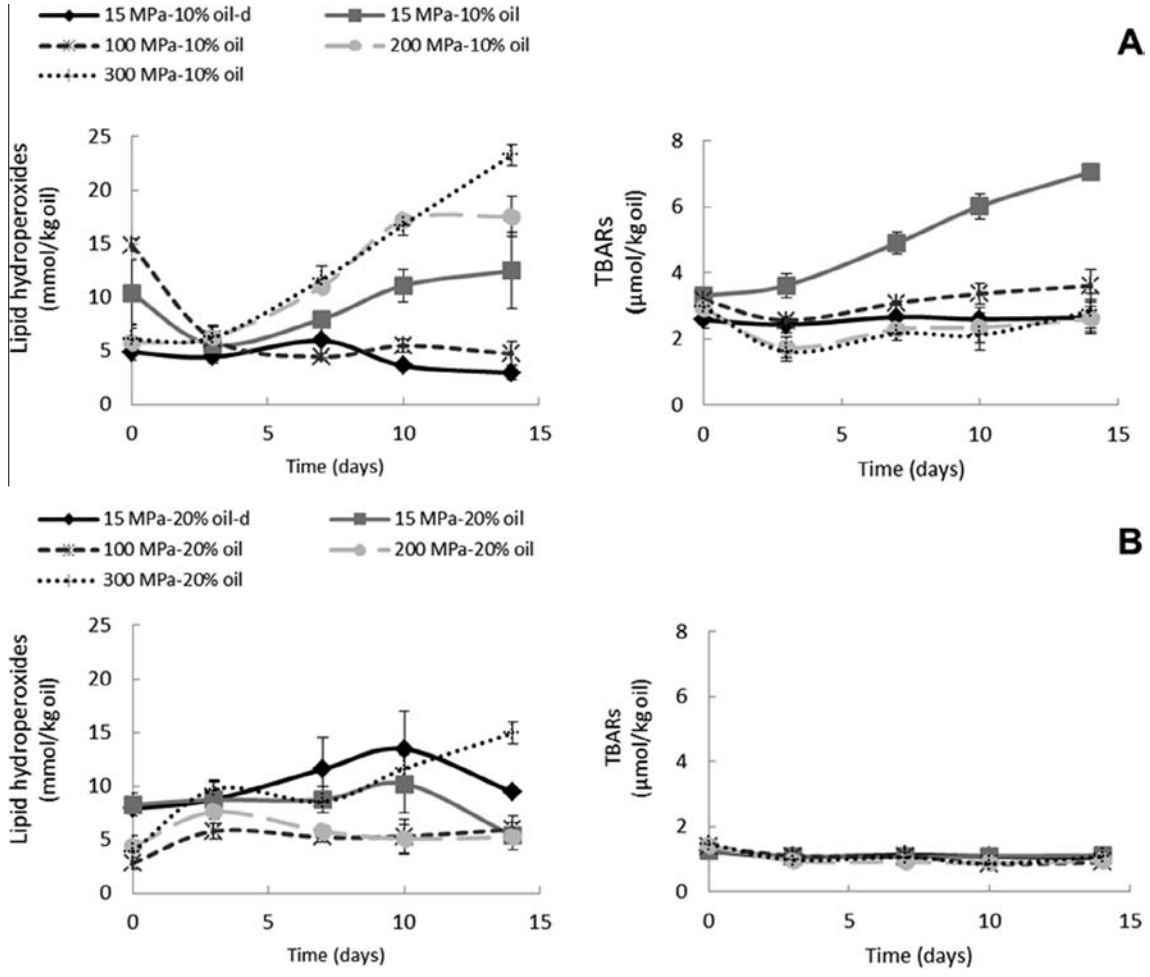

Fig. 4. Lipid hydroperoxides (mmol/kg oil) and TBARs (µmol/kg oil) of emulsions treated by conventional homogenization stabilized by non-heated (15 MPa) and heated SPIs (15 MPa – d) and emulsions treated by Ultra-High Pressure Homogenization and stabilized by untreated SPI under different homogenizing pressures (100–300 MPa). The protein concentration in continuous phase was 4% (w/v), and the volume oil fraction 10% (v/v) for A, and 20% (v/v) for B. Bars represent the standard error of the mean.

same range, owing to other factors involved (ca. pressure applied, surface load), better oxidative stability of emulsions containing 20% (v/v) oil was more evident when observing the TBARs values. In particular, UHPH emulsions containing 20% (v/v) of soybean oil homogenized at 100 and 200 MPa exhibited the best lipid oxidation stability, showing the higher ΔP percentages and clear protein tails at the interface (Table 1, Fig 3b,D). These emulsions exhibited a significantly higher specific interfacial area than their counterpart CH emulsion (Table 1 $P < 0.05$). These results indicate that a higher interfacial area is not the only determining factor favouring lipid oxidation, as other authors have recently confirmed (Hebishy et al., 2015). In the literature reviewed, higher levels of hydroperoxides were found in soy protein-stabilized emulsions in the range of 0–160 mmol/kg oil, albeit those emulsions contained antioxidants (Cui et al., 2014; Wan, Wang, Wang, Yang, & Yuan, 2013; Wan et al., 2014). Additionally, this study found lower TBARs values for all the emulsions compared to other soybean oil-in-water emulsions containing 10–20% (v/v) oil in the literature (Cheng, Xiong, & Chen, 2010; Huang, Lu, Wang, & Wu, 2011; Shao & Tang, 2014), indicating little formation of secondary oxidation products.

3.3.2. Decrease of lipid oxidation with surface protein concentration

The amount of protein used to stabilize emulsions is generally higher than the actual amounts required just to cover the interfacial area (Berton-Carabin et al., 2014; McClements, 2005). The main reason is the poor solubility that commercial protein isolates show. Consequently, a substantial fraction of the proteins remains unadsorbed, and therefore partitions in the water and oil-phase have to be carefully considered. In this study, 4% (w/v) of SPI was applied. UHPH and CH emulsions stabilized with non-heated SPI containing 20% (v/v) oil showed significantly more protein coverage in the interfaces than their counterparts containing 10% (v/v) oil ($P < 0.05$) (Table 1). Then, oil-phase volume fraction amount not only has an effect on better oxidative stability of emulsions containing 20% (v/v), but also acts to increase SPC. A graphical scheme illustrating the relationship among lipid oxidation and interfacial properties of the various emulsions is represented in Fig. 5. Particularly, UHPH emulsions containing 20% (v/v) oil showed less primary and secondary oxidation products. In parallel, unadsorbed proteins can induce depletion flocculation, leading to the physical and chemical destabilization of the emulsion (Berton-Carabin et al., 2014). The emulsions oxidized at higher extent were CH and UHPH emulsion treated at 100 MPa containing 10% oil (Fig. 1). They exhibited significantly smaller SPC values than their counterparts, as well as higher amounts of unadsorbed proteins in the continuous phase (Table 1). So that extensively depletion flocculation occurred in the presence of aggregates, as

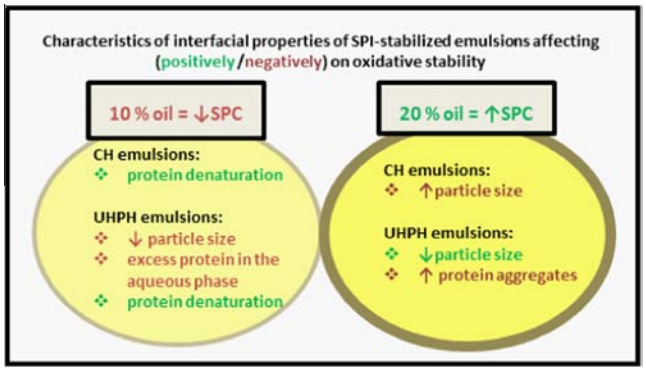

Fig. 5. Graphical scheme for correlations between lipid oxidation and interfacial properties of the emulsions.

it is shown in Figs. 2A and 3A, leading to higher lipid oxidation rate over time (Fig. 4). This is supported by higher hydroperoxides/TBARs content in CH emulsions and TBARs content in UHPH emulsions containing 10% oil, compared to their homologous emulsions containing 20% oil. Furthermore, another factor influencing the higher lipid oxidation of UHPH emulsion containing 10% oil, compared to their homologous emulsions containing 20% oil, may be the significantly ($P < 0.05$) higher SSA values (Table 1). UHPH emulsion with 10% oil also showed poorer physical stability compared to other formulations (Fernández-Ávila et al., 2015). It is therefore likely that such connections exist between good physical and oxidative stability.

3.3.3. Effect of pressure level and heat treatment of proteins on lipid oxidation

The homogenization equipment, as well as the pressure applied, affects the relative adsorption of soy proteins (in particular β-conglycinin and glycinin), leading to different interfacial compositions (Malaki Nik, Wright, & Corredig, 2011; Molina, Defaye, & Ledward, 2002). UHPH technology seemed to have an antioxidant effect on emulsions containing 20% (v/v) oil by reducing the initial content of hydroperoxides (8 mmol/kg oil) in half, as seen in fresh emulsions (day 1). This trend was not fully observed in emulsions containing 10% (v/v) oil, as the UHPH emulsion treated at 100 MPa experienced greater primary oxidation on fresh emulsions. Lipid oxidation compounds may lead to desorption of proteins from interface and contribute to physical destabilization of the system (Genot, Meynier, & Riaublanc, 2003), as hydroperoxides from linoleic acid, methyl linoleate, and trilinolein are more surface-active than their nonperoxidized counterparts (Nuchi, Hernandez, McClements, & Decker, 2002). In fact, when comparing physical stability of similar UHPH emulsions, the emulsion treated at 100 MPa showed the poorest physical stability (Fernández-Ávila et al., 2015). Nevertheless, UHPH technology also reduced the initial hydroperoxides content (10 mmol/kg oil) by half on fresh emulsions containing 10% (v/v) oil (day 1) treated with 200 and 300 MPa (Fig. 4). The sinuosity manner of the TBARs at day 3 for these emulsions, instead of a constant increase or decrease during time, may be related to a start in the observation of an increase in peroxides. Afterwards, TBARs of these emulsions kept constant over time. This result may be explained by the fact that UHPH oil-in-water emulsions stabilized by 3% glycinin and treated at 200 and 300 MPa undergoes partial or total denaturation of proteins (Floury et al., 2002). Denaturation temperature of major soybean proteins (β-conglycinin and glycinin) occurs at 75 and 95 °C, respectively (Keerati-u-rai & Corredig, 2010). As UHPH emulsions homogenized at 100 MPa and containing 10% (v/v) oil experienced 51.5 ± 2.1 °C at the exit of the homogenizer valve, total denaturation did not occur. Additionally, it has been previously reported that UHPH at 350 MPa strongly decreases the solubility of 2% glycinin dispersions, but it remains preserved at relatively moderate homogenization pressure (<250 MPa). It can thus be suggested that, taking into account other factors of the emulsion (amount of protein and oil-phase volume fraction, specific surface area, pH, soluble or non-soluble protein attached at the interface, e.g.), denaturation could play a positive role on lipid oxidation stability. This might be supported by different results of hydroperoxides and TBARs content in CH emulsions containing 10% oil (Fig. 4). Those prepared with the heated SPI, exhibited an improved primary lipid oxidation, even though the same SPC values were obtained within emulsions. A possible explanation for these lower levels of oxidation products is that hydrophobic interactions between heated protein and lipophilic oxidation products might have increased due to the exposure of hydrophobic residues after thermal processing of protein dispersions (95 °C, 15 min). In fact, Tong, Sasaki, McClements, and Decker (2000) found that the heating of whey

protein increased the exposure of reduced sulfhydryl groups, particularly at 80 and 90 °C for 15 min. As reduced sulfhydryl groups act as free radical scavengers, antioxidant activity of salmon oil emulsions stabilized with the heated whey protein was improved. Conversely, heated SPI emulsions containing 20% oil in this work showed no remarkable improvement on lipid oxidation compared to those stabilized with non-heated SPI, due to lower interfacial protein accumulation and EAI indices (Table 1). Another possible reason why there was any observable improvement in lipid oxidation of emulsions containing 20% by previously heating the SPI dispersions might be related with the lower protein content remaining unadsorbed in the aqueous phase, compared to emulsions with 10% oil. Thus, the excess of proteins and hydrophobic residues caused by heating in the aqueous phase of those emulsions (Table 1) might have decreased lipid oxidation, by interacting with metal ions, or by scavenging free-radicals and then decreasing lipid oxidation (Berton-Carabin et al., 2014; Sun & Gunasekaran, 2009).

4. Conclusions

UHPH technology is suitable to produce SPI-stabilized submicron emulsions containing soybean oil with a higher oxidative stability compared to emulsions obtained by CH. Lipid oxidation of emulsions was strongly dependent on volume oil-phase fraction and protein attachment at the oil droplets, since the more stable emulsions exhibited the highest protein percentage at the interfaces (UHPH emulsions containing 20% (v/v) of soybean oil and homogenized at 100 and 200 MPa). Indeed, lipid oxidation was negatively affected when CH and UHPH emulsions homogenized at 100 and 200 MPa did not contain enough oil content to bind emulsifier (10% of soybean oil). In those emulsions, it has been suggested that the presence of a larger amount of protein aggregates in the continuous phase accelerated lipid oxidation. However, oxidative stability was negatively affected in the emulsions treated at 300 MPa due to the displacement of the protein to rearrange into aggregates as a result of the higher temperatures reached at the exit of the UHPH-valve. Nevertheless, our results also show heating the soy protein isolate dispersions before CH treatment improved oxidative stability of emulsions containing 10% (v/v) of soybean oil. Taken together, these findings suggest the advantages for lipid oxidation in promoting UHPH technology.

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References

Amador-Espejo, G. G., Suàrez-Berencia, A., Juan, B., Bárcenas, M. E., & Trujillo, A. J. (2014). Effect of moderate inlet temperatures in ultra-high-pressure homogenization treatments on physicochemical and sensory characteristics of milk. *Journal of Dairy Science, 97*(2), 659–671.

Berton-Carabin, C. C., Ropers, M.-H., & Genot, C. (2014). Lipid oxidation in oil-in-water emulsions: Involvement of the interfacial layer. *Comprehensive Reviews in Food Science and Food Safety, 13*(5), 945–977.

Bissegger, S. (2007). *Production of bioactive soy peptides* Dissertation.Canada: University of Waterloo.

Bos, M. A., & van Vliet, T. (2001). Interfacial rheological properties of adsorbed protein layers and surfactants: A review. *Advances in Colloid and Interface Science, 91*(3), 437–471.

Cheng, Y., Xiong, Y. L., & Chen, J. (2010). Antioxidant and emulsifying properties of potato protein hydrolysate in soybean oil-in-water emulsions. *Food Chemistry, 120*(1), 101–108.

Cruz, N. S., Capellas, M., Jaramillo, D. P., Trujillo, A. J., Guamis, B., & Ferragut, V. (2009). Soymilk treated by ultra high-pressure homogenization: Acid coagulation properties and characteristics of a soy-yogurt product. *Food Hydrocolloids, 23*(2), 490–496.

Cui, Z., Kong, X., Chen, Y., Zhang, C., & Hua, Y. (2014). Effects of rutin incorporation on the physical and oxidative stability of soy protein-stabilized emulsions. *Food Hydrocolloids, 41*, 1–9.

Desrumaux, A., & Marcand, J. (2002). Formation of sunflower oil emulsions stabilized by whey proteins with high-pressure homogenization (up to 350 MPa): effect of pressure on emulsion characteristics. *International Journal of Food Science and Technology, 37*(3), 263–269.

Dumay, E., Chevalier-Lucia, D., Picart-Palmade, L., Benzaria, A., Gràcia-Julià, A., & Blayo, C. (2013). Technological aspects and potential applications of (ultra) high-pressure homogenisation. *Trends in Food Science & Technology, 31*(1), 13–26.

EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies) (2010). Scientific Opinion on the substantiation of health claims related to conjugated linoleic acid (CLA) isomers and contribution to the maintenance or achievement of a normal body weight, increase in lean body mass.... *EFSA Journal, 8*(10), 26. 1794.

Faraji, H., McClements, D. J., & Decker, E. A. (2004). Role of continuous phase protein on the oxidative stability of fish oil-in-water emulsions. *Journal of Agricultural and Food Chemistry, 52*(14), 4558–4564.

Fernandez-Avila, C., Arranz, E., Guri, A., Trujillo, A. J., & Corredig, M. (2015). Vegetable protein isolate-stabilized emulsions for enhanced delivery of conjugated linoleic acid in Caco-2 cells. *Food Hydrocolloids, 55*, 144–154.

Fernández-Ávila, C., Escriu, R., & Trujillo, A. J. (2015). Ultra-high pressure homogenization enhances physicochemical properties of soy protein isolate-stabilized emulsions. *Food Research International, 75*, 357–366.

Floury, J., Desrumaux, A., & Legrand, J. (2002). Effect of ultra-high-pressure homogenization on structure and on rheological properties of soy protein-stabilized emulsions. *Journal of Food Science, 67*(9), 3388–3395.

Genot, C., Meynier, A., & Riaublanc, A. (2003). Lipid oxidation in emulsions. In A. Kamal-Eldin (Ed.), *Lipid oxidation pathways* (pp. 190–244). Illinois: AOCS Press.

Gunstone, F. D., & Martini, S. (2010). Chemical and physical deterioration of bulk oils and shortenings, spreads and frying oils. In L. H. Skibsted, J. Risbo, & M. L. Andersen (Eds.), *Chemical deterioration and physical instability of food and beverages* (pp. 413–438). UK: Woodhead Publishing Limited.

Hebishy, E., Buffa, M., Guamis, B., Blasco-Moreno, A., & Trujillo, A.-J. (2015). Physical and oxidative stability of whey protein oil-in-water emulsions produced by conventional and ultra high-pressure homogenization: Effects of pressure and protein concentration on emulsion characteristics. *Innovative Food Science & Emerging Technologies, 32*, 79–90.

Hu, M., Julian McClements, D., & Decker, E. A. (2004). Impact of chelators on the oxidative stability of whey protein isolate-stabilized oil-in-water emulsions containing ω-3 fatty acids. *Food Chemistry, 88*(1), 57–62.

Huang, P.-H., Lu, H.-T., Wang, Y.-T., & Wu, M.-C. (2011). Antioxidant activity and emulsion-stabilizing effect of pectic enzyme treated pectin in soy protein isolate-stabilized oil/water emulsion. *Journal of Agricultural and Food Chemistry, 59*(17), 9623–9628.

Kargar, M., Spyropoulos, F., & Norton, I. T. (2011). The effect of interfacial microstructure on the lipid oxidation stability of oil-in-water emulsions. *Journal of Colloid and Interface Science, 357*(2), 527–533.

Keerati-u-rai, M., & Corredig, M. (2009). Heat-induced changes in oil-in-water emulsions stabilized with soy protein isolate. *Food Hydrocolloids, 23*(8), 2141–2148.

Keerati-u-rai, M., & Corredig, M. (2010). Heat-induced changes occurring in oil/water emulsions stabilized by soy glycinin and β-conglycinin. *Journal of Agricultural and Food Chemistry, 58*(16), 9171–9180.

Keerati-u-rai, M., Wang, Z., & Corredig, M. (2011). Adsorption of soy protein isolate in oil-in-water emulsions: Difference between native and spray dried isolate. *Journal of the American Oil Chemists' Society, 88*(10), 1593–1602.

Kinsella, J. E. (1979). Functional properties of soy proteins. *Journal of the American Oil Chemists' Society, 56*(3), 242–258.

Malaki Nik, A., Wright, A. J., & Corredig, M. (2011). Impact of interfacial composition on emulsion digestion and rate of lipid hydrolysis using different in vitro digestion models. *Colloids and Surfaces B Biointerfaces, 83*(2), 321–330.

Matthäus, B. (2010). Oxidation of edible oils. In E. A. Decker, R. J. Elias, & D. J. McClements (Eds.), *Oxidation in foods and beverages and antioxidant applications. Management in different industry sectors* (Vol. 2, pp. 183–238). UK: Woodhead Publishing Limited.

McClements, D. J. (2005). *Food emulsions: Principles, practice, and techniques* (2nd ed.). Boca Raton, FL: CRC Press.

McClements, D. J., & Li, Y. (2010). Structured emulsion-based delivery systems: Controlling the digestion and release of lipophilic food components. *Advances in Colloid and Interface Science, 159*(2), 213–228.

Molina, E., Defaye, A. B., & Ledward, D. A. (2002). Soy protein pressure-induced gels. *Food Hydrocolloids, 16*(6), 625–632.

Nuchi, C. D., Hernandez, P., McClements, D. J., & Decker, E. A. (2002). Ability of lipid hydroperoxides to partition into surfactant micelles and alter lipid oxidation

rates in emulsions. *Journal of Agricultural and Food Chemistry, 50*(19), 5445–5449.
Pearce, K. N., & Kinsella, J. E. (1978). Emulsifying properties of proteins: Evaluation of a turbidimetric technique. *Journal of Agricultural and Food Chemistry, 26*(3), 716–723.
Poliseli-Scopel, F. H., Hernández-Herrero, M., Guamis, B., & Ferragut, V. (2014). Sterilization and aseptic packaging of soymilk treated by ultra high pressure homogenization. *Innovative Food Science & Emerging Technologies, 22*, 81–88.
Shao, Y., & Tang, C.-H. (2014). Characteristics and oxidative stability of soy protein-stabilized oil-in-water emulsions: Influence of ionic strength and heat pretreatment. *Food Hydrocolloids, 37*, 149–158.
Sørensen, G., & Jørgensen, S. S. (1996). A critical examination of some experimental variables in the 2-thiobarbituric acid (TBA) test for lipid oxidation in meat products. *Zeitschrift Für Lebensmittel-Untersuchung Und-Forschung, 202*(3), 205–210.
Sun, C., & Gunasekaran, S. (2009). Effects of protein concentration and oil-phase volume fraction on the stability and rheology of menhaden oil-in-water emulsions stabilized by whey protein isolate with xanthan gum. *Food Hydrocolloids, 23*(1), 165–174.
Tong, L. M., Sasaki, S., McClements, D. J., & Decker, E. A. (2000). Antioxidant activity of whey in a salmon oil emulsion. *Journal of Food Science, 65*(8), 1325–1329.
Walker, R., Decker, E. A., & McClements, D. J. (2015). Development of food-grade nanoemulsions and emulsions for delivery of omega-3 fatty acids: Opportunities and obstacles in the food industry. *Food & Function, 6*(1), 42–55.
Wan, Z.-L., Wang, J.-M., Wang, L.-Y., Yang, X.-Q., & Yuan, Y. (2013). Enhanced physical and oxidative stabilities of soy protein-based emulsions by incorporation of a water-soluble stevioside-resveratrol complex. *Journal of Agricultural and Food Chemistry, 61*(18), 4433–4440.
Wan, Z.-L., Wang, J.-M., Wang, L.-Y., Yuan, Y., & Yang, X.-Q. (2014). Complexation of resveratrol with soy protein and its improvement on oxidative stability of corn oil/water emulsions. *Food Chemistry, 161*, 324–331.
Waraho, T., Cardenia, V., Decker, E. A., & McClements, D. J. (2010). Lipid oxidation in emulsified food products. In E. A. Decker, R. J. Elias, & D. J. McClements (Eds.), *Oxidation in foods and beverages and antioxidant applications. Management in different industry sectors* (Vol. 2, pp. 306–343). UK: Woodhead Publishing Limited.

CHAPTER 5: Vegetable protein isolate-stabilized emulsions for enhanced delivery of conjugated linoleic acid in Caco-2 cells

[Experiment 3]

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Vegetable protein isolate-stabilized emulsions for enhanced delivery of conjugated linoleic acid in Caco-2 cells

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ABSTRACT

Developing edible delivery systems which offer higher protection and release of bioactive constituents is a current challenge in the food industry. The ability of oil-in-water emulsions (20% oil) stabilized by soy or pea protein isolates (4%) to deliver conjugated linoleic acid (CLA, 6%), was studied. The emulsions were prepared by conventional homogenization (550 bar) using one or five homogenization passes. The physicochemical properties of the emulsions were determined, as well as loading capacity and oxidative stability. The emulsions were subjected to *in vitro* digestion, and tested on absorptive Caco-2 cells. The presence of CLA isomers was followed throughout the process. When comparing similar treatments, soy protein isolate emulsions showed smaller particle size distributions than emulsions prepared with pea protein isolates. Emulsions containing soy proteins showed preferential adsorption of the α' subunit of conglycinin and the A_1, A_2, A_4 subunits of glycinin on the oil droplets. All emulsions protected the encapsulated CLA better than the non-emulsified control in which CLA was oxidized during storage, as well as after *in vitro* digestion and delivery in Caco-2 cells. Similar percentages of bioaccessibility and bioavailability of CLA were found for all the emulsion treatments. The results obtained here open new prospects for using oil-in-water emulsions as structured emulsion-based delivery systems to be used in functional foods containing CLA with health enhancing properties.

1. Introduction

The enrichment of foods with lipophilic bioactives, such as omega-3 fatty acids, carotenoids and phytosterols, is an increasingly important area in the food industry, especially in light of the increased tendency for consumption of low-fat food, which in turn decreases the intake of lipophilic bioactives (Gao et al., 2014). A number of emulsion-based technologies could be used as edible delivery systems of these compounds by the food, medical, and pharmaceutical industries, including conventional emulsions, multiple emulsions, multilayer emulsions, solid lipid particles, and filled hydrogel particles. Emulsion technology is particularly suited for the design and fabrication of delivery systems for encapsulating bioactive lipids (McClements, Decker, & Weiss, 2007; McClements & Li, 2010). To create emulsions kinetically stable for a reasonable period of time, stabilizers such as emulsifiers or texture modifiers need to be added to prevent gravitational separation, flocculation, coalescence and Oswald ripening (McClements, 2005). In this sense, commercial protein isolates are commonly used as emulsifiers. However, they exhibit poor solubility compared to other protein fractions or high-soluble isolates obtained in the laboratory (Keerati-u-rai & Corredig, 2010; Wang et al., 2012).

Within bioactive compounds of lipophilic nature, conjugated linoleic acids (CLAs) are a mixture of positional and geometric isomers of linoleic acid with conjugated double bonds. The CLAs are found naturally in foods derived from ruminant animals, meat, or dairy products. The most representative CLA isomers are c9,t11-18:2 and t10,c12*c*-18:2. CLA has been shown to exert various potent physiological functions such as anticarcinogenic, antiobese, antidiabetic and antihypertensive properties (Koba & Yanagita, 2014; Moon, Lee, Chung, Choi, & Cho, 2008). In recent years, there has been an increasing interest to incorporate CLA into various food products, the most common being dairy products (Campbell, Drake, & Larick, 2003; Jimenez, Garcia, & Beristain, 2008). However, CLA exhibits very poor chemical stability presenting autoxidation and isomerization during thermal processing,

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0268-005X/

such as UHT, which decrease its nutritional value. To overcome these problems, it is necessary to protect/encapsulate CLA in an efficient delivery system, like an oil-in-water emulsion. Gum arabic was found to be a suitable emulsifier for CLA-in-water emulsions (Yao et al., 2013). However, to date the research made with this bioactive compound has focused mainly on microencapsulation by drying processes rather than oil-in-water-emulsions (see, for example, Choi, Ryu, Kwak, & Ko, 2010; Costa et al., 2015; Lalush, Bar, Zakaria, Eichler, & Shimoni, 2004).

There is still significant debate on what the best strategies for CLA protection are since it has been demonstrated that these molecules can decompose to furan fatty acids in the presence of air. Furan fatty acids may have toxicological properties and it has been suggested that encapsulation procedures using spray drying may not be adequate (Buhrke, Merkel, Lengler, & Lampen, 2012). Gao et al. (2014) successfully developed soy lipophilic protein nanoparticles (LPP) as a novel delivery vehicle for conjugated linoleic acids. Vegetable proteins, such as soy protein, are rarely used to deliver hydrophobic compounds, in spite of the gained interest for sourcing more sustainable protein isolates for food product development.

Oil-in-water emulsions containing CLA could be used as ingredients in functional foods; however, efficient delivery of the biological compound and its bioefficacy in the emulsion system needs to be demonstrated. Previous research demonstrated that LPP can protect CLA from oxidation and also show a sustained release profile (Gao et al., 2014). However, so far, no studies evaluated CLA bioavailability in oil-in-water emulsion systems using human intestinal cell models such as Caco-2 cells. *In vitro* experiments, such as the uptake by cell cultures (*e.g., Caco-2 cells*) that mimic the human intestinal epithelium, provide useful insights into possible physicochemical mechanisms that occur during digestion and absorption of lipids (McClements & Li, 2010).

The efficiency of encapsulation, the release and bioefficacy of CLA encapsulated in oil-in-water emulsions stabilized with vegetable proteins have yet to be reported. The aim of this study was to investigate the effect of different oil in water emulsions stabilized by soy and pea protein isolates on the delivery of CLA. Commercially available soy and pea protein isolates were used, to increase the relevance of this research to the food industry (Nishinari, Fang, Guo, & Phillips, 2014). The CLA bioefficacy was studied using *in vitro* digestion and a Caco-2 intestinal cell absorption model.

2. Materials and methods

2.1. Materials

A commercial soy protein isolate (SPI) (PRO-FAM 974) was purchased from Lactotecnia (Barcelona, Spain). The composition of this commercial SPI according to manufacturer was: 90% protein, 4% fat, 6% moisture, and less than 5% ash (dry basis, w/w). Pea protein isolate (PPI) (Nutralys®F85M) was purchased from Roquette Frères S.A. (Lestrem, France). The composition of this commercial PPI according to manufacturer was: 85% protein, 7% moisture, and less than 5% ash (dry basis, w/w). Solubility of both protein isolates at neutral pH was <40%. Soybean oil was purchased from Sigma–Aldrich Corporation (Oakville, ON, Canada). A free fatty acid mixture high in isomers of CLA (Neobee®CLA80) was purchased from Stepan Specialty Products LLC (Maywood, USA), containing 80.4% of total CLA. The two main isomers present were c9,t11-CLA and t10,c12-CLA, in a 50:50 ratio. Other CLA isomers (*cis,cis* and *trans,trans*) were present in minor concentrations (<1.2%). Neobee®CLA80 was obtained from natural safflower oil by a gentle, proprietary process without antioxidants added. All other chemicals used were of analytical or better grade. Pepsin (P7000), pancreatin (P1750), phospholipase A2 (P6534) and bile salts (B8631) were obtained from Sigma–Aldrich.

2.2. Preparation of oil-in-water emulsions

Oil-in-water emulsions (20% oil, v/v) containing conjugated linoleic acid (CLA, 6%) and soybean oil were emulsified with a fixed protein content of SPI or PPI (4%, w/v). Firstly, the stock protein dispersion (4%, w/v) was prepared by dispersing the protein in ultrapure water and stirring for 1 h at 40 °C. Protein dispersions were stored overnight at 4 °C to allow complete hydration. A coarse emulsion was prepared by mixing the protein dispersion with the oil using a hand-held homogenizer (Polytron PT 1200, Kinematica, Fisher Sci., Mississagua, ON, Canada) at 15,000 rpm for 1 min. Conventional homogenization was then carried out at 550 bar for one and five passes using Emulsiflex C5, Avestin (Ottawa, ON, Canada). Emulsions were separated using ultracentrifugation (45 min at 50,000 g and at 25 °C) (OptimaTM LE-80K, with a Ti-45 rotor, Beckman–Coulter, Mississauga, Ontario, Canada). Cream was carefully removed from the top layer and dried on a filter paper, then resuspended in ultrapure water to the initial volume fraction. The serum phase was withdrawn using a syringe and filtered through 0.45 μm filter (Millipore, Billerica, MA, USA). Fresh emulsions were immediately used for further analysis and stored at 4 °C with 0.02% (w/v) of sodium azide to determine physico-chemical stability.

2.3. Determination of oil droplet size distribution

The particle size distribution of the emulsions was measured using static light scattering (Mastersizer 2000, Malvern Instruments, Worcestershire, UK) using water as the dispersing agent. The refractive indexes of soy oil and water were taken as 1.47 and 1.33, respectively. Emulsion samples were diluted in distilled water until an appropriate obscuration was obtained. Emulsion samples were also diluted in 2 g/L sodium dodecyl sulfate (SDS) at least 30 min before light scattering analyses to check for the presence of aggregated or coalesced droplets that could be dissociated by SDS (Pearce & Kinsella, 1978). The surface-weighted mean diameter ($d_{3,2}$, μm) and the volume-weighted mean diameter ($d_{4,3}$, μm) were determined on the fresh emulsions, the emulsions were kept at 4 °C during 10 days and the digestates of the fresh emulsions.

2.4. SDS-PAGE

Aliquots (100 μL) of sample (emulsions, serum and cream phases) were treated with 200 μL of extraction buffer, containing 50 mM Tris–HCl, 5 M urea, 1% SDS, 4% 2-mercaptoethanol, pH 8.0. The samples were equilibrated at room temperature for an hour then centrifuged (Eppendorf, Brinkmann Instruments, Westbury, NY, USA) at 10,000 *g* for 10 min, to separate the oil phase before mixing the aqueous extract with 200 μL of the electrophoresis buffer, which was composed by 125 mM Tris–HCl, 5 M Urea, 1% SDS, 20% Glycerol, 4% 2-mercaptoethanol, pH 6.8. Samples were then heated at 95 °C for 5 min. After cooling to room temperature, aliquots (5 μL) of all protein samples were loaded onto 12.5% polyacrylamide gel with 4% stacking gel in Bio-Rad mini-protein electrophoresis (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) for proteins separation. Gels were fixed and stained using Bio-Rad Coomassie blue R-250 stain solution (45% methanol, 10% acetic acid and 0.10% Blue R-250) followed by de-staining using 45% methanol, 45% ultrapure water and 10% acetic acid solution then gel was scanned using a SHARP JX-330 scanner (Amersham Biosciences, Quebec, Canada) and the bands were analyzed using

image analysis software (ImageMaster® 1D, Version 2.0, Amersham Biosciences).

2.5. Quantification of CLA isomers

An alkali plus acid-catalyzed methylation method was carried out to quantify the CLA isomers (Moltó-Puigmartí, Castellote, & López-Sabater, 2007). Briefly, saponification with $NaOCH_3$/MeOH (2500 μL) and esterification procedure (2500 μL of BF_3) was taken. Fatty acid methyl esters (FAME) were analyzed by gas chromatography, using an automated Agilent 6890 GC system (Agilent, Palo Alto, CA, USA) equipped with a flame ionization detector (FID). FAME was separated on CP-Sil 88 capillary column (100 m × 0.25 mm i.d. × 0.20 μm). Operating conditions were as follows: injector port temperature 250 °C; hydrogen as carrier gas at a linear pressure of 17.7 psi; split ratio 1:10 or 1:0.1 and injection volume 1 μL or 2 μL for emulsions and digestates, or Caco-2 cell monolayer samples, respectively. The detector temperature was set to 255 °C, with H_2 flow 40.0 mL/min and air flow 450 mL/min. A programmed temperature gradient was used for the chromatographic separation. The run started at 70 °C for 4 min, and then a gradient of 13 °C/min was applied to reach 175 °C. After 27 min at this temperature, the temperature was risen to 215 °C applying a gradient of 4 °C/min, and finally held there for 31 min, for a total run time of 80 min (Kramer et al., 2004, 1997). Gas-chromatographic peaks were identified by comparing the peaks' retention times to those of a standard FAME mix as well as of a linoleic acid methyl ester isomer mix. CLA peaks were quantified using individual pure standards c9,t11 and t10,c12-CLA. The c9,t11 and t10,c12-CLA isomers were also confirmed by spiking the standards in o/w emulsions containing soybean oil. Calibration curves of t9,c11 and t10,c12-CLA were constructed using pure standards (in a range of 0.05–1 μg/μL for the emulsions and digestates samples and 0.01–0.5 μg/μL for the different Caco-2 cell monolayers phases) containing 50 μL of the internal standard solution (C23:0, 0.2 μg/μL) and were found to be linear, with correlation coefficients >0.985. Other isomers products (*cis,cis* and *trans,trans*-CLA) derived from the main isomers (t9,c11-CLA and t10,c12-CLA) were quantified with t10,c12-CLA due to closer retention time. The ratio percentages between t9,c11; t10,c12; *cis,cis* and *trans,trans*-CLA were also determined. The initial CLA sample, was analyzed to confirm its composition, and it was composed by 49% c9,t11; 48% *t10,c12*; 0.45% *cis,cis* and 1.9% *trans,trans*.

2.6. CLA encapsulation

The content of CLA loaded in o/w emulsions was determined by GC-FID technique in the emulsions (initial CLA isomer) and the cream phase resuspended in water (recovered CLA isomer). The amount of loaded CLA per 100 g of initial CLA (in emulsions)—loading capacity (LC) was thus calculated from Eq. (1):

$$\%LC = \frac{C_{recovered\ CLA\ isomer}}{C_{initial\ CLA\ isomer}} \times 100 \quad (1)$$

where *C* is the concentration.

2.7. Peroxide value of emulsions

Emulsions with sodium azide (0.02% w/v) were stored at 37 °C for 20 days. Fresh samples and samples after 10 and 20 days of storage were collected to further analysis. The same mixture of oils (soybean oil and CLA) was used for the control. The formation of lipid peroxides was evaluated according to the AOAC Official Method 965.33 (Hortwitz, 2002). Briefly, 25 mL CH_3COOH–$CHCl_3$ was added into 1.50 ± 0.05 g test portion, and the mixture was stirred to destroy the emulsifier-trapping material. Then 1 mL saturated KI solution was added, followed by vigorously shaking 1 min, and the mixture was held in a dark place for 5 min. Then 75 mL H_2O was added to terminate the reaction. It was slowly titrated with 0.0394 M $Na_2S_2O_3$ with vigorous shaking. Subsequently, drops of 1% starch solution was added, titration and shaking was continued to release all I_2 from $CHCl_3$ layer, until blue just disappeared. Peroxide value (Pv) was calculated as follows from Eq. (2):

$$Pv_{(\text{milliequivalent peroxide/kg oil or fat})} = \frac{V \times N \times 1000}{g\ sample} \quad (2)$$

where *V* is the volume (mL) of the $Na_2S_2O_3$ solution used and N is its normality. A blank was titrated to adjust the Pv of samples.

2.8. Release of CLA isomers

2.8.1. In vitro gastro-duodenal digestion

The *in vitro* digestion experiments were performed according to the INFOGEST method (Minekus et al., 2014), with minor modifications. In brief, oil-in-water emulsions and the CLA-control (the CLA without being protected by an emulsification process) were initially dispersed in simulated gastric fluids. The samples were incubated with the gastric fluids containing pepsin (25,000 U/mL) at 37 °C, and at pH 2 in a 250 rpm shaking water bath for 1 h. The duodenal digestion was then initiated by the addition of simulated duodenal fluids containing pancreatin (5 mg/mL), and phospholipase A_2 (5 μL of 6.7 mg/mL). Simulated bile fluids containing bile salts (0.4 m M) and phospholipids (1 mM) were also added. The duodenal phase of digestion was also simulated at 37 °C for 2 h in a 250 rpm shaking water bath at pH 7. Digestion of the samples was stopped with the same medium that we use to cultivate the cells. We chose the dilution 1:40, prior cytotoxic experiments using sulforhodamine B (SBR) assay demonstrated that this dilution was the minimum with no toxic effect (data not shown). Digestion samples were collected and analyzed by GC-FID as described above.

2.8.2. Free Fatty Acid (FFA) determination

The amount of free fatty acids (FFA) liberated after *in vitro* digestion was quantified as described elsewhere (Malaki Nik, Wright, & Corredig, 2011). In brief, the FFAs were extracted under acidic conditions after duodenal digestion. The amount of FFA was determined using a colorimetric method (NEFA kit) by measuring absorbance at λ_{max} of 550 nm (UV-VIS microplate spectrophotometer, Spectramax plus, Molecular Devices, CA, USA) and by reference to a standard curve prepared using oleic acid ranging from 0.1 to 2 mM.

2.8.3. Transport studies through the Caco-2 cell monolayer

Caco-2 cell line was provided from the Canadian Research Institute for Food Safety (CRIFS) Culture Collection (Food Science, University of Guelph, ON, Canada). The cells were cultured in DMEM (Sigma–Aldrich) supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, 1% non-essential aminoacids and 2 mM L-glutamine (Invitrogen, Canada Inc., Burlington, ON, Canada) at 37 °C in at humidified atmosphere containing 5% CO_2.

For transport experiments, Caco-2 cells were seeded in 12-well Transwell® plates (0.4 μm pore size, inserts of 1.2 cm diameter, BD Biosciences, Mississauga, ON, Canada) at a density of 6×10^4 cells per insert. The cells were maintained for 21 days until they reach full confluency and the complete monolayer was formed. The culture medium was replaced every second day. To ensure the

integrity of the monolayer was maintained the transepithelial electrical resistance (TEER) (Evon World Precision Instruments, Sarasota, FL, USA) was measured every other day.

Apical and basolateral compartments were washed with PBS to remove any cell debris and then incubated respectively with 425 μL and 1500 μL of DMEM without FBS. The cells were incubated at 37 °C for 30 min prior to experiments to equilibrate the monolayers. Digestates were added in the apical compartment at a dilution ratio of 1:40 (sample: medium, v/v) and incubated for 4 h at 37 °C, 5% CO_2. The viability of the monolayers was assessed by measuring the TEER value before and after 1, 2 and 4 h of experiment. Then the cell lysates (collected in PBS), apical and basolateral samples were collected and stored at − 20 °C till analyzed by GC-FID.

The amount of bioaccessible CLA *in vitro* per 100 g of initial CLA (in digestates of emulsions) was thus calculated from Eq. (3):

$$\%\ \text{Bioaccessible isomer} - \text{CLA} = \frac{C_{\text{CLA isomer in cells compartment}}}{C_{\text{CLA isomer in digestates}}} \times 100 \quad (3)$$

Also, the amount of bioavailable CLA *in vitro* per 100 g of initial CLA (in digestates of emulsions)—was therefore calculated from Eq. (4):

$$\%\ \text{Bioavailable isomer} - \text{CLA} = \frac{C_{\text{CLA isomer in basolateral compartment}}}{C_{\text{CLA isomer in digestates}}} \times 100 \quad (4)$$

where C is the concentration.

2.9. Statistical analyses

Descriptive statistics, mean and standard deviation, were listed for each variable in this study. In order to evaluate each variable of emulsions among type of protein (SPI or PPI) and passes of homogenization (one and five passes), a General Linear Model was performed. The statistical analysis was performed using the SPSS® v17.0 package to a 95% level of significance and Tukey adjustment was performed for multiple comparisons of the means ($P < 0.05$). Also, the Student's *t*-test for paired samples analysis was carried out to compare the changes between day 0, 10 and 20. Significance was defined as $P < 0.05$ for a two-sided test. Experiments were performed in duplicate as separate, independent runs.

3. Results and discussion

3.1. Physical characteristics of emulsions

3.1.1. Particle size distribution

The diameter distribution of SPI and PPI stabilized emulsions is shown in Fig. 1, for samples before and after digestion. While emulsions prepared using only 1 pass through the homogenizer showed a large diameter, between 10 and <100 μm, those prepared with five passes through the homogenizer showed smaller particle size distributions (Fig. 1A and C, for emulsions before digestion). These emulsions had a major peak for diameters of about 14 μm, a value significantly higher than for the same emulsions prepared with five passes. The droplets covered with soy protein isolate after five homogenization passes showed the smallest particle size ($d_{4,3}$ and $d_{3,2}$, $P < 0.05$), and they were smaller of the same emulsions prepared with PPI (Table 1). It is widely known that the more an emulsion passes through the homogenization device, the less recoalescence of droplets will occur (Floury, Desrumaux, & Lardières, 2000; Schultz, Wagner, Urban, & Ulrich, 2004). After one pass through the homogenizer, the emulsions, regardless of the type of protein used to stabilize the system, were extensively flocculated. This is clearly shown by the smaller particle size distribution measured after treatment with SDS (Fig. 1A and C). The residual larger droplets in the emulsions prepared with five passes were also disrupted using SDS, indicating that in this case also some bridging flocculation remained for both SPI and PPI emulsions.

The emulsions treated only with one pass through the homogenizer showed a gel-like behavior, due to the extensive flocculation, and this was not the case for the five passes emulsions, which showed to be shear-thinning (data not shown). With respect to the physical stability of the emulsions after 10 days, only the soy protein isolate emulsion treated at one pass had signs of destabilization, and this was clearly shown with an increase in particle size ($P < 0.05$).

The emulsions were subjected to *in vitro* digestion, and their particle size distribution is shown in Fig. 1B and D. In the case of SPI stabilized emulsions, at five passes, there were no significant changes in $d_{4,3}$ and $d_{3,2}$ ($P > 0.05$). The particle size of the SPI emulsions prepared with one pass also showed a similar particle size distribution, albeit shifted to smaller diameter, possibly due to the presence of bile salts, disrupting some of the bridging flocs of the emulsions. In the case of PPI emulsions prepared with five passes, flocculation after digestion was observed. These differences in particle size distribution between the emulsions may cause differences in the fatty acid release and the delivery and absorption of CLA.

3.1.2. Protein composition at the interface

To determine the differences in the protein adsorption at the interface, SDS-PAGE analysis was carried out on emulsions and cream samples. Differences in the distribution of the proteins in the cream phase would indicate differences in the protein adsorption. The major protein bands were quantified by laser scanning densitometry and the ratios between the main protein fractions present in the emulsions and cream phases of the SPI and PPI emulsions obtained after five passes through the homogenizer are shown in Table 2 and Fig. 2.

In the case of soy protein stabilized emulsions, the major fractions associated with the interface were α′ subunit of conglycinin, as well as the A and B subunits of glycinin. Specifically, α′ subunit of conglycinin and A_1, A_2, A_4 subunits of glycinin in SPI emulsion showed to be at a higher ratio compared to the emulsions ($P < 0.05$). These results were in contrast with previous reports (Keerati-u-rai & Corredig, 2009) for 1% SPI oil-in-water emulsions. In that case, there was no difference in the subunit distribution between the oil droplets and the serum phase. The higher protein level in the emulsion as well as their different protein history (with a lower solubility index for the proteins used in this study) may be the cause for the discrepancy.

For PPI stabilized emulsions, the protein subunits that were present the most at the interface were legumin α and vicilin (Table 2 and Fig. 2). There were no statistically significant differences in the intensity of the protein bands in the cream or emulsion samples (Table 2).

The difference in behavior between SPI and PPI stabilized emulsions was caused by the difference in the amount of protein necessary to obtain a full coverage of the interface, and the aggregation state of the protein. It has been previously reported that 0.5–2.0% highly soluble soy protein fractions can stabilize emulsions containing 10–20% oil (Floury, Desrumaux, & Legrand, 2002; Keerati-u-rai & Corredig, 2009). However, the commercial SPI used in this study had lower solubility than that reported in the

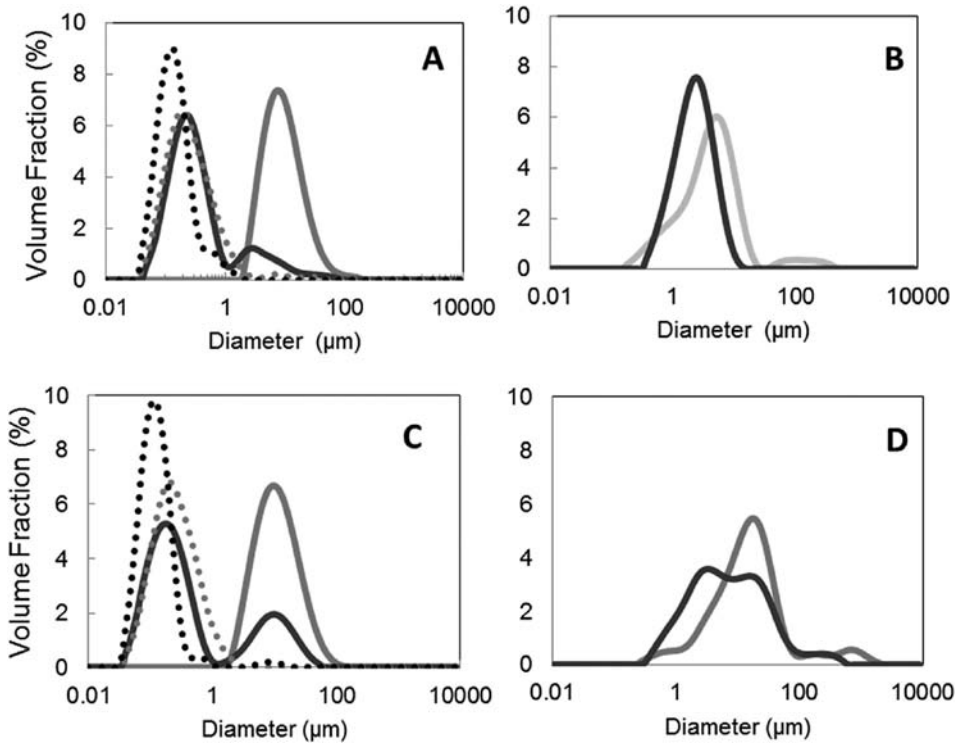

Fig. 1. Size distribution profiles of oil in water emulsions stabilized with soy protein isolate (A and B) and pea protein isolate (C and D) homogenized with one (grey) or five (black) passes of homogenization. Emulsions were measured fresh (A and C) or after digestion (B and D). Dotted lines are the same emulsions after treatment with 2% SDS. Samples are representative of two independent experiments.

Table 1
Average diameter ($d_{4,3}$, d3.2) of emulsions containing soybean oil and CLA (20%, v/v) stabilized with 4% (w/v) SPI or PPI, and prepared using 1 or 5 passes. Each emulsion contained 6% (v/v) of CLA. The particle size is also shown for the same emulsions after digestion. Within a column, different letters indicate statistical significant differences ($P < 0.05$).* Indicate statistically significant differences with storage. Differences were calculated using Student's t-test. Standard deviations are also indicated.

Sample	Treatment	$d_{4,3}$ (μm)		$d_{3,2}$ (μm)	
		day 0	day 10	day 0	day 10
Emulsion	SPI 1	11.8 ± 0.8^{b}	$18.7 \pm 1.2^{a*}$	7.6 ± 0.8^{b}	$10.9 \pm 0.8^{a*}$
	SPI 5	1.9 ± 0.2^{c}	2.0 ± 0.4^{b}	0.21 ± 0.01^{c}	0.21 ± 0.01^{b}
	PPI 1	16.9 ± 2.3^{a}	22.3 ± 7.3^{a}	9.3 ± 0.4^{a}	10.7 ± 2.9^{a}
	PPI 5	4.2 ± 0.4^{c}	6.7 ± 3.9^{ab}	0.22 ± 0.03^{c}	0.18 ± 0.04^{b}
Digestates	SPI 1	11.0 ± 1.8^{b}		1.4 ± 0.8^{b}	
	SPI 5	5.2 ± 1.7^{b}		1.0 ± 0.6^{b}	
	PPI 1	37 ± 14^{a}		3.5 ± 1.7^{a}	
	PPI 5	45 ± 8^{a}		2.8 ± 0.2^{ab}	

literature (Keerati-u-rai & Corredig, 2010; Wang et al., 2012), and similarly, PPI isolates also did not show good solubility. Hence, in both cases, a higher protein content was needed to stabilize the emulsion. The particle size distribution clearly showed that the emulsion droplets showed some extent of flocculation even after 5 homogenization passes, possibly due to the presence of large protein aggregates bridging between the droplets.

3.1.3. CLA loading capacity (%)

There were no significant differences in the loading capacity of c9t11 and t10c12-CLA isomers within different emulsions (Fig. 3). Both CLA isomers showed the same recovery of the CLA initially added into emulsions. However, fresh SPI emulsions prepared with 5 homogenization passes showed a higher recovery of CLA isomers compared to the same emulsion obtained with one pass of homogenization or the emulsions stabilized with PPI. It is important to note that a physically stable emulsion is needed to produce an effective encapsulation of lipids (Hu, Julian McClements, & Decker, 2004; McClements & Li, 2010; Zhang et al., 2014). Furthermore, evaluating CLA concentration in cream phases, significant differences were seen for all CLA isomers between SPI and PPI emulsions prepared with 5 homogenization passes, in which the emulsion stabilized with PPI had a lower concentration (Fig. 4). The SPI-stabilized emulsion treated with five passes showed the smallest average diameter and the smallest increase in $d_{4,3}$ after 10 days of storage, and this is cause to the higher % loading of c9t11-CLA and t10c12-CLA isomers (both 71 ± 18%).

It was hypothesized that the CLA encapsulated in these emulsions could be protected from oxidation and isomerization of CLA. Very few studies are available on the loading of CLA in oil-in-water emulsions. It has been previously reported (Costa et al., 2015) that CLA could be loaded in PPI inclusions, and the loading capacity reported was lower than that measured in this work (Fig. 3).

A smaller loading capacity for CLA has been reported also in freeze-dried and spray-dried matrices, compared to the fresh emulsions measured in this study, with values below 15% (w/w) (Choi et al., 2010; Park et al., 2002). Other authors have studied the complexes formed between CLA and other components such amylose and β-cyclodextrin, such complexes improved the efficiency of the delivery of CLA (Lalush et al., 2004; Yang, Gu, & Zhang, 2009).

Recently CLA was encapsulated in soy lipophilic protein nanoparticles (LPP) by ultrasonication and coating the particles with sodium caseinate (Gao et al., 2014). The encapsulation efficiency of such systems was about 90%. It is possible to hypothesize that, in the case of SPI and PPI emulsions, complexes may also form between CLA and the protein complexes, precipitating during

Table 2
% Area for different polypeptide bands measured in the emulsions and their cream phases prepared with soy (SPI) and pea protein isolate (PPI). Each protein fraction was measured in two separate experiments, after homogenization with five passes at 550 Bar. *P*-value ($P < 0.05$) is for emulsions and their cream phases (emulsion and cream) differences using Student's t-test for paired samples analysis, and significant differences are indicated by *.

Emulsifier	Protein fractions	% band density emulsion	% band density cream phase	*P* value
SPI	α′	15.1 ± 0.4	20.6 ± 0.7	0.01*
	α	4.9 ± 0.1	3.6 ± 1.2	0.25
	β	7.87 ± 0.05	7.0 ± 0.6	0.17
	A_3	8.1 ± 0.4	7.5 ± 0.2	0.20
	A_1, A_2, A_4	24.4 ± 0.5	28.7 ± 0.8	0.02*
	B	39.5 ± 0.6	32.7 ± 3	0.09
PPI	Lipoxygenase	5.6 ± 4	2.6 ± 2.0	0.44
	Convicilin	8.6 ± 1.1	8.8 ± 0.5	0.84
	$Vicilin_1$	12 ± 3.7	20.3 ± 0.6	0.10
	Legumin α	16.8 ± 3.8	28.2 ± 2.2	0.07
	$Vicilin_2$	14.6 ± 0.4	14.2 ± 0.1	0.30
	$Vicilin_3$	4.6 ± 0.6	6.1 ± 0.3	0.09
	$Vicilin_4$	5.8 ± 2.4	4.2 ± 0.9	0.45
	Legumin β_1	20.4 ± 2.8	14.4 ± 0.2	0.10
	Legumin β_2	29.4 ± 5.1	25.7 ± 0.4	0.42

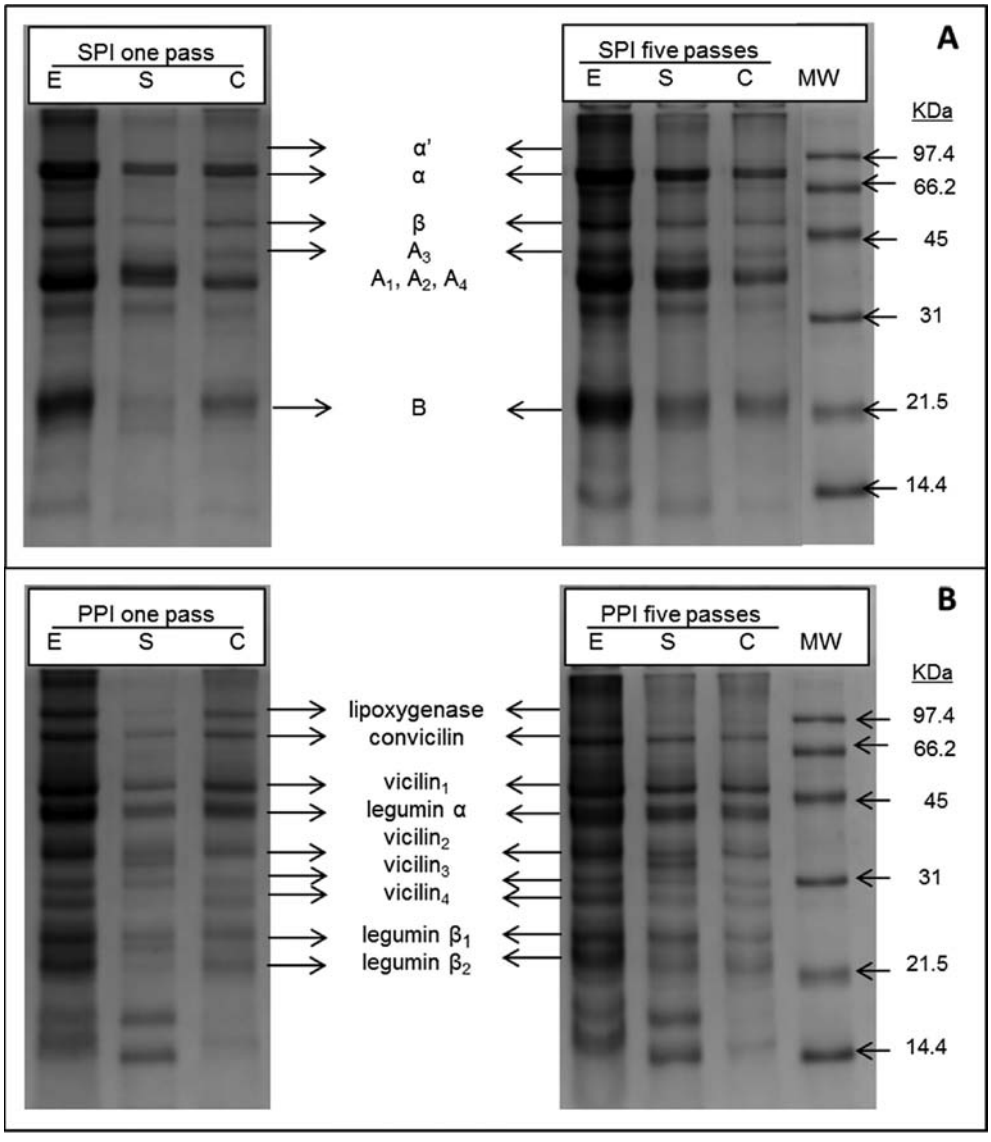

Fig. 2. SDS-PAGE profiles under reducing conditions of SPI emulsions (A) and PPI emulsions (B) containing CLA and homogenized at one or five passes (E), their serum phases (S) and cream phases (C). MW: protein markers.

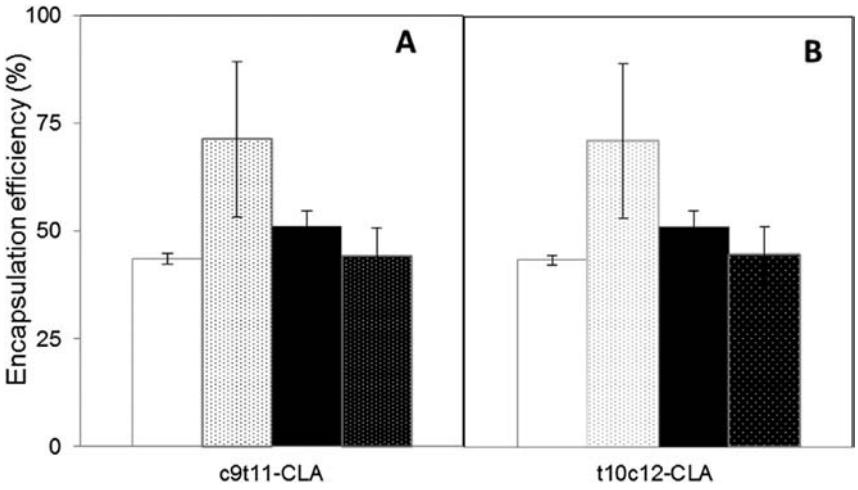

Fig. 3. Amount of c9t11-CLA (A) and t10c12-CLA (B) recovered in the fresh oil-in-water emulsions. Emulsions were stabilized with soy protein isolate (white bars) or pea protein isolate (black bars), and homogenized with one (solid bars) or five passes (dotted bars). Data are shown as average ± SD of two independent experiments.

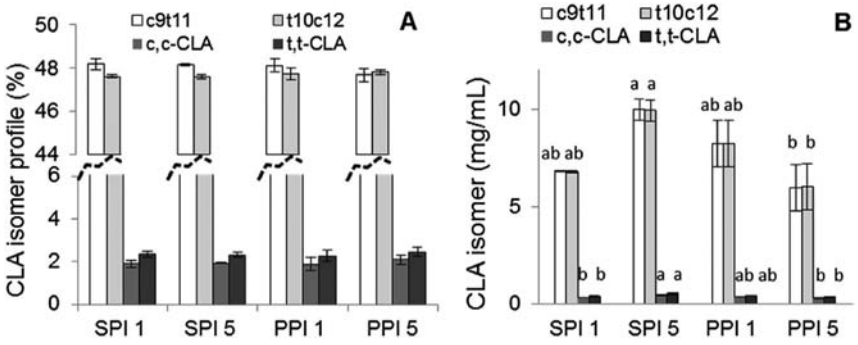

Fig. 4. Chemical stability of CLA in cream phases. CLA isomer profile (%) of cream phases of fresh emulsions (A) and the quantitation of each CLA isomer (B). Data are represented as the average ± SD of two separate experiments. Means with different letters indicate significant differences ($P < 0.05$).

centrifugation, leading to incomplete recovery of CLA in the cream phase of the emulsions.

3.2. Chemical stability of the emulsions

The double bonds of CLA may be in positions 7,9; 8,10; 9,11; 10,12; or 11,13 along the 18 carbon chain. Amongst the isomers, t9t11-CLA and t10c12-CLA isomers have been linked to health benefits (Koba & Yanagita, 2014). The CLA free fatty acid oil used in this study is rich in those isomers, but further transformation may occur during processing (Martínez-Monteagudo, Saldaña, Torres, & Kennelly, 2012).

The stability of CLA in emulsions is shown in Fig. 5, by comparing the concentration of the main isomers in fresh emulsions and in the emulsions after storage at 4 °C for 10 days. There were no changes in the isomer CLA profiles and their CLA content in emulsions with storage (Fig. 5). During oxidation, free radicals react with molecular oxygen to form hydroperoxides. It is virtually impossible to have an unsaturated hydrocarbon compound completely free of peroxides (Martínez-Monteagudo et al., 2012; Moon et al., 2008). It is therefore desirable to avoid these compounds or to keep them apart, for example, by controlling the surface charge on emulsion droplets (Hu et al., 2004). Fig. 6 illustrates the peroxide values of the emulsions, as well as of the control (soybean oil with CLA added) as a function of time of storage at 37 °C. The peroxide value of all the emulsions remained under 10 meq O_2/kg fat after 20 days of storage at 37 °C, with no significant differences ($P > 0.05$) among treatments (Fig. 6). In contrast, the peroxide values for the control increased significantly and reached a plateau after 10 days. The relatively low content of peroxides in the emulsions is not surprising, since CLA can act as antioxidant, capturing free radicals responsible for peroxide formation during lipid oxidation (Fagali & Catalá, 2008; Martínez-Monteagudo et al., 2012). The findings suggested that the oxidative stability of the CLA-soybean oil emulsions was preserved in the emulsions compared to free oil control.

3.3. In vitro digestion

Fig. 7 illustrates the changes that occurred to the CLA isomers after *in vitro* digestion. No significant differences ($P > 0.05$) were found in the CLA isomer profile among emulsions after digestion (Fig. 7). However, the CLA-control showed significant ($P < 0.05$) increased percentages of isomerization for *trans,trans* isomers compared to emulsions containing CLA, even though *trans,trans* CLA isomers are more stable in air (Moon et al., 2008).

The CLA concentration of different isomers in the digestates was also evaluated, to avoid misleading interpretations by the isomer CLA profile using internal normalization, and the concentration of the isomers is summarized in Fig. 7B. No significant differences were seen for isomerization products from t9c11 and c10t12-CLA bioactive compounds between different treatments. However,

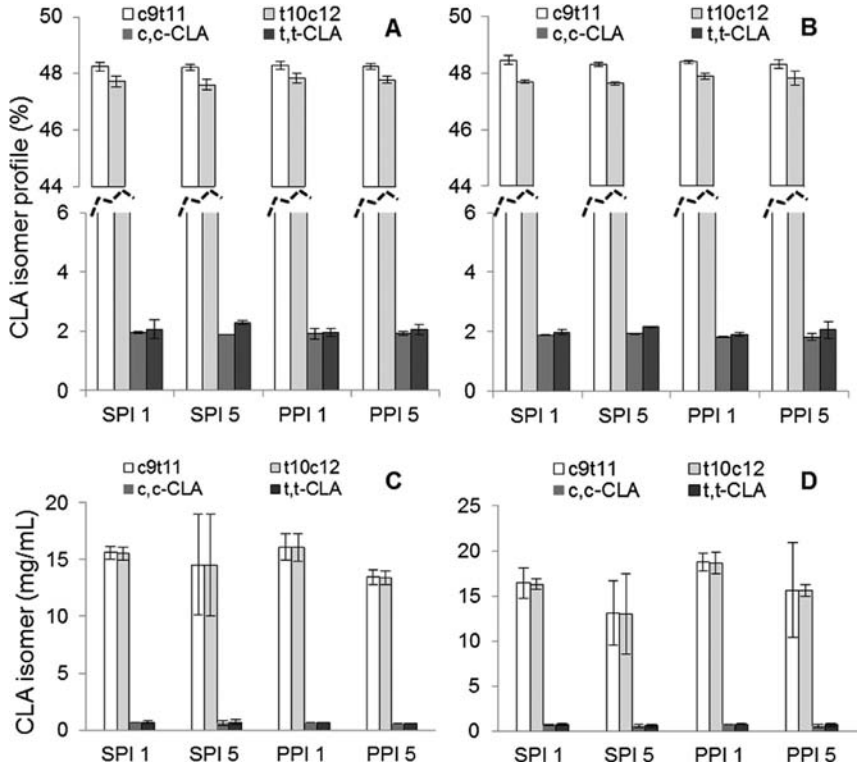

Fig. 5. Chemical stability of CLA in emulsions. CLA isomer profile (%) of freshly emulsions (A) and emulsions stored at 4 °C during 10 days (B), and the quantitation of each CLA isomer of freshly emulsions (C) and emulsions stored at 4 °C during 10 days (D). Data are represented as the mean ± SD.

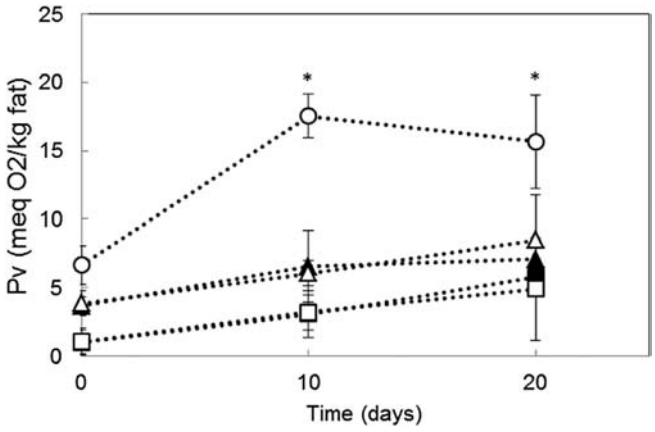

Fig. 6. Peroxide value of emulsions and the control (CLA and soybean oil; ○) stored at 37 °C during 20 days (D). Emulsions were stabilized with soybean protein isolate (■ □) or pea protein isolate (▲ △), homogenized for one (■ ▲) or five passes (□ △). Data are represented as the mean ± SD. * Indicates significant differences between treatments ($P < 0.05$) at 10 and 20 days.

slight changes in the two major groups of CLA isomers were observed in the digestates of the CLA-control (free CLA), which had a lower concentration of these isomers than in the emulsions. Thus, these results are in concordance with oxidation or losses of CLA isomers in oil that is not protected in an emulsion delivery system (Fig. 7). This indicated that c9t11-CLA and t10c12-CLA isomers are more susceptible to oxidative degradation than isomerization, as other authors have also indicated (Moon et al., 2008). In general, emulsions clearly protected t9c11-CLA and t10c12-CLA isomers from transformation and oxidation.

The release of CLA isomers could have been affected by the interfacial composition of the emulsions after the digest process. It has been previously characterized that bile salts have an important role in the duodenal phase for the total displacement of proteins from the interface even without the presence of phospholipids (Malaki Nik, Corredig, & Wright, 2011; Malaki Nik, Wright, et al., 2011; Maldonado-Valderrama, Wilde, Macierzanka, & Mackie, 2011). SDS-PAGE of digestates indicated a complete hydrolysis of all soy and pea polypeptides (data not shown). The extent of lipolysis of the emulsions was also measured after digestion. Emulsions-stabilized with SPI (4%, w/v) at one and five passes of homogenization presented FFA release percentages of 23.3 ± 0.7 and 22 ± 1.5%, respectively. In parallel, emulsions-stabilized with PPI (4%, w/v) at one and five passes of homogenization showed 25.3 ± 0.3 and 24.8 ± 3.1, respectively. The extent of lipolysis of the oil (soybean oil and CLA) in these emulsions did not vary between treatments suggesting that the *in vitro* digestibility of the oil within the emulsions would not be affected by the type of protein present at the interface (SPI or PPI), and that the particle size of the emulsions did not affect the final lipolysis. This fact is in concordance with complete hydrolysis of all soy and pea polypeptides after digestion of the emulsions. Other authors reported >80% lipid hydrolysis values for emulsions containing 10% of soybean oil and 1, 5% (w/v) SPI (Malaki Nik, Corredig, et al., 2011; Malaki Nik, Wright, et al., 2011). The smaller FFA values obtained in this study compared to those found by other authors, can be explained by the differences in the composition of the *in vitro* gastric and intestinal fluids, as well as the high amount of protein adsorbed at the interface, which may act as physical barrier at the lipid droplet surface during digestion, decreasing the extent of lipolysis. Malaki Nik, Corredig, et al. (2011), Malaki Nik, Wright, et al. (2011) showed that the presence of bile salts in the emulsions led to disruption of the flocs formed in the gastric phase and a significant decrease in mean droplet diameter was observed by the end of the duodenal stage.

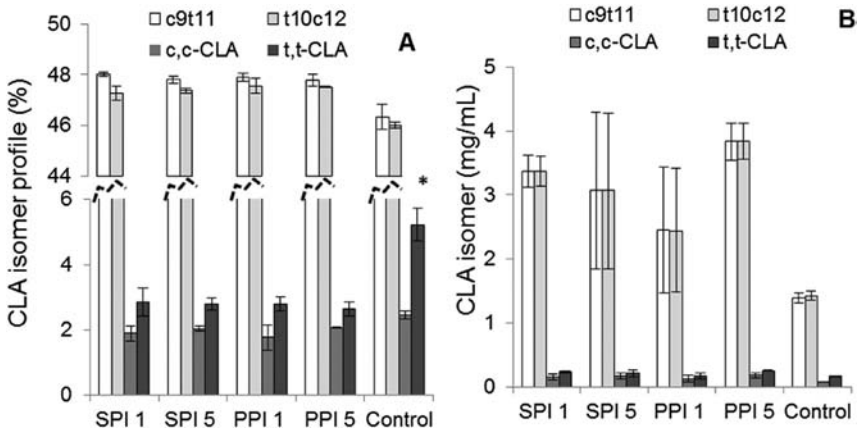

Fig. 7. Chemical stability of CLA in digestates of the fresh emulsions. CLA isomer profile (%) of emulsions after being digested (A) and the quantitation of each CLA isomer (B). Data are represented as the mean ± SD. * It indicates significant differences between treatments ($P < 0.05$).

However, as we used lower concentration of bile salts (0.4 mM) in the duodenal phase compared to these authors, which could have not been enough to disrupt flocs during digestion process, as particle size measurements showed in most of the emulsions after digestion process (Table 1). Also, larger particle size might be explained by less interfacial activity of mixed bile salts-phospholipids compared to bile salts alone, as previously suggested (Malaki Nik, Corredig, et al., 2011; Malaki Nik, Wright, et al., 2011). Then, larger particle size of the emulsions in this study could have led to the reduction of the lipolysis rate. It is well understood that changes in composition of the emulsions can be used to modulate free fatty acid release in the gastrointestinal tract, and affect satiety and release of bioactives (Guri & Corredig, 2014; McClements & Li, 2010).

3.4. Bioaccessibility and bioavailability of CLA isomers

The transport of CLA isomers after digestion and absorption was investigated using a human intestinal cell culture model, by quantifying CLA in the cells and basolateral compartments. Fig. 8 summarizes the concentration of CLA recovered in the cells and basolateral compartment after 4 h of incubation, showing the percentages of the bioaccessibility and bioavailability of CLA. These values were calculated dividing the concentration which remained in the compartments by the amount in the digestates of the emulsions (Eqs. (3) and (4)).

All the treatments showed very similar CLA bioaccessibility results from the main CLA isomers (c9t11 and t10c12) in digestates of the emulsions loaded into the human intestinal cell culture model, except for the higher concentration recovered in the CLA-control digestates. The results clearly suggested that when present in the emulsion, there is a delayed absorption of the CLA in the cells with a lower recovery after 4 h. In concordance with oxidation/isomerization analysis of CLA-control after digestion, there was an extensive absorption and possibly in cell metabolism of CLA for control samples, where CLA was unentrapped.

After absorption, there were no significant differences in the amount of t9c11-CLA recovered in the basolateral fraction, between the treatments and the CLA-control ($P > 0.05$). This may indicate that albeit there was a higher concentration of CLA in the cell for control, the CLA was also metabolized to a higher extent. Only less than 1% of each CLA isomer from digestates of emulsions loaded to the cell media was recovered in the basolateral phase. On the other hand, a higher concentration of t10c12 was found in the basolateral phase for control than for the emulsion treatments. In this case also, the amounts recovered were around 1.5%. There was no significant difference between the emulsion treatments.

CLA bioactive isomers solubilized in oil droplets exhibited minimal duodenal release, but instead remained protected from the gastric and duodenal environment, to later be released after absorption and transport through Caco-2 monolayers.

Recently, nanoemulsified CLA in an obesity rat model had

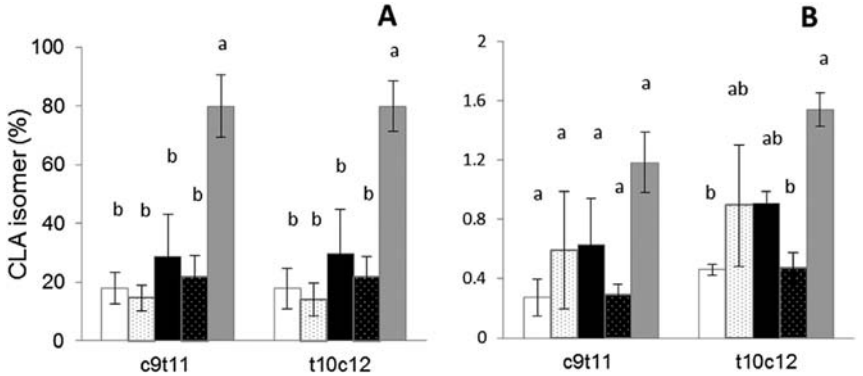

Fig. 8. Bioaccessibility (A, measured in the cell) and bioavailability (B, as measured in the basolateral phase after transport) of the main CLA isomers (c9t11 and t10c12) in digestates after absorption and transport through Caco-2 monolayers. Emulsions were stabilized with soy protein isolate (white bars) or pea protein isolate (black bars), and homogenized with one (solid bars) or five passes (dotted bars). Grey bars show control experiments (oil − no emulsion). Data are represented as the average ± SD of two separate experiments. Means with different letters indicate significant differences ($P < 0.05$).

greater antiobesity effect than in the non-emulsified CLA-treated group (Kim, Park, Kweon, & Han, 2013). However, chemical stability of different CLA isomers among non-emulsified CLA diet and nanoemulsified CLA (with lecithin) diet and its possible effect on metabolism was not assessed. In this respect, we have shown that the oil-in-water emulsion formulations protected the encapsulated CLA better than the corresponding CLA-control, and the longer residence times may also be beneficial for controlled delivery. In that manner, further work needs to be done to establish whether CLA chemical stability preservation with emulsifiers widely used in the food industry, as protein isolates, is correlated to health benefits to the human body.

4. Conclusions

This research demonstrated the importance of oil in water emulsions as delivery matrices for highly hydrophobic bioactive compounds. This work was undertaken to design different oil-in-water emulsions formulations to evaluate CLA delivery. All emulsions protected CLA from oxidation during storage, and after *in vitro* digestion, absorption and transport through Caco-2 monolayers compared to non-emulsified CLA control. Both SPI and PPI isolates were effective in stabilizing the emulsions encapsulating and delivering CLA. The emulsions showed a similar *in vitro* digestibility, and similar results of CLA absorption and transport through Caco-2 monolayers.

Over the last few decades, CLA have been intensively investigated in numerous *in vivo* and *in vitro* studies. However, the bioactive main isomers of CLA are already available as food supplements in the market, and the risks and benefits associated with supplementation are currently under discussion. Auto-oxidation of CLA in the presence of molecular oxygen leads to isomerization of CLA into *cis*,*cis* and *trans*,*trans*-isomers through the Caco-2 cells. The results of this study indicate that isomers form during storage and digestion and further investigation is needed to evaluate the risks that these isomers may cause on the human body. Novel-technologies of encapsulation of CLA are necessary to protect the main biological CLA isomers and oil-in-water emulsions seem to be a suitable solution. It is important to note that the concentrations used in this study would be in the range of what is necessary to deliver a sufficient amount of CLA to be nutritionally significant.

To the best of our knowledge this was the first attempt in which CLA was preserved into oil-in-water emulsions stabilized with vegetable protein isolates. The findings of this study have a number of important implications for future practice in the food industry for development of novel functional foods.

Acknowledgments

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References

Buhrke, T., Merkel, R., Lengler, I., & Lampen, A. (2012). Absorption and metabolism of cis-9, trans-11-CLA and of its oxidation product 9,11-furan fatty acid by Caco-2 cells. *Lipids, 47*(4), 435–442.

Campbell, W., Drake, M. A., & Larick, D. K. (2003). The impact of fortification with conjugated linoleic acid (CLA) on the quality of fluid milk. *Journal of Dairy Science, 86*(1), 43–51.

Choi, K.-O., Ryu, J., Kwak, H.-S., & Ko, S. (2010). Spray-dried conjugated linoleic acid encapsulated with Maillard reaction products of whey proteins and maltodextrin. *Food Science and Biotechnology, 19*(4), 957–965.

Costa, A. M. M., Nunes, J. C., Lima, B. N. B., Pedrosa, C., Calado, V., Torres, A. G., et al. (2015). Effective stabilization of CLA by microencapsulation in pea protein. *Food Chemistry, 168*, 157–166.

Fagali, N., & Catalá, A. (2008). Antioxidant activity of conjugated linoleic acid isomers, linoleic acid and its methyl ester determined by photoemission and DPPH techniques. *Biophysical Chemistry, 137*(1), 56–62.

Floury, J., Desrumaux, A., & Lardières, J. (2000). Effect of high-pressure homogenization on droplet size distributions and rheological properties of model oil-in-water emulsions. *Innovative Food Science & Emerging Technologies, 1*(2), 127–134.

Floury, J., Desrumaux, A., & Legrand, J. (2002). Effect of ultra-high-pressure homogenization on structure and on rheological properties of soy protein-stabilized emulsions. *Journal of Food Science, 67*(9), 3388–3395.

Gao, Z.-M., Zhu, L.-P., Yang, X.-Q., He, X.-T., Wang, J.-M., Guo, J., et al. (2014). Soy lipophilic protein nanoparticles as a novel delivery vehicle for conjugated linoleic acid. *Food & Function, 5*(6), 1286–1293.

Guri, A., & Corredig, M. (2014). *Food Structures, digestion and health*. Elsevier.

Hortwitz, W. (2002). AOAC official method 965.33, Peroxide value of oils and fats. In *Official Methods of Analysis of AOAC International* (17th ed.).

Hu, M., Julian McClements, D., & Decker, E. A. (2004). Impact of chelators on the oxidative stability of whey protein isolate-stabilized oil-in-water emulsions containing ω-3 fatty acids. *Food Chemistry, 88*(1), 57–62.

Jimenez, M., Garcia, H. S., & Beristain, C. I. (2008). Sensory evaluation of dairy products supplemented with microencapsulated conjugated linoleic acid (CLA). *LWT – Food Science and Technology, 41*(6), 1047–1052.

Keerati-u-rai, M., & Corredig, M. (2009). Heat-induced changes in oil-in-water emulsions stabilized with soy protein isolate. *Food Hydrocolloids, 23*(8), 2141–2148.

Keerati-u-rai, M., & Corredig, M. (2010). Heat-Induced changes occurring in oil/water emulsions stabilized by soy glycinin and β-conglycinin. *Journal of Agricultural and Food Chemistry, 58*(16), 9171–9180.

Kim, D., Park, J.-H., Kweon, D.-J., & Han, G. D. (2013). Bioavailability of nanoemulsified conjugated linoleic acid for an antiobesity effect. *International Journal of Nanomedicine, 8*, 451–459.

Koba, K., & Yanagita, T. (2014). Health benefits of conjugated linoleic acid (CLA). *Obesity Research & Clinical Practice, 8*, e525–532.

Kramer, J. K. G., Cruz-Hernandez, C., Deng, Z., Zhou, J., Jahreis, G., & Dugan, M. E. R. (2004). Analysis of conjugated linoleic acid and trans 18:1 isomers in synthetic and animal products. *The American Journal of Clinical Nutrition, 79*(6), 1137S–1145S.

Kramer, J. K., Fellner, V., Dugan, M. E., Sauer, F. D., Mossoba, M. M., & Yurawecz, M. P. (1997). Evaluating acid and base catalysts in the methylation of milk and rumen fatty acids with special emphasis on conjugated dienes and total trans fatty acids. *Lipids, 32*(11), 1219–1228.

Lalush, I., Bar, H., Zakaria, I., Eichler, S., & Shimoni, E. (2004). Utilization of amylose-lipid complexes as molecular nanocapsules for conjugated linoleic Acid. *Biomacromolecules, 6*(1), 121–130.

Malaki Nik, A. M., Corredig, M., & Wright, A. J. (2011). Release of lipophilic molecules during in vitro digestion of soy protein-stabilized emulsions. *Molecular Nutrition & Food Research, 55*(Suppl. 2), S278–S289.

Malaki Nik, A., Wright, A. J., & Corredig, M. (2011). Impact of interfacial composition on emulsion digestion and rate of lipid hydrolysis using different in vitro digestion models. Colloids and Surfaces. B. *Biointerfaces, 83*(2), 321–330.

Maldonado-Valderrama, J., Wilde, P., Macierzanka, A., & Mackie, A. (2011). The role of bile salts in digestion. *Advances in Colloid and Interface Science, 165*(1), 36–46. http://dx.doi.org/10.1016/j.cis.2010.12.002.

Martínez-Monteagudo, S. I., Saldaña, M. D. A., Torres, J. A., & Kennelly, J. J. (2012). Effect of pressure-assisted thermal sterilization on conjugated linoleic acid (CLA) content in CLA-enriched milk. *Innovative Food Science & Emerging Technologies, 16*, 291–297.

McClements, D. J. (2005). *Food emulsions: Principles, practice, and techniques*. CRC Press Inc.

McClements, D. J., Decker, E. A., & Weiss, J. (2007). Emulsion-based delivery systems for lipophilic bioactive components. *Journal of Food Science, 72*(8), R109–R124.

McClements, D. J., & Li, Y. (2010). Structured emulsion-based delivery systems: controlling the digestion and release of lipophilic food components. *Advances in Colloid and Interface Science, 159*(2), 213–228.

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., et al. (2014). A standardised static in vitro digestion method suitable for food – an international consensus. *Food & Function, 5*(6), 1113–1124.

Moltó-Puigmartí, C., Castellote, A. I., & López-Sabater, M. C. (2007). Conjugated linoleic acid determination in human milk by fast-gas chromatography. *Analytica Chimica Acta, 602*(1), 122–130.

Moon, H.-S., Lee, H.-G., Chung, C.-S., Choi, Y.-J., & Cho, C.-S. (2008). Physico-chemical modifications of conjugated linoleic acid for ruminal protection and oxidative stability. *Nutrition & Metabolism, 5*(1), 16.

Nishinari, K., Fang, Y., Guo, S., & Phillips, G. O. (2014). Soy proteins: a review on composition, aggregation and emulsification. *Food Hydrocolloids, 39*, 301–318.

Park, C. W., Kim, S. J., Park, S. J., Kim, J. H., Kim, J. K., Park, G. B., et al. (2002). Inclusion complex of conjugated linoleic acid (CLA) with cyclodextrins. *Journal of Agricultural and Food Chemistry, 50*(10), 2977–2983.

Pearce, K. N., & Kinsella, J. E. (1978). Emulsifying properties of proteins: evaluation

of a turbidimetric technique. *Journal of Agricultural and Food Chemistry, 26*(3), 716–723.
Schultz, S., Wagner, G., Urban, K., & Ulrich, J. (2004). High-pressure homogenization as a process for emulsion formation. *Chemical Engineering & Technology, 27*(4), 361–368.
Wang, J.-M., Xia, N., Yang, X.-Q., Yin, S.-W., Qi, J.-R., He, X.-T., et al. (2012). Adsorption and dilatational rheology of heat-treated soy protein at the oil-water interface: relationship to structural properties. *Journal of Agricultural and Food Chemistry, 60*(12), 3302–3310.
Yang, Y., Gu, Z., & Zhang, G. (2009). Delivery of bioactive conjugated linoleic acid with self-assembled amylose-CLA complex. *Journal of Agricultural and Food Chemistry, 57*(15), 7125–7130.
Yao, X., Xu, Q., Tian, D., Wang, N., Fang, Y., Deng, Z., et al. (2013). Physical and chemical stability of gum arabic-stabilized conjugated linoleic acid oil-in-water emulsions. *Journal of Agricultural and Food Chemistry, 61*(19), 4639–4645.
Zhang, Y., Tan, C., Abbas, S., Eric, K., Zhang, X., Xia, S., et al. (2014). The effect of soy protein structural modification on emulsion properties and oxidative stability of fish oil microcapsules. *Colloids and Surfaces. B, Biointerfaces, 120*, 63–70.

CHAPTER 6: Enhanced stability of emulsions treated by Ultra-High Pressure Homogenization for delivering conjugated linoleic acid in Caco-2 cells

[Experiment 4]

Enhanced stability of emulsions treated by Ultra-High Pressure Homogenization for delivering conjugated linoleic acid in Caco-2 cells

1. Introduction

There have been major advances in the design and fabrication of functional food products to improve performance, maintain well-being and prevent the development of chronic diseases, such as heart disease, cancer, hypertension and obesity (McClements, Decker, Park, & Weiss, 2009; McClements, 2013). Functional lipids, such as conjugated linoleic acid (CLA), are derived from ruminant animals in beef and milk fat. As those natural products contain CLA in relatively low levels, it has been successfully incorporated into foods to boost its functional effects (Jimenez, Garcia, & Beristain, 2008; Martínez-Monteagudo, Saldaña, Torres, & Kennelly, 2012; Nasrabadi, Goli, & Nasirpour, 2015).

CLA has been shown to exert various potent physiological functions to reduce carcinogenesis, atherosclerosis, adverse effects of immune stimulation and body fat, and an increase in lean body mass (LBM) (Gaullier et al., 2004; Koba & Yanagita, 2013). Even though CLA involves a number of geometric and positional isomers, current research has tended to focus on the c9,t11-18:2 and t10,c12c-18:2 isomers. Both isomers have been supplemented in a free (CLA-FFA) or triglyceride form by capsules and both are able to reduce body fat mass in healthy overweight adults (Gaullier et al., 2004). After 1 year of supplementation, the CLA-FFA group had significantly higher LBM than did the placebo group ($P < 0.05$), whereas LBM in the CLA-triacylglycerol group did not differ significantly from that in the placebo group.

Anyway, individuals may prefer to consume a medical/functional food every day, rather than taking capsules or pills (Fasinu, Pillay, Ndesendo, du Toit, & Choonara, 2011). In the recent years, different delivery systems containing CLA have been applied, such as oil-in-water emulsions and microencapsulation by spray drying processes (Choi, Ryu, Kwak, & Ko, 2010; Costa et al., 2015; Fernandez-Avila, Arranz, Guri, Trujillo, & Corredig, 2015; Nasrabadi et al., 2015). Despite spray drying efficacy for encapsulation, CLA suffers from several major drawbacks as it presents very poor chemical stability during thermal processing and can decompose to furan fatty acids in

the presence of air (Buhrke, Merkel, Lengler, & Lampen, 2012). Furan fatty acids may have toxicological properties and it has been suggested that encapsulation procedures using emulsion-based delivery systems may be the most suitable (Fernandez-Avila et al., 2015; Gao et al., 2014). Our previous research studied different vegetable protein-stabilized oil-in-water emulsions prepared by conventional homogenization (550 bar) that protected CLA-FFA from oxidation during storage, and after *in vitro* digestion, absorption and transport through Caco-2 monolayers compared to non-emulsified CLA-FFA control (Fernandez-Avila et al., 2015).

Ultra-High Pressure Homogenization (UHPH) has been proposed as a novel antioxidative technology to produce stable submicron emulsions (Fernandez-Avila & Trujillo, 2016; Hebishy, Buffa, Guamis, Blasco-Moreno, & Trujillo, 2015). UHPH technology modifies particle size and proteins structure owing to different phenomena occurring at the exit of the UHPH valve, such as high turbulence, cavitation and shear stress (Dumay et al., 2013; Fernández-Ávila, Escriu, & Trujillo, 2015). The most significant improvements of UHPH emulsions are their physical and microbiological stabilities. Actually, recent research at our laboratory has allowed to develop aseptically packaged beverages by UHPH-processing at 300 MPa, which allow maintaining products in stock (Amador-Espejo, Suàrez-Berencia, Juan, Bárcenas, & Trujillo, 2014; Poliseli-Scopel, Hernández-Herrero, Guamis, & Ferragut, 2014).

Application of UHPH in obtaining submicron emulsions with CLA and their incorporation into dairy products could be suitable to boost CLA functional effects. However, oral bioavailability of many lipophilic bioactives, such as CLA, is relatively low due to their poor water-solubility, permability and/or susceptibility to chemical and biochemical degradation within the gastrointestinal tract (McClements, 2013). In this sense, efficient delivery of the lipophilic compound and its bioefficacy in the UHPH emulsion system needs to be demonstrated.

The release and bioefficacy of CLA encapsulated in oil-in-water emulsions stabilized with UHPH, as well as the physico-chemical, oxidative and microbiological stability of emulsions have yet to be reported. In previous research, similar UHPH formulations were screened (100-300 MPa) and the UHPH emulsion containing 20% soybean oil and

treated at 200 MPa exhibited the best physical and oxidative stability (Fernández-Ávila et al., 2015; Fernandez-Avila et al., 2016). The aim of this study was to investigate the effect of different oil-in-water emulsions stabilized by soy protein isolate and homogenized by CH or UHPH on the delivery of CLA in a free form, which is believed to be absorbed in a large extent to reach the site of action. High-temperature-short-time conditions (HTST, 72 ˚C for 20 s) were also applied for another batch of CH emulsions to extend their shelf life. Commercially available soy protein isolates as emulsifiers were used, to increase the relevance of this research to the food industry. The CLA bioefficacy was studied using *in vitro* digestion and a Caco-2 intestinal cell absorption model.

2. Materials and methods

2.1. Materials

A commercial SPI (PRO-FAM 974) was purchased from Lactotecnia (Barcelona, Spain). The composition of this commercial SPI according to manufacturer was: ≥90% protein, <4% fat, <6% moisture, and less than 5% ash (dry basis, w/w). SPI PRO-FAM 974 has acid character and the isoelectric point is 4.6 (Kinsella, 1979) due to the high content of glutamic acid (Glu, 19.2%) and aspartic acid (Asp, 11.5%). Solubility of PRO-FAM 974 at pH = 7 is 39.5% (Bissegger, 2007). Soybean oil was purchased from Gustav Heess (Barcelona, Spain). Peroxide value of the soybean oil was below 10 meq O_2/kg and the acidity index was below 0.5 mg KOH/g. A free fatty acid (FFA) mixture high in isomers of CLA (Neobee®CLA80) was kindly donated from Stepan Specialty Products LLC (Maywood, USA), containing 80.4% of total CLA. The two main isomers present were c9,t11-CLA and t10,c12-CLA in a free form, in a 50:50 ratio. Other CLA isomers (*cis,cis* and *trans,trans*) were present in minor concentrations (<1.2%). Neobee®CLA80 was obtained from natural safflower oil by a gentle, proprietary process without antioxidants added. Peroxide value of CLA was below 10 meq O_2/kg. All other chemicals used were of analytical or better grade. Pepsin (P7000), pancreatin (P1750), phospholipase A2 (P6534) and bile salts (B8631) were obtained from Sigma-Aldrich.

2.2. Preparation of oil-in-water CLA emulsions

Oil-in-water emulsions were prepared with a fixed content of SPI (4%, w/v) and oil (20%, v/v), containing CLA (6%, v/v) and soybean oil (14%, v/v). Firstly, the stock protein dispersion (4%, w/v) was prepared by dispersing SPI in deionised water by using a high-speed dispersing unit at a rate of about 250 rpm for 1 h at 25 ˚C. Protein dispersions were stored overnight at 4 ˚C to allow complete hydration. Protein dispersions and oil were equilibrated at 20 ˚C (inlet temperature) before mixing. Pre-emulsions (or coarse emulsions) were prepared by mixing the protein dispersions with the soybean oil using a rotor-stator emulsifying unit (model Diax 900, Heidolph, Kehlheim, Germany) at 15000 rpm for 4 min. The coarse emulsions were further homogenised through a high-pressure homogenizer or by conventional homogenization (Stansted Benchtop Homogenizator nG12500, Stansted Fluid Power Ltd., Essex, UK). The emulsions were treated at 200 MPa (single-stage) by the high-pressure homogenizer (flow rate of 8 L/h) provided with a high-pressure ceramic needle-seat valve, and at 15 MPa with a ceramic ball-seat valve (conventional homogenization treatments, single-stage). Pre-emulsions were passed through both devices with an inlet temperature (T_{in}) of 20 ˚C. The outlet temperature of emulsions were controlled by a heat exchanger (Inmasa, Reus, Spain) located immediately after the high-pressure valve or ball-seat valve. During treatments, the T_{in}, the temperature after the high pressure valve (T_1), and outlet temperature were monitored. For another batch of CH emulsions, a heat treatment was also conducted applying high-temperature-short-time conditions (72 ˚C for 20 s; CH-HTST emulsion). Samples were maintained in refrigeration at 4 ˚C and all the analysis were carried out on freshly emulsions. Microbiological analyses, rheological properties, physical stability, chemical stability of CLA and lipid oxidation of emulsions were evaluated over time. Sampling of CH was in a shorter period of time because the lower effect of the low-pressure treatment on shelf-life: day 0, 3, 7 and 10. In the case of CH-HTST and UHPH emulsions, sampling was on day 0, 15, 40, 65, 90, 115, 140 and 165. Sampling ended when counts were >6 log UFC/g. The emulsions pH was evaluated during all sampling by electrode immersion with a micro pH-potenciometer (model 2001; Crison Instruments SA, Alella, Spain).

2.3. Determination of oil droplet size distribution

The particle size distribution in the fresh 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. The optical parameters used were: a refractive index of 1.475 for the soybean oil and a refractive index of 1.332 for the water. The surface-weighted mean diameter ($d_{3.2}$, μm) and volume-weighted mean diameter ($d_{4.3}$, μm) were determined.

2.4. Microbiological analysis

To determine the microbial load obtained of emulsions after treatments, the following microbial groups were enumerated in duplicate: psychrotrophic, spore, and *Enterobacteriaceae* counts. Total psychrotrophic count was determined by the pour-plate method in plate count agar (Oxoid Ltd., Basingstoke, UK) and incubated for 5 days at 21°C. To determine the spore load, emulsions were heated for 10 min at 85 °C and then enumerated by the pour-plate method in plate count agar (Oxoid Ltd.) and incubated for 48 h at 30 °C. *Enterobacteriaceae* were enumerated on violet red bile agar (Oxoid Ltd.) by pour plating, and incubated for 24 h at 37 °C. When emulsions counts were under the detection limit, the emulsions sterility was evaluated by bacteria enumeration in plate count agar after emulsion incubation at 55 and 30°C for 7 and 15 d, respectively. Microbial load was calculated as logarithms of colony counts of the treated samples. Microbiological analysis for pshycrotrophic count in emulsions was performed until the end of their shelf life (> 6 log).

2.5. Rheological measurements

Rheological measurements were performed using a controlled stress rheometer (Haake Rheo Stress 1, Thermo Electron Corporation, Karlsruhe, Germany) using a cone (1º, 60 mm diameter) and plate geometry probe at 21 ˚C. Flow curves (shear stress compared to shear rate) were determined at increasing and decreasing shear rates

between 0.001 s^{-1} and 100.0 s^{-1} in 1 min (up and down flow curves). Flow curves were fitted to the rheological model Herschel-Bulkley: $\tau = \tau_o + K (\dot{\gamma})^n$ and the consistency coefficient (K, mPa × s^n), flow behavior index (n) and initial shear stress (τ_o) were obtained.

2.6. Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) technique was used in fluorescence mode for obtaining high-resolution optical images of fresh emulsions. The protein was stained by the fluorescein isothiocyanate dye (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 (5 mL) were dyed with one drop of each dye. Then, 3 drops of emulsions labeled were transferred to microscope slides with concave cavities, covered with a cover slip and sealed to prevent evaporation. The confocal microscope (Leica SP5, Heidelberg, Germany) was equipped with an oil-coupled Leica objective with a 63× augmentation and a numerical aperture of 1.4. Images were acquired in 2 channels simultaneously as 1,024 × 1,024 pixel slices in the horizontal x-y plane along the z plane. Excitation wavelengths for FITC and Nile red channels were 488 and 561 nm and emission wavelengths were 500-550 and 600-775 nm, respectively.

2.7. Physical stability

The stability of emulsions was determined through the use of the optical analyser Turbiscan MA 2000 (Formulaction, Toulouse, France). The Turbiscan measures backscattered and transmitted light as a function of the axial tube coordinate and time by means of two synchronous optical sensors that respectively receive light transmitted through the sample and light backscattered by the sample. The light source is an electro luminescent diode in the near infrared (λ_{air} = 850 nm). Therefore, any destabilisation phenomenon happening in a given sample will have an effect on its values of backscattering or transmission intensities during its ageing. In the present study, freshly emulsions were filled into the Turbiscan tube after UHPH and CH

treatments and stored in quiescent conditions at 4 ˚C. Destabilization kinetics was evaluated by measuring Turbiscan Stability Index (TSI) using the Turbisoft 2.0 software (Formulaction, 2015). TSI is an algorithm that takes any change (destabilization phenomena) into account. It compares mathematically the intensity of light toward the complete cell height, based on a scan-to-scan difference during ageing, calculated using the following Eq. (1):

$$TSI = \sum_i \frac{\sum h\,|scan_i - scan_{i-1}|}{H} \quad (1)$$

Then, for every scan in the history of your sample, a new point is added to its destabilization kinetics, representing its TSI for this ageing time.

2.8. Analysis of lipid oxidation

2.8.1. Lipid hydroperoxides content

The formation of lipid peroxides was evaluated according to the method of Hu, McClements, & Decker (2004). Lipid hydroperoxide concentrations (mmol/L of emulsion) were determined using a standard curve made from cumene hydroperoxide. Afterwards, data was expressed in mmol/kg of oil in order to differentiate between emulsions with low and high volume oil-phase fractions regarding the formation of hydroperoxides.

2.8.2. Thiobarbituric acid-reactive substances (TBARs)

To determine the formation of secondary oxidation products, TBARs technique was performed. It detects the formation of malondialdehyde, which reacts with thiobarbituric acid (TBA). Malondialdehyde or TBARs content of the emulsions upon storage was determined using a process as described by Sørensen & Jørgensen (1996), with a few modifications. In brief, emulsions (3 mL) were mixed with 6 mL of TCA reagent (7.5%, w/v) and were vortexed. The resultant mixtures were filtered with a 1 µm microporous membrane after 20 min of the TCA addition. The filtered (2 mL) were placed in test tubes containing TBA reagent (0.8%, w/v) and they were mixed by vortexing. The resultant mixtures were heated in a water bath (95 ˚C) for 15 min, and

then cooled immediately in an ice-bath to slow down the reaction. The absorbance of the final extracts was recorded at 532 nm. The TBARs concentration (µmol/L of emulsion) was determined according to a standard curve with 1, 1, 3, 3-tetraethoxypropane. Again, data was expressed in µmol/kg of oil to consider the effect of oil content on lipid oxidation.

2.9. Release of CLA isomers and quantification

2.9.1. In vitro gastro-duodenal digestion

The *in vitro* digestion experiments were performed for oil-in-water emulsions and the CLA-control (CLA without being protected by an emulsification process) according to the INFOGEST method with minor modificacions (Fernandez-Avila et al., 2015). Digestion of the samples was stopped with the same medium that we use to cultivate the cells. We chose the dilution 1:80, prior cytotoxic experiments using sulforhodamine B (SBR) assay demonstrated that this dilution was the minimum with no toxic effect (data not shown). Digestion samples were collected and analysed by GC-FID as described below.

2.9.2. Transport studies through the Caco-2 cell monolayer

Caco-2 cell line was provided from the Celltec UB Culture Collection (University of Barcelona, Spain). The cells were cultured in DMEM (Sigma-Aldrich) supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, 1% non-essential aminoacids and 2 mM L-glutamine (Sigma-Aldrich) at 37 ˚C in at humidified atmosphere containing 5% CO_2.

For transport experiments, Caco-2 cells were seeded in 12-well Corning®Transwell® plates (0.4 µm size pore polycarbonate membrane inserts of 1.2 cm diameter, Sigma-Aldrich) at a density of 6×10^4 cells per insert. The cells were maintained for 21 days until they reach full confluency and the complete monolayer was formed. The culture medium was replaced every second day. To ensure the integrity of the monolayer was

maintained the transepithelial electrical resistance (TEER) (Evon World Precision Instruments, Sarasota, FL, USA) was measured every other day.

Apical and basolateral compartments were washed with PBS to remove any cell debris and then incubated respectively with 425 µL and 1500 µL of DMEM without FBS. The cells were incubated at 37 ˚C for 30 min prior to experiments to equilibrate the monolayers. Digestates were added in the apical compartment at a dilution ratio of 1:80 (sample: medium, v/v) and incubated for 2 h at 37 ˚C, 5% CO_2. The viability of the monolayers was assessed previously by measuring the TEER value just before experiment. Then the cell lysates (collected in PBS), apical and basolateral samples were collected and stored at – 80 ˚C till analysed by GC-FID or GC-MS.

The amount of non-absorbed CLA *in vitro* per 100 g of initial CLA (in digestates of emulsions) was thus calculated from Eq. (2):

$$\%\ \text{Non absorbed isomer} - \text{CLA} = \frac{C_{CLA\ isomer\ in\ apical\ compartment}}{C_{CLA\ isomer\ in\ digestates}} \times 100 \qquad (2)$$

Also, the amount of bioaccessible CLA *in vitro* per 100 g of initial CLA (in digestates of emulsions)—was therefore calculated from Eq. (3):

$$\%\ \text{Bioaccessible isomer} - \text{CLA} = \frac{C_{CLA\ isomer\ in\ cells\ compartment}}{C_{CLA\ isomer\ in\ digestates}} \times 100 \qquad (3)$$

Also, the amount of bioavailable CLA *in vitro* per 100 g of initial CLA (in digestates of emulsions)—was therefore calculated from Eq. (4):

$$\%\ \text{Bioavailable isomer} - \text{CLA} = \frac{C_{CLA\ isomer\ in\ basolateral\ compartment}}{C_{CLA\ isomer\ in\ digestates}} \times 100 \qquad (4)$$

where *C* is the concentration.

2.9.3. Quantification of CLA isomers

An alkali plus acid-catalyzed methylation method was carried out to quantify the CLA isomers (Moltó-Puigmartí, Castellote, & López-Sabater, 2007). Briefly, saponification with $NaOCH_3$/MeOH (2500 µL) and esterification procedure (2500 µL of BF_3) was taken. Fatty acid methyl esters (FAME) were analyzed by gas chromatography, using an

automated Agilent 6890 GC system (Agilent, Wilmington, DL, USA) equipped with a flame ionization detector (FID). FAME was separated on CP-Sil 88 capillary column (100 m × 0.25 mm i.d.× 0.20 µm). Operating conditions were performed as previously described (Fernandez-Avila et al., 2015). Gas-chromatographic peaks were identified by comparing the peaks' retention times to those of a standard FAME mix as well as of a linoleic acid methyl ester isomer mix. CLA peaks were quantified using individual pure standards c9,t11 and t10,c12-CLA. Calibration curves of t9,c11 and t10,c12-CLA were constructed using pure standards (in a range of 0.05-1 µg/µL for the digestates and 0.01-0.5 µg/µL for the different Caco-2 cell monolayers phases) containing 50 µL of the internal standard solution (C19:0, 0.1 and 0.01 µg/µL respectively) and were found to be linear, with correlation coefficients >0.99. Other isomers products (*cis,cis* and *trans,trans*-CLA) derived from the main isomers (t9,c11-CLA and t10,c12-CLA) were quantified with t10,c12-CLA due to closer retention time.

Quantification of CLA isomers on basolateral fractions were performed using an Agilent 6890 gas chromatograph coupled to a 5975 MSD mass spectrometer (Agilent Technologies, Palo Alto, Calif, U.S.A.) in order to increase the sensitivity of the determination. Same column and operating conditions were carried out. Both Scan and SIM modes were performed. SIM mode was used for quantification in order to obtain lower detection limits. For each analyte, the following MS ions were monitored; internal standard methyl ester (298.55 and 312.55), CLA methyl esters (280.45 and 294.45), methyl 8-(5-hexyl-2-furyl)-octanoate (methyl ester of furan fatty acid, 294.45 and 308.45). CLA was identified by relative retention time of every chromatographic peak and by comparing their mass spectra with those of mass spectra from Wiley 6.0 library.

2.10. Statistical analyses

Descriptive statistics, mean and standard deviation, were listed for each variable in this study. In order to evaluate the physical stability of emulsions among type of emulsion (CH, CH-HTST or UHPH), one-way ANOVA was performed. The statistical analysis was performed using the SPSS® v17.0 package to a 95% level of significance and Tukey

adjustment was performed ($P< 0.05$). Also, the Student's t-test for paired samples analysis was carried out to compare the changes of particle size between fresh emulsions and digestates in Table 1 and between day 0 and day 7 (for CH emulsions) or day 15 (for CH-HTST and UHPH emulsions) in Figure 4 and Figure 5A,C. Significance was defined as $P < 0.05$ for a two-sided test. Experiments were performed in duplicate as separate, independent runs.

3. Results and Discussion

3.1. Microbiological inactivation

The initial bacterial load of coarse emulsions was 3.6 ± 0.5 log cfu/mL. In this study, a reduction of 0.9 ± 0.5 log cfu/mL bacteria count was achieved with the CH treatment. In contrast, in the CH-HTST and UHPH treatment at 200 MPa with T_{in} = 20 °C, total microbial counts appeared to be under the detection level. For all treatments at day 1, spores and *Enterobacteriaceae* counts were below the detection level. The absence of *Enterobacteriaceae* counts indicated good manufacturing practices during the process at the pilot plant and further storage. Sterility test (see Section 2.7) results showed that UHPH-treated emulsions were sterile in comparison to CH-HTST emulsions. Other studies performed by UHPH also obtained sterility conditions but applying 300 MPa together with high T_{in}: 75 ˚C and 85 ˚C in milk (Amador-Espejo et al., 2014) and 80 ˚C for soybean milk (Poliseli-Scopel et al., 2014). Contrary, UHPH-treated emulsions in this work were sterile at 200 MPa using lower T_{in} (20 ˚C). Our results are in line with the absence of spore counts, as they are dormant differentiated cells that are very resistant to heat and other physical and chemical stresses (Diels & Michiels, 2006). The exact mechanism how UHPH inactivates microorganisms has not yet been fully elucidated as there many factors involved. High pressure and velocity gradients, shear stresses, turbulence, shocks and cavitation phenomena occurring through the UHPH-valve could induce mechanical disruption and/or at least alteration of cell membranes (Dumay et al., 2013). All these factors along with higher T_{in} of samples will increase temperature at the exit of the UHPH-valve (T_1), provoking a very short time thermal effect which could also induce lethality of microorganisms (Roig-Sagués et al., 2009).

As commented previously, our lab allowed to develop aseptically packaged beverages by UHPH processing at 300 MPa, in which T_1 were above 130 ˚C, so that thermal effect might have been the main responsible of sterility. In the present study, UHPH treatment at 200 MPa reached a T_1 of 83 ˚C (Table 1). Nevertheless, this moderate temperature (83 ˚C) along with the pressure applied at the UHPH-valve (200 MPa), were effective conditions to inactivate the microrganisms present in the emulsion.

Table 1. Average diameter ($d_{3.2}$, $d_{4.3}$) of emulsions containing soybean oil and CLA (20%, v/v) stabilized with 4% (w/v) SPI and homogenized by conventional homogenization (CH, 15 MPa), conventional homogenization (15 MPa) plus high-temperature short-time pasteurization (CH-HTST) and Ultra-High Pressure homogenization at 200 MPa. The particle size is also shown for the same emulsions after digestion comparing with a CLA-control (free CLA).

Emulsion treatment			Particle Size of emulsions		Particle Size of digestates	
Sample name	MPa	T_1 (˚C)	$d_{3.2}$ (µm)	$d_{4.3}$ (µm)	$d_{3.2}$ (µm)	$d_{4.3}$ (µm)
CH	15	43.5 ±2.1	0.41 ± 0.06[a]	3.15 ± 1.28[a]	0.65 ± 0.10[a]*	2.67 ± 0.59[a]
CH-HTST	15	43.5 ±2.1	0.47 ± 0.08[a]	5.05 ± 1.48[a]	0.71 ± 0.01[a]*	2.81 ± 0.26[a]
UHPH	200	82.5 ± 0.7	0.26 ± 0.01[b]	0.69 ± 0.12[b]	0.74 ± 0.08[a]*	2.87 ± 0.16[a]*

[a-b] Within a column, different letters indicate statistical significant differences (P<0.05).

* Indicate statistically significantly differences for $d_{3.2}$ and $d_{4.3}$ after digestion calculated using Student's t-test. Standard deviations are also indicated.

Characteristics of food matrix, such as fat content and viscosity, had also been reported in the literature to influence microbial inactivation (Diels, Callewaert, Wuytack, Masschalck, & Michiels, 2005; Roig-Sagués et al., 2009)**.** Roig-Sagués *et al.* (2009) inoculated 7.0 log cfu/mL of *Listeria monocytogenes* to milk samples differing in fat content, and were further processed to a single stage of UHPH treatment at 200-400 MPa. In that work it was concluded that the amount of fat (0.3, 3.6, 10 and 15%) had a stronger effect on *L. monocytogenes* inactivation in comparison to the temperature reached at the exit of the valve, which resulted in <90 ˚C for all the treatments. In this sense, the high amount of oil amount (20 %, v,v) included in the CLA emulsions treated by UHPH in the present work could has helped on bacteria count inactivation . Furthermore, the possible antimicrobial effect of CLA has to be considered as well. Some researchers used an *in vitro* assay procedure to show which FFAs derived from whey cream specifically inhibit the germination of *Candida albicans*.

Myristoleic acid, palmitoleic acid and CLA were found to be the most active fatty acids inhibiting *Candida albicans* germination (Clément, Tremblay, Lange, Thibodeau, & Belhumeur, 2007). In whole milk and skim milk, potassium (K)-CLA was bacteriostatic and prolonged the lag phase of *L. monocytogenes* especially at 4 °C (Wang & Johnson, 1992).

Responsible bacteria of deterioration of refrigerated food are mainly psychrotrophic, as they can significantly grow at cold temperatures. Then, in order to evaluate shelf-life of other non-sterile emulsions (CH and CH-HTST), psychrotrophic counts were performed every 3-4 days, as emulsions were stored at 4 ˚C. Emulsion treated by CH presented a shelf life between 7 (3.6 ± 1.1 log cfu/mL) and 10 days, (>6 cfu/mL). For another batch of CH emulsions, HTST was performed. HTST is a light pasteurization which was focused to reduce bacterial load in emulsions. CH-HTST emulsion maintained its shelf life until day 18 (5,66 ± 1.65 cfu/mL of total psychrotrophic count), as counts increased >6 cfu/mL at day 22.

3.2. Physical characteristics and stability of emulsions

3.2.1. Particle size distribution

The diameter distribution of soy protein isolate-stabilized emulsions is shown in Figure 1, for samples before and after digestion.

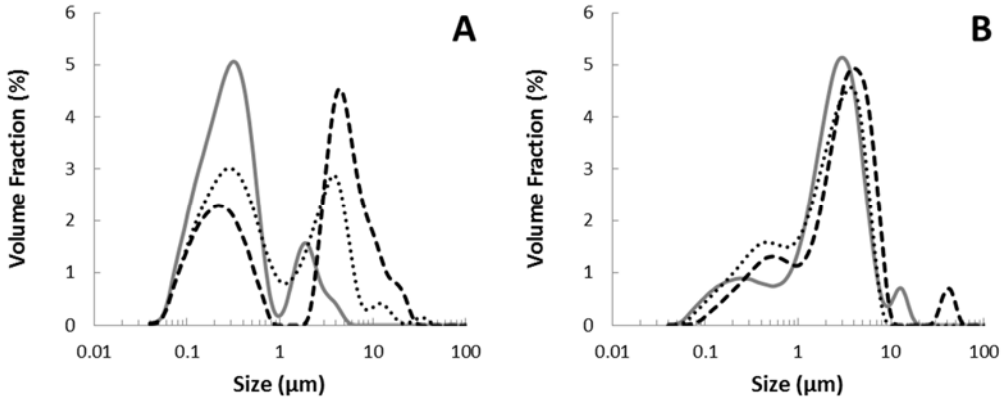

Figure 1. Size distribution profiles of CLA emulsions stabilized with Ultra-High Pressure Homogenization (grey), conventional homogenization (dotted black) and conventional homogenization plus high-temperature short-time pasteurization (dashed black). Emulsions were measured fresh (A) or after digestion (B). Samples are representative of two independent experiments.

While emulsions prepared using CH through the homogenizer showed a large diameter and bimodal distribution, between 0.1 and <10 μm, those homogenized with UHPH showed smaller particle size distributions (Fig. 1A, for emulsions before digestion). Compared to CH emulsion, CH-HTST emulsion showed different ratio for major peaks between 0.1 and <20 μm, in which the larger peak was the most abundant. However, UHPH emulsions presented a major peak of 300 nm but also a minor peak for diameters of about 2 μm, a value significantly lower than for the same emulsions prepared with CH. This is confirmed by the smallest particle size for submicron UHPH fresh emulsions compared to both CH-treated emulsions ($d_{3.2}$ and $d_{4.3}$, $P < 0.05$) (Table 1).

The emulsions were subjected to *in vitro* digestion and all their digestates showed similar particle size distribution, as shown in Fig. 1B. The digestate of UHPH sample presented a major peak ~ 2 μm compared to the other treatments ~ 3 μm. A minor peak ~ 200 nm is visible for UHPH emulsion, in which other treatments appeared to be shifted ~ 500 nm. Additionally, CH-HTST emulsion showed a minor peak for diameters of about 40 μm. Comparing the digestates with their counterparts emulsions, there were significant changes in $d_{3.2}$ ($P < 0.05$). Also, $d_{4.3}$ was significantly different for UHPH emulsion ($P < 0.05$). Thus, flocculation occurred due to chemical interaction during *in vitro* digestion.

3.2.2. Microstructure, rheological properties and physical stability of emulsions

Figure 2 shows the microstructure of the emulsions studied by CLSM with a 63ˣ augmentation. Micrographs show the oil droplets dyed in red and the protein aggregates in green. As it was expected, UHPH reduced the mean particle size in emulsions compared to CH and CH-HTST emulsions. This is confirmed by results from Table 1, in which UHPH samples shows the smallest particle size ($d_{3.2} = 0.26 \pm 0.01$ and $d_{4.3} = 0.69 \pm 0.12$). UHPH emulsions showed more homogenous particles than the other formulations, and it is also confirmed by larger $d_{4.3}$ values in CH and CH-HTST ($P < 0.05$, Table 1). Moreover, HTST pasteurization induced coalescence (Fig. 2A) as it is represented with a surface plot of the red channel and more protein aggregates (Fig.

2B). This is also confirmed by the increase in particle size of CH-HTST emulsions among CH emulsions (Table 1). Therefore HTST affected negatively the microstructure of the CH emulsions.

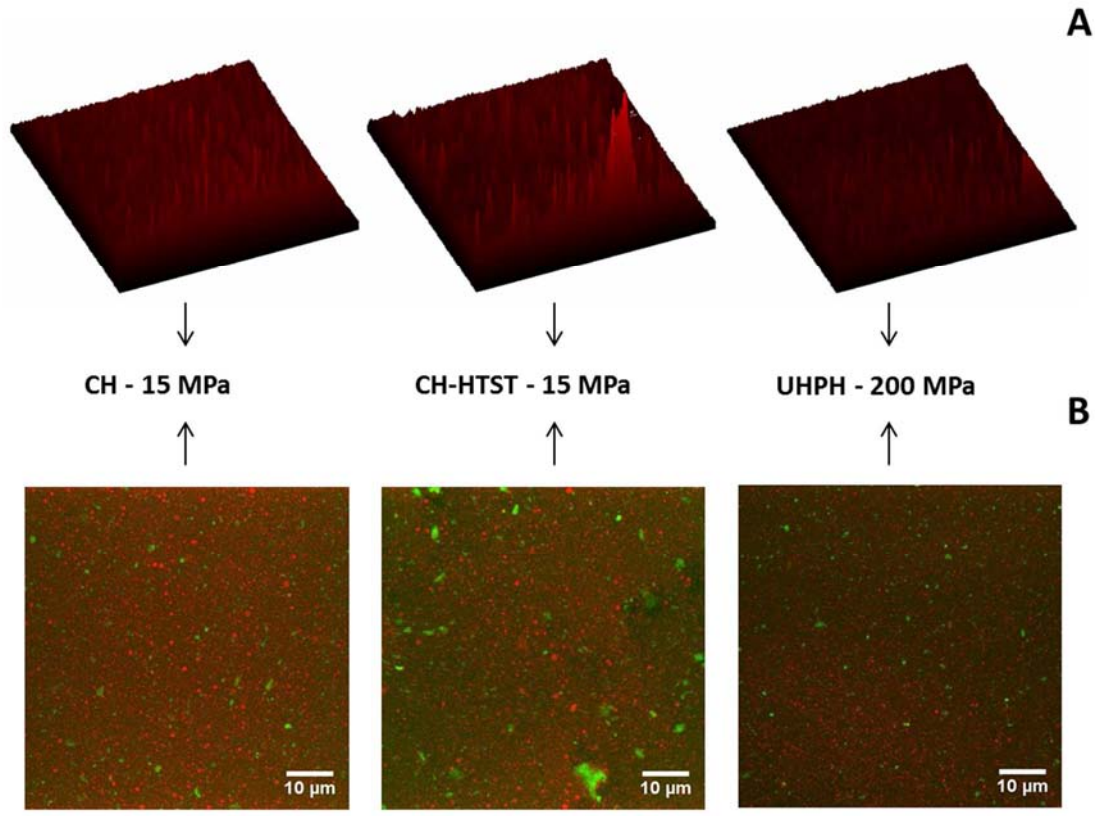

Figure 2. Confocal micrographs of fresh CLA emulsions stabilized with conventional homogenization (CH - 15 MPa), conventional homogenization plus high-temperature short-time pasteurization (CH-HTST - 15 MPa) and Ultra-High Pressure Homogenization (UHPH). The maximum intensity projection of 3D z-stacks (B) and their red channels in a surface plot (A) were shown. Red colour represents the oil phase of emulsions (A) and green colour represents the proteins. Samples are representative of two independent experiments.

Particle size strongly influenced colloidal interaction between particles and thus rheological behaviour of emulsions, as UHPH emulsions with smaller particle size (Table 1) showed shear-thinning behaviour and CH and CH-HTST emulsions showed gel-like behaviour (Fig. 3). Although consistency (K) and initial shear stress (τ_o) of CH-HTST and UHPH fresh emulsions were not significantly different ($P > 0.05$, Fig 3B,C), they showed different behaviour, as UHPH emulsion did not present thixotropic behaviour (Fig 3D). Thixotropic behaviour is considered when the shape of the upward and downward flow curves are different, so that CH and CH-HTST emulsions presented

thixotropic behavior, and therefore droplets were aggregated by weak forces (McClements, 2005). We suggest that the absence of differences of K and τ_o between CH and CH-HTST emulsions might be due to the rupture of protein network by heating during HTST (P > 0.05, Fig 3B,C).

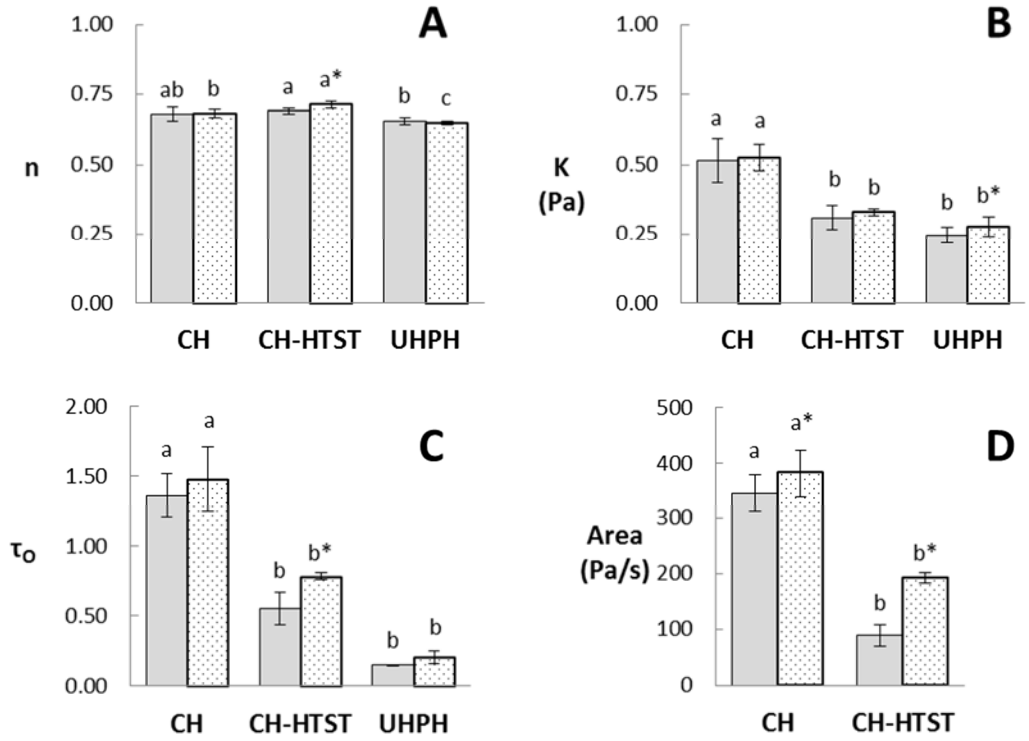

Figure 3. Mean ± SD values of rheological properties of CLA emulsions stabilized with conventional homogenization (CH - 15 MPa), conventional homogenization plus high-temperature short-time pasteurization (CH-HTST - 15 MPa) and Ultra-High Pressure Homogenization (UHPH). The flow behaviour (n), the consistency index (K) and the initial shear stress (τ_o) of all emulsions were determined. The Area under the curve of emulsions which experimented thixotropic behaviour was also analysed. CH fresh emulsions (grey bars) are compared with their last day of shelf life (dotted bars, CH – 7 days and CH-HTST – 15 days) (A,C). Sterile UHPH emulsions were also analysed at 15 days. Within treatments, different letters indicate statistical significant differences (P<0.05). * Indicate statistically significantly differences between fresh and aged emulsions calculated using Student's t-test.

The rupture of the protein network in the CH-HTST gel-like emulsion is confirmed by the presence of protein aggregates in confocal micrographs (Fig. 2B). In the literature, different flow behaviour and consistency index were obtained for UHPH emulsions stabilized with soy protein isolate and homogenized at 200 MPa containing 20 % (v/v) of soybean oil (n = 0.76 ± 0.01 and K = 114 mPa, respectively) (Fernández-Ávila et al., 2015). In the present study, CLA emulsions were more viscous (K = 250 mPa) and presented a flow behaviour of 0.66 ± 0.01 (Table 2). Indeed, viscosity of CLA emulsions

in this study (6% CLA in a FFA form and 14% soybean oil) increased because colloidal structures of such dispersed systems are different from those where triacylglycerol are only present in emulsions. In fact, FFAs are surface-active, form micelles or mixed micelles with surfactants or, when mixed with a nonpolar oil, preferentially locate at the oil droplet surface (Berton-Carabin, Ropers, & Genot, 2014).

Table 2. Mean ± SD values of pH and rheological parameters of emulsions containing soybean oil and CLA (20%, v/v) stabilized with 4% (w/v) SPI and homogenized by Ultra-High Pressure homogenization at 200 MPa. The flow behaviour (n), the consistency index (K) and the initial shear stress (τ_O) of all the emulsions were determined. The Area under the curve of emulsions which experimented thixotropic behaviour was also analysed.

Days	pH	Rheological parameters			
		n	K (Pa)	τ_O	Area (Pa/s)
1	6.7 ± 0.0	0.66 ± 0.01	0.25 ± 0.03^{b}	0.15 ± 0.01^{d}	ND*
15	6.6 ± 0.0	0.65 ± 0.01	0.28 ± 0.04^{ab}	0.21 ± 0.05^{cd}	ND
40	6.7 ± 0.1	0.65 ± 0.01	0.28 ± 0.03^{ab}	0.22 ± 0.04^{bcd}	34.21 ± 9.88^{b}
65	6.6 ± 0.0	0.65 ± 0.00	0.30 ± 0.02^{ab}	0.26 ± 0.03^{abc}	42.43 ± 13.41^{ab}
90	6.6 ± 0.0	0.65 ± 0.00	0.30 ± 0.02^{ab}	0.31 ± 0.05^{ab}	51.96 ± 17.47^{ab}
115	6.5 ± 0.0	0.65 ± 0.01	0.30 ± 0.03^{ab}	0.32 ± 0.05^{abc}	54.52 ± 15.19^{ab}
140	6.5 ± 0.0	0.65 ± 0.01	0.32 ± 0.03^{a}	0.32 ± 0.04^{ab}	56.40 ± 9.01^{ab}
165	6.5 ± 0.0	0.66 ± 0.00	0.31 ± 0.03^{ab}	0.30 ± 0.08^{a}	74.87 ± 19.67^{a}

$^{a-d}$ Within a column, different letters indicate statistical significant differences (P<0.05).

* ND = Not determined.

Emulsions are unstable colloidal systems that undergo many destabilization phenomenon (sedimentation, clarification, creaming and coalescence) as a result of various factors, such as lack of surfactant to cover the interface, coexistence of small and big droplets and attractive forces (McClements, 2005). Figure 4 shows the global destabilisation phenomenon kinetics in the emulsions versus ageing time, according to the TSI. It sums all the variations detected in the samples in terms of size and/or concentration to qualify the best homogenization technique for a given emulsion. The higher is the TSI, the worse is the stability. TSI values of both emulsions treated by CH were not high enough to play a stabilising role, compared to UHPH technology which decreases significantly the kinetics of any destabilisation phenomenon. HTST showed

no influence on the global stabilization of the CH samples, despite coalescence observed in CLSM micrographs (Fig 2A). Additionally, the kinetics destabilisation phenomenon of UHPH emulsion during longer storage was suitable until 90 days (Fig. 4), and it could related to the increase in τ_o and the area (Pa/s) under the curve, indicating thixotropic behaviour since day 40 and thus more weak colloid interactions (Table 2).

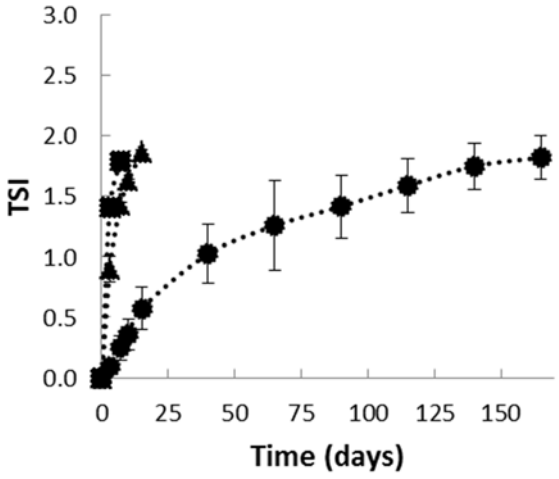

Figure 4. Physical stability of CLA emulsions stored at 4˚C during 165 days measured by Turbiscan Stability Index (TSI). Emulsions were stabilized with conventional homogenization (■), conventional homogenization plus high- temperature short-time pasteurization (▲) and Ultra-High Pressure Homogenization (●). Data are represented as the mean ± SD of two separate experiments.

3.3. Determination of primary and secondary lipid oxidation of emulsions

Lipid oxidation is the main reason for the deterioration of fats and oils (Matthäus, Decker, Elias, & McClements, 2010). It is the result of the reaction of molecular oxygen with unsaturated fatty acid, such as CLA and soybean oil, resulting in unsaturated hydroperoxides (Hu et al., 2004). As they are unstable molecules, they break down into other secondary oxidation products, particularly aldehydes, responsible for undesirable odours and flavours (Sørensen & Jørgensen, 1996).

In the literature reviewed, a higher levels of hydroperoxides were found for soy protein-stabilized emulsions in comparison to those obtained in this study (Fig. 5), albeit those emulsions contained antioxidants and they were performed by

temperature-induced oxidation (Cui, Kong, Chen, Zhang, & Hua, 2014; Wan, Wang, Wang, Yang, & Yuan, 2013; Wan, Wang, Wang, Yuan, & Yang, 2014). Recently, it has been found that lipid oxidation of similar UHPH emulsions containing soybean oil was strongly dependent on volume oil-phase fraction and protein attachment at the oil droplets (Fernandez-Avila et al., 2016). The hydroperoxides content of CLA emulsions in the present study (Fig. 5A,B) are in line with those of Fernandez-Avila et al., (2016). However, TBARs content was almost 10-fold larger than similar UHPH emulsions containing soybean oil and UHPH-treated at 200 MPa, as the inclusion of FFA induces more lipid oxidation.

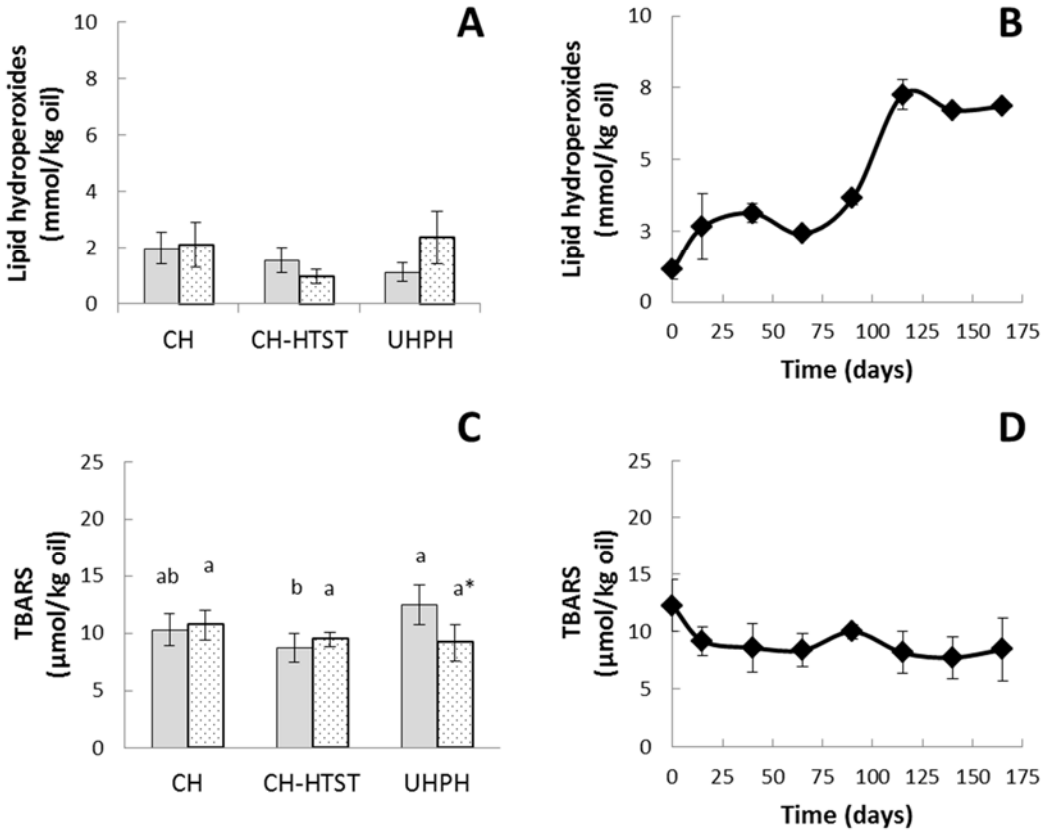

Figure 5. Lipid hydroperoxides (mmol/kg oil) TBARs (µmol/kg oil) of CLA emulsions stabilized with conventional homogenization (CH - 15 MPa), conventional homogenization plus high-temperature short-time pasteurization (CH-HTST - 15 MPa) and Ultra-High Pressure Homogenization (UHPH). CH fresh emulsions (grey bars) are compared with their last day of shelf life (dotted bars, CH – 7 days and CH-HTST – 15 days) (A,C). Sterile UHPH emulsions were also analysed at 15 days and further stored at 4˚C during 165 days (B,D). Data are represented as the average ± SD of two separate experiments. Within treatments, different letters indicate statistical significant differences (P<0.05). * Indicate statistically significantly differences between fresh and aged emulsions calculated using Student's t-test.

In the present study, no significant differences were found for hydroperoxides content between fresh emulsions (Fig. 5A). However, UHPH emulsion exhibited higher content of TBARs than the CH-HTST ($P < 0.05$), although they showed similar results after 15 days ($P > 0.05$) (Fig. 5C). CH-HTST and UHPH emulsions exhibited different particle size, as UHPH showed the smallest particle size (Table 1, $P < 0.05$). It is believed that an increased oxidation rate may also be due to an increased interfacial area, by increasing the contact between the oil and peroxides (Berton-Carabin et al., 2014). From these results it seems clear that the interfacial area is not the only determining factor on favouring lipid oxidation, as other authors have recently confirmed (Fernandez-Avila et al., 2016; Hebishy et al., 2015).

Lipid oxidation compounds may lead to desorption of proteins from interface and contribute to physical destabilization of the system (Genot, Meynier, Riaublanc, & Kamal-Eldin, 2003), as hydroperoxides from linoleic acid, methyl linoleate, and trilinolein are more suface-active than their nonperoxidized counterparts (Nuchi, Hernandez, McClements, & Decker, 2002). Hydroperoxides content of UHPH emulsion after 90 days increased steadily until 115 days, when primary oxidation products reached a steady state (Fig. 5B). Similar trend was observed on the decrease of physical stability after 90 days (Fig. 4). It is therefore likely that such connections exist between physical and oxidative stability. Anyway, we consider that emulsions could be cold stored (4 ˚C) in stock until 165 days as they are expected to be diluted 10 folds in a beverage emulsion system, where peroxide value would remain below 5 mmol O_2/kg oil in the beverage (~ 8 mmol/kg oil is present in UHPH emulsions at day 165). Additionally, TBARs content in UHPH emulsion kept steady during storage and confined lower values compared to other soybean oil-in-water emulsions containing 10-20% (v/v) of oil in the literature reviewed (Cheng, Xiong, & Chen, 2010; Huang, Lu, Wang, & Wu, 2011), indicating little formation of secondary oxidation products.

Some authors reported that the presence of FFAs in emulsified oil was shown to increase lipid oxidation, which could have been related to their location at the interface and the increased negative net charge of the oil droplets, which is postulated to attract metal ions onto the oil droplet surface (Waraho, Cardenia, Rodriguez-Estrada, McClements, & Decker, 2009; Waraho, McClements, & Decker, 2011).

However, it has previously suggested that complexes may also form between CLA and soy protein isolate, as incomplete recovery of CLA (< 90% encapsulation efficiency) after ultracentrifugation was reported for similar CLA emulsions (Fernandez-Avila et al., 2015).

3.4. Conjugated linoleic acid uptake

The transport of CLA isomers after digestion and absorption was investigated using a human intestinal cell culture model, by quantifying CLA in the apical, cells and basolateral compartments. Digestates were added in the apical compartment at a dilution ratio of 1:80 and incubated for 2 h at 37 ˚C, according to previous cytotoxicology results in which viability of cells was above 95 % (data not shown). Fig. 6 summarizes the concentration of CLA recovered in each compartment, showing the percentages of bioavailability of CLA (Eqs. (1), (2) and (3)). All the treatments showed very similar CLA uptake results from the main CLA isomers (c9t11 and t10c12) in digestates of the emulsions loaded into the apical compartment ($P > 0.05$, Fig. 6A). Similar CLA uptake into the cells compartment was also observed ($P > 0.05$, Fig. 6B), along with a higher trend of the CLA-control (CLA non-emulsified). This trend, unless it is non-significant, it is in concordance with previous research which compared different vegetable protein-stabilized oil-in-water emulsions containing the same amount of CLA (Fernandez-Avila et al., 2015). In that work authors suggested that when CLA was present in the emulsion rather than in the control (CLA non-emulsified), there was a delayed absorption in the cells with a lower recovery after 4 h. Thus, the non-emulsified CLA could have experimented an extensive absorption and possibly in cell metabolism, where CLA was unentrapped. The results obtained from CLA uptake into basolateral compartment are shown in Fig. 6C. All treatments presented similar c9t11-CLA uptake into the basolateral compartment ($P > 0.05$). However, t10c12-CLA was fully metabolized into other *trans,trans*-CLA isomers. Moreover, furan fatty acids determined as oxidation products of CLA were not detected in the basolateral compartment.

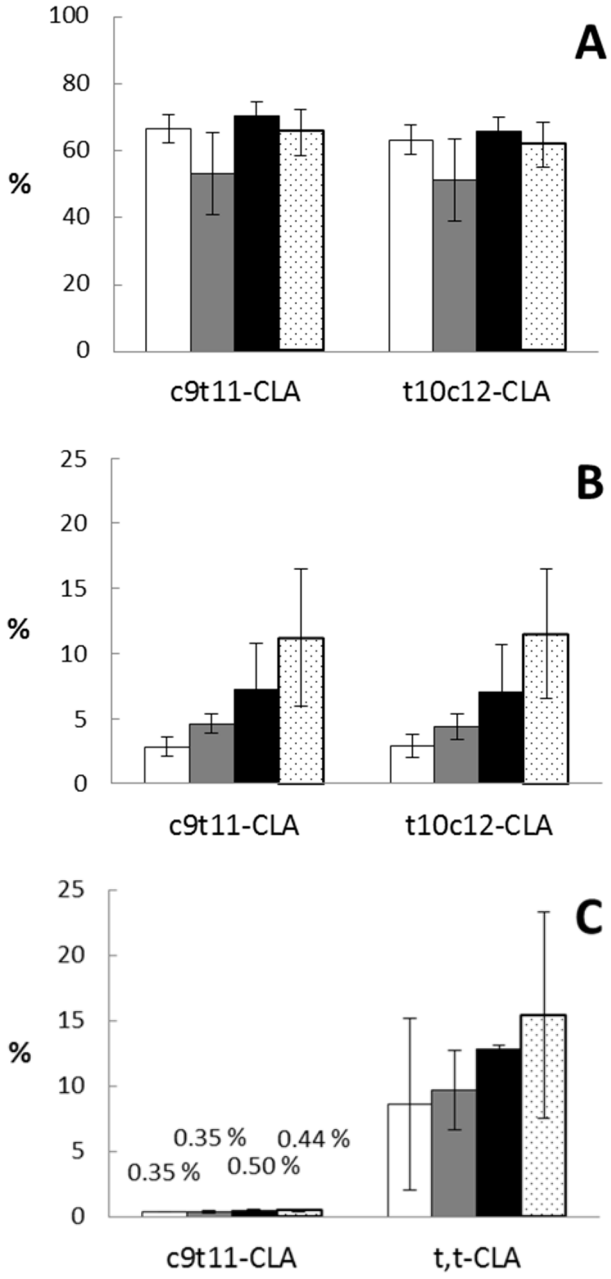

Figure 6. Non absorbed (A, measured in the apical phase), bioaccessibility (B, measured in the cell) and bioavailability (C, as measured in the basolateral phase after transport) of the CLA isomers (c9t11, t10c12 and trans,trans-CLA) in digestates after absorption and transport through Caco-2 monolayers. CLA emulsions were homogenized with Ultra-High Pressure Homogenization (white bars), conventional homogenization (grey bars) and conventional homogenization plus high- temperature short-time pasteurization (black bars). Dotted bars show control experiments (oil – no emulsion). Data are represented as the average ± SD of two separate experiments.

4. Conclusions

This dissertation has investigated the importance of oil in water emulsions treated by UHPH as delivery matrices for highly hydrophobic and prone to oxidation bioactive compounds, such as free-CLA. In this investigation, the aim was to assess the stability of UHPH emulsions containing CLA to evaluate their delivery, comparing with other oil-in-water emulsions treated by the conventional homogenization method.

It has been shown that the emulsion treated by UHPH presented the smallest particle size and more homogeneous microstructure. However, all emulsions exhibited a similar particle size after *in vitro* digestion, and similar results of CLA absorption and transport through Caco-2 monolayers. The UHPH treatment with an inlet temperature of 20 ˚C proved to be efficient in achieving total microbial inactivation and then emulsion sterility (200 MPa). During storage, UHPH emulsions appeared to be more physically stable than CH and CH-HTST, and similar results within all treatments were found for oxidative stability. Considering that the FFA mixture of CLA and the CLA in a triglyceride form have comparable biocompatibilities, the current study has only examined CLA in a free form, as it is more prone to oxidation. In this sense, as no antioxidants were added, the emulsion-delivery system was critical on CLA preservation. Particularly, we consider UHPH emulsions could stay in refrigeration as a stock for a longer period of time than 5-6 months, corresponding to the sampling duration in this study.

Also, this study has an added value as the free fatty acid form of CLA is just ingested in capsules, and individuals may prefer to consume the nutraceutical into the supplemented food. Anyway, it would be interesting to assess the bioavaliability of CLA in a triglyceride form as well, as it is available in the market. The present study, however, makes several noteworthy contributions to emulsion-delivery systems field. Novel technologies for CLA encapsulation are necessary to protect the main biological CLA isomers and UHPH technology showed promising results. To the best of our knowledge this was the first attempt in which CLA was preserved into oil-in-water emulsions treated by UHPH, leading to sterile products. The findings of this study have

a number of important implications for future practice in the food industry for inclusion of nutraceuticals into functional foods.

5. References

Amador-Espejo, G. G., Suàrez-Berencia, A., Juan, B., Bárcenas, M. E., & Trujillo, A. J. (2014). Effect of moderate inlet temperatures in ultra-high-pressure homogenization treatments on physicochemical and sensory characteristics of milk. *Journal of Dairy Science*, *97*(2), 659–71.

Berton-Carabin, C. C., Ropers, M.-H., & Genot, C. (2014). Lipid Oxidation in Oil-in-Water Emulsions: Involvement of the Interfacial Layer. *Comprehensive Reviews in Food Science and Food Safety*, *13*(5), 945–977.

Bissegger, S. (2007). Production of bioactive soy peptides. Dissertation. University of Waterloo, Canada.

Buhrke, T., Merkel, R., Lengler, I., & Lampen, A. (2012). Absorption and metabolism of cis-9, trans-11-CLA and of its oxidation product 9,11-furan fatty acid by Caco-2 cells. *Lipids*, *47*(4), 435–442.

Cheng, Y., Xiong, Y. L., & Chen, J. (2010). Antioxidant and emulsifying properties of potato protein hydrolysate in soybean oil-in-water emulsions. *Food Chemistry*, *120*(1), 101–108.

Choi, K.-O., Ryu, J., Kwak, H.-S., & Ko, S. (2010). Spray-dried conjugated linoleic acid encapsulated with Maillard reaction products of whey proteins and maltodextrin. *Food Science and Biotechnology*, *19*(4), 957–965.

Clément, M., Tremblay, J., Lange, M., Thibodeau, J., & Belhumeur, P. (2007). Whey-derived free fatty acids suppress the germination of Candida albicans in vitro. *FEMS Yeast Research*, *7*(2), 276–85.

Costa, A. M. M., Nunes, J. C., Lima, B. N. B., Pedrosa, C., Calado, V., Torres, A. G., & Pierucci, A. P. T. R. (2015). Effective stabilization of CLA by microencapsulation in pea protein. *Food Chemistry*, *168*, 157–66.

Cui, Z., Kong, X., Chen, Y., Zhang, C., & Hua, Y. (2014). Effects of rutin incorporation on the physical and oxidative stability of soy protein-stabilized emulsions. *Food Hydrocolloids*, *41*, 1–9.

Diels, A. M. J., Callewaert, L., Wuytack, E. Y., Masschalck, B., & Michiels, C. W. (2005). Inactivation of Escherichia coli by high-pressure homogenisation is influenced by fluid viscosity but not by water activity and product composition. *International Journal of Food Microbiology*, *101*(3), 281–91.

Diels, A. M. J., & Michiels, C. W. (2006). High-pressure homogenization as a non-thermal technique for the inactivation of microorganisms. *Critical Reviews in*

Microbiology, *32*(4), 201–16.

Dumay, E., Chevalier-Lucia, D., Picart-Palmade, L., Benzaria, A., Gràcia-Julià, A., & Blayo, C. (2013). Technological aspects and potential applications of (ultra) high-pressure homogenisation. *Trends in Food Science & Technology*, *31*(1), 13–26.

Fasinu, P., Pillay, V., Ndesendo, V. M. K., du Toit, L. C., & Choonara, Y. E. (2011). Diverse approaches for the enhancement of oral drug bioavailability. *Biopharmaceutics & Drug Disposition*, *32*(4), 185–209.

Fernandez-Avila, C., Arranz, E., Guri, A., Trujillo, A. J., & Corredig, M. (2015). Vegetable protein isolate-stabilized emulsions for enhanced delivery of conjugated linoleic acid in Caco-2 cells. *Food Hydrocolloids*, *55*, 144–154.

Fernández-Ávila, C., Escriu, R., & Trujillo, A. J. (2015). Ultra-High Pressure Homogenization enhances physicochemical properties of soy protein isolate-stabilized emulsions. *Food Research International,* 75, 357–366.

Fernandez-Avila, C., & Trujillo, A. J. (2016). Ultra-High Pressure Homogenization improves oxidative stability and interfacial properties of soy protein isolate-stabilized emulsions. *Food Chemistry (in press).*

Gao, Z.-M., Zhu, L.-P., Yang, X.-Q., He, X.-T., Wang, J.-M., Guo, J., … Yin, S.-W. (2014). Soy lipophilic protein nanoparticles as a novel delivery vehicle for conjugated linoleic acid. *Food & Function*, *5*(6), 1286–93.

Gaullier, J.-M., Halse, J., Høye, K., Kristiansen, K., Fagertun, H., Vik, H., & Gudmundsen, O. (2004). Conjugated linoleic acid supplementation for 1 y reduces body fat mass in healthy overweight humans. *The American Journal of Clinical Nutrition*, *79*(6), 1118–25.

Genot, C., Meynier, A., & Riaublanc, A. (2003). Lipid oxidation in emulsions. In A. Kamal-Eldin (Ed.), *Lipid Oxidation Pathways (pp. 190–244)*. Illinois: AOCS Press.

Gunstone, F. D., & Martini, S. (2010). Chemical and physical deterioration of bulk oils and shortenings, spreads and frying oils. In L. H. Skibsted, J. Risbo, & M. L. Andersen (Eds.), *Chemical Deterioration and Physical Instability of Food and Beverages (pp. 413–438)*. UK: Woodhead Publishing Limited.

Hu, M., Julian McClements, D., & Decker, E. A. (2004). Impact of chelators on the oxidative stability of whey protein isolate-stabilized oil-in-water emulsions containing ω-3 fatty acids. *Food Chemistry*, *88*(1), 57–62.

Huang, P.-H., Lu, H.-T., Wang, Y.-T., & Wu, M.-C. (2011). Antioxidant activity and emulsion-stabilizing effect of pectic enzyme treated pectin in soy protein isolate-stabilized oil/water emulsion. *Journal of Agricultural and Food Chemistry*, *59*(17),

9623–8.

Jimenez, M., Garcia, H. S., & Beristain, C. I. (2008). Sensory evaluation of dairy products supplemented with microencapsulated conjugated linoleic acid (CLA). *LWT - Food Science and Technology*, *41*(6), 1047–1052.

Kinsella, J. E. (1979). Functional properties of soy proteins. *Journal of the American Oil Chemists' Society*, *56*(3), 242–258. Koba, K., & Yanagita, T. (2014). Health benefits of conjugated linoleic acid (CLA). *Obesity Research & Clinical Practice, 8,* 525–532.

Lalush, I., Bar, H., Zakaria, I., Eichler, S., & Shimoni, E. (2004). Utilization of amylose-lipid complexes as molecular nanocapsules for conjugated linoleic Acid. *Biomacromolecules*, *6*(1), 121–30.

Martínez-Monteagudo, S. I., Saldaña, M. D. a., Torres, J. A., & Kennelly, J. J. (2012). Effect of pressure-assisted thermal sterilization on conjugated linoleic acid (CLA) content in CLA-enriched milk. *Innovative Food Science & Emerging Technologies*, *16*, 291–297.

Matthäus, B. (2010). Oxidation of edible oils. In E. A. Decker, R. J. Elias, & D. J. McClements (Eds.), *Oxidation in foods and beverages and antioxidant applications. Volume 2: Management in different industry sectors (pp. 183–238)*. UK: Woodhead Publishing Limited.

McClements, D. J. (2005). *Food Emulsions: Principles, Practice, And Techniques* (2nd ed.). Boca Raton, FL: CRC Press.

McClements, D. J. (2013). Utilizing food effects to overcome challenges in delivery of lipophilic bioactives: structural design of medical and functional foods. *Expert Opinion on Drug Delivery*, *10*(12), 1621–32.

McClements, D. J., Decker, E. A., Park, Y., & Weiss, J. (2009). Structural design principles for delivery of bioactive components in nutraceuticals and functional foods. *Critical Reviews in Food Science and Nutrition*, *49*(6), 577–606.

Moltó-Puigmartí, C., Castellote, A. I., & López-Sabater, M. C. (2007). Conjugated linoleic acid determination in human milk by fast-gas chromatography. *Analytica Chimica Acta*, *602*(1), 122–130.

Nasrabadi, M. N., Goli, S. A. H., & Nasirpour, A. (2015). Stability assessment of conjugated linoleic acid (CLA) oil-in-water beverage emulsion formulated with Acacia and xanthan gums. *Food Chemistry*, *199*, 258–264.

Nuchi, C. D., Hernandez, P., McClements, D. J., & Decker, E. A. (2002). Ability of lipid hydroperoxides to partition into surfactant micelles and alter lipid oxidation rates in emulsions. *Journal of Agricultural and Food Chemistry*, *50*(19), 5445–9.

Poliseli-Scopel, F. H., Hernández-Herrero, M., Guamis, B., & Ferragut, V. (2014). Sterilization and aseptic packaging of soymilk treated by ultra high pressure homogenization. *Innovative Food Science & Emerging Technologies, 22,* 81–88..

Roig-Sagués, A. X., Velázquez, R. M., Montealegre-Agramont, P., López-Pedemonte, T. J., Briñez-Zambrano, W. J., Guamis-López, B., & Hernandez-Herrero, M. M. (2009). Fat content increases the lethality of ultra-high-pressure homogenization on Listeria monocytogenes in milk. *Journal of Dairy Science*, *92*(11), 5396–402.

Sørensen, G., & Jørgensen, S. S. (1996). A critical examination of some experimental variables in the 2-thiobarbituric acid (TBA) test for lipid oxidation in meat products. *Zeitschrift Für Lebensmittel-Untersuchung Und -Forschung*, *202*(3), 205–210.

Wan, Z.-L., Wang, J.-M., Wang, L.-Y., Yang, X.-Q., & Yuan, Y. (2013). Enhanced physical and oxidative stabilities of soy protein-based emulsions by incorporation of a water-soluble stevioside-resveratrol complex. *Journal of Agricultural and Food Chemistry*, *61*(18), 4433–40.

Wan, Z.-L., Wang, J.-M., Wang, L.-Y., Yuan, Y., & Yang, X.-Q. (2014). Complexation of resveratrol with soy protein and its improvement on oxidative stability of corn oil/water emulsions. *Food Chemistry*, *161*, 324–31.

Wang, L. L., & Johnson, E. A. (1992). Inhibition of Listeria monocytogenes by fatty acids and monoglycerides. *Appl. Envir. Microbiol.*, *58*(2), 624–629.

Waraho, T., Cardenia, V., Rodriguez-Estrada, M. T., McClements, D. J., & Decker, E. A. (2009). Prooxidant mechanisms of free fatty acids in stripped soybean oil-in-water emulsions. *Journal of Agricultural and Food Chemistry*, *57*(15), 7112–7.

Waraho, T., McClements, D. J., & Decker, E. A. (2011). Impact of free fatty acid concentration and structure on lipid oxidation in oil-in-water emulsions. *Food Chemistry*, *129*(3), 854–9.

CHAPTER 7: Enhancement of physicochemical and sensory characteristics of a UHT milk-based product enriched with conjugated linoleic acid emulsified by Ultra-High Pressure Homogenization

[Experiment 5]

Enhancement of physicochemical and sensory characteristics of a UHT milk-based product enriched with conjugated linoleic acid emulsified by Ultra-High Pressure Homogenization

1. Introduction

There is a growing public interest in functional food products, as they offer great potential to improve health and/or help prevent certain diseases when taken as part of a balanced diet and healthy lifestyle (Alasalvar & Bolling, 2015; Sikand, Kris-Etherton, & Boulos, 2015). In the past decade, there has been an increasing interest in the incorporation of lipophilic bioactives, such as conjugated linoleic acid (CLA) into various food products, the most common being dairy products (Campbell, Drake, & Larick, 2003; Jimenez, Garcia, & Beristain, 2008).

CLA is a mixture of positional and geometric isomers of octadecadienoic acid (cis9,cis12 18:2) and the double bonds, which reduce its oxidative stability, are adjacent with no interceding methylene group (Bauman & Lock, 2006). It is naturally found in dairy and beef products from ruminant animals at very low concentrations, and it is necessary to prepare CLA by chemical processes for further applications in foodstuffs (Yettella, Henbest, & Proctor, 2011). Among all CLA isomers, the most representative are c9,t11-18:2 (rumenic acid) and t10,c12-18:2, which show different predominant health effects (Bhattacharya, Banu, Rahman, Causey, & Fernandes, 2006). In general, CLA has been found to confer many potential health benefits, such as anticarcinogenic, antiobese, antidiabetic, antihypertensive properties, atherosclerosis reduction and immune response enhancement (Koba & Yanagita, 2013). Although CLA it is not approved by the EFSA in E.U. (EFSA Panel, 2010), in the U.S., CLA status was approved as GRAS (generally recognized as safe) in 2008. Currently, it is claimed that “use of CLA-rich oil in certain specified foods within the general categories of soy milk, meal replacement beverages and bars, milk products and fruit juices, at a level of 1.5 g CLA per serving, is exempt”(Kim, Kim, Kim, & Park, 2016).

CLA exhibits very poor water solubility and therefore should be encapsulated within colloidal delivery systems, such as oil-in-water emulsions. Among the methods used to protect CLA, different strategies have been used, such as lipophilic nanoparticles (Gao et al., 2014), spray-drying (Choi, Ryu, Kwak, & Ko, 2010; Costa et al., 2015; He et al., 2016), and emulsions produced by conventional homogenization (Fernandez-Avila, Arranz, Guri, Trujillo, & Corredig, 2015) or High-Pressure Homogenization (Heo, Kim, Pan, & Kim, 2016). CLA also presents poor chemical stability, presenting autoxidation and isomerization to *trans,trans*-CLA during thermal processing in naturally CLA enriched milk (121 °C, 15 min) (Rodríguez-Alcalá, Alonso, & Fontecha, 2014). Consequently, thermal processing decrease nutritional value of enriched CLA products and may cause off-flavours (Campbell et al., 2003). To overcome these problems, it is important to develop effective delivery systems to encapsulate and protect CLA.

In particular, the emergent UHPH technology was successfully applied (0-350 MPa) in different milks (cow, goat and ewe) and the absent of significant modifications on the naturally CLA isomers profile were reported (Rodríguez-Alcalá et al., 2009), which showed the potential of UHPH on preserving low concentrations of naturally present CLA (<1 g CLA/100 mL). Recently, the ability of oil-in-water emulsions (20% oil) treated by UHPH and CH and stabilized by soy protein isolate (SPI, 4%) to deliver CLA (6%) has been studied by Fernandez-Avila & Trujillo (2015). It was found that UHPH treatment produced sterile emulsions and better physical and oxidative stabilities while maintaining similar percentages of CLA bioavailability, in comparison to CH-treated emulsions, suggesting the potential use of UHPH technology for delivering CLA in functional foods. Lately, lecithin-nanoemulsification of CLA in a free fatty acid form (FFA) enhanced the antiobesity effecs in rats (Kim, Park, Kweon, & Han, 2013), and recent evidence confirms the role of improved *in vivo* bioavailability of those nanoemulsions (Heo et al., 2016). Although lecithins are already widely used as natural emulsifiers and have been gaining increasing interest as natural antioxidants, they can readily react with prooxidants (e.g. transition metals), thus serving as lipid oxidation substrates and resulting in the development of off-flavours (Cui & Decker, 2016). For this reason, another kind of emulsifier such as SPI along with UHPH could be a suitable emulsification procedure (Fernandez-Avila & Trujillo, 2016), as UHPH produces

different interactions between molecules masking certain aromas and flavours (Innocente, Marchesini, & Biasutti, 2014), and could even prevent oxidation of CLA-FFA form, which is more susceptible to heat treatment than CLA-TAG (triglyceride form) (Heo et al., 2016).

The efficiency of CLA encapsulation in oil-in-water emulsions stabilized with vegetable proteins and their incorporation in a food-grade milk-based product have yet to be reported. The aim of the study was to investigate the physicochemical stability and sensory characteristics of different UHT milk-based products, in which were incorporated a soybean oil emulsion enriched with CLA-FFA treated by UHPH (200 MPa) in comparison to CH (15 MPa).

2. Materials and methods

2.1. Materials

A commercial SPI (PRO-FAM 974) was purchased from Lactotecnia (Barcelona, Spain). The composition of this commercial SPI according to manufacturer was: ≥90% protein, < 4% fat, < 6% moisture, and < 5% ash (dry basis, w/w). SPI PRO-FAM 974 has acid character and its isoelectric point is 4.6 (Kinsella, 1979) due to the high content of glutamic acid (Glu, 19.2%) and aspartic acid (Asp, 11.5%). Solubility of PRO-FAM 974 at pH = 7 is 39.5% (Bissegger, 2007). Soybean oil was purchased from Gustav Heess (Barcelona, Spain). Peroxide value of the soybean oil was below 10 meq O_2/kg and the acidity index was below 0.5 mg KOH/g. A free fatty acid (FFA) mixture high in isomers of CLA (Neobee®CLA80) was kindly donated from Stepan Specialty Products LLC (Maywood, USA), containing 80.4% of total CLA. The two main isomers present were c9, t11-CLA and t10.c12-CLA in a free form, in a 50:50 ratio. Other CLA isomers (*cis,cis* and *trans,trans*) were present in minor concentrations (<1.2%). Neobee®CLA80 was obtained from natural safflower oil by a gentle process without antioxidants added. Peroxide value of CLA was below 10 meq O_2/kg. All other chemicals used were of analytical or better grade.

2.2. Preparation of oil-in-water CLA emulsions

Oil-in-water emulsions were prepared with a fixed content of SPI (4%, w/v) and oil (20%, v/v), containing CLA (6%, v/v) and soybean oil (14%, v/v). Firstly, the stock protein dispersion (4%, w/v) was prepared by dispersing SPI in deionised water by using a high-speed dispersing unit at a rate of about 250 rpm for 1 h at 25 °C. Protein dispersions were stored overnight at 4 °C to allow complete hydration. Protein dispersions and oil were equilibrated at 20 °C (inlet temperature) before mixing. Pre-emulsions (or coarse emulsions) were prepared by mixing the protein dispersions with the soybean oil using rotor stator emulsifying unit (model Diax 900; Heidolph, Kehlheim, Germany) at 15000 rpm for 2 min. The coarse emulsions were further homogenised by conventional homogenization (CH) or Ultra-High Pressure Homogenization (UHPH) producing the corresponding emulsions (CH-E and UHPH-E). UHPH emulsions were treated at 200 MPa (single-stage) by an UHPH-Ypsicon equipment (Ypsicon Advanced Technologies, S.L., Barcelona, Spain) with a flow rate of 150 L/h and provided with a high-pressure ceramic needle-seat valve. CH emulsions were homogenized at 25 MPa with a flow rate of 250-350 L/h, using Tetra Alex® Homogenizer (Model S05 A, Tetra Pak Processing Components AB, Lund, Sweden) with a cobalt carbide mushroom valve. Pre-emulsions were passed through both devices with an inlet temperature (T_{in}) of 25 °C. The outlet temperature of UHPH emulsions was controlled by a heat exchanger located immediately after the high-pressure valve. During UHPH treatments, the T_{in}, the temperature after the high pressure valve (T_1), and outlet temperature were monitored.

2.3. Production of milk-based product

To produce the milk-based products, skim milk was mixed with CH emulsion or UHPH emulsion, in a ratio of 1:10 (emulsion:skim milk), to obtain fat content of 2% in the products (CH-M or UHPH-M). Concentrations used in the final product would be in the range of what is necessary to deliver a sufficient amount of CLA to be nutritionally significant (0.6% CLA; 600 mg/100 mL). To both products indirect UHT treatment was applied in a tubular indirect Finamat heat exchanger (model 6500/010; GEA Finnah

GmbH, Ahaus, Germany) at 140 °C for 4 s. Final milk-based products (CH-P and UHPH-P) were aseptically packed in 200 mL Tetra Brick Aseptic slim containers using aseptic packaging line (TBA9, Tetra Pak Processing Components AB). Sterility test was evaluated by bacteria enumeration in plate count agar after CH-P and UHPH-P incubation at 55 and 30 °C for 7 and 15 d, respectively. Both products were confirmed to be sterile. Products were held at 21 °C during 4 months. Physicochemical composition of the fresh milk-based products was evaluated and their rheological properties, colour, pH, physical stability, proteolysis and lipid oxidation were evaluated over time.

2.4. Determination of oil droplet size distribution

The particle size distribution in the emulsions (CH-E and UHPH-E), the mixing of the emulsions and the skim milk before UHT treatment (CH-M and UHPH-M) and the final milk-based product samples (CH-P and UHPH-P) was determined after each process, using a Beckman Coulter laser diffraction particle size analyzer (LS 13 320 series, Beckman Coulter, Fullerton, CA, USA). However, particle size of CH-E and UHPH-E was analyzed after 24 h. All emulsion samples were diluted in distilled water until an appropriate obscuration was obtained in the diffractometer cell. The optical parameters used were: a refractive index of 1.475 for the soybean oil and a refractive index of 1.332 for the water. The surface-weighted mean diameter ($d_{3.2}$, μm) and volume-weighted mean diameter ($d_{4.3}$, μm) were determined.

2.5. Physicochemical composition

Fat content was measured by Gerber method (ISO 2446:2008/IDF 226:2008). Total protein content was determined by measuring total nitrogen using the Kjeldahl method (ISO 8968-3:2004/IDF 20-3:2004). Total solids content was enumerated based on a mass fraction of substances remaining after the heating process (ISO 6731:2010/IDF 21:2010).

2.6. Rheological measurements

Rheological measurements were performed using a controlled stress rheometer (Haake Rheo Stress 1, Thermo Electron Corporation, Karlsruhe, Germany) using a cone (1°, 60 mm diameter) and plate geometry probe at 21 °C. Flow curves (shear stress compared to shear rate) were determined at increasing and decreasing shear rates between 0.001 s^{-1} and 100.0 s^{-1} in 1 min (up and down flow curves). Flow curves were fitted to the Newton model, $\tau = \eta\gamma$, where τ is the shear stress (Pa), η is the viscosity (Pa × s), and γ is the shear rate (s^{-1}).

2.7. Colour

The colour values of the milk-based products were measured using a Hunter Lab colorimeter (MiniScan XE Hunter Associates Laboratory Inc., USA). Colour coordinates were measured with an illuminant of D65 and a standard observer of 10° and the colorimeter was calibrated against white and black tile standards. Data was acquired in the CIELab colour space, where L* value represents the lightness with values from 0 (black) to 100 (white), which indicates a perfect reflecting diffuser. The *a** and *b** axes have no specific numerical limits and represent chromatic components. The *a** values are a measure of redness (higher positive values indicate a redder colour, higher negative values indicate a greener colour); *b** values are a measure of yellowness (higher positive values indicate a more yellow colour, higher negative values indicate a bluer colour). Data were used then to calculate the total colour difference $\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$ formula equation. ΔL^*, Δa^* and Δb^* are the differences in the tristimulus coordinates between L*, a* and b* of the samples over time and the standard (day 0) of CH-P and UHPH-P, which gives an idea of the influence of the homogenization treatment contributing to the overall colour.

2.8. Physical stability

The optical characterization of milk-based products was analysed using a Turbiscan® MA 2000 (Formulaction, Toulouse, France), which is composed by a reading head

(pulsed near-IR light source; λ = 850 nm, and two synchronous detectors of transmission and backscattering) which scans the sample glass tube. Milk-based product samples were placed in a cylindrical glass cell, under sterile conditions, on the day of production. Product destabilization (sedimentation and creaming) was analysed. When backscattered light is in the range of 0% to 100%, propagation of light goes through a clear to an opaque dispersion, respectively. Thus, if backscattering is nearly 100%, it indicates sedimentation or creaming. Turbiscan Stability Index (TSI) in the bottom, middle and top of the glass cell was measured to rank the stability of CH-P and UHPH-P. At the bottom and the top of the Turbiscan glass tube, BS signal increase/decrease if the concentration increases/decreases; and in the middle, BS signal evolves, because of the size variation.

2.9. Proteolysis

The pH 4.6-soluble fraction of milk-based products was obtained by isoelectric casein precipitation. Briefly, samples were centrifuged at 20.000 *g* for 10 min at 21 °C to separate the fat content. The top cream layer was removed and the pH of skim milk-based products (1 mL) was adjusted to 4.6 by adding 15 µL of acetic acid of 33.3% (v/v), followed by addition of 15 µL of 3.33 M sodium acetate. The mix was centrifuged at 2800 *g* for 5 min and 21 °C, and the supernatant was collected. From this fraction the total free amino acids (FAA) was determine by the cadmium-ninhydrin method of Folkertsma and Fox (1992), measuring the total FAA concentrations (mg Leu/mL milk) at 507 nm and using a standard curve made from leucine (0.002-0.04 mg Leu/mL).

2.10. Analysis of lipid oxidation

The formation of lipid hydroperoxides was evaluated according to the method of (Hu et al.,2004). In brief, aliquots (0.3 mL) of the emulsions, after storage of a specific incubation period, were mixed with 1.5 mL of isooctane/2-propanol (3:1, v/v) by vortexing. The organic solvent phase of the mixtures was collected by centrifugation at 1000 *g* for 2 min. The organic solvent phase (200 µL) was added to 2.8 mL of a methanol:1-butanol mixture (2:1, v/v), followed by addition of 15 µL of 3.94 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 resultant solutions were measured at 510 nm 20 min after addition of the iron. Lipid hydroperoxide concentrations (mmol/L of emulsion) were determined using a standard curve made from cumene hydroperoxide (0.5-5 mmol/L).

To determine the formation of secondary oxidation products, thiobarbituric acid-reactive substances (TBARs) technique was performed. It detects the formation of malondialdehyde, which reacts with thiobarbituric acid (TBA). Malondialdehyde or TBARs content of the emulsions upon storage at 37 °C for 14 days was determined using a process as described by Sørensen & Jørgensen (1996), with a few modifications. In brief, emulsions (3.0 mL) were mixed with 6.0 mL of trichloroacetic acid (TCA) reagent (7.5%, w/v) and were vortexed. The resultant mixtures were filtered with a 1 µm microporous membrane after 20 min of the TCA addition. The filtered (2.0 mL) were placed in test tubes containing TBA reagent (0.8%, w/v) and they were mixed by vortexing. The resultant mixtures were heated in a water bath (95 ^{o}C) for 15 min, and then cooled immediately in an ice-bath to slow down the reaction. The absorbance of the final extracts was recorded at 532 and 450 nm (A_{450}). The TBARs concentration (mmol/L of emulsion) was determined according to a standard curve with hexanal.

2.11. Sensorial analysis

Milk characteristics were evaluated according to the recommended protocol proposed by the ISO 22935-2:2009 (IDF 99-2:2009) for the sensory evaluation of milk products. The trained sensory panel considered the attributes listed in Table 1.

Table 1. Sensory attributes for which the panel was trained.

Odour	Appearance	Mouthfeel/Texture	Flavour
Global intensity	Cream layer	Viscosity	Global intensity
Cooked*	Protein/fat flocks	Presence of aggregates	Grassy
Grassy	Colour		Oxidized
Oxidized	Sedimentation		Rancid
Foreign			Bitter
			Foreign

Sensory panel was constituted by 13 individuals from "Departament de Ciència Animal i dels Aliments" (Facultat de Veterinària, Universitat Autònoma de Barcelona) familiarized with dairy products. Nonetheless, they were trained for the identification of specific odour and flavour attributes, as indicated in Table 2 and the literature reviewed (Pereda, 2009; Poliseli-Scopel, 2012).

Table 2. Preparation of defects in milk for the panel sensory training.

Defect in milk	Preparation
Cooked	Heating the milk based product containing CLA close boiling point (85 °C) and then cooled rapidly.
Oxidized	Addition of 4 drops of 1 mg/L 2-nonenal and 10 mg/L 2-heptenal in ethanol to the milk based product containing CLA.
Rancid	Addition of 6 drops of 1% butyric acid in ethanol to 250 ml of a milk based product containing CLA.
Bitter	Addition of 0.06% caffeine into a milk based product containing CLA.

Instructions for the judge's panel test were as follows. To evaluate the product appearance, the package was opened it with a scissors cutting the whole traverse-welding. Next, phase separation, protein or fat aggregates, creaming and were evaluated.

Immediately, milk content was carefully poured in a plastic cup to evaluate flavour characteristics. The precipitates presence at bottom of the empty package was pointed. To each attribute notation, the following scale, which gives the deviation magnitude regarding to a standard sample, was given: (0) inappreciable, (1) minimal, (2) remarkable, (3) considerable, and (4) very considerable deviation in comparison to the established sensorial specification. Both CH and UHPH-milk based products were randomly employed as standard.

2.12. Statistical analyses

Descriptive statistics, mean and standard deviation, were listed for each variable in this study. In order to evaluate the physico-chemical stability of the milk-based products containing CLA over time, one-way ANOVA was performed. The statistical analysis was performed using the SPSS® v17.0 package to a 95% level of significance and Tukey adjustment was performed ($P < 0.05$). Also, the Student's t-test for paired samples analysis was carried out to compare the changes between samples (CH and UHPH).

Significance was defined as $P < 0.05$ for a two-sided test. Experiments were performed in duplicate as separate independent runs.

3. Results and discussion

3.1. Particle size of of milk-based products

Figure 1 shows the effect of UHPH (200 MPa) and CH (15 MPa) treatments on particle size distribution in emulsions (E), the milk-based products containing those emulsions before UHT (M) and the final UHT milk-based products (P). The particle size distribution curve of CH-E showed a bimodal particle distribution between 0.07 and >100 µm, whereas UHPH-E showed a monomodal distribution and smaller particle size distribution, between 0.05 and <5 µm (Fig. 1A and B for emulsions). CH-E was characterized by a main peak of about 20 µm due to larger particle size and flocculated particles, as this emulsion showed a gel-like behaviour (data not shown). In contrast, UHPH-E was characterized by a main peak of about 0.7 µm and a second minor peak that could correspond to small particle aggregates.

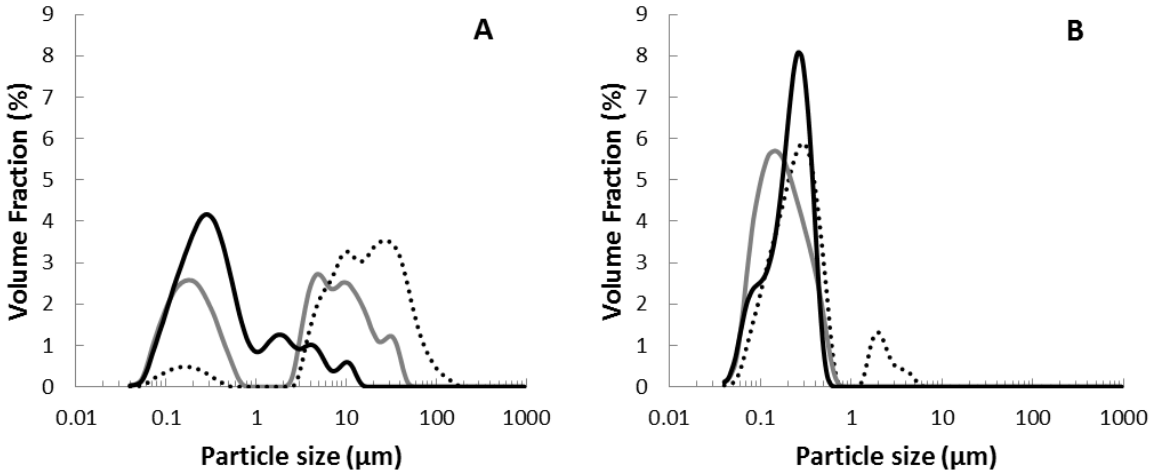

Figure 1. Size distribution profiles of emulsions (CH-E and UHPH-E; dotted line), the mixing of the emulsions and the skim milk before UHT treatment (CH-M and UHPH-M; grey line) and the final milk-based product (CH-P and UHPH-P; black line) samples treated by conventional homogenization at 25 MPa (A) and Ultra-High Pressure homogenization at 200 MPa (B).

When comparing UHPH-E treated by Ypsicon equipment in this study (pilot-plant scale) in comparison with the same emulsion formulation treated by Stansted Benchtop

Homogenizator nG12500 (lab scale) (Fernández-Avila et al., 2015), a minor volume fraction (5%) is found for the main peak, even though particle size analysis of UHPH-E in this study were determined after 24 h. Possibly, the smaller T_1 obtained by the Ypsicon-UHPH in this study (75 °C) in comparison to those obtained by the Stansted Benchtop Homogenizator (83 °C) could have avoided coalescence of oil droplets.

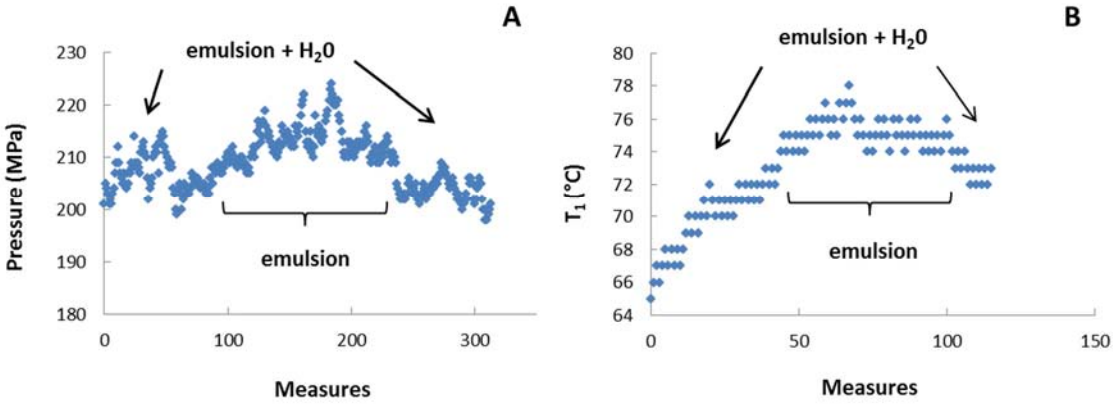

Figure 2. Pressure (MPa) (A) and temperature (B) reached in the high-pressure valve (T1) in emulsions treated by Ultra-High Pressure Homogenization (UHPH) at 200 MPa. Measures of emulsions passing through the UHPH-valve are represented with curly brackets.

Before UHT treatment, changes in particle size distribution could be observed when emulsions were mixed with skim milk. For UHPH-M the particle distribution changed from bimodal to monomodal, which size comprised between 0.03 and <0.7 µm and was characterised by a main peak of about 0.2 µm. In the case of the CH-M, a change in the typical bimodal distribution was visible, with a dramatic increase in the first peak intensity and a displacement of the second peak (Fig. 1A and B). Finally, curves of size distribution of CH-P showed a polydisperse distribution profile with the displacement of the second group of larger particles due to the possible disruption of particles owing to turbulences created during the UHT treatment. In a similar way, UHPH-P showed a displacement of the peak into the narrowest distribution (Fig. 1A and B).

The particle size values ($d_{3,2}$ and $d_{4,3}$) for E, M and P samples treated by CH and UHPH are listed in Table 3.

Table 3. Mean values ± SD of $d_{3.2}$ and $d_{4.3}$ indexes of emulsions (E), the mixing of the emulsions and the skim milk before UHT treatment (M) and the final UHT milk-based product samples (P) treated by conventional homogenization (CH) and Ultra-High Pressure Homogenization (UHPH).

Stage treatment	Particle size			
	$d_{3,2}$ (μm)		$d_{4,3}$ (μm)	
	CH	UHPH	CH	UHPH
E	$3.25^{a,x}$ ± 1.86	$0.21^{a,y}$ ± 0.01	$36.64^{a,x}$ ±14.73	$1.72^{a,y}$ ± 1.43
M	$0.34^{b,x}$ ± 0.02	$0.15^{b,y}$ ± 0.00	$7.67^{b,x}$ ± 0.65	$0.23^{b,y}$ ± 0.04
P	$0.28^{b,x}$ ± 0.01	$0.16^{b,y}$ ± 0.03	$1.73^{c,x}$ ± 0.65	$0.24^{b,y}$ ± 0.01

$^{a, b}$ Means within a column for each treatment with a different superscript were significantly different ($P < 0.05$).

$^{x, y}$ Means within a row for each parameter with a different superscript were significantly different ($P < 0.05$).

Particle size values ($d_{3,2}$ and $d_{4,3}$) were significantly ($P < 0.05$) reduced in CH-M and UHPH-M samples in comparison to their counterpart E samples (Table 3), but not significant changes were found in CH-P and UHPH-P, in comparison to their respective M samples, as an exception for $d_{4,3}$ in CH-P ($P < 0.05$). As expected, the reduction in $d_{3,2}$ and $d_{4,3}$ values, achieved in UHPH samples was higher ($P < 0.05$) than that obtained by the CH treatment.

Other researchers have also reported greater reduction of particle size achieved by UHPH at 200 MPa compared to CH in O/W emulsions containing vegetable oils and whey protein isolate as emulsifier (Hebishy, Buffa, Guamis, Blasco-Moreno, & Trujillo, 2015), soy milk (Poliseli-Scopel, Hernández-Herrero, Guamis, & Ferragut, 2012), almond milk (Valencia-Flores, Hernández-Herrero, Guamis, & Ferragut, 2013) and tiger nut milk (Codina-Torrella, Guamis, Ferragut, & Trujillo, 2016).

3.2. Physicochemical composition of milk-based products

Chemical composition of UHT milk-based products containing CH and UHPH emulsions are showed in Table 4. No significant ($P > 0.05$) differences can be observed between both treatments; therefore, homogenization process did not influence in protein, fat and total solid content of CH-P and UHPH-P.

Table 4. Mean values ± SD of protein, fat and total solid content in UHT milk-based products containing CLA-emulsions treated by conventional homogenization (CH-P) or by Ultra-High Pressure Homogenization (UHPH-P).

UHT milk-based product	Chemical composition		
	Protein (%)	Fat (%)	Total solid (%)
CH-P	3.31 ± 0.08	1.65 ± 0.00	9.88 ± 0.05
UHPH-P	3.32 ± 0.00	1.65 ± 0.00	10.08 ± 0.00

The pH of milk-based products was unaffected by the homogenization treatment applied, showing acidic pH values closed to neutrality at day 1 (Table 5). However at 30, 60 and 120 days of storage, the pH of CH-P was significantly lower ($P < 0.05$) than the pH values of UHPH-P. By comparing CH-P and UHPH-P, the pH has an slightly but significant ($P < 0.05$) decreased in both products during the storage time at 21 ^{o}C up to day 90, although a not significant increase in the pH values at 120 days of storage was evidenced Table 5.

Table 5. Mean values ± SD of pH UHT milk-based products containing CLA-emulsions treated by conventional homogenization (CH-P) and Ultra-high Pressure Homogenization (UHPH-P).

Time (days)	pH	
	CH-P	UHPH-P
1	$6.33^{a} \pm 0.02$	$6.36^{a} \pm 0.04$
30	$6.28^{ab,y} \pm 0.01$	$6.32^{ab,x} \pm 0.01$
60	$6.29^{a,y} \pm 0.04$	$6.32^{ab,x} \pm 0.03$
90	$6.18^{c} \pm 0.04$	$6.24^{c} \pm 0.01$
120	$6.22^{bc,y} \pm 0.04$	$6.27^{bc,x} \pm 0.02$

^{a,b,c} Means within a column for each treatment with a different superscript were significantly different ($P < 0.05$).

$^{x, y}$ Means within a row for each parameter with a different superscript were significantly different ($P < 0.05$).

3.3. Viscosity of milk-based products

Viscosity affects the flow conditions in dairy processes and the mouthfeel sensory attributes. Table 6 shows the viscosity evolution (mPa × s) of UHT milk-based products containing CH and UHPH emulsions during storage at 21 °C. Values measured in CH-P

showed a slightly but significant ($P < 0.05$) decrease of viscosity during storage from day 60. Nevertheless, the UHPH-P viscosity values also indicated a slight decrease, but in this case not significant ($P > 0.05$). By comparing CH-P and UHPH-P no significant ($P > 0.05$) differences could be observed, although UHPH-P had in generallower values than CH-P during storage.

Table 6. Evolution of viscosity (mPa × s) of UHT milk-based products containing CLA-emulsions treated by conventional homogenization (CH-P) and Ultra-high Pressure Homogenization (UHPH-P) during storage at 21 °C.

Time (days)	Viscosity	
	CH-P	UHPH-P
1	$2.084^{a} \pm 0.039$	$2.020^{a} \pm 0.050$
30	$2.011^{ab} \pm 0.014$	$1.968^{a} \pm 0.088$
60	$1.965^{b} \pm 0.069$	$1.954^{a} \pm 0.004$
90	$1.960^{b} \pm 0.060$	$1.958^{a} \pm 0.058$
120	$1.972^{b} \pm 0.023$	$1.984^{a} \pm 0.114$

^{a,b} Means within a column for each treatment with a different superscript were significantly different ($P < 0.05$).

3.4. Colour of milk-based products

The influence of treatments applied and storage time on the colour of CH-P and UHPH-P is shown in Table 7. For both products, lightness (L*) and colour intensity (a* and b*) presented a significant ($P < 0.05$) increased tendency during storage time. L* values increased during the storage time, however the increase was only significant up to 60 days of storage. From 90 days of storage, UHPH-P showed higher L* values (whiter colour) than CH-P. Significant differences ($P < 0.05$) in a* value between CH-P and UHPH-P were only detected at day 1, being CH-P greener than UHPH-P. Significant differences ($P < 0.05$) in b* values between CH-P and UHPH-P were only detected from day 60, with CH-P being more yellowness than UHPH-P.

To compare general colour changes in the products the total colour difference (ΔE) was measured over time (Table 7). The ΔE of both products during storage was significantly ($P < 0.05$) different up to day 60, being the ΔE of UHPH-P during storage higher than CH-P.

Table 7. Colour parameters[1] of UHT milk-based products containing CLA-emulsions produced by conventional homogenization (CH-P) and Ultra-high Pressure Homogenization (UHPH-P).

Time (days)	Colour parameters							
	L*		a*		b*		$\Delta E^{day\ 1}$	
	CH-P	UHPH-P	CH-P	UHPH-P	CH-P	UHPH-P	CH-P	UHPH-P
1	$36.66^{b} \pm 1.06$	$35.99^{c} \pm 0.27$	$-1.37^{ab,y} \pm 0.14$	$-1.28^{a,x} \pm 0.15$	$3.53^{b} \pm 0.51$	$3.10^{b} \pm 0.46$	-	-
30	$37.06^{b} \pm 0.40$	$38.20^{b} \pm 0.61$	$-1.62^{b} \pm 0.11$	$-1.64^{b} \pm 0.05$	$4.55^{a} \pm 0.30$	$4.40^{a} \pm 0.10$	$0,97^{b,y} \pm 0,14$	$3,53^{b,x} \pm 1,61$
60	$38.74^{a} \pm 0.40$	$39.61^{a} \pm 1.12$	$-1.24^{a} \pm 0.16$	$-1.14^{a} \pm 0.13$	$4.93^{a,x} \pm 0.21$	$4.53^{a,y} \pm 0.16$	$3,90^{a,y} \pm 1,98$	$8,20^{a,x} \pm 3,62$
90	$37.56^{ab,y} \pm 0.72$	$38.37^{ab,x} \pm 0.27$	$-1.26^{a} \pm 0.16$	$-1.16^{a} \pm 0.09$	$4.85^{a,x} \pm 0.35$	$4.68^{a,y} \pm 0.27$	$2,27^{ab} \pm 1,31$	$4,32^{ab} \pm 0,28$
120	$38.04^{ab,y} \pm 0.34$	$38.34^{ab,x} \pm 0.33$	$-1.33^{ab} \pm 0.14$	$-1.18^{a} \pm 0.07$	$4.76^{a,x} \pm 0.20$	$4.36^{a,y} \pm 0.18$	$2,31^{ab,y} \pm 1,33$	$3,64^{b,x} \pm 0,61$

[1]Mean values ± SD of colour parameters. ΔE was calculated taking into account day 1 as reference sample.

[a, b] Means within a column for each treatment with a different superscript were significantly different ($P < 0.05$).

[x, y] Means within a row for each parameter with a different superscript were significantly different ($P < 0.05$).

3.5. Physical stability of milk-based products

Figure 3 shows the turbidity profile for CH-P and UHPH-P. At the top and bottom of the vial, creaming and sedimentation are represented by an intense orange, respectively. As noted, creaming layer in CH-P appeared before 1 month and increased during the storage time.

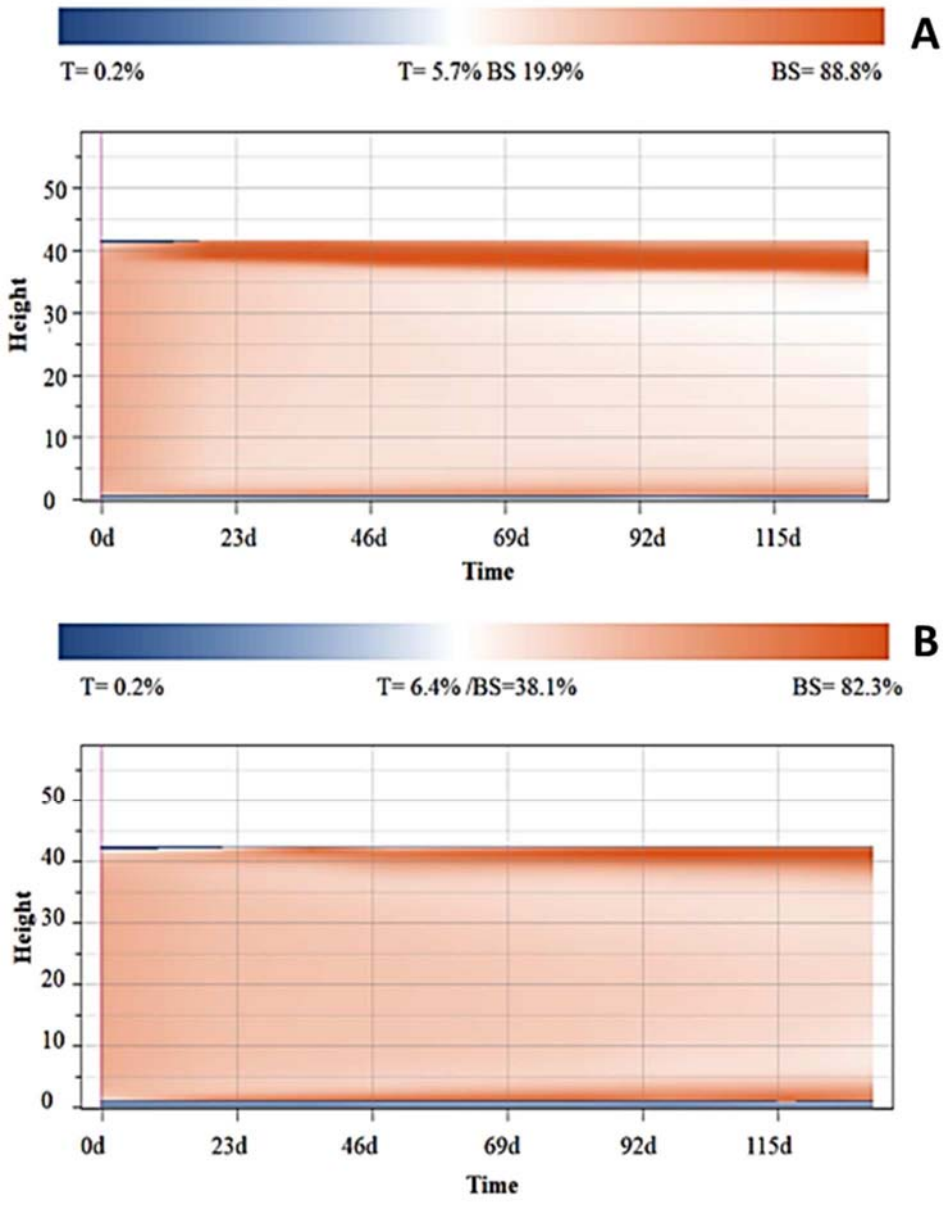

Figure 3. Evolution in backscattering (BS) and transmission (T) of UHT milk-based products containing CLA-emulsions treated by conventional (A) or Ultra-high Pressure (B) homogenization during storage time.

In the middle of the glass cell, some clarification was observed in CH-P compared to UHPH-P (represented by the appearance of a diffuse white coloration), which is due to migration of the particles toward the surface container (Fig. 3A). These clarification phenomena may be related to physical instability of the product and produce a significant ($P < 0.05$) viscosity decrease after 60 days along storage time in CH-P (Table 6). Figure 4 represents the TSI of the bottom, middle and top of the samples, which is a more accurate tool to distinguish the more physically stable product. CH-P was more unstable than UHPH-P due to larger extent in creaming and sedimentation during storage, as a result of the larger particle size compared to UHPH-P (Fig. 1, Table 3).

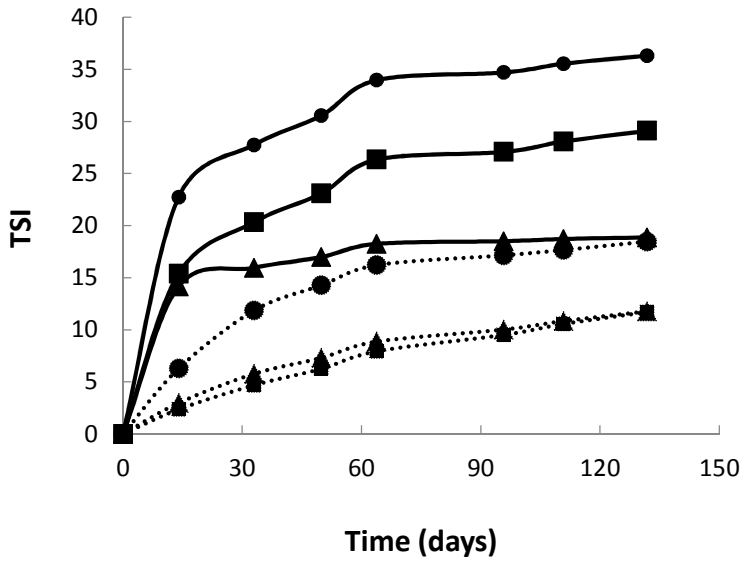

Figure 4. Turbiscan Stability Index (TSI) in the bottom (▲), middle (■) and top (●) of UHT milk-based products containing CLA-emulsions treated by conventional (solid lines) or Ultra-High Pressure (dotted lines) Homogenization during storage time.

3.6. Proteolysis in milk-based products

Proteolysis of UHT milk is mainly caused by indigenous proteases and by those produced by psychotrophic bacteria during cold storage of raw milk (Datta & Deeth, 2003; Pereda, Ferragut, Buffa, Guamis, & Trujillo, 2008). Plasmin (PL) and its inactive precursor plasminogen are the main proteolytic enzymes in milk, associated with the casein micelle and the milk fat globule membrane, that can survive severe heat treatments (Bastian & Brown, 1996; Enright et. al, 1999). In general, PL hydrolyses mainly β- and α_{s2}-caseins, and more slowly α_{s1}-casein, and its activity, in general, generates sensory problems in commercial sterile milks, such as bitter and astringent

flavours (Santos et al., 2003; Datta & Deeth, 2003). In this study, the FAA concentration determined in CH-P and UHPH-P showed an oscillatory tendency around 0.05-0.07 mg Leu/mL and there were no significant differences between products ($P > 0.05$). These results suggest an insignificant or absence activity of plasmin and/or of other exogenous proteases, and a similar propensity to proteolysis in both UHT products.

3.7. Lipid oxidation of milk-based products

Lipid oxidation is the main reason for the deterioration of fats and oils and consists in the reaction of molecular oxygen with unsaturated fatty acids which are prone to oxidation, such as CLA, resulting in unsaturated hydroperoxides (Gunstone & Martini, 2010; Rodríguez-Alcalá et al., 2014). Lipid hydroperoxides are unstable primary oxidation products which can further decompose to a wide range of volatile and non-volatile secondary products, particularly aldehydes, responsible for undesirable odours and flavours (McClements & Decker, 2000). Results obtained for primary oxidation in Table 8 showed an oscillatory behaviour in both milk-based products, due to the fact that they can be quickly transformed into secondary oxidation products. By comparing both UHT products, significant differences ($P < 0.05$) were detected from 90 days of storage, being the level of lipid hydroperoxides lower for UHPH-P than for CH-P.

Table 8. Mean values ± SD of lipid hydroperoxides (mmol/L product) of UHT milk-based products containing CLA-emulsions treated by conventional homogenization (CH-P) and Ultra-High Pressure Homogenization (UHPH-P).

Time (days)	Lipid hydroperoxides (mmol/L product)	
	CH-P	UHPH-P
1	$0.575^{a} \pm 0.010$	$0.592^{a} \pm 0.019$
30	$0.535^{b} \pm 0.017$	$0.525^{b} \pm 0.006$
60	$0.292^{e} \pm 0.021$	$0.280^{e} \pm 0.008$
90	$0.442^{c,x} \pm 0.015$	$0.415^{c,y} \pm 0.006$
120	$0.375^{d,x} \pm 0.006$	$0.320^{d,y} \pm 0.018$

$^{a-e}$ Means within a column for each treatment with a different superscript were significantly different ($P < 0.05$).

^{x,y} Means within a row for each parameter with a different superscript were significantly different ($P < 0.05$).

In TBARS method, the pink complex that exhibits a maximum absorbance at 532 nm due to the reaction between TBA and MDA (produced from lipid hydroperoxide decomposition) was not observed. Instead, the reaction formed a yellow colour, with a maximum absorption at 450 nm. It has been previously observed that interfering peak at 450 nm is due to other lipid oxidation products (such as alkenals, alkadienals, ketones, and other aldehydes) (Fenaille, Mottier, Turesky, Ali, & Guy, 2001; Hedegaard et al., 2006). In this study, storage did not affect TBARS for any of the products. In fact, A_{450} remained 0.04 ± 0.00 for all measurements. Moreover, the differences between CH-P and UHPH-P along storage were not found to be significant ($P > 0.05$). These results are in line with those of Hedegaard et al., (2006), who evaluated TBARS (A_{450} = 0.07) and hexanal (SPME-GC-FID) of 3 types of bovine milk with different fatty acid profiles, in which development of secondary oxidation products was non-significant. In contrast, other researchers reported higher TBA values for a CLA (3.5%) O/W non-dairy beverage emulsion containing acacia gum (10%) and xanthan gum (0.3%) (Nasrabadi, Goli, & Nasirpour, 2015). Nevertheless, TBA values were obtained at 535 nm and higher CLA content was employed in comparison to the present study (0.6% CLA in CH-P and UHPH-P). More recently, dairy matrix interferences have been identified in the TBARS method in flavoured phytosterol-enriched drinking yogurts, such as lactose and lactic acid (Semeniuc, Mandrioli, Rodriguez-Estrada, Muste, & Lercker, 2016). For these reasons, it was refused to report the TBARs content as hexanal in this study, and only A_{450} measures have been expressed, owing to overestimated hexanal levels by milk-based matrix components. Thus, TBARS must be considered to be a less valuable method, in comparison to SPME-GC/MS, as other authors have successfully reported the characterization of various volatile compounds in UHPH milk (Pereda et al., 2008).

3.7.1. Sensory attributes and preference test for CLA milk-based products

The trained sensory panel evaluated the sensory characteristics listed in Table 1 during 4 months (Fig. 5). Each month (M0-M4), sensory attributes (Fig. 5A-C) and a preference test of milk-based products (Fig. 5D) was undertaken, in order to distinguish which organoleptic characteristics affected consumers acceptability.

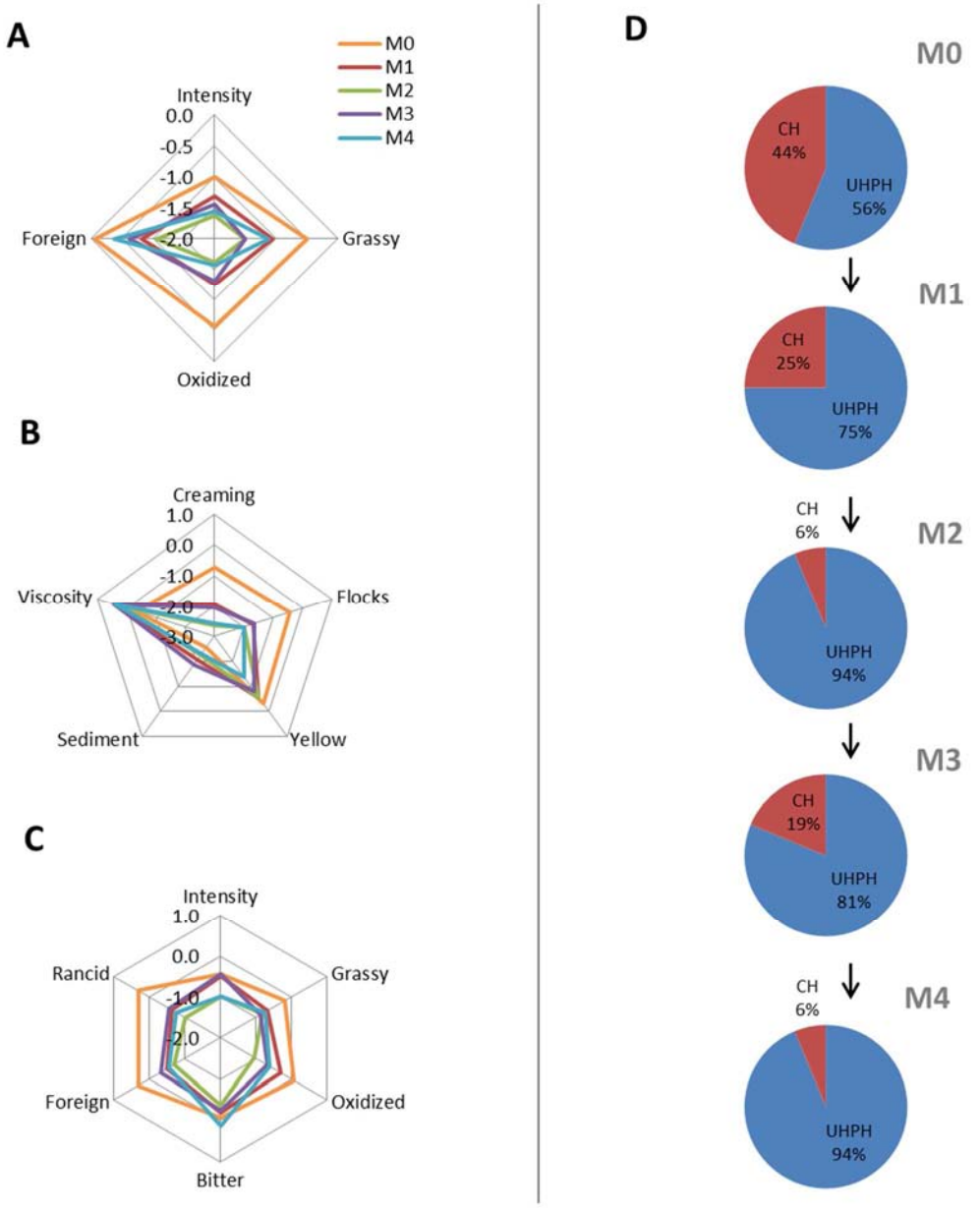

Figure 5. Sensorial attributes evaluated in the milk-based products containing CLA emulsified by Ultra-High Pressure Homogenization (UHPH-P) or by conventional homogenization (CH-P): odour (A), appearance (B) and flavour (C). Results have been represented as the UHPH-P in comparison to CH-P (standard). Preference test (D) was undertaken since the initial storage time (M0) until 4 months (M4).

In the initial sensory panel (M0), minimal differences were shown between products (CH-P and UHPH-P) for odour (Fig. 5A) and flavour (Fig. 5C) attributes, which corresponded with the absence of a preference for a product (44% people preferred the CH-P and 56% the UHPH-P) (Fig. 5D).

Almost no appreciable differences were agreed on viscosity, and this was in line with no statistical differences ($P > 0.05$) between CH-P and UHPH-P along storage measured by instrumental analysis (Table 6).

Among products, the differences in creaming and fat/protein flocks were considerable after 1 month of storage. The observation of creaming was in line with TSI results at the top of the Turbican tube, in which CH-P showed more than 2 folds of cream layer than UHPH-P (Fig. 4). Also, as TSI shows, the kinetics of creaming kept steady after 3 months, which is related to the lack of evolution in the sensory analysis over storage (Fig. 5B). Moreover, CH-P considerably presented more sedimentation than UHPH-P since the first sensory analysis. Although the bottom of Turbiscan cell containing UHPH-P presented a TSI around 10, no signs of sedimentation were observed by the panelists during storage. Probably, the smaller oil droplets formed during emulsification in UHPH-P (Fig. 1, Table 3) were enough to avoid sedimentation, as more protein can be loaded owing to higher specific surface area (McClements, 2005). In this sense, larger particle size in CH-P was detrimental as it might be the cause of sedimentation, due to an excess of proteins remaining in the continuous phase. During storage, sensorial analysis of UHPH-P was characterized with a minimal decrease in the yellowness intensity in comparison to CH-P (Fig. 5B), which was confirmed by their higher L* values after 3 months and lower b* values after 2 months of storage (Table 7).

At the end of the storage (M4), the intensity of odour and its grassy and oxidized attributes decreased for UHPH-P (Fig. 5A). In concordance, the intensity of flavour and its foreign, grassy, oxidized and rancid attributes also decreased after 4 months (Fig. 5C). Oxidized notes (oxidized, foreign and grassy attributes) could be related with the lower lipid hydroperoxides content in UHPH-P ($P < 0.05$) compared to CH-P (Table 8). However, the minimal differences for less rancidity in UHPH-P after 1 month of storage were not consistent as panelists stated the difficulty in evaluating the net effect of each sample. Also, no appreciable differences were noticed on bitterness along storage, and this was in line with no statistical differences in proteolysis (mg Leu/mL) between CH-P and UHPH-P along storage.

The results obtained in the preference test of both products showed a very considerable preference for UHPH-P after one month of storage (75%) with increasing tendency (94% of preference after 4 months), which corresponded to the considerable signs of physical destabilization phenomenon (Fig. 3 and 4) and the increase in off-flavour perception for CH-P during storage (Fig. 5A,C). However, panelists stated that they would not consume any product (CH-P and UHPH-P) as the rancidity flavour could not be fully masked. For these reason, further research should be done to investigate the sensory acceptability of similar formulations including CLA-TAG, as Heo et al. (2016) have confirmed similar *in vivo* bioavailability between lecithin-nanoemulsions containing CLA-FFA or CLA-TAG.

4. Conclusions

This manuscript has investigated the importance of oil-in-water emulsions treated by UHPH as preservative matrices against heat treatment (UHT) for highly hydrophobic and prone to oxidation bioactive compounds, such as free-CLA. In this investigation, the aim was to assess the stability of UHPH emulsions containing soybean oil and CLA added to skim milk for producing a milk-based product, comparing with other oil-in-water emulsions treated by the conventional homogenization method.

It has been reported that the UHT milk-based product containing the Ultra-High Homogenization Pressure CLA-emulsion (UHPH-P) presented smaller particle size and better physical stability compared to the homologous product with the conventional homogenization CLA-emulsion (CH-P). Particularly, creaming rate of UPHP-P was much slower than in CH-P. After 3 months of storage, UHPH-P appeared to be more oxidatively stable than CH-P.

In order to assess the impact of CLA fortification on the consumers' acceptance, sensory characteristics of the developed UHT milk-based products were also evaluated. The results obtained in the preference test of both products showed a very considerable preference for UHPH-P after one month of storage (>75%), which corresponded to the considerable signs of creaming for CH-P, its remarkable increase in intensity, grassy and oxidized odour, as well as the minimal increase in intensity,

foreign, grassy and oxidized flavour. In future investigations it might be more convenient to use a different CLA less prone to oxidation (triglyceride from) in order to assess an improvement on sensory characteristics. Also, further research should be done to investigate the effect of both milk-based products on *in vivo* bioavailability, as the repercussion of soy protein isolate as delivery system and different homogenization techniques have yet to be reported.

5. References

Alasalvar, C., & Bolling, B. W. (2015). Review of nut phytochemicals, fat-soluble bioactives, antioxidant components and health effects. *The British Journal of Nutrition*, *113 Suppl* , S68–78.

Bastian, E. D., & Brown, R. J. (1996). Plasmin in milk and dairy products: An update. *International Dairy Journal*, *6*(5), 435–457.

Bauman, D. E., & Lock, A. L. (2006). Conjugated Linoleic Acid: Biosynthesis and Nutritional Significance. In *Advanced Dairy Chemistry Volume 2 Lipids* (pp. 93–136). Boston, MA: Springer US.

Bhattacharya, A., Banu, J., Rahman, M., Causey, J., & Fernandes, G. (2006). Biological effects of conjugated linoleic acids in health and disease. *The Journal of Nutritional Biochemistry*, *17*(12), 789–810.

Bissegger, S. (2007). Production of bioactive soy peptides. Dissertation. University of Waterloo, Canada.

Campbell, W., Drake, M. A., & Larick, D. K. (2003). The impact of fortification with conjugated linoleic acid (CLA) on the quality of fluid milk. *Journal of Dairy Science*, *86*(1), 43–51.

Choi, K.-O., Ryu, J., Kwak, H.-S., & Ko, S. (2010). Spray-dried conjugated linoleic acid encapsulated with Maillard reaction products of whey proteins and maltodextrin. *Food Science and Biotechnology*, *19*(4), 957–965.

Codina-Torrella, I., Guamis, B., Ferragut, V., & Trujillo, A. J. (2016). Potential application of ultra-high pressure homogenization in the physico-chemical stabilization of tiger nuts' milk beverage. *Innovative Food Science & Emerging Technologies (in press)*.

Costa, A. M. M., Nunes, J. C., Lima, B. N. B., Pedrosa, C., Calado, V., Torres, A. G., & Pierucci, A. P. T. R. (2015). Effective stabilization of CLA by microencapsulation in pea protein. *Food Chemistry*, *168*, 157–66.

Cui, L., & Decker, E. A. (2016). Phospholipids in foods: prooxidants or antioxidants? *Journal of the Science of Food and Agriculture*, *96*(1), 18–31.

Datta, N., & Deeth, H. C. (2003). Diagnosing the cause of proteolysis in UHT milk. *LWT - Food Science and Technology*, *36*(2), 173–182.

EFSA Panel on Dietetic Products, N. and A. (NDA). (2010). Scientific Opinion on the substantiation of health claims related to conjugated linoleic acid (CLA) isomers and contribution to the maintenance or achievement of a normal body weight (ID 686, 726,

1516, 1518, 2892, 3165), increase in lean body mass (ID 49. *EFSA Journal 2010*, *8*(10).

Enright, E., Patricia Bland, A., Needs, E. C., & Kelly, A. L. (1999). Proteolysis and physicochemical changes in milk on storage as affected by UHT treatment, plasmin activity and KIO3 addition. *International Dairy Journal*, *9*(9), 581–591.

Fenaille, F., Mottier, P., Turesky, R. J., Ali, S., & Guy, P. A. (2001). Comparison of analytical techniques to quantify malondialdehyde in milk powders. *Journal of Chromatography. A*, *921*(2), 237–45.

Fernandez-Avila, C., Arranz, E., Guri, A., Trujillo, A. J., & Corredig, M. (2015). Vegetable protein isolate-stabilized emulsions for enhanced delivery of conjugated linoleic acid in Caco-2 cells. *Food Hydrocolloids*, *55*, 144–154.

Fernandez-Avila, C., Trujillo, A.J. "Ultra-High Pressure Homogenization enhances physical, microbiological and oxidative stability of emulsions containing conjugated linoleic acid*",* oral communication presented at *2nd UK Hydrocolloids Symposium*, Birmingham (UK), 10th September (2015).

Fernandez-Avila, C., & Trujillo, A. J. (2016). Ultra-High Pressure Homogenization improves oxidative stability and interfacial properties of soy protein isolate-stabilized emulsions. *Food Chemistry, 75*, 357–366*.*

Gao, Z.-M., Zhu, L.-P., Yang, X.-Q., He, X.-T., Wang, J.-M., Guo, J., … Yin, S.-W. (2014). Soy lipophilic protein nanoparticles as a novel delivery vehicle for conjugated linoleic acid. *Food & Function*, *5*(6), 1286–93.

Gunstone, F. D., & Martini, S. (2010). Chemical and physical deterioration of bulk oils and shortenings, spreads and frying oils. In L. H. Skibsted, J. Risbo, & M. L. Andersen (Eds.), *Chemical Deterioration and Physical Instability of Food and Beverages (pp. 413–438)*. UK: Woodhead Publishing Limited.

He, H., Hong, Y., Gu, Z., Liu, G., Cheng, L., & Li, Z. (2016). Improved stability and controlled release of CLA with spray-dried microcapsules of OSA-modified starch and xanthan gum. *Carbohydrate Polymers*, *147*, 243–250.

Hebishy, E., Buffa, M., Guamis, B., Blasco-Moreno, A., & Trujillo, A. J. (2015). Physical and oxidative stability of whey protein oil-in-water emulsions produced by conventional and ultra high-pressure homogenization: Effects of pressure and protein concentration on emulsion characteristics. *Innovative Food Science and Emerging Technologies*, *32*, 79–90.

Hedegaard, R. V., Kristensen, D., Nielsen, J. H., Frøst, M. B., Østdal, H., Hermansen, J. E., … Skibsted, L. H. (2006). Comparison of Descriptive Sensory Analysis and Chemical Analysis for Oxidative Changes in Milk. *Journal of Dairy Science*, *89*(2), 495–504.

Heo, W., Kim, J. H., Pan, J. H., & Kim, Y. J. (2016). Lecithin-Based Nano-emulsification Improves the Bioavailability of Conjugated Linoleic Acid. *Journal of Agricultural and*

Food Chemistry, *64*(6), 1355–1360.

Hu, M., Julian McClements, D., & Decker, E. A. (2004). Impact of chelators on the oxidative stability of whey protein isolate-stabilized oil-in-water emulsions containing ω-3 fatty acids. *Food Chemistry*, *88*(1), 57–62.

IDF 21:2010. Milk, cream and evaporated milk – Determination of total solids content (Reference method). IDF Standard 21:2010/ISO 6731. International Dairy Federation, Brussels, Belgium.

IDF 226:2008. Milk – Determination of fat content. IDF Standard 226:2008/ISO 2446:2008. International Dairy Federation, Brussels, Belgium.

IDF 185:2002. Milk and milk products – Determination of nitrogen content. IDF Standard 185:2002/ISO 14891. International Dairy Federation, Brussels, Belgium.

IDF 99-2:2009. Milk and milk products - Sensory analysis - Part 2: Recommended methods for sensory evaluation. IDF Standard 99-2:2009/ISO 22935-2:2009. International Dairy Federation, Brussels, Belgium.

Innocente, N., Marchesini, G., & Biasutti, M. (2014). Effect of high-pressure homogenisation on the retention of selected aroma compounds in model dairy emulsions. *International Journal of Food Science & Technology*, *49*(9), 1992–2000.

Jimenez, M., Garcia, H. S., & Beristain, C. I. (2008). Sensory evaluation of dairy products supplemented with microencapsulated conjugated linoleic acid (CLA). *LWT - Food Science and Technology*, *41*(6), 1047–1052.

Kim, D., Park, J.-H., Kweon, D.-J., & Han, G. D. (2013). Bioavailability of nanoemulsified conjugated linoleic acid for an antiobesity effect. *International Journal of Nanomedicine*, *8*, 451–9.

Kim, J. H., Kim, Y., Kim, Y. J., & Park, Y. (2016). Conjugated Linoleic Acid: Potential Health Benefits as a Functional Food Ingredient. *Annual Review of Food Science and Technology*, *7*, 221–44.

Kinsella, J. E. (1979). Functional properties of soy proteins. *Journal of the American Oil Chemists' Society*, *56*(3), 242–258.

Koba, K., & Yanagita, T. (2013). Health benefits of conjugated linoleic acid (CLA). *Obesity Research & Clinical Practice*. doi:10.1016/j.orcp.2013.10.001

McClements, D., & Decker, E. (2000). Lipid Oxidation in Oil-in-Water Emulsions: Impact of Molecular Environment on Chemical Reactions in Heterogeneous Food Systems. *Journal of Food Science*, *65*(8), 1270–1282.

McClements, D. J. (2005). *Food Emulsions: Principles, Practice, And Techniques* (2nd ed.). Boca Raton, FL: CRC Press.

Nasrabadi, M. N., Goli, S. A. H., & Nasirpour, A. (2015). Stability assessment of conjugated linoleic acid (CLA) oil-in-water beverage emulsion formulated with Acacia and xanthan gums. *Food Chemistry*, *199*, 258–264.

Pereda, J. (2009). Utilización de la Ultra Alta Presión por Homogenización como alternativa al tratamiento de pasteurización para la obtención de leche de consumo. (Thesis) Bellaterra: Universitat Autònoma de Barcelona.

Pereda, J., Ferragut, V., Buffa, M., Guamis, B., & Trujillo, A. J. (2008). Proteolysis of ultra-high pressure homogenised treated milk during refrigerated storage. *Food Chemistry*, *111*(3), 696–702.

Pereda, J., Jaramillo, D. P., Quevedo, J. M., Ferragut, V., Guamis, B., & Trujillo, A. J. (2008). Characterization of volatile compounds in ultra-high-pressure homogenized milk. *International Dairy Journal*, *18*(8), 826–834.

Poliseli-Scopel, F. H. (2012). Study of the Ultra High Pressure Homogenization (UHPH) technology for producing high quality soymilk. (Thesis) Bellaterra: Universitat Autònoma de Barcelona.

Poliseli-Scopel, F. H., Hernández-Herrero, M., Guamis, B., & Ferragut, V. (2012). Comparison of ultra high pressure homogenization and conventional thermal treatments on the microbiological, physical and chemical quality of soymilk. *LWT - Food Science and Technology*, *46*(1), 42–48.

Rodríguez-Alcalá, L. M., Alonso, L., & Fontecha, J. (2014). Stability of fatty acid composition after thermal, high pressure, and microwave processing of cow milk as affected by polyunsaturated fatty acid concentration. *Journal of Dairy Science*, *97*(12), 7307–7315.

Rodríguez-Alcalá, L. M., Harte, F., & Fontecha, J. (2009). Fatty acid profile and CLA isomers content of cow, ewe and goat milks processed by high pressure homogenization. *Innovative Food Science & Emerging Technologies*, *10*(1), 32–36.

Santos, M. V, Ma, Y., Caplan, Z., & Barbano, D. M. (2003). Sensory threshold of off-flavors caused by proteolysis and lipolysis in milk. *Journal of Dairy Science*, *86*(5), 1601–1607.

Semeniuc, C. A., Mandrioli, M., Rodriguez-Estrada, M. T., Muste, S., & Lercker, G. (2016). Thiobarbituric acid reactive substances in flavored phytosterol-enriched drinking yogurts during storage: formation and matrix interferences. *European Food Research and Technology*, *242*(3), 431–439.

Sikand, G., Kris-Etherton, P., & Boulos, N. M. (2015). Impact of functional foods on prevention of cardiovascular disease and diabetes. *Current Cardiology Reports*, *17*(6), 39.

Sørensen, G., & Jørgensen, S. S. (1996). A critical examination of some experimental variables in the 2-thiobarbituric acid (TBA) test for lipid oxidation in meat products. *Zeitschrift Für Lebensmittel-Untersuchung Und -Forschung*, *202*(3), 205–210.

Valencia-Flores, D. C., Hernández-Herrero, M., Guamis, B., & Ferragut, V. (2013). Comparing the effects of ultra-high-pressure homogenization and conventional thermal treatments on the microbiological, physical, and chemical quality of almond beverages. *Journal of Food Science*, *78*(2), E199–205.

Yettella, R. R., Henbest, B., & Proctor, A. (2011). Effect of antioxidants on soy oil conjugated linoleic acid production and its oxidative stability. *Journal of Agricultural and Food Chemistry*, *59*(13), 7377–7384.

CHAPTER 8: General discussion

General discussion

1. Preliminary analysis and results

In this section, a brief discussion of the general effects produced by UHPH in comparison to CH is given for all O/W emulsions studied (emulsions without CLA, CLA-emulsions and the final milk-based products which have the CLA-emulsions incorporated).

Initially, the implementation of different analytical techniques was carried out. The modification of TBARS method was applied in order to improve time analysis (**Experiment 2**), as the incubation temperature after TBA addition was applied at 95 °C during 10 min. Also, differential scanning calorimetry (DSC) method for denaturation analysis of soy protein isolate (SPI) was performed and no conclusive results were obtained (data not shown). Additionally, different methylation procedures for gas chromatography (GC) analysis of CLA were applied, in order to choose the most suitable technique avoiding a severe isomerization of c9,t11-CLA and t9,c11-CLA.

In order to decide the % of ingredients in emulsion formulations (**Experiments 1 and 2**), an experiment was developed consisting in the production of emulsions with different contents of soy protein isolate (SPI, 2 and 4%) and soybean oil (10 and 20%) using SPI heated (95 °C, 15 min) and non-heated.

1.1. Optimization of the methylation procedure and GC analysis of CLA

1.1.1. Statement of the problem

Few direct methylation methods, which do not require previous lipid extraction and shorten analysis time, have been successfully developed and applied for the analysis of fatty acids and CLA analysis (Bondia-Pons, Moltó-Puigmartí, Castellote, & López-Sabater, 2007; Moltó-Puigmartí, Castellote, & López-Sabater, 2007). Optimization of the methylation procedure requires control of the reaction temperature and time as well as control of the reagents themselves in order to achieve complete methylation of CLA isomers and minimize isomerization and formation of methoxy artifacts (Moltó-Puigmartí et al., 2007). The GC techniques with mass spectrometry (MS) and silver-ion

HPLC (Ag+-HPLC), normally using highly polar cyanosilicone capillary columns such as CP-Sil 88 (100 m × 0.25 mm × 0.20 µm), are currently the most popular tools for elucidating the CLA-isomer profile and are mandatory for a complete analysis of all CLA isomers (Kramer et al., 2004). The CP-Sil 88 is based on a stabilized, highly substituted cyanopropyl siloxane phase and due to its highly polar properties is able to effectively separate on small structural differences of many positional fatty acid methyl esters (FAME) isomers (Roach, Mossoba, Yurawecz, & Kramer, 2002). The aim of this work was to caracterize and quantify the two most important bioactive isomers from CLA (c9,t11-CLA and t10,c12-CLA) and its degradation/isomerization due to methylation procedure so CG-FID characterization appeared to be a suitable technique.

1.1.2. Experimental design

For testing the most suitable methylation procedure in this thesis, four alkali plus acid-catalyzed methods were followed. Firstly, the method of Moltó-Puigmartí et al. (2007) was carried out and some modifications were tested: a decrease on the volumes for saponification $NaOCH_3$/MeOH (500 µL) and esterification (750 µL of BF_3) steps was taken. Secondly, the method of Enke et al. (2011) was carried out and a decrease on the temperature from 100 °C to 80 °C was tested. Finally, extraction procedure was carried out by hexane in all methods. In order to test the method which minimized most the degradation/isomerization of CLA isomers, aliquotes of Tonalin® containing c9,t11- and t10,c12-CLA (50:50) were skipped (2 µg/µL) to a sample of soybean oil, into culture tubes containing 10µL of the internal standard solution (C11:0, 1 µg/µL). Reagents and standards are previously designated in **Experiments 3 and 4.** The linoleic acid methyl ester isomer mix contained 75-90% of CLA (c9,t11-CLA and t9,c11-CLA: 42%; t10,c12-CLA: 44%; c10,c12-CLA: 10%; and t9,t11-CLA: 5%). FAME were separated on a CP-Sil 88 capillary column (100 m × 0.25 mm × 0.20 µm), by GC-FID (**Experiments 3 and 4**). Operating conditions were followed as previously specified in **Experiments 3 and 4**. GC peaks were identified by comparing the retention times of samples FAME with those of the standard FAME mix and linoleic acid methyl ester isomer mix. The c9,t11- and t10,c12-CLA isomers were identified using Tonalin® and confirmed by spiking the soybean oil with them.

1.1.3. Results

Figure 1 shows the identification of peaks in a GC-FID chromatogram of a pure standard of CLA in a CP-Sil 88 column of 100 m.

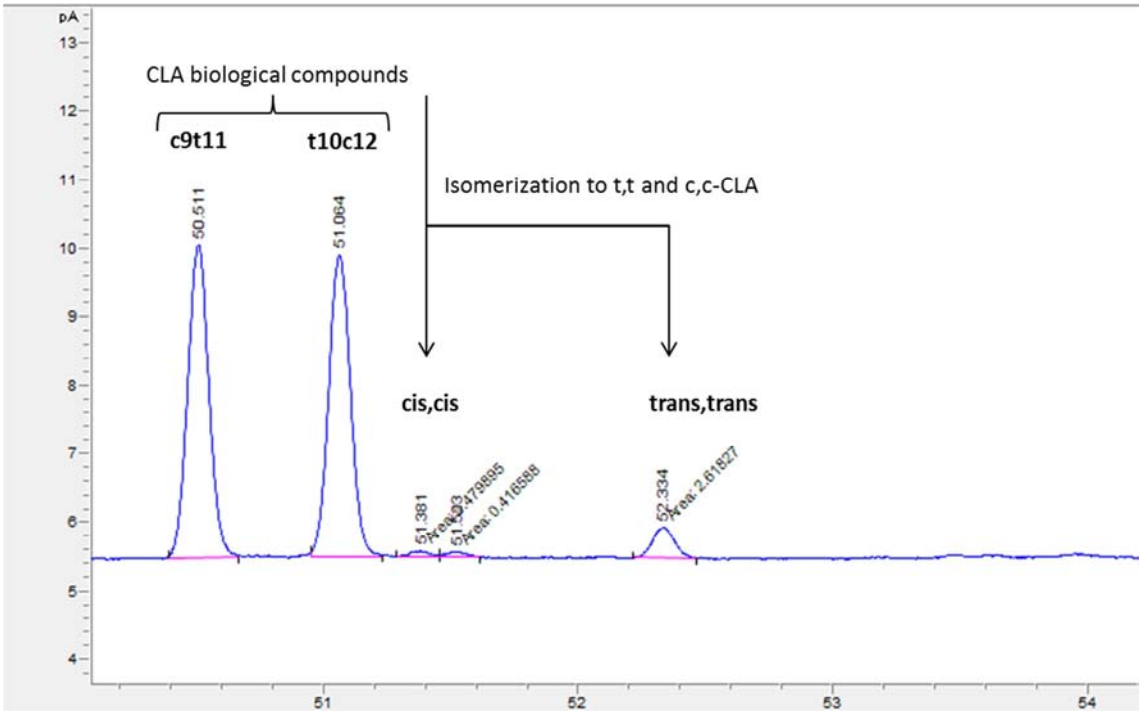

Figure 1. CLA peaks identification of a pure CLA standard (chromatogram from **Experiment 3**) and their isomers quantified.

The most appropriate methylation method to determine c9,t11 and t10,c12-CLA was the one which used 2.5 mL of $NaOCH_3$/MeOH + BF_3 at 80 °C during 10 and 3 min, respectively. This method has shown the best abundance of those CLA isomers as well as less isomerization to c10,c12-CLA and t9,t11-CLA (Table 1).

Table 1. Percentage of c9,t11-CLA, t10,c12-CLA and their isomerization to t9,t11-CLA, c10,c12-CLA obtained under different methylation in soybean oil supplemented with Tonalin®.

Methylating agent	**$NaOCH_3$/MeOH + BF_3**		**NaOH/MeOH + BF_3**	
Conditions	80 °C (10 min + 3 min) and 2.5 mL $NaOCH_3$/MeOH and BF_3	80 °C (10 min + 3 min) and 500 µL $NaOCH_3$/MeOH and 750 µL BF_3	100 °C (5 min + 5 min) and 2 mL NaOH and BF_3	80 °C (5 min + 5 min) and 2 mL NaOH and BF_3
c9,t11-CLA	33.03	34.80	36.36	38.20
t10,c12-CLA	53.21	46.40	40.91	45.05
c10,c12-CLA	n.d.	n.d.	n.d.	n.d.
t9,t11-CLA	13.76	18.79	22.73	16.67

According to Moltó-Puigmartí et al. (2007), similar % of isomerization of the c9,t11-CLA (5,27%) and t10,c12-CLA (8,49%) isomers were obtained. Thus, it was considered that those percentages were assumable in order to achieve a complete methylation of fatty acids in biological samples, such as the permeates from Caco-2 cells, because it is well known that at 80 °C, fatty acids are completely converted into their FAME. It was also observed that c9,t11-CLA isomerized to t10,c12-CLA isomer. The greater the incubation temperature comparing methods of methylation with NaOH, the greater the amount of t9,t11-isomers and c9,t11/t10,c12 isomerization produced. Also, it is important to methylate with an excess of reagents as it can be seen comparing methods of $NaOCH_3$. After the most suitable method was chosen, other aspects like limits of detection (LOD) and quantification (LOQ) were performed in **Experiments 3 and 4**.

1.2. Formulation of SPI emulsions

In order to choose the % of protein, a production of emulsions with different contents of SPI (2 and 4%) and a fixed % of soybean oil (20%) was performed. The results of particle size are presented in Table 2.

Table 2. Particle size ($d_{4.3}$ and $d_{3.2}$ values) of UHPH emulsions (100, 200 and 300 MPa) containing 20% of soybean oil and stabilized with 2 or 4% of SPI.

Pressure (MPa)	$d_{3.2}$		$d_{4.3}$	
	2% SPI	4% SPI	2% SPI	4% SPI
100	0.61 ± 0.04	0.42 ± 0.00	3.09 ± 0.15	0.98 ± 0.00
200	0.52 ± 0.00	0.30 ± 0.00	2.20 ± 0.19	1.27 ± 0.05
300	0.37 ± 0.01	0.28 ± 0.01	1.84 ± 0.77	1.07 ± 0.37

Average size diameters ($d_{3.2}$) of UHPH emulsions stabilized with 2% SPI were about 2-fold larger than those UHPH emulsions stabilized with 4% SPI. Such a large particle size is due to the fact that a 2% SPI was insufficient to stabilize the great specific surface area generated during emulsification in UHPH (Table 2). Moreover, an increase in $d_{4.3}$ indicates flocculation phenomenon, which was especially observed in emulsions stabilized with 2% SPI. Thus, a 4% of protein was chosen for formulation of CH (15 MPa) and UHPH (100, 200 and 300 MPa) emulsions (**Experiments 1 and 2**). This percentage (4%) was also chosen for emulsions containing 10% soybean oil

(**Experiments 1 and 2**), as an excess of proteins in the aqueous phase could decrease lipid oxidation, by interacting with metal ions, or by scavenging free-radicals (Berton-Carabin, Ropers, & Genot, 2014). Also, the same formulations, that appeared to be more physically and oxidatively stable (4% of SPI and 20% soybean oil), were compared to their homologous conventional emulsions (55 MPa) with pea protein isolate (PPI) including 6% CLA (**Experiment 3**). In addition, 1 or 5 passes of homogenization were tested, so the so-called emulsions are SPI1, SPI5, PPI1 and PPI5. It has been previously reported that 0.5-2.0% highly soluble soy protein fractions can stabilize emulsions containing 10-20% oil (Floury, Desrumaux, & Legrand, 2002; Keerati-u-rai & Corredig, 2009). However, the commercial SPI used in this thesis exhibited lower solubility (**Experiments 1-5**). Furthermore, this higher protein content in comparison to the literature was needed to stabilize the SPI5 emulsion, which was confirmed with the better attachment of α' subunit of conglycinin and A1, A2, A4 subunits of glycinin in interfaces (cream phase), as they showed to be at a higher ratio compared to the emulsions ($P < 0.05$) (**Experiment 3**).

2. Effect of Ultra-High Pressure Homogenization (UHPH) compared to conventional homogenization (CH) on stability of emulsions

As it has been described in the *Literature Review* of this thesis, during emulsification, oil droplets break-up in smaller ones and its higher specific area covers with protein particles. Homogenization treatment is responsible on the amount of material covering oil droplets, their particle size, as well as stability of emulsions during storage.

CH emulsions, at both oil concentrations (10 and 20% soybean oil), exhibited the largest particle size (500 nm) followed by emulsions stabilized by UHPH at 100, 200 and 300 MPa (370-230 nm) (**Experiment 1**). When conventionally treated (55 MPa) emulsions (20% oil) stabilized by SPI and PPI (4%) contained 6% of CLA and one homogenization pass was performed (SPI1 and PPI1), particle size was around 8 and 9 µm, respectively (**Experiment 3**). In this study, when 5 passes were applied (SPI5 and PPI5), a similar particle size (around 215 nm) than UHPH CLA-emulsions was observed (260 nm) (**Experiment 4**). Nevertheless, the same CLA-emulsions containing SPI and treated by low-homogenizing pressure (15 MPa) resulted in smaller particle size (410

nm) than those treated at 55 MPa at one pass of homogenization (**Experiment 3**). This might be owing to differences in the geometries and material of the valves (Donsì, Sessa, & Ferrari, 2012). All emulsions studied (emulsions without CLA, CLA-emulsions and the final milk-based products which have the CLA-emulsions incorporated) exhibited bimodal distribution (**Experiments 1-5**), although increasing the homogenization pressure in UHPH emulsions almost shifted the bimodal curve to monomodal with a narrower size distribution, compared to CH emulsions. A significant increase in polydispersity of emulsions was observed when the pressure increased from 100-200 to 300 MPa at both oil concentrations (**Experiment 1**), as this was also confirmed by the increase in $d_{4.3}$ values (**Experiment 2**), which is an indicator of flocculation. It has been suggested that protein aggregates are produced by partial or total denaturation of SPI owing to the increase of the temperature (≥100 °C) at the exit of the UHPH valve when 300 MPa is applied (Floury et al., 2002). The resulting protein aggregates could have induced flocculation leading to large tails of flocs, as it was only confirmed by their presence in 300 MPa-treated emulsions with 10% soybean oil and by their absence after changing the environmental conditions with 2% SDS (**Experiment 1**). Protein denaturation might have led to a decrease in consistency of emulsions, as the highest consistency coefficient value was observed in emulsions treated at 100 MPa and were gradually reduced up to 300 MPa (**Experiment 1**). However, this was not applicable when comparing CH and UHPH CLA-emulsions for partial denaturation of SPI (**Experiment 3**), as the smaller particle size play an important role increasing viscosity (Hebishy, 2013).

2.1. Physical stability

Physical stability of emulsions in this thesis has been determined by differences in particle size over storage and Turbiscan analysis. After 10 days, among conventionally treated (55 MPa) CLA-emulsions from **Experiment 3** (SPI1, SPI5, PPI1 and PPI5), only the SPI1 emulsion showed an increase in particle size ($P < 0.05$). A physically stable emulsion is needed to produce an effective encapsulation of lipids (McClements & Li, 2010); as an example, the SPI5 showed the smallest increase in $d_{4.3}$ after 10 days and it

has been related to the higher % loading of c9t11-CLA and t10c12-CLA isomers (both 71 ± 18%).

However, Turbiscan seems a better tool for physical destabilization analysis, as it can analyse different phenomenon at the same time (Herrera, 2012; Juliano et al., 2011). Turbiscan backscattering profiles from **Experiment 1** showed that UHPH emulsions were more physically stable than CH emulsions as a result of the smaller particle size. Not only the homogenization process has a great influence on physical stability over time, but also the viscosity. In general, viscosity increases when increasing droplet concentration (Sun & Gunasekaran, 2009), and this was supported by the Newtonian behaviour of emulsions containing 10% oil compared to the pseudoplastic behaviour showed at 20% oil (**Experiment 1**). Thus, oil content and viscosity strongly affected the physical stability of emulsions against creaming, since the more stable emulsions exhibited the highest shear-thinning behaviour, which were the UHPH emulsions containing 20% (v/v) of soybean oil and homogenized at 100 and 200 MPa. Also, the higher emulsifying activity index (EAI) of SPI and the adsorbed protein percentages at the interfaces of oil droplets enhanced a better physical stability of those emulsions (**Experiment 2**). Moreover, in that study, the EAI emerged as a reliable predictor of the SPC technique, which requires much more time consuming.

When conventionally treated (15 MPa) emulsions (20% oil) stabilized by SPI (4%) contained CLA (6% in a FFA form), a gel-like behaviour was observed (**Experiment 4**). A possible explanation on the increase of viscosity in CLA-emulsions compared to the previous emulsions (**Experiment 1**) could be related with the location of CLA in the emulsion, as FFAs are surface-active preferentially locate at the oil droplet surface (Berton-Carabin et al., 2014). When evaluating profile backscatering between CLA-emulsions from **Experiment 4**, no differences were found, as they were more physically stable than their counterpart emulsions without CLA from **Experiment 1**, because higher consistency values increase resistance against destabilization phenomenon (Hebishy, Buffa, Guamis, Blasco-Moreno, & Trujillo, 2015). For this reason TSI was used in **Experiment 4**, as it sums all the variations detected in the samples in terms of size and/or concentration, which appeared to be a more sensitive tool. TSI values of both emulsions treated by CH (non-pasteurized and pasteurized)

were not high enough to play a stabilising role, compared to UHPH technology which significantly decreased the kinetics of any destabilisation phenomenon. This kinetics was suitable until 3 months, where thixotropic behaviour occurred on day 40, as CH-treated CLA-emulsions exhibited, and subconsequently more weak colloid interactions (McClements, 2005).

In a previous study it was reported that heating a highly soluble SPI before homogenization resulted in all the protein subunits to be present at the interface in an aggregated form (Keerati-u-rai & Corredig, 2009). In contrast, our results showed that the heating of SPI (95 °C, 15 min) prior emulsification process did not increase SPC and increased the protein aggregates in the continuous phase, as TEM micrographs showed (**Experiments 1 and 2**). These facts could have led to a greater rate in creaming (**Experiment 1**). In fact, most of the properties for commercially SPI used in this thesis, such as solubility, are similar to heat-treated soy proteins (see *Section 1.4* in the *Literature Review*).

2.2. Oxidative and CLA chemical stability

Oxidative stability in emulsions without CLA and treated by UHPH was improved, when compared to CH (**Experiment 2**). Lipid oxidation decreased with surface protein concentration, as well as with oil-phase volume fraction. This finding supports previous research when oil volume fraction increased from 5 to 40% (Kargar, Spyropoulos, & Norton, 2011; Sun & Gunasekaran, 2009). The excess of protein in the continuous phase in UHPH emulsions treated at 100 MPa (**Experiment 2**) led to depletion flocculation (**Experiment 1**), which accelerates physico-chemical destabilization of emulsions (Berton-Carabin et al., 2014). It can thus be suggested that, taking into account other factors of the emulsion, such as smaller specific surface area (**Experiment 2**), partial denaturation could also play a positive role on lipid oxidation stability, as a previous study confirmed that the solubility of 2% glycinin dispersions remained preserved at relatively moderate homogenization pressure (<250 MPa) (Floury et al., 2002). In fact, heating the SPI (95 °C, 15 min) before emulsification of CH emulsion containing 10% soybean oil improved its oxidative stability, probably due to the exposure of more hydrophobic groups, as reduced sulfhydryl groups act as free

radical scavengers (Tong, Sasaki, McClements, & Decker, 2000). It is important to note that results of enhanced oxidative stability of UHPH emulsions (100 and 200 MPa) with 20% soybean oil (**Experiment 2**) were clearly in line with the best physical stability among all emulsions (**Experiment 1**).

The findings of emulsions (55 MPa) that contained 6% CLA from **Experiment 3** (SPI1, SPI5, PPI1 and PPI5) indicated that the oxidative stability of the CLA-soybean oil emulsions was preserved in the emulsions compared to free oil control after 10 days ($P < 0.05$). Also, the chemical stability of CLA in emulsions stored at 4 °C during 10 days showed no significant differences ($P > 0.05$).

When adding 6% CLA in a FFA form in the formulations from **Experiments 1 and 2** that exhibit the best physical and oxidative stability (UHPH emulsion treated at 200 MPa and CH emulsions containing 20% oil), primary oxidation products decreased in half (hydroperoxides; A = 510 nm), and secondary oxidation products enlarged three times (TBARS; A = 532 nm) (**Experiment 4**). However, those TBARS in the UHPH emulsion did not develop further over storage (5 months in refrigeration), in comparison to lipid hydroperoxides (8 mmol/kg oil). This value is acceptable when emulsions are diluted 10 folds in a beverage emulsion system and still remains under harsh conditions of a sanitation system, such as UHT, which it will be further discussed in the *Section 3* of this general discussion.

2.3. Microbial stability

Microbial analysis for CH- and UHPH-treated CLA-emulsions was evaluated over time in refrigeration (4 °C) by using psychrotrophic bacteria as microbial indicator of emulsion spoiling, although other microbial groups (*Enterobacteriaceae* and total spores) were also evaluated on fresh emulsions (**Experiment 4**). The initial bacterial load of coarse CLA-emulsions was 3.6 ± 0.5 log cfu/mL. A reduction of 0.9 ± 0.5 log cfu/mL bacteria count was achieved with the CH treatment (15 MPa). For another batch of CH emulsion, a HTST pasteurization was applied, and it presented a shelf life between 7 (3.6 ± 1.1 log cfu/mL) and 10 days (>6 cfu/mL). Furthermore, in UHPH samples bacterial growth appeared to be under the limits of detection, for which reason

sterility test was carried out. Results showed that UHPH CLA-emulsions were sterile. For all treatments at day 1, spores and *Enterobacteriaceae* counts were below the detection level. The moderate temperature (83 °C) at the exit of the valve in UHPH treatment along with the pressure applied (200 MPa) seems to be effective conditions to inactivate the microorganisms present in the emulsion. Other factors such as matrix characteristics of the existing emulsions (CLA presence, fat content and viscosity) have also been suggested to influence on microbial inactivation (Clément, Tremblay, Lange, Thibodeau, & Belhumeur, 2007; Roig-Sagués et al., 2009).

2.4. *In vitro* digestion

During *in vitro* digestion, no significant differences were seen for t9c11 and c10t12-CLA and their isomerization products (*cis,cis* and *trans,trans*-CLA) between different homogenization and formulation treatments (**Experiments 3 and 4**). However, matrix interactions in emulsions affected the CLA release, as the free CLA (non-emulsified control) showed a tendency to be oxidized. This could be explained by the small free fatty acid release percentages of emulsions in **Experiment 3**, as well as the high amount of protein adsorbed at the interface in similar emulsions without CLA (**Experiment 2**), which may decrease the extent of lipolysis. These results are in concordance with other studies in which lipid digestion is reduced when matrices are more viscous (Li, Hu, Du, Xiao, & McClements, 2011; Li & McClements, 2011). In turn, the reduction of the lipolysis rate can affect the release of bioactives and induce satiety (Guri & Corredig, 2014).

2.5. Caco-2 cells uptake

Similar percentages of CLA bioaccessibility (measured in the cell) and bioavailability (measured in the basolateral phase) in Caco-2 cells were found for all emulsion treatments (**Experiments 3 and 4**). These results are in line with those of Heo, Kim, Pan, & Kim (2016), who compared lecithin-nanoemulsions containing both CLA forms (CLA-triclycerides; TG, and CLA-FFA) and no differences in bioavailability were observed. Their emulsions also exhibited different particle size: 70 and 230 nm, respectively. However they directly applied the emulsions to the Caco-2 cells, instead

of submitting the emulsions through in vitro digestions for further application of the digestates. Results from **Experiment 4** are in line with those from **Experiment 3** owing to the highest concentration recovered in the CLA-control digestates in the cell. However, in the basolateral phase, the main CLA bioactive compounds in **Experiment 4** were not delivered to the consumer in the intended state (c9,t11-CLA and t9,c11-CLA), as more isomerization to *trans,trans*-CLA was produced in comparison to conventional CLA-emulsions from **Experiment 3.** Nevertheless, time of incubation and dilution of digestates were different for both experiments: 4 and 2 h; and 1:40 and 1:80 (for **Experiments 3 and 4**, respectively). Possibly, more isomerization was produced when digestates of emulsions were more diluted (**Experiment 4**). Changes on such conditions for **Experiment 4** were applied owing to % of cell bioavailability (Table 3). Comparing to **Experiment 3** (4 h of incubation time and dilution of 1:40), digestates of emulsions were diluted at 1:80 and less incubation time was applied, as a consequence of poor **%** cell viability when CLA-emulsions were treated by UHPH (200 MPa).

Table 3. Sulforhodamine B assay (SRB) for cytotoxicity screening. A >80% cell viability is considered for further analysis of Caco-2 cells uptake at corresponding dilution and incubation time. Results are shown for both productions analysed.

Treatment	Dilution and incubation time					
	1:80 – 2 h		1:60 – 2 h		1:40 – 2 h	
	Viability	Effect	Viability	Effect	Viability	Effect
UHPH-1	100.0	0.0	72.6	27.4	20.3	79.7
UHPH-2	105.7	-5.7	89.2	10.8	21.1	78.9
CH-HTST-1	NA	NA	97.8	2.2	92.4	7.6
CH-HTST-2	NA	NA	106.1	-6.1	87.2	12.8
CH-1	NA	NA	103.6	-3.6	96.8	3.2
CH-2	NA	NA	101.3	-1.3	111.5	-11.5
CLA-1	NA	NA	87.9	12.1	49.8	50.2
CLA-2	NA	NA	95.6	4.4	94.1	5.9

* NA= not applicable.

In order to discard the presence of inhibitors in such emulsions due to UHPH effect, sterility test was carried at dilutions of -3 of emulsion and no existing colonies were found. Also, and according to the manufacturer, trypsin inhibitors during SPI processing were inactivated. For this reason, a possible effect of the gel-like matrix of the CH CLA-emulsion compared to its counterpart UHPH may be responsible of the higher **%** cell viability values (>80%). As previously stated in the *Literature Review*, gels

reduce *in vitro* digestion (Li et al., 2011; Li & McClements, 2011). In fact, a higher rate in the digestion leads to higher toxic compounds to cells, which increases cytotoxicity (Arranz, Corredig, & Guri, 2016).

3. Effect of UHPH compared to CH on stability of CLA-emulsions incorporated in a UHT milk-based product

CLA concentrations present in the developed CH-P and UHPH-P products (UHT milk-based products containing CH and UHPH CLA-emulsions) are in the range of what is necessary to deliver a sufficient amount to be nutritionally significant: ~3 g/day (Kim, Kim, Kim, & Park, 2016). As CLA-emulsions were diluted 10 folds in the skim milk, the final UHT milk-based product contained 0.6% CLA and 1 brick (200 mL) would deliver 1.2 g CLA/serving.

However, it is of vital importance to assess that CLA bioactives are delivered to the consumer in the intended state and concentration to accomplish the claimed health effect. In **Experiments 3 and 4**, stability of CLA and oxidative stability of emulsions during storage was confirmed. For this reason, they resulted to be potential delivery systems to be incorporated in a UHT milk-based product, and UHPH CLA-emulsions showed promising results owing to physical and microbial stability. Also, as CLA-FFA is more prone to oxidation than its triglyceride form, it would rather distinguish possible differences in preservation between CH and UHPH before UHT. Before mixing CLA-emulsions with the skim milk for the UHT treatment, all CLA-emulsions exhibited bimodal distribution (**Experiment 5**). However, UHT treatment in UHPH-P completely shifted the bimodal curve to monomodal with a narrower size distribution, compared to CH-P. This result would have probably occurred owing to shear stress and turbulence occurring through the pipes, even though the homogenization valve of homogeniser of UHT equipment remained open in order to avoid an additional homogenization to UHPH and CH CLA-emulsions during the heat treatment.

3.1. Physical stability

The final products (**Experiment 5**) exhibited visible destabilization phenomena to the human eye (creaming and sedimentation) in comparison to CLA-emulsions (**Experiment 4**), as they were diluted 10 folds. UHPH-P exhibited better colloidal stability compared to CH-P, and this was attributed to the monomodal size distribution of oil droplets (Fig. 2).

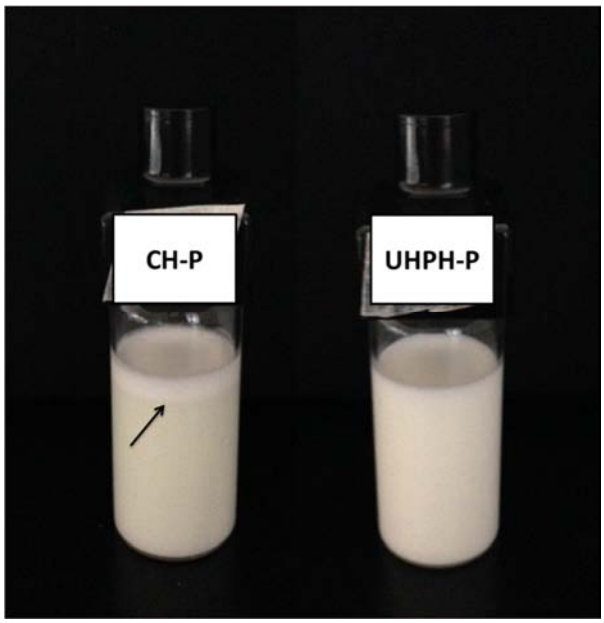

Figure 2. Turbiscan glass vials filled with the final UHT milk-based products from **Experiment 5** (CH-P and UHPH-P) after 5 months of storage at 21 °C.

3.2. Oxidative stability

Primary oxidation products of CH-P and UHPH-P (**Experiment 5**) were expressed per liter of the final product. When comparing the concentration of lipid hydroperoxides (~2 mmol/kg oil) of their emulsions (**Experiment 4**), with those of the final product (~0.4 mmol/L product = 20 mmol/kg oil), lipid hydroperoxides in CH-P and UHPH-P increased 10 folds (**Experiment 5**). An increased oxidation rate may also be due to the decrease in volume oil-phase fraction (10-fold smaller) and subsequently reduced viscosity (**Experiment 2**), as well as the prooxidant activity of CLA-FFA by co-oxydising the triacylglycerol in the bulk oil of the emulsion (Waraho, McClements, & Decker, 2011). Additionally, TBARs content in both final products kept steady during storage,

but the results appeared to be inconsistent due to matrix effects of the milk (Semeniuc, Mandrioli, Rodriguez-Estrada, Muste, & Lercker, 2016). However, UHPH was a suitable technology to preserve CLA in emulsions during storage (**Experiment 4**) and it could be a potential homogenization technique for production of fortified milk with CLA, as previously suggested by Rodríguez-Alcalá, Harte, & Fontecha (2009) in naturally CLA-enriched milks.

3.3. Sensorial analysis

In general, UHPH-P was more accepted by the panellists along storage mainly by its better colloidal stability (**Experiment 5**). After 1 month of storage at 21 °C, a 75% of panellists preferred, which % increased along the 5 months. However, both products were not well-accepted by the sensory panel, mostly due to the impossibility of encapsulating free CLA to reduce its rancidity. Nevertheless, UHPH-P masked off-flavours caused by oxidation.

As previously stated, CLA-FFA form is more susceptible to oxidation than CLA-TAG (Heo et al., 2016). For this reason, it would be interesting to assess the same emulsions studied in this thesis with CLA-TAG. In fact, recent evidence confirms no differences in *in vivo* bioavailability between lecithin-nanoemulsions containing CLA-TAG and CLA-FFA (Heo et al., 2016). It was previously demonstrated that such emulsions with CLA-FFA enhanced the antiobesity effecs in rats (Kim, Park, Kweon, & Han, 2013). Thus, it is hypothesized that the homologous emulsions containing TAG could exert a similar effect, along with sensory characteristics improved, as CLA-TAG is less prone to oxidation than CLA-FFA.

4. References

Arranz, E., Corredig, M., & Guri, A. (2016). Designing food delivery systems: challenges related to the in vitro methods employed to determine the fate of bioactives in the gut. *Food Funct (In press).*

Berton-Carabin, C. C., Ropers, M.-H., & Genot, C. (2014). Lipid Oxidation in Oil-in-Water Emulsions: Involvement of the Interfacial Layer. *Comprehensive Reviews in Food Science and Food Safety*, *13*(5), 945–977.

Bondia-Pons, I., Moltó-Puigmartí, C., Castellote, A. I., & López-Sabater, M. C. (2007). Determination of conjugated linoleic acid in human plasma by fast gas chromatography. *Journal of Chromatography A*, *1157*(1–2), 422–429.

Clément, M., Tremblay, J., Lange, M., Thibodeau, J., & Belhumeur, P. (2007). Whey-derived free fatty acids suppress the germination of Candida albicans in vitro. *FEMS Yeast Research*, *7*(2), 276–85.

Donsì, F., Sessa, M., & Ferrari, G. (2012). Effect of Emulsifier Type and Disruption Chamber Geometry on the Fabrication of Food Nanoemulsions by High Pressure Homogenization. *Industrial & Engineering Chemistry Research*, *51*(22), 7606–7618.

Hebishy, E. H. (2013). 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. Universitat Autònoma de Barcelona. Thesis (Bellaterra): Universitat Autonòma de Barcelona.

Enke, U., Jaudszus, A., Schleussner, E., Seyfarth, L., Jahreis, G., & Kuhnt, K. (2011). Fatty acid distribution of cord and maternal blood in human pregnancy: special focus on individual trans fatty acids and conjugated linoleic acids. *Lipids in Health and Disease*, *10*(1), 247.

Floury, J., Desrumaux, A., & Legrand, J. (2002). Effect of Ultra-high-pressure Homogenization on Structure and on Rheological Properties of Soy Protein-stabilized Emulsions. *Journal of Food Science*, *67*(9), 3388–3395.

Guri, A., & Corredig, M. (2014). Chapter 17 – Dairy Materials as Delivery Tools for Bioactive Components in Dairy Platforms. In *Food Structures, Digestion and Health* (pp. 465–488).

Hebishy, E., Buffa, M., Guamis, B., Blasco-Moreno, A., & Trujillo, A.-J. (2015). Physical and oxidative stability of whey protein oil-in-water emulsions produced by conventional and ultra high-pressure homogenization: Effects of pressure and

protein concentration on emulsion characteristics. *Innovative Food Science & Emerging Technologies 32, 79–90*.

Heo, W., Kim, J. H., Pan, J. H., & Kim, Y. J. (2016). Lecithin-Based Nano-emulsification Improves the Bioavailability of Conjugated Linoleic Acid. *Journal of Agricultural and Food Chemistry*, *64*(6), 1355–1360.

Herrera, M. (2012). *Analytical Techniques for Studying the Physical Properties of Lipid Emulsions*. New York: Springer.

Juliano, P., Kutter, A., Cheng, L. J., Swiergon, P., Mawson, R., & Augustin, M. A. (2011). Enhanced creaming of milk fat globules in milk emulsions by the application of ultrasound and detection by means of optical methods. *Ultrasonics Sonochemistry*, *18*(5), 963–73.

Kargar, M., Spyropoulos, F., & Norton, I. T. (2011). The effect of interfacial microstructure on the lipid oxidation stability of oil-in-water emulsions. *Journal of Colloid and Interface Science*, *357*(2), 527–533.

Keerati-u-rai, M., & Corredig, M. (2009). Heat-induced changes in oil-in-water emulsions stabilized with soy protein isolate. *Food Hydrocolloids*, *23*(8), 2141–2148.

Kim, D., Park, J.-H., Kweon, D.-J., & Han, G. D. (2013). Bioavailability of nanoemulsified conjugated linoleic acid for an antiobesity effect. *International Journal of Nanomedicine*, *8*, 451–9.

Kim, J. H., Kim, Y., Kim, Y. J., & Park, Y. (2016). Conjugated Linoleic Acid: Potential Health Benefits as a Functional Food Ingredient. *Annual Review of Food Science and Technology*, *7*, 221–44.

Kramer, J. K. G., Cruz-Hernandez, C., Deng, Z., Zhou, J., Jahreis, G., & Dugan, M. E. R. (2004). Analysis of conjugated linoleic acid and trans 18:1 isomers in synthetic and animal products. *The American Journal of Clinical Nutrition*, *79*(6), 1137S–1145S.

Li, Y., Hu, M., Du, Y., Xiao, H., & McClements, D. J. (2011). Control of lipase digestibility of emulsified lipids by encapsulation within calcium alginate beads. *Food Hydrocolloids*, *25*(1), 122–130.

Li, Y., & McClements, D. J. (2011). Controlling lipid digestion by encapsulation of protein-stabilized lipid droplets within alginate–chitosan complex coacervates. *Food Hydrocolloids*, *25*(5), 1025–1033.

McClements, D. J. (2005). *Food Emulsions: Principles, Practice, And Techniques*. (2nd ed.). Boca Raton, FL: CRC Press.

McClements, D. J., & Li, Y. (2010). Structured emulsion-based delivery systems: controlling the digestion and release of lipophilic food components. *Advances in Colloid and Interface Science*, *159*(2), 213–28.

Moltó-Puigmartí, C., Castellote, A. I., & López-Sabater, M. C. (2007). Conjugated linoleic acid determination in human milk by fast-gas chromatography. *Analytica Chimica Acta*, *602*(1), 122–130.

Roach, J. A. G., Mossoba, M. M., Yurawecz, M. P., & Kramer, J. K. G. (2002). Chromatographic separation and identification of conjugated linoleic acid isomers. *Chromatography of Fatty Acids*, *465*(1–2), 207–226.

Rodríguez-Alcalá, L. M., Harte, F., & Fontecha, J. (2009). Fatty acid profile and CLA isomers content of cow, ewe and goat milks processed by high pressure homogenization. *Innovative Food Science & Emerging Technologies*, *10*(1), 32–36.

Roig-Sagués, A. X., Velázquez, R. M., Montealegre-Agramont, P., López-Pedemonte, T. J., Briñez-Zambrano, W. J., Guamis-López, B., & Hernandez-Herrero, M. M. (2009). Fat content increases the lethality of ultra-high-pressure homogenization on Listeria monocytogenes in milk. *Journal of Dairy Science*, *92*(11), 5396–402.

Semeniuc, C. A., Mandrioli, M., Rodriguez-Estrada, M. T., Muste, S., & Lercker, G. (2016). Thiobarbituric acid reactive substances in flavored phytosterol-enriched drinking yogurts during storage: formation and matrix interferences. *European Food Research and Technology*, *242*(3), 431–439.

Sun, C., & Gunasekaran, S. (2009). Effects of protein concentration and oil-phase volume fraction on the stability and rheology of menhaden oil-in-water emulsions stabilized by whey protein isolate with xanthan gum. *Food Hydrocolloids*, *23*(1), 165–174.

Tong, L. M., Sasaki, S., McClements, D. J., & Decker, E. A. (2000). Antioxidant Activity of Whey in a Salmon Oil Emulsion. *Journal of Food Science*, *65*(8), 1325–1329.

Waraho, T., McClements, D. J., & Decker, E. A. (2011). Impact of free fatty acid concentration and structure on lipid oxidation in oil-in-water emulsions. *Food Chemistry, 129*(3), 854–9.

CHAPTER 9: Conclusions

Conclusions

1. One of the more significant findings to emerge from this thesis is that Ultra-High Pressure Homogenization (UHPH) could be successfully employed in the food industry as a novel stabilization technique for O/W emulsions. In particular, UHPH (using 100, 200 and 300 MPa) was suitable to produce soy protein isolate-stabilized (4%) submicron emulsions containing soybean oil (10 and 20%) with a high physical stability against clarification in the bottom and creaming in the top, when compared to emulsions obtained by conventional homogenization (CH; using 15 MPa).

2. The best physical stability of UHPH emulsions was strongly dependent on the viscosity (shear-thinning behaviour), the increase in volume oil fraction (from 10% to 20%), the pressure applied at 100 and 200 MPa, the low particle size and polydispersity, the higher emulsifying activity index (EAI) of SPI, the adsorbed protein percentages at the interfaces of oil droplets and the moderate temperatures reached at the exit of the UHPH-valve (<77 °C) which could have led to partial denaturation.

3. The oxidative stability of UHPH emulsions was strongly reliant on the physical stability, as both emulsions treated at 100 and 200 MPa with 20% oil exhibited the best oxidative and physical stability. In the same manner, oxidative stability of those UHPH emulsions was strongly dependent on the same parameters, listed above, that contribute to enhance their physical stability.

4. Heating SPI dispersions (95 °C, 15 min) before CH treatments did not improve physical stability of CH emulsions either containing 10 or 20% soybean oil, due to the fact that the increase of protein aggregates in the continuous phase could have led to flocculation, and consequently increased the creaming kinetics. The same reason has been attributed to the UHPH emulsion treated at 100 MPa containing 10% oil, owing to a lack of greater oil content and specific surface area values, and consequently more unadsorbed protein remained in the

aqueous phase. In contrast, heating SPI dispersions before CH treatments improved oxidative stability of CH emulsions containing 10% oil.

5. O/W CH emulsions (14% soybean oil) tested with different vegetable protein isolates (4% soy or pea) and homogenized at 55 MPa, with 1 or 5 passes, protected CLA (6%) from oxidation and isomerization during storage, and after *in vitro* digestion and uptake in Caco-2 monolayers compared to non-emulsified CLA control.

6. All CH emulsions (20% oil: 6% CLA and 14% soybean oil) homogenized at 55 MPa showed a similar *in vitro* digestibility, and similar results of CLA absorption and transport through Caco-2 monolayers. When applying UHPH technology (200 MPa) to SPI-stabilized emulsion, the main biological CLA isomers (*c*9*t*11-CLA and *t*10*c*12-CLA) were similarly bioavailable, taking into account the differences in dilution ratio of the digestates and the incubation rate of Caco-2 cells.

7. It was also shown that the UHPH treatment (200 MPa) enhanced physical stability of CLA emulsions (20% oil: 6% CLA and 14% soybean oil) due to smaller particle size and a highly homogeneous microstructure, produced sterile emulsions and maintained the oxidative stability of emulsions over 3 months, in comparison to their homologous CH emulsions (15 MPa). In contrast, the shelf life of CH emulsions was about one week, and applying a plus high-temperature short-time pasteurization extended it just one week more.

8. The best physical stability of UHT milk-based products (2% oil) was presented when UHPH emulsions (20% oil: 6% CLA and 14% soybean oil) were incorporated (UHPH-P) in a ratio of 1:10 (emulsion:skim milk), in comparison to the counterpart CH emulsion in the final product (CH-P) during 4 months of storage at 21 °C. The insignificant sedimentation and creaming in UHPH-P was strongly dependent on the low particle size and monomodal size distribution.

9. When sensory characteristics of UHT milk-based products were evaluated, creaming observation in CH-P by panelists was correlated to the analytical

determination during storage. The better physical of UHPH-P stability in comparison to CH-P was a determining factor for a very considerable preference for this product during storage.

10. The oxidative stability of UHPH-P was dependent on the physical stability, as it exhibited the best oxidative and physical stability compared to CH-P.

11. UHPH masked off-flavours caused by CLA oxidation through UHT for UHPH-P; however, both final products (UHPH-P and CH-P) were not well-accepted by panelists owing to the non-encapsulated rancidity flavour.