

Article

# Characterization of Whey Protein Oil-in-Water Emulsions with Different Oil Concentrations Stabilized by Ultra-High Pressure Homogenization

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**Abstract:** In this study, the effect of ultra-high-pressure homogenization (UHPH: 100 or 200 MPa at 25 °C), in comparison to colloid mill (CM: 5000 rpm at 20 °C) and conventional homogenization (CH: 15 MPa at 60 °C), on the stability of oil-in-water emulsions with different oil concentrations (10, 30 or 50 g/100 g) emulsified by whey protein isolate (4 g/100 g) was investigated. Emulsions were characterized for their microstructure, rheological properties, surface protein concentration (SPC), stability to creaming and oxidative stability under light (2000 lux/m<sup>2</sup>). UHPH produced emulsions containing lipid droplets in the sub-micron range (100–200 nm) and with low protein concentrations on droplet surfaces. Droplet size ( $d_{3.2}$ ,  $\mu\text{m}$ ) was increased in CH and UHPH emulsions by increasing the oil concentration. CM emulsions exhibited Newtonian flow behaviour at all oil concentrations studied; however, the rheological behaviour of CH and UHPH emulsions varied from Newtonian flow ( $n \approx 1$ ) to shear-thinning ( $n < 1$ ) and thixotropic behaviour in emulsions containing 50% oil. This was confirmed by the non-significant differences in the  $d_{4.3}$  ( $\mu\text{m}$ ) value between the top and bottom of emulsions in tubes left at room temperature for nine days and also by a low migration velocity measured with a Turbiscan LAB instrument. UHPH emulsions showed significantly lower oxidation rates during 10 days storage in comparison to CM and CH emulsions as confirmed by hydroperoxides and thiobarbituric acid-reactive substances (TBARS). UHPH emulsions treated at 100 MPa were less oxidized than those treated at 200 MPa. The results from this study suggest that UHPH treatment generates emulsions that have a higher stability to creaming and lipid oxidation compared to colloid mill and conventional treatments.

**Keywords:** ultra-high pressure homogenization (UHPH); whey protein; submicron emulsions; physical and oxidative stabilities

## 1. Introduction

Oils can become oxidized during processing, distribution and handling, particularly if they are highly unsaturated [1,2]. Lipid oxidation in formulated foods is therefore a major concern to food producers. Modern consumers have a preference for 'clean label' food products with little to no additives present, e.g., synthetic antioxidants and emulsifiers; thus, it is important to develop new processing technologies that reduce the need of using stabilizing additives.

Oil-in-water (O/W) submicron/nano-emulsions have been studied in order to encapsulate lipophilic molecules in the food, pharmaceutical and cosmetic sectors [3,4]. Emulsion stability depends on a range of complex mechanisms, including flocculation, coalescence, Ostwald ripening and phase separation. However, it is well known that one of the key factors determining stability is the average size of fat globules and their size distribution [5]. Submicron (nano) emulsions are systems containing droplets with diameters below 1  $\mu\text{m}$  and are known to have a high physical stability [6]. Producing emulsions with small droplet sizes and narrow size distributions can be achieved through the application of a large amount of energy, or the use of surfactants, or the combination of both. Low-energy emulsification processes, such as phase-inversion temperature techniques, require the use of large quantities of surfactants; thus, they cannot be used for large-scale industrial production [7]. High-energy emulsification utilizes mechanical devices, such as high-pressure homogenizers and microfluidizers or sonication equipment, that are being used in industrial production. These technologies generate intense disruptive forces that break up the oil and water phases and lead to the formation of small oil droplets [8]. Ultra-high-pressure homogenization (UHPH) is a non-thermal processing technique, which works at significantly higher pressures ( $\geq 200$  MPa) than high-pressure homogenization (150–200 MPa) or microfluidization (20–150 MPa) [9,10].

The physical characteristics and emulsion stability of nutritional formulations were evaluated on a pilot-scale HPH unit [11]. The authors reported that homogenization at pressures from 100–150 MPa yielded more shear-thinning fluids with increased physical stability, representing an opportunity to reduce the concentration of stabilizers in dairy beverages. In another study, UHPH (100–300 MPa) was compared to conventional homogenization at 15 MPa and was found to produce oil-in-water emulsions containing 4.0% ( $w/v$ ) of soy protein isolate (SPI) and soybean oil (10% and 20%,  $v/v$ ) with small  $d_{3.2}$  ( $\mu\text{m}$ ) values and great physical stability [12]. Kuhn and Cunha [13] studied the effects of homogenization pressure and the number of homogenization cycles on the physicochemical characteristics of emulsions containing flaxseed oil and whey protein isolate. The authors reported that these emulsions showed good physical stability without phase separation after nine days of storage, when homogenized at high-pressure (20–100 MPa) with 1–7 homogenization cycles.

Proteins are ingredients widely used in food emulsions as emulsifiers/stabilizers due to their amphiphilic character. Proteins are able to form an interfacial layer that generates repulsive forces (e.g., steric and electrostatic) between oil droplets, which plays an important role in stabilizing the droplets against flocculation and coalescence during long-term storage [14]. Whey protein isolates (WPI) are widely used as emulsifiers to enhance the formation and stability of (O/W) emulsions [15,16]. Whey proteins are also known to inhibit lipid oxidation by preventing pro-oxidants from accessing the droplets [17].

The oil volume fraction has a great influence on the physicochemical and viscoelastic properties of emulsions by affecting droplet size distribution, creaming, oxidative stability and rheological properties [18]. Few data are available concerning the combined effects of varying dynamic high-pressure treatments and the oil volume fraction on emulsion rheology and physical stability. Cortés-Muñoz et al. [19] studied oil concentrations of 15%, 30% and 45% and pressures up to 300 MPa in O/W emulsions stabilized by whey protein isolate (4%), reporting that optimal droplet breakdown was observed for emulsions with 30% of oil ( $w/w$ ) treated with homogenization pressures  $\geq 200$  MPa. Flourey et al. [20] reported that increasing oil concentration resulted in a larger mean droplet diameter at constant homogenizing conditions due to the limitation of the emulsifier. These researchers revealed that the emulsions containing less than 20% of oil followed Newtonian behaviour ( $n = 1$ ) regardless of the homogenization pressure applied; however, emulsions containing more than 20% of oil and homogenized at 20 or 150 MPa showed shear-thinning behaviours ( $n < 1$ ). Flourey et al. [20] also showed that the application of increasing homogenization pressures resulted in high oil content emulsions (>40%) transitioning from shear-thinning behaviour (at 20 MPa) to Newtonian behaviour (at 300 MPa).

It is well established that emulsions with small droplet sizes and high specific surface areas are very sensitive to lipid oxidation [21]. Many other factors may affect the oxidative stability of

nano-emulsions, such as the physical structure of emulsions, the level and type of emulsifier(s) or the bulk oil-phase [22]. However, there are few studies that have investigated the oxidative deterioration of these emulsions, including those containing high oil concentrations.

In a previous work of the present authors [23], submicron emulsions with high physical and oxidative stabilities were obtained using 1 or 2 g WPI /100 g and 100 MPa homogenization pressure or 4 g WPI /100 g and 200 MPa homogenization pressure. Fernandez-Avila and Trujillo [24] reported that soy protein isolate stabilized emulsions containing 20% of oil (*w/v*), rather than 10% (*w/v*), with homogenization pressures of 100 or 200 MPa from the point of view of oxidation. The researchers attributed this high oxidative stability to the large quantity of oil and the high protein load at the surface. However, to the knowledge of the present authors, no reports on the effect of oil concentration and homogenization pressure on the oxidative stability of whey protein-stabilized emulsions have been referred in the literature. Further research is required to establish the relationship between UHPH treatment of emulsions and the stability to lipid oxidation. The objective of this study was to evaluate the effect of homogenization pressures (100–200 MPa) and oil concentration (10, 30 and 50 g/100 g) on the structure, rheological properties, physical and oxidative stabilities of emulsions containing 4 g whey protein isolate/100 g, in comparison with those produced by colloid mill and conventional homogenization.

## 2. Materials and Methods

### 2.1. Materials

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

Refined sunflower and olive oils were purchased from Gustav Heess Company (Barcelona, Spain). The characteristics and composition of oils according to the producer were: density (20 °C) = 0.921 and 0.913; acid value = 0.09 (mg KOH/g) and 0.11%; peroxide value (meq O<sub>2</sub>/kg) = 0.02 and 0.5; absorbance (270 nm) = not determined and 0.29; unsaponifiable (% m/m) = <0.05% and <1.5%; C16:0 (%) = 6.34 and 11.94; C18:0 (%) = 3.97 and 3.30; C18:1 (%) = 26.65 and 75.23; C18:2 (%) = 61.02 and 6.75; C18:3 (%) = 0 and 0.38; for sunflower and olive oils, respectively.

### 2.2. Preparation of Emulsions

#### 2.2.1. Experimental Design

A completely randomized factorial design was applied to study the influence of homogenization pressure and oil concentration on the physical and oxidative stability of the emulsions. This design was used with three homogenization methods (colloid mill (CM), conventional homogenization (CH) and ultra-high pressure homogenization (UHPH)). By varying the shear rate (5000 rpm for CM) or homogenization pressure (15 MPa for CH and 100, 200 MPa for UHPH) and oil concentration (10, 30 and 50 g/100 g) according to Table 1, twelve samples were formulated. Prepared samples were stored in glass bottles under refrigeration (4 °C) until physical analyses. Oxidation analyses were carried out on the first and last day of a 10-day storage at 10 °C in clear glass bottles under light (2000 lux/m<sup>2</sup>).

**Table 1.** Experimental design used in emulsion formulation. CM, colloid mill; CH, conventional homogenization.

Treatments	(Homogenization Method)	Homogenization Level	(Oil Content %)	Inlet Temperature (°C)
1	CM	5000 rpm	10	20
2	CM	5000 rpm	30	20
3	CM	5000 rpm	50	20
4	CH	15 MPa	10	60
5	CH	15 MPa	30	60
6	CH	15 MPa	50	60
7	UHPH	100 MPa	10	25
8	UHPH	100 MPa	30	25
9	UHPH	100 MPa	50	25
10	UHPH	200 MPa	10	25
11	UHPH	200 MPa	30	25
12	UHPH	200 MPa	50	25

### 2.2.2. Preparation of Protein Dispersions

WPI dispersions containing 4 g WPI/100 g were prepared in deionized water using agitation with a high speed mechanical blender (Frigomat, Guardamiglio, Italy) at a rate of 250 rpm at 20 °C. Protein dispersions with pH  $\approx$  6.5–7 (MicropH 2001, Crison, Alella, Spain) were stored overnight at 4 °C to allow protein hydration.

### 2.2.3. Homogenization Treatments

After rehydration, protein dispersions of 4 g WPI/100 g and different oil concentrations (10, 30 and 50 g/100 g) were equilibrated at 20 °C before mixing. Pre-emulsions (or coarse emulsions) were prepared by mixing the oily dispersed phase (3 parts sunflower:1 part olive oil) with the aqueous continuous phase containing WPI at room temperature to give a total volume of 40 L. The mixture was stirred for 5 min using a colloid mill homogenizer (E. Bachiller B, S.A, Barcelona, Spain) at maximum power (5000 rpm) to obtain the CM emulsions and further processed into CH and UHPH emulsions as follows.

Conventional homogenization of the CM emulsions was performed using an APV Rannie Copenhagen Series Homogenizer (Model 40.120 H, single-stage hydraulic valve assembly, Copenhagen, Denmark) with  $T_{in}$  of 60 °C at 15 MPa (CH emulsions).

CM emulsions were treated by UHPH using a Stansted high-pressure homogenizer (Model/DRG number FPG 11,300:400 Hygienic Homogenizer, Stansted Fluid Power Ltd., Harlow, UK) with a flow rate of 120 L/h as indicated by the manufacturer. Two spiral-type heat exchangers (Garvía, Barcelona, Spain) located behind the second valve were used to cool the emulsion immediately after the HP-valve to minimize temperature retention in the emulsion after treatment, which may affect emulsion composition. Emulsions were UHPH-treated at pressures of 100 and 200 MPa (single-stage) with an inlet temperature ( $T_{in}$ ) of 25 °C (UHPH emulsions). Throughout the experiment, the  $T_{in}$ , the temperature after the homogenization valve ( $T_1$ ) and the temperature of the outlet product ( $T_2$ ) were monitored.

The experiment by each preparation method in each study was repeated three times.

## 2.3. Emulsion Measurements and Analyses

### 2.3.1. Droplet Size Distribution

The droplet size distribution of the different emulsions was determined just after sample preparation using a Beckman Coulter laser diffraction particle size analyser (LS 13 320 series, Beckman Coulter, Fullerton, CA, USA), as described by Hebshy et al. [23]. Emulsion samples were diluted in distilled water until an appropriate obscuration was obtained in the diffractometer cell. An optical model based on the Mie theory of light scattering by spherical droplets was applied by

using the following conditions: real refractive index of the oil mixture (sunflower oil:olive oil (3:1)), which was obtained by a refractometric measurement (Spectronic Instruments, Inc., Rochester, NY, USA), 1.471; refractive index of fluid (water), 1.332; the refractive index of the protein was assumed to be 0 [25]; imaginary refractive index, 0; pump speed, 20%. The volume weighted mean diameter ( $d_{4.3}$ ,  $\mu\text{m}$ ), surface-weighted mean diameter ( $d_{3.2}$ ,  $\mu\text{m}$ ) and specific surface area (SSA,  $\text{m}^2/\text{mL}$ ) were determined. Each diluted sample was analysed at least four times in succession to obtain a mean size distribution curve and the corresponding mean values.

### 2.3.2. Surface Protein Concentration

The surface protein concentration of oil droplets was determined according to the method of Desrumaux and Marcand [26], as described by Hebishy et al. [23]. Briefly, the cream layer was isolated by centrifugation and clarified. The protein content of the isolated purified protein layers was determined in triplicate by the Dumas method with a Leco FP-528 nitrogen/protein instrument (Leco Corp., St. Joseph, MI, USA), estimating crude protein content as  $N \times 6.38$ .

### 2.3.3. Rheological Measurements

Rheological measurements were performed at 25 °C using a controlled stress rheometer (Haake Rheo Stress 1, Thermo Electron Corporation, Karlsruhe, Germany) with a parallel plate geometry probe (1°, 60 mm diameter). To avoid any structure destruction, samples were left standing for 5 min at 25 °C in order to reach equilibrium. Flow curves were fitted to the Ostwald de Waele rheological model:  $\tau = K \cdot \dot{\gamma}^n$ , and the consistency coefficient ( $K$ ,  $\text{mPa} \times \text{s}$ ) and flow behaviour index ( $n$ ) were obtained. Rheological measurements were carried out in triplicate.

### 2.3.4. Physical Stability

The physical stability of emulsions was assessed by measuring the  $d_{4.3}$  ( $\mu\text{m}$ ) value at the top or at the bottom of the emulsion tubes stored for 9 days. Measurements were performed in triplicate using the laser diffraction particle size analyser (LS 13 320 series, Beckman Coulter, Fullerton, CA, USA), as detailed before (Section 2.3.1).

The stability of emulsions was also determined with a vertical scan analyser Turbiscan MA 2000 (Formulation, Toulouse, France), as reported by Hebishy et al. [23]. This equipment allows the optical characterization of any type of dispersion. The light source is an electro-luminescent diode in the near-infrared ( $\lambda_{\text{air}} = 850 \text{ nm}$ ). Any change due to a variation of the droplet size (flocculation, coalescence) or a local variation of the volume fraction (migration phenomena: creaming, sedimentation) is detected. Under backscattering mode, Turbiscan measures the light backscattered by the sample, which is directly dependent on the droplet mean diameter, at pre-set intervals (30 min for CM emulsions, 3 days for CH and UHPH emulsions) over a selected period of time (5 h for CM emulsions and 17 days for CH and UHPH emulsions). The migration rate or velocity ( $V$ ;  $\mu\text{m}/\text{min}$ ) of the clarification front was also calculated using Turbisoft software in order to follow the kinetics of the creaming phenomenon.

### 2.3.5. Emulsion Microstructure

In order to assess the microstructure of emulsions, emulsion samples were observed under a transmission electron microscope with a Jeol 1400 (Jeol Ltd., Tokyo, Japan) equipped with a Gatan Ultrascan ES1000 CCD Camera. Samples were prepared according to Cruz et al. [27], as described by Hebishy et al. [23].

### 2.3.6. Stability of Emulsions to Photo-Oxidation

For the determination of primary oxidation products, lipid hydroperoxides were measured by mixing 0.3 mL of emulsion with 1.5 mL of isooctane/2-propanol (3:1,  $v/v$ ) by vortexing (10 s,

three times) and isolating the organic solvent phase by centrifugation at  $1000\times g$  for 2 min. The organic solvent phase (200  $\mu\text{L}$ ) was added to 2.8 mL of methanol/1-butanol (2:1, *v/v*), followed by 15  $\mu\text{L}$  of 3.97 M ammonium thiocyanate and 15  $\mu\text{L}$  of ferrous iron solution (prepared by mixing 0.132 M  $\text{BaCl}_2$  and 0.144 M  $\text{FeSO}_4$ ). The absorbance of the solution was measured at 510 nm, 20 min after the addition of the iron [28]. Hydroperoxide content was expressed as absorbance (A510).

For the determination of secondary oxidation products, thiobarbituric acid-reactive substances (TBARS) were determined according to an adapted method of McDonald and Hultin [29]. The emulsion (1.0 mL) was combined with 2.0 mL of TBA solution (prepared by mixing 15 g of trichloroacetic acid, 0.375 g of thiobarbituric acid, 1.76 mL of 12 N HCl and 82.9 mL of  $\text{H}_2\text{O}$ ) in test tubes and placed in a boiling water bath for 15 min. The tubes were allowed to cool to room temperature for 10 min, and then, the coloured solution was separated by filtration through glass wool. The absorbance was measured at 532 nm. Concentrations of TBARS were calculated from a standard curve prepared using 1,1,3,3-tetraethoxypropane.

#### 2.4. Statistical Analyses

Statistical analyses were performed using SAS System<sup>®</sup> v9.2 (SAS Institute Inc., Cary, NC, USA), with a nominal significance level of 5% ( $p < 0.05$ ) and Tukey adjustment for multiple comparisons of means. In order to evaluate the physical and oxidative stabilities depending on the type of emulsion (CM, CH or UHPH) and the concentration of oil (10%, 30% and 50%), a general linear model with repeated measures was performed. The rheological index, consistency coefficient, hydroperoxides and TBARS values were compared between the CM, CH and UHPH emulsions. The other parameters (d3.2, SSA and SPC) were compared only between CH and UHPH emulsions, excluding CM emulsions due to the wide variation of data; these three parameters were compared among the three oil levels in CM emulsions.

### 3. Results and Discussion

#### 3.1. Droplet Size Distribution

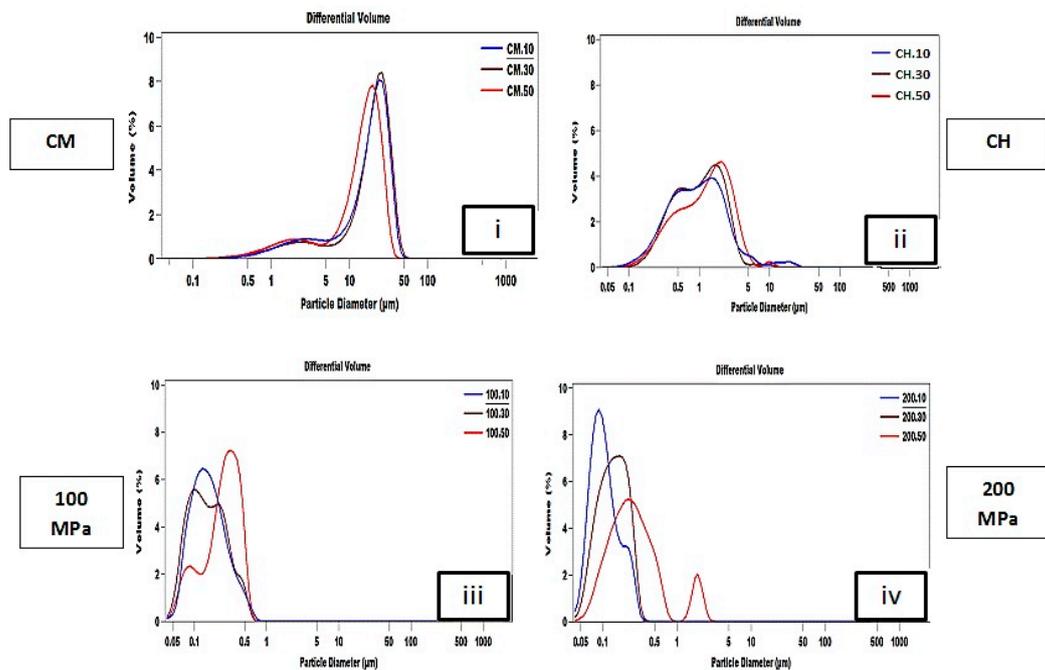
The droplet size (d3.2,  $\mu\text{m}$ ) and specific surface area (SSA,  $\text{m}^2/\text{mL}$ ) for emulsions containing 4 g WPI/100 g and different oil concentrations (10, 30 and 50 g/100 g) are shown in Table 2 and Figure 1A. CM emulsions, at all oil concentrations, had large droplet sizes ( $\sim 6 \mu\text{m}$ ), as can be observed in the TEM micrograph (Figure 1B(i)). When increasing from 10%–30% and to 50%, oil concentration significantly affected ( $p < 0.05$ ) droplet size in CM emulsions, decreasing d3.2 ( $\mu\text{m}$ ), as can be seen from their size distribution curves (Figure 1A).

Applying the conventional homogenization at 15 MPa significantly decreased ( $p < 0.05$ ) the droplet size to the nano range. However, greater d3.2 ( $\mu\text{m}$ ) were observed when the oil content increased from 10%–30% and 50% (Table 2), as evidenced by TEM images (Figure 2A–C). This phenomenon was accompanied by a concomitant and significant change ( $p < 0.05$ ) in the surface protein concentration of CH emulsions (Table 2).

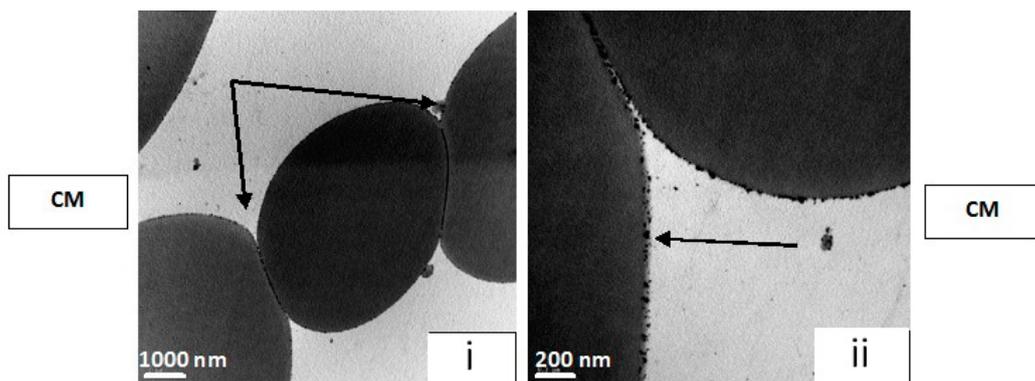
Concerning the effect of UHPH, treatments performed at 200 MPa were more effective in reducing droplet size than those at 100 MPa, as can be seen from TEM images (Figure 2G–L). Similar results have been obtained when applying the same homogenization pressures to soymilk or cow milk [27,30]. A previous study of the present authors showed that although pressures of up to 200 MPa were effective in decreasing the droplet size of emulsions with 4 g WPI/100 g (*w/v*) and 20 g/100 g of oil content, increasing the pressure to 300 MPa tended to increase the droplet size [23].

UHPH emulsions treated at 100 MPa and containing 10% of oil exhibited small droplet sizes (Table 2) and monomodal distributions (Figure 1A(iii)). As the oil content was increased, droplets became larger with a concomitant decrease of their SSA. However, these changes were only significant ( $p < 0.05$ ) in emulsions containing 50% oil, whose distribution curves changed to bimodal. The increase in droplet size may be attributed to a high degree of flocculation (Figure 2I).

As the oil content increased to 30% in emulsions treated at 200 MPa, the UHPH was able to produce monomodal distributions (Figure 1A(iv)), but was unable to achieve a narrow size distribution. Increasing the oil content to 50% in emulsions treated at 200 MPa clearly shifted the curve to bimodal with a wider size distribution (Figure 1A(iv)). As can be observed from the TEM images (Figure 2J–L), flocculation or coalescence is likely to be responsible for the large droplet sizes observed in emulsions treated at 200 MPa containing 50% oil. The bimodal distribution in these emulsions may be due to an over-processing phenomena affecting the flocculation of the newly-created fine droplets in the homogenization chamber or during pressure release. This phenomenon may happen when the surfactant concentration is not sufficient to cover the newly-created surface [31].



(A)

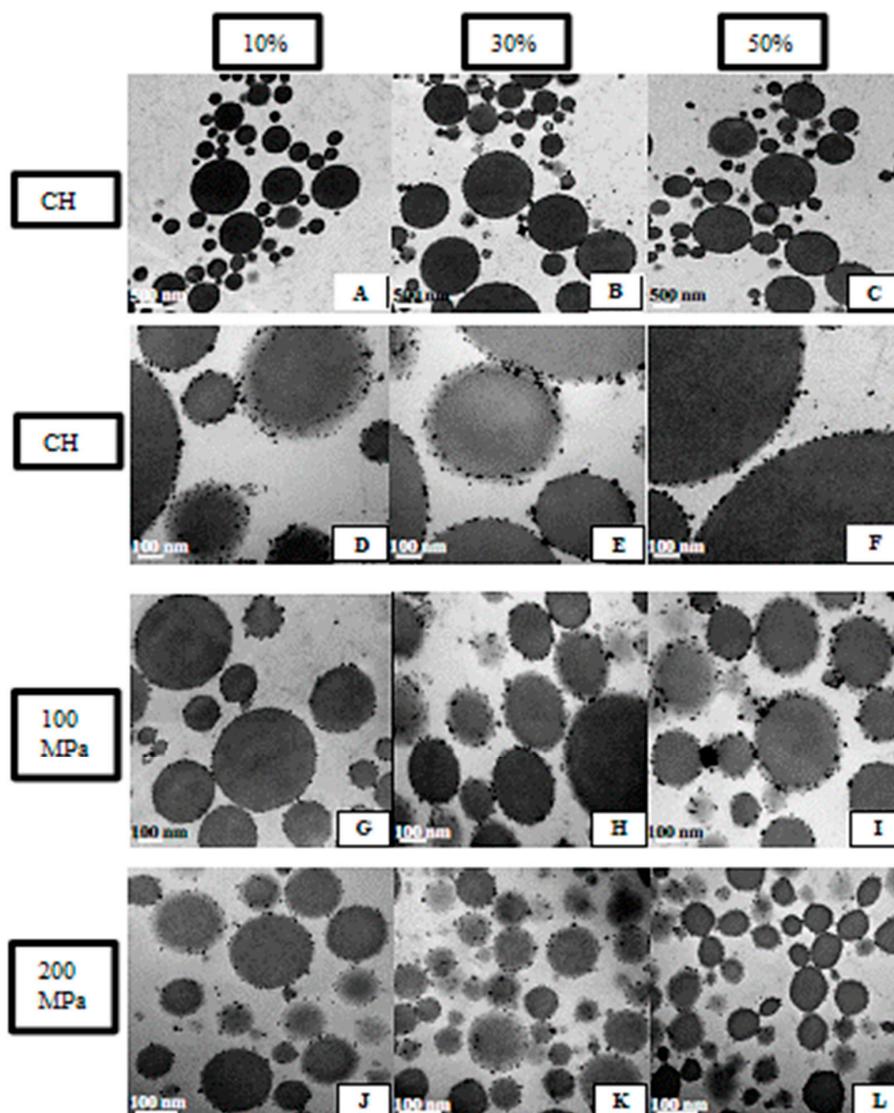


(B)

**Figure 1.** (A) Droplet size distribution curves measured by light scattering of oil-in-water (O/W) emulsions containing 10%, 30% and 50% oil plus 4% whey protein isolates (WPI) processed by (i) CM, (ii) CH and UHPH at (iii) 100 and (iv) 200 MPa; (B) TEM images of O/W emulsions stabilized by CM (i,ii) containing 30% oil (i)  $\times 10,000$  and (ii)  $\times 50,000$ , respectively.

Several reasons may explain the increase of droplet size with increasing volume fraction: (1) higher oil concentrations increase the emulsion viscosity, and thereby, droplet disruption might become more

difficult [32]; (2) an inadequate amount of protein in the aqueous phase may cause some aggregation of fat globules [33]; and (3) the rate of collision frequency and thus coalescence frequency may increase as the oil content increases [34]. The results of the present study are in agreement with the results that have been reported for emulsions containing different oil concentrations with whey proteins as emulsifiers. Cortés-Muñoz et al. [19] studied the submicron emulsion characteristics using 15%–45% of oil and pressures up to 300 MPa. They reported that the best droplet breakdown was achieved when the pressure used was less than 225 MPa with 30% of oil. These researchers attributed this result to the increase in the fluid viscosity at the valve gap outlet since it may shift the flow pattern from turbulent to transitional, reducing therefore cavitation and impact phenomena and limiting droplet re-agglomeration and coalescence. Fernández-Ávila et al. [12] studied the effect of UHPH (100–300 MPa) on the physicochemical properties of oil-in-water emulsions prepared with 4.0% (*w/v*) of soy protein isolate and soybean oil (10% and 20%, *v/v*). The authors reported that at a constant emulsification pressure,  $d_{3.2}$  ( $\mu\text{m}$ ) rose with increasing oil content.



**Figure 2.** TEM images of emulsions containing 10%, 30% and 50% oil and WPI 4% stabilized by CH at 15 MPa (A–C)  $\times 100,000$  and (D–F)  $\times 25,000$  and by UHPH  $\times 100,000$  at 100 MPa (G–I) and 200 MPa (J–L), respectively.

### 3.2. Surface Protein Concentration

Table 2 shows the amount of protein adsorbed at the emulsion droplet surface. High protein amounts ( $\text{mg}/\text{m}^2$ ) were observed in CM emulsions, as a result of high droplet size and low SSA. The amount of protein adsorbed at the interface of an emulsion droplet suggests the state of the protein adsorbed at the interface. If the protein load is  $\sim 1 \text{ mg}/\text{m}^2$ , which is the case of UHPH emulsions; it indicates that the protein molecules are fully unfolded. If the protein load is  $1\text{--}3 \text{ mg}/\text{m}^2$  (as for CH emulsions), a monolayer of globular proteins may be present or unfolded molecules may be adsorbed in the conformation of trains, loops and tails. A protein load above  $5 \text{ mg}/\text{m}^2$  (such as CM emulsions) indicates adsorption of aggregates of proteins or multilayers of proteins [35–37].

At first glance, it would seem reasonable to think that UHPH emulsions had lower protein load than CM and CH emulsions since they had significantly lower ( $p < 0.05$ ) surface protein concentrations. However, and taking into account the SSA of droplets, which was significantly higher for UHPH compared with both CM and CH, the amount of surface protein per volume (millilitre) would be much higher in UHPH emulsions (41 and  $53.51 \text{ mg}/\text{mL}$  at 100 and 200 MPa, respectively) than in CM and CH emulsions ( $23.30$  and  $25.80 \text{ mg}/\text{mL}$ , respectively). This may be attributed to the increased spreading and rearrangement of adsorbed protein molecules at the interface. High homogenization pressures may modify the properties of any protein and especially serum proteins by modifying their 3D structures [38] and thus facilitate their adsorption at the interface, as suggested by Dalgleish [36].

Concerning the effect of oil content on the SPC, as shown in Table 2, increasing oil concentration in CM emulsions resulted in a strong linear decrease in the SPC, probably due to the decrease in the droplet size and the concomitant increase in the SSA. The higher the surface area formed during homogenization, the higher the amount of protein needed to obtain a full coverage of the droplets. In CH emulsions, increasing the oil content from 10%–30% resulted in a significant increase in the SPC, possibly due to the significant increase ( $p < 0.05$ ) in the droplet size and the decrease in the SSA. But further increases in the oil content to 50% decreased significantly ( $p < 0.05$ ) the SPC, which might be due to the limited protein availability to cover the newly-created interface. In fact, TEM images (Figure 2D–F) showed a dense interfacial layer very apparent in emulsions containing 10% and 30% oil, but imperceptible in those containing 50% oil. SPC reduction as oil content increases may be the predominant factor that promoted flocculation. Considering UHPH, both at 100 and 200 MPa, the oil concentration did not have any significant effect on the SPC.

### 3.3. Rheological Behaviour

Low viscosities and Newtonian behaviour were observed for all CM emulsions and for CH emulsions containing only 10% or 30% oil, which could be explained by a low degree of interactions between droplets. However, high consistency coefficient ( $K$ ) values and shear-thinning behaviour or pseudo-plasticity were observed in CH emulsions when the oil concentration increased to 50% (Table 2). In the case of UHPH emulsions, the apparent viscosity increased and could be explained by an increase in droplet interactions.

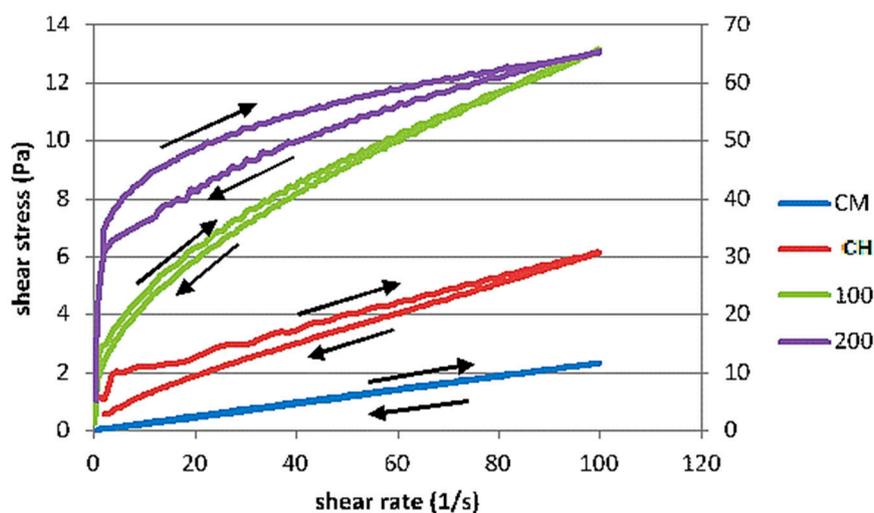
**Table 2.** Mean  $\pm$  SD of droplet size distribution (d3.2,  $\mu\text{m}$ ), specific surface area (SSA,  $\text{m}^2/\text{mL}$ ), surface protein concentration (SPC,  $\text{mg}/\text{m}^2$ ), rheological characteristics (flow and consistency indices) and physical creaming stability (d4.3,  $\mu\text{m}$ ) value at the top and bottom of tube after 9 days of storage at room temperature) of O/W emulsions containing 4% ( $w/w$ ) whey protein isolate plus sunflower and olive oils (10%, 30% and 50%) and prepared by colloid mill (CM), conventional homogenization (15 MPa) and ultra-high pressure homogenization at 100 and 200 MPa.

Pressure (MPa)	Oil Content (%)	Droplet Size		Rheological Behaviour		Surface Coverage	Creaming Stability	
		d3.2 ( $\mu\text{m}$ )	Specific Surface Area SSA ( $\text{m}^2/\text{mL}$ )	Consistency Coefficient (K) $\text{mPa} \times \text{s}$	Flow Behaviour Index ( $n$ )	Surface Protein Concentration (SPC) $\text{mg}/\text{m}^2$	Emulsion Stability after 9 Days (d4.3)	
							Top	Bottom
CM	10	6.656 $\pm$ 0.654 <sup>A</sup>	0.862 $\pm$ 0.061 <sup>B</sup>	0.0016 $\pm$ 0.0001 <sup>h</sup>	0.968 $\pm$ 0.020	27.04 $\pm$ 7.17 <sup>A</sup>	ND	
	30	6.132 $\pm$ 0.166 <sup>A</sup>	0.979 $\pm$ 0.027 <sup>A,B</sup>	0.0025 $\pm$ 0.0008 <sup>g,h</sup>	1.105 $\pm$ 0.087	12.87 $\pm$ 0.17 <sup>B</sup>	ND	
	50	5.151 $\pm$ 0.215 <sup>B</sup>	1.193 $\pm$ 0.015 <sup>A</sup>	0.0185 $\pm$ 0.0051 <sup>f</sup>	1.045 $\pm$ 0.038	6.85 $\pm$ 0.95 <sup>C</sup>	ND	
CH	10	0.559 $\pm$ 0.055 <sup>b</sup>	10.89 $\pm$ 1.139 <sup>e</sup>	0.0017 $\pm$ 0.0001 <sup>h</sup>	0.984 $\pm$ 0.012	2.37 $\pm$ 0.41 <sup>c</sup>	0.907 $\pm$ 0.037 <sup>b</sup>	0.487 $\pm$ 0.061 <sup>c,*</sup>
	30	0.746 $\pm$ 0.107 <sup>a</sup>	8.853 $\pm$ 0.521 <sup>e</sup>	0.0051 $\pm$ 0.0018 <sup>g</sup>	0.973 $\pm$ 0.021	4.69 $\pm$ 0.44 <sup>a</sup>	1.204 $\pm$ 0.053 <sup>a,b</sup>	0.775 $\pm$ 0.046 <sup>b,*</sup>
	50	0.699 $\pm$ 0.036 <sup>a</sup>	8.537 $\pm$ 0.450 <sup>e</sup>	0.5299 $\pm$ 0.0696 <sup>d</sup>	0.596 $\pm$ 0.152	3.65 $\pm$ 0.13 <sup>b</sup>	1.411 $\pm$ 0.194 <sup>a</sup>	1.436 $\pm$ 0.191 <sup>a</sup>
UHPH 100 MPa	10	0.134 $\pm$ 0.006 <sup>e</sup>	45.00 $\pm$ 2.072 <sup>b</sup>	0.0017 $\pm$ 0.0001 <sup>h</sup>	0.984 $\pm$ 0.013	0.92 $\pm$ 0.04 <sup>d</sup>	0.175 $\pm$ 0.018 <sup>d</sup>	0.145 $\pm$ 0.015 <sup>f,g,*</sup>
	30	0.141 $\pm$ 0.007 <sup>d,e</sup>	43.52 $\pm$ 1.836 <sup>b,c</sup>	0.4037 $\pm$ 0.0008 <sup>d,e</sup>	0.973 $\pm$ 0.020	0.85 $\pm$ 0.01 <sup>d</sup>	0.186 $\pm$ 0.016 <sup>d</sup>	0.183 $\pm$ 0.014 <sup>e,f</sup>
	50	0.188 $\pm$ 0.022 <sup>c,d</sup>	34.24 $\pm$ 2.259 <sup>c,d</sup>	2.8961 $\pm$ 0.7420 <sup>b</sup>	0.437 $\pm$ 0.086	1.16 $\pm$ 0.02 <sup>d</sup>	0.267 $\pm$ 0.021 <sup>c,d</sup>	0.240 $\pm$ 0.039 <sup>d,e</sup>
UHPH 200 MPa	10	0.103 $\pm$ 0.006 <sup>e</sup>	60.81 $\pm$ 1.903 <sup>a</sup>	0.0020 $\pm$ 0.0003 <sup>h</sup>	0.983 $\pm$ 0.016	0.88 $\pm$ 0.07 <sup>d</sup>	0.117 $\pm$ 0.009 <sup>d</sup>	0.121 $\pm$ 0.004 <sup>g</sup>
	30	0.123 $\pm$ 0.010 <sup>e</sup>	51.38 $\pm$ 2.380 <sup>a,b</sup>	1.1960 $\pm$ 0.0168 <sup>c</sup>	0.882 $\pm$ 0.087	1.06 $\pm$ 0.14 <sup>d</sup>	0.191 $\pm$ 0.069 <sup>d</sup>	0.188 $\pm$ 0.068 <sup>e,f</sup>
	50	0.214 $\pm$ 0.033 <sup>c</sup>	31.55 $\pm$ 0.339 <sup>d</sup>	8.3300 $\pm$ 1.108 <sup>a</sup>	0.284 $\pm$ 0.076	1.18 $\pm$ 0.13 <sup>d</sup>	0.422 $\pm$ 0.118 <sup>c</sup>	0.531 $\pm$ 0.237 <sup>c</sup>

<sup>a-h</sup> Different letters in the same column (capital letters (A,B, ... ) for comparison between CM emulsions and small letters (a,b, ... ) to compare between CH and UHPH emulsions) indicate significant differences between treatments (General Linear Models (GLM) with repeated measures,  $p < 0.05$ ). Tukey correction was applied. \* The sign indicates that the differences between the d4.3 ( $\mu\text{m}$ ) at the top or at the bottom of emulsions are significant per level of pressure and oil concentration (Mann-Whitney-Wilcoxon test,  $p < 0.05$ ). ND, not determined.

With respect to the effect of oil concentration on the rheological flow behaviour, Newtonian behaviour ( $n \approx 1$ ) and low viscosities were observed in UHPH emulsions containing 10% ( $w/w$ ) oil; however, when the oil content was increased to 30% ( $w/w$ ), the  $K$  values significantly increased ( $p < 0.05$ ) with a slight change in the rheological profile to shear-thinning behaviour in emulsions homogenized at 200 MPa ( $n = 0.88$ ), but not in those treated at 100 MPa. An increase of oil concentration to 50% resulted in a large increase in the  $K$  value, especially in emulsions treated at 200 MPa, and the flow index was altered to a high degree of pseudo-plasticity. Similar trends in the rheological characteristics have been reported [20] for emulsions prepared using UHPH and the same emulsifier (1.5% whey proteins) with oil contents varying between 10% and 50% and [19] using 4% whey proteins and 15%–45% oil. In a study by Bellalta et al. [39], WPI emulsions sonicated at a nominal power level of 100 W for 180 s and containing 50%–55% oil showed Newtonian behaviour, but a further increase in the oil content to 60% changed the rheological behaviour to shear-thinning. They reported that, in emulsions with lower oil contents, the droplets are far apart and the inter-droplet interactions are relatively weaker. As oil content increases, the droplets are closer and the number density of droplets (number of droplets per unit volume of emulsions at a given dispersed phase volume fraction) increases. As a consequence, the mean distance of separation between droplets decreases, where London-van der Waals forces of attraction between droplets are dominant, leading to packing of the oil droplets and inter-droplet interactions and collisions. The flocculated droplets immobilize a significant amount of the continuous phase within themselves, which is released as the shear rate (or shear stress) increases. As a result, the effective dispersed-phase concentration decreases, which causes a decrease in the viscosity and the shear-thinning effect [40].

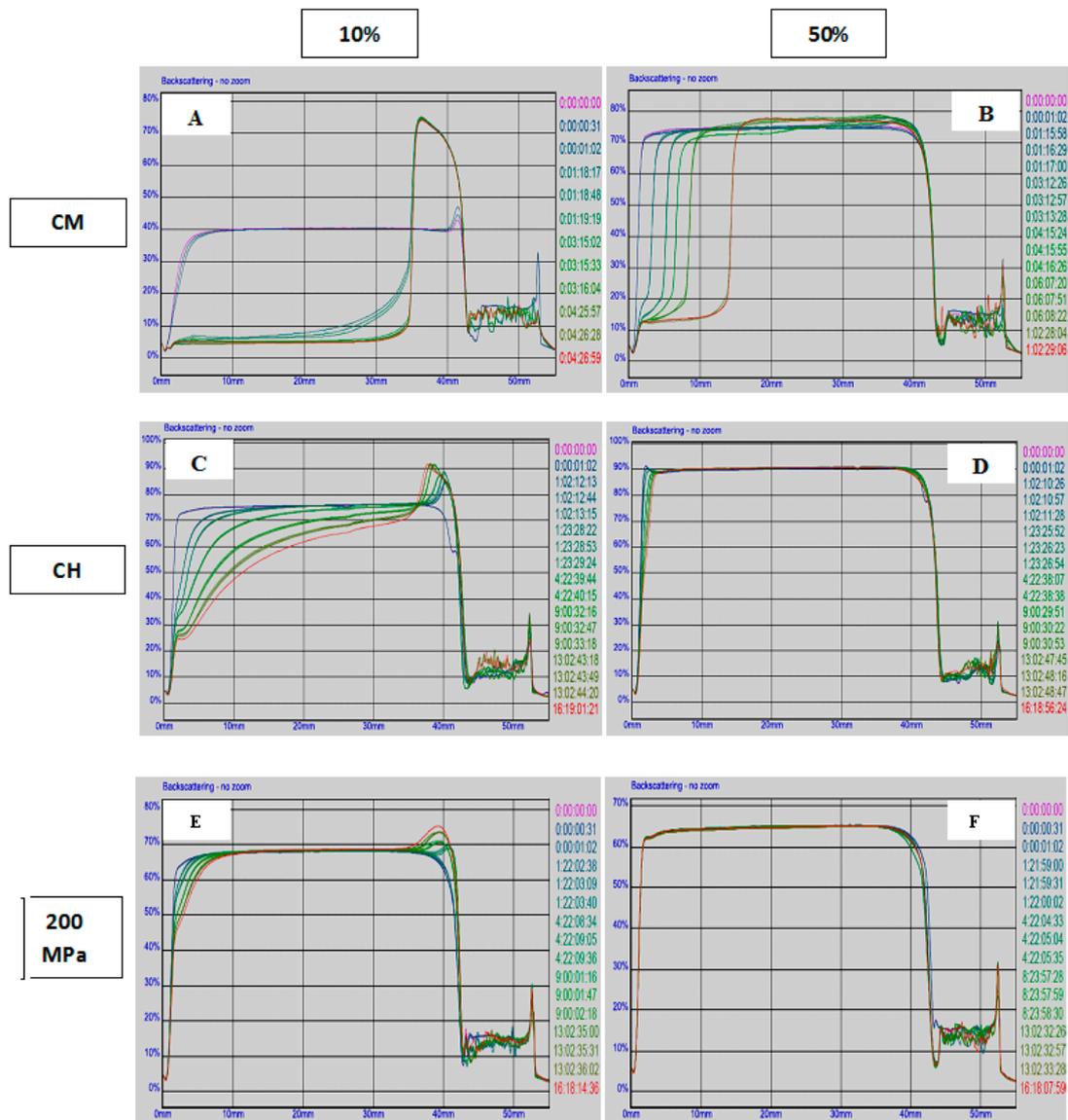
UHPH emulsions prepared with 50% ( $w/w$ ) oil presented not only a shear-thinning behaviour, but also a thixotropic behaviour, as evidenced by hysteresis in Figure 3. The thixotropic behaviour of an emulsion indicates the existence of a structure that breaks down while shearing at a constant shear rate as a function of time followed by a gradual recovery when the shear is removed [41]. In this type of time-dependent fluid, a hysteresis loop can be observed when the sample is subjected to increasing and then reducing shear. CH and UHPH emulsions with 50% oil content had a thixotropic behaviour, observed as a loop between the up and down curves, i.e., the samples behaved differently before and after shearing in the following order: 200 MPa > CH > 100 MPa (Figure 3).



**Figure 3.** Hysteresis loops for O/W emulsions containing 50% oil and WPI 4% stabilized by CM (blue line), CH at 15 MPa (red line) and by UHPH at 100 MPa (green line) and 200 MPa (purple line).

### 3.4. Stability of Emulsions to Creaming

Creaming stability results using the  $d_{4.3}$  ( $\mu\text{m}$ ) value at the top and bottom of emulsions stored at room temperature for nine days are presented in Table 2. The light scattering fingerprints obtained by Turbiscan analysis of CM, CH and UHPH emulsions with oil concentrations of 10% and 50%  $w/w$  are shown in Figure 4.



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

Low creaming stability could be observed in CM emulsions. Possible reasons for this could be their high droplet size (the larger the droplets, the faster the creaming [42]), their high interfacial tension between oil droplets because no protective protein layer exists surrounding the oil droplets, their low viscosity and their high coalescence rate, as previously described. A great variation in physical stability with time could be observed in the backscattering profiles for CM emulsions containing 10% oil, with the emulsion being totally separated after  $\sim 1$  h. CM emulsions containing 50% oil exhibited

higher stability to creaming and lower separation rate with time. These results were validated by the migration velocity  $V(t)$  ( $\mu\text{m}/\text{min}$ ) of droplets to the top of the samples, which was calculated with the Turbiscan equipment. Migration velocity was reduced in emulsions containing 50% oil ( $37.1 \mu\text{m}/\text{min}$ ), in comparison to those containing 10% and 30% oil ( $273.6$  and  $79.3 \mu\text{m}/\text{min}$ , respectively). This higher stability may be explained by a significantly lower droplet size ( $p < 0.05$ ), higher viscosity and better protein coverage of CM emulsions containing 50% oil.

Data in Table 2 and Figure 4C–F show the physical stability of CH and UHPH emulsions. CH emulsions exhibited high stability to creaming, especially those containing 30% and 50% oil, presenting lower migration velocity of droplets ( $120.4$ ,  $18.6$  and  $17.7 \mu\text{m}/\text{min}$  for CH emulsions containing 10%, 30% and 50% oil, respectively). Creaming was visually observed in CH emulsions containing 10% and 30% oil, but not in emulsions containing 50% oil. These results were also confirmed by the  $d_{4.3}$  ( $\mu\text{m}$ ) value at the top or at the bottom of emulsions, where differences were significant ( $p < 0.05$ ) in emulsions containing 10% and 30% oil, but not in those containing 50% oil (Table 2). Conventional emulsions are thermodynamically unstable systems because of the positive free energy associated with the contact of oil and water phases, as manifested by a relatively high positive interfacial tension [43].

All UHPH emulsions remained fully turbid along the tube during the 17 days of storage without any visual changes or phase separation. Generally, UHPH led to excellent oil droplet stability to creaming for all model emulsions. For instance, the migration velocity value for UHPH emulsions treated at 200 MPa and containing 10% oil ( $15.2 \mu\text{m}/\text{min}$ ) was much lower than those of CM and CH emulsions with the same oil content ( $273.6$  and  $120.4 \mu\text{m}/\text{min}$ , respectively). UHPH-treated emulsions at 200 MPa seemed to be much more stable than those treated at 100 MPa.  $d_{4.3}$  ( $\mu\text{m}$ ) values at the top and at the bottom of UHPH emulsions indicated a slight creaming effect in emulsions with 10% oil treated at 100 MPa compared to those containing 30% and 50% oil (Table 2). However, all emulsions treated at 200 MPa were more stable to creaming independently of oil concentration with no significant differences ( $p < 0.05$ ) between top and bottom  $d_{4.3}$  ( $\mu\text{m}$ ) values (Table 2).

High-pressure homogenization improves the creaming stability of emulsions by decreasing droplet size according to Stoke's law, which in turn increases the density of droplets and concomitantly the viscosity of the emulsion, slowing down the droplet movement [44]. The obtained results for UHPH emulsions are in agreement with the results found by Cortés-Muñoz et al. [19]. These researchers observed a slight creaming effect in emulsions with 15% ( $w/w$ ) of oil, in comparison to those containing 30% and 45%, and treated at 100–150 MPa. However, UHPH treatment at 200 MPa led to excellent oil droplet stability to creaming and coalescence, especially when a high oil concentration was used. Other studies [12] reported that UHPH emulsions treated at 100 MPa, compared to 200 and 300 MPa, exhibited greater clarification and creaming, which also supports the results of the present study.

Increasing the oil concentration significantly reduced creaming ( $p < 0.05$ ). As might be expected, larger droplets result in less stable emulsions. However, for the same droplet sizes, oil content also plays an important role [45]. These authors reported that a larger amount of oil in the emulsion (40%) resulted in a more stable system in comparison to a small oil amount (20%). For 20% O/W emulsions with larger droplets, the crystallization was promoted, producing emulsions with large fat crystals, which may result in partial coalescence in the emulsion [46]. However, crystallization was promoted in 40% O/W emulsions with a small droplet size where only small fat crystals are formed through a homogeneous nucleation, which in combination with the emulsifier, stabilizes the interface of the emulsion droplets [47]. Another explanation for the lower tendency to creaming in emulsions containing higher amounts of oil, either in the conventional or in the UHPH emulsions, could be the increase in the viscosity of the continuous phase that surrounds the oil droplets, restricting the movement of droplets. Cortés-Muñoz et al. [19] reported high stability at higher oil concentrations than at lower oil concentrations. Fernández-Ávila [12], who also found similar results to those of the present study, reported that UHPH emulsions containing 20% ( $v/v$ ) oil were more stable to creaming than those containing 10% ( $v/v$ ) oil. They also reported that after more than five months of cold

storage, emulsions containing 20% (*v/v*) and treated at 100 or 200 MPa did not show any differences in creaming. This fact was explained by the lack of larger aggregates in fresh emulsions, as well as the depletion flocculation of protein-coated droplets by unadsorbed proteins in the aqueous phase.

### 3.5. Oxidative Stability

Hydroperoxide content and TBARS values of 4% of WPI emulsions containing different oil concentrations and treated by either CM, CH or UHPH are shown in Table 3. The results of the present study showed that CM emulsions, which had large droplet sizes and low SSA values, were more prone to oxidation than the corresponding CH and UHPH emulsions. Similar results have been reported in a previous study with emulsions produced under the same conditions with whey protein isolate [23]. Atarés et al. [48], using a high-pressure jet homogenizer at 30 MPa, evaluated the structure and oxidative stability of O/W emulsions formulated with whey protein and sunflower oil in the presence of flavonoid rutin. It was found that high-pressure homogenization, through droplet size reduction, stabilized the emulsions against both creaming and oil oxidation. These studies suggest that SSA is not the only determining factor of the oxidative stability of emulsions.

High levels of primary and secondary oxidation products were observed in CM emulsions. The high hydroperoxide value in combination with the high levels of TBARS obtained, especially those CM emulsions containing 10% or 50% oil, indicates the progression of oxidation from a primary to a secondary state. This high sensitivity of CM emulsions to lipid oxidation could be due to their low protein coverage (Figure 1B(ii)) and high coalescence rate in comparison to CH and UHPH emulsions, as previously described.

CH emulsions exhibited an intermediate oxidation level between CM and UHPH emulsions. At Day 10 of storage, CH emulsions containing 10% and 50% oil presented significantly higher ( $p < 0.05$ ) amounts of hydroperoxides in comparison to those containing 30% oil. Furthermore, the secondary oxidation products also increased as the oil content increased, although the differences in TBARS were not significant ( $p < 0.05$ ) between different oil concentrations.

UHPH emulsions presented the best oxidative stability. When comparing UHPH pressures, although not significant ( $p < 0.05$ ), emulsions treated at 100 MPa seem to be slightly less oxidized than those treated at 200 MPa. All UHPH treatments independently of oil content showed a significant increase ( $p < 0.05$ ) in hydroperoxide content between Day 1 and Day 10 of storage, except for UHPH treatment at 200 MPa of emulsions with 10% oil. However, taking into account the TBARS values, the latter sample was the only UHPH treatment at 200 MPa showing a significant increase ( $p < 0.05$ ) during storage. The higher oxidation rate observed in UHPH emulsions treated at 200 MPa, compared to those treated at 100 MPa, could be due to the decrease in the ability of whey proteins to protect the oil droplets with increasing pressure of the treatment, which may be related to the increase in the product temperature that takes place during UHPH treatment at the outlet of the homogenization valve.

Similar results were found in a previous study of the present authors [23] on systems containing WPI at different concentrations (1%, 2% and 4%) with 20% oil, in which UHPH-treated emulsions at 100 MPa were more stable to oxidation than those treated at 200 or 300 MPa. It was reported that treating emulsions at 100 MPa caused partial denaturation and/or aggregation of whey proteins resulting in the dissociation of large aggregates, which in turn resulted in an increased surface hydrophobicity of  $\beta$ -Lg. This increase in hydrophobicity may allow more of the protein to pack at the surface of the emulsion droplets, thereby providing better protection against oxidation.

Over a 10-day storage, all samples showed an increase in primary oxidation products, except for emulsions with 10% oil treated at 200 MPa. However, a significant increase ( $p < 0.05$ ) was only observed in emulsions with 50% oil treated at 100 MPa and emulsions with 10% and 30% oil treated at 200 MPa. This indicates that increasing the oil content to 50% negatively affects the oxidative stability at this UHPH pressure.

**Table 3.** Mean  $\pm$  standard deviation of the amount of hydroperoxides (absorbance at 510 nm) and thiobarbituric acid-reactive substances (TBARS) ( $\mu\text{g/mL}$ ) of O/W emulsions containing 4% ( $w/w$ ) of whey protein isolate plus sunflower and olive oils (10%, 30% and 50%) and prepared by colloid mill (CM), conventional homogenization (15 MPa) and ultra-high pressure homogenization at 100 and 200 MPa.

Pressure (MPa)	Oil content (%)	Hydroperoxides (ABS 510 nm)			TBARS ( $\mu\text{g/mL}$ )		
		Day 1	Day 10	Difference (Day 10-Day 1)	Day 1	Day 10	Difference (Day 10-Day 1)
CM	10	0.017 $\pm$ 0.004 <sup>c</sup>	0.326 $\pm$ 0.195 <sup>a,b</sup>	0.309 $\pm$ 0.198 <sup>a,*</sup>	0.097 $\pm$ 0.016 <sup>a</sup>	0.143 $\pm$ 0.017 <sup>a,b</sup>	0.046 $\pm$ 0.031 <sup>a,b,*</sup>
	30	0.039 $\pm$ 0.005 <sup>a,b,c</sup>	0.333 $\pm$ 0.026 <sup>a,b</sup>	0.294 $\pm$ 0.022 <sup>a,*</sup>	0.060 $\pm$ 0.003 <sup>b,c,d</sup>	0.079 $\pm$ 0.003 <sup>c,d,e</sup>	0.020 $\pm$ 0.004 <sup>a,b,c,d,*</sup>
	50	0.078 $\pm$ 0.003 <sup>a</sup>	0.433 $\pm$ 0.063 <sup>a</sup>	0.356 $\pm$ 0.063 <sup>a,*</sup>	0.100 $\pm$ 0.007 <sup>a</sup>	0.160 $\pm$ 0.022 <sup>a</sup>	0.061 $\pm$ 0.017 <sup>a,*</sup>
CH	10	0.039 $\pm$ 0.015 <sup>a,b,c</sup>	0.252 $\pm$ 0.032 <sup>a,b</sup>	0.213 $\pm$ 0.047 <sup>a,b,*</sup>	0.054 $\pm$ 0.002 <sup>b,c,d</sup>	0.075 $\pm$ 0.007 <sup>c,d,e</sup>	0.021 $\pm$ 0.007 <sup>a,b,c,d,*</sup>
	30	0.041 $\pm$ 0.023 <sup>a,b,c</sup>	0.178 $\pm$ 0.012 <sup>c</sup>	0.137 $\pm$ 0.034 <sup>a,b,*</sup>	0.058 $\pm$ 0.004 <sup>b,c,d</sup>	0.092 $\pm$ 0.009 <sup>c,d</sup>	0.034 $\pm$ 0.006 <sup>a,b,c,d,*</sup>
	50	0.057 $\pm$ 0.029 <sup>a,b</sup>	0.245 $\pm$ 0.048 <sup>a,b</sup>	0.187 $\pm$ 0.074 <sup>a,b,*</sup>	0.068 $\pm$ 0.005 <sup>b,c</sup>	0.110 $\pm$ 0.035 <sup>b,c</sup>	0.041 $\pm$ 0.034 <sup>a,b,c,*</sup>
UHPH 100 MPa	10	0.022 $\pm$ 0.004 <sup>b,c</sup>	0.027 $\pm$ 0.004 <sup>c,d</sup>	0.005 $\pm$ 0.004 <sup>b,c,*</sup>	0.052 $\pm$ 0.004 <sup>c,d</sup>	0.044 $\pm$ 0.003 <sup>e</sup>	-0.08 $\pm$ 0.003 <sup>e,*</sup>
	30	0.026 $\pm$ 0.006 <sup>b,c</sup>	0.034 $\pm$ 0.0008 <sup>c,d</sup>	0.009 $\pm$ 0.006 <sup>b,c,*</sup>	0.051 $\pm$ 0.004 <sup>c,d</sup>	0.040 $\pm$ 0.008 <sup>e</sup>	-0.011 $\pm$ 0.008 <sup>e,*</sup>
	50	0.028 $\pm$ 0.009 <sup>b,c</sup>	0.033 $\pm$ 0.009 <sup>c,d</sup>	0.0049 $\pm$ 0.004 <sup>b,c,*</sup>	0.041 $\pm$ 0.009 <sup>d</sup>	0.053 $\pm$ 0.008 <sup>d,e</sup>	0.012 $\pm$ 0.006 <sup>b,c,d,*</sup>
UHPH 200 MPa	10	0.025 $\pm$ 0.004 <sup>b,c</sup>	0.024 $\pm$ 0.003 <sup>d</sup>	0.00 $\pm$ 0.0016 <sup>c</sup>	0.054 $\pm$ 0.008 <sup>b,c,d</sup>	0.088 $\pm$ 0.006 <sup>c,d</sup>	0.034 $\pm$ 0.011 <sup>a,b,c,d,*</sup>
	30	0.019 $\pm$ 0.006 <sup>b,c</sup>	0.037 $\pm$ 0.003 <sup>c,d</sup>	0.018 $\pm$ 0.004 <sup>b,*</sup>	0.059 $\pm$ 0.008 <sup>b,c,d</sup>	0.066 $\pm$ 0.013 <sup>d,e</sup>	0.006 $\pm$ 0.017 <sup>b,c,d</sup>
	50	0.032 $\pm$ 0.006 <sup>b,c</sup>	0.038 $\pm$ 0.004 <sup>c</sup>	0.006 $\pm$ 0.004 <sup>b,c,*</sup>	0.072 $\pm$ 0.007 <sup>b</sup>	0.072 $\pm$ 0.004 <sup>c,d,e</sup>	0.00 $\pm$ 0.009 <sup>c,d</sup>

<sup>a-c</sup> Different letters in the same column indicate significant differences between treatments (GLM with repeated measures,  $p < 0.05$ ). Tukey correction was applied. \* The sign indicates that the differences in oxidation products between Day 10 and Day 1 are significant per level of pressure and oil concentration (Wilcoxon test,  $p < 0.05$ ).

The obtained results in agreement with those previously reported for safflower oil [44], canola oil [49], menhaden oil [34] and walnut oil [50], in which the oil-phase volume fraction played a dominant role in determining the oxidative stability, increasing the oil content negatively affected the oxidative stability of emulsions. Sun and Gunasekaran [34] found that 40% (*v/v*) O/W emulsions had slightly higher peroxide values than 20% *v/v* O/W emulsions in the 0.2% WPI-stabilized emulsions. Gharibzahedi et al. [50], studying different concentrations of walnut oil, observed that the emulsions with high oil contents had the highest peroxide values.

#### 4. Conclusions

The results of the present study revealed the potential of the UHPH technology in the production of submicron emulsions with high physical and oxidative stability, including those containing large concentrations of oil (e.g., up to 50% oil).

Emulsions produced with a colloid mill were more prone to creaming and oxidation compared to those produced by conventional homogenization and UHPH. Increasing oil concentration in the former emulsions significantly decreased ( $p < 0.05$ ) droplet size and improved creaming stability. On the contrary, for conventional homogenization and UHPH emulsions, an increase in the droplet size was observed as oil content increased from 10%–30% and 50%. Furthermore, increases in oil concentration in these emulsions caused a change in the rheological behaviour from Newtonian to shear-thinning flow (with evidence of thixotropy). High oxidative stability was achieved with conventional homogenization and UHPH compared to colloid milling. UHPH emulsions with 30% oil treated at 100 MPa presented the best oxidative stability of all of the emulsions studied. Conventional homogenization resulted in higher levels of hydroperoxides than UHPH during storage, but similar levels of TBARS. The high physical and oxidative stability of UHPH-treated submicron emulsions suggests that UHPH might have a great potential in increasing the shelf-life of food products rich in polyunsaturated fatty acids.

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