Impact of Oil Phase Concentration on Physical and Oxidative Stability of Oil-In-Water Emulsions Stabilized by Sodium Caseinate and Ultra-High Pressure Homogenization

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ABSTRACT

In the present study, oil-in-water emulsions were formulated using 5.0% (w/v) of sodium caseinate (SC) and different oil concentrations (10-30 %, v/v) by conventional homogenization (CH) and ultra-high pressure homogenization (UHPH, 200-300 MPa). The effect of oil concentration and pressure of treatment on emulsions characteristics and stability was studied. Emulsions were characterized assessing their microstructure, droplet size distribution, rheological properties, emulsifying activity index, creaming stability by Turbiscan®, and photo-oxidation. UHPH emulsions, especially those treated at 200 MPa, showed smaller droplet size and greater physical stability than CH emulsions. In addition, emulsions containing higher oil volume fractions (20 and 30%) exhibited greater physical and oxidative stability. UHPH emulsions treated at 200 MPa and containing 20% oil content were the most stable emulsions against physical separation and photo-oxidation. These results show that UHPH is a potential technology to enhance the physical and oxidative stability of emulsions containing sodium caseinate as emulsifier for several applications.

Keywords: Submicron emulsions, ultra-high pressure homogenization, conventional homogenization, sodium caseinate, oil concentration.

1. Introduction

Emulsions form part of most commercial food products, including simple (e.g., milk) and sophisticated (e.g., mayonnaise) food systems. An emulsion is a mix of two non-miscible phases, which can be mixed by reducing droplet size using a proper emulsifier with the aid of a mechanical treatment such as homogenization.

In the last decade, there is a high interest in using emulsion-based systems for the delivery of bioactive compounds. Emulsions with large droplet size (i.e. conventional emulsions; >1 μm) have poor physical and oxidative stability when compared to submicron/nano emulsions [1]. Gravitational forces can be reduced when emulsion droplet size decreases, preventing flocculation, creaming or sedimentation [2].

The formation of sub-micron emulsions requires high-energy inputs. Current equipment used for emulsion preparation includes microfluidizers, sonicators or (ultra) high-pressure homogenizers [3] and conventional homogenizers [1]. Ultra-High Pressure Homogenization (UHPH) is a powerful technology that has been used to produce nano stable emulsions (< 1 μm) [1, 4-8]. In previous studies carried out in our laboratory [1, 6-8] using dairy proteins ingredients (sodium caseinate and whey protein isolate) and soy proteins, UHPH was capable of producing submicron emulsions with an improved physical and oxidative stability. Fernandez-Avila and Trujillo [9] applied UHPH (200 MPa) to obtain submicron emulsions enriched in conjugated linoleic acid (CLA, 6%, v/v) and stabilized by soy protein isolates (4%, w/v) to be incorporated into UHT milk. The authors reported that UHPH produced emulsions with low droplet size, high physical and oxidative stability during months and enhanced CLA delivery.

Subsequent to homogenization, the oil and water phases tend to separate. Proteins, when used as emulsifiers in the emulsion preparation, are adsorbed to the interface between oil

and water during homogenization, which reduces the interfacial tension between oil and water phases and prevents coalescence [10]. Proteins also play an important role as inhibitors of lipid oxidation [2]. Sodium caseinate (SC), a milk protein product, can protect oil droplets against coalescence through electrostatic and steric repulsion [11].

The choice of the oil concentration to be used in the emulsion formulation is critical as it has an eminent effect on emulsion structure and stability [12]. Different authors [4, 13, 14] studied physical stability of concentrated emulsions produced by UHPH. However, to the best of our knowledge, the effect of different oil concentrations on oxidative stability of emulsions prepared by UHPH and milk proteins has been only reported in a recently published work using whey protein isolate [8].

In a previous research [7], UHPH emulsions, in comparison to conventional homogenization, were screened (100-300 MPa) using SC at different protein levels (1 - 5%, w/v) using a mixture of sunflower and olive oils (20%, v/v). It was concluded that UHPH treatment (200 and 300 MPa) was capable of producing sodium caseinate (5%, w/v) emulsions with improved physical and oxidative stability. The objective of the present study is to characterize UHPH emulsions with different oil concentrations (10, 20 and 30%) emulsified by sodium caseinate (5%), in comparison to colloid mill and conventional homogenization.

Materials and Methods

Materials

Sodium caseinate was obtained from Zeus Quimica (Sodium Caseinate 110, Barcelona, Spain). The physico-chemical characteristics, as indicated by the producer were: moisture = 5.73%; granulometry (% < 300 mm) = 99.99; pH = 6.7; sediment at 70 °C (%) = 0.05;

minerals = 3.52%; MAT (N x 6.38) = 90%; fat = 1 %; density = 0.42. Refined sunflower and olive oils were purchased from Gustav Heess Company (Barcelona, Spain). The characteristics and composition of oils according to the producer are detailed in Hebishy et al. [8].

Preparation of Emulsions

Experimental Design

The effect of homogenization methods, pressure, and oil content on emulsion stability was studied using a completely randomized factorial design. Twelve formulations were produced and stored in glass bottles (4 °C) for physical analyses Oxidative stability was examined during 10-days storage period at 10 °C in samples stored under light (2000 lux/m^2).

Preparation of Protein Dispersions

Protein dispersions (5%, w/v; pH ≈ 6.5-7) were prepared in deionized water at 20 °C using a pilot-scale high speed (250 rpm) mechanical blender (Frigomat, Guardamiglio, Italy). The solutions were then placed at 4°C overnight to facilitate rehydration and equilibration of minerals.

Homogenization Treatments

After overnight rehydration, protein dispersions were equilibrated at 20 °C and mixed with the oil phase; sunflower and olive oil (3:1). Pre-emulsion (CM emulsion) was formed by mixing protein dispersion with oil using a colloid mill high-shear system (E. Bachiller B, S.A, Barcelona, Spain) during 5 min (5000 rpm).

CM emulsions were homogenized using APV Rannie Copenhagen Series Conventional Homogenizer (Model 40.120 H, single-stage hydraulic valve assembly, Copenhagen,

Denmark) at 15 MPa (CH emulsions).

UHPH emulsions were formed by passing CM emulsions through a Stansted high-pressure homogenizer with a flow rate of 120 L/h (Model/DRG number FPG 11,300:400 Hygienic Homogenizer, Stansted Fluid Power Ltd., Harlow, UK). Emulsions were cooled immediately after the HP-valve using two spiral-type heat exchangers (Garvía, Barcelona, Spain) in order to minimize temperature retention. Emulsions were UHPH-treated for single-stage at two different pressures (200 and 300 MPa) with an inlet temperature (Tin) of 25 °C. The inlet and outlet temperatures were monitored for the whole duration of the experiment.

The experiment was repeated three times.

Emulsion Measurements and Analyses

Droplet Size Distribution

Emulsions droplet size distribution was measured the same day of preparation, as described by Hebishy et al. [1] using a Beckman Coulter laser diffraction particle size analyser (LS 13 320 series, Beckman Coulter, Fullerton, CA, USA) by applying an optical model according to the Mie theory of light scattering. Emulsions were diluted in distilled water to get an appropriate obscuration. Samples were analysed at least four times and droplet size indices (d4.3 and d3.2, μm) and specific surface area (SSA, m^2/mL) were determined.

Rheological Measurements

Rheological measurements were performed in triplicate using a controlled stress rheometer (Haake Rheo Stress 1, Thermo Electron Corporation, Karlsruhe, Germany) with a parallel plate geometry [15] probe (1°, 60 mm diameter) at 25 °C. Before starting the experiment, the emulsion loaded to the rheometer was allowed to stand for 5 min in order to reach

equilibrium and to avoid any structure destruction. Ostwald de Waele rheological model: $\tau = K\dot{\gamma}^n$ was used to fit the flow curves, and the consistency coefficient (K, mPa × s) and flow behaviour index (n) were obtained.

Emulsifying properties

Emulsifying activity index (EAI) value was determined based on the method of Pearce and Kinsella. [16] with a minor modification. Briefly, aliquots (100 µl) of samples were diluted by 0.1% (w/v) SDS solution to give appropriate absorbance after which the absorbance was measured using a UV-visible spectrophotometer (CECIL model 9000 series, Cambridge, UK) at 500 nm. EAI value was calculated from the equation (Eq. 1) below as proposed by Cameron et al. [17].

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

where (DF) is the dilution factor (i.e. 250 times for CM emulsions and 2500 times for CH and UHPH emulsions), (A) is the spectorophotometric absorbance at 500 nm, (C) is the weight of protein per unit volume of aqueous phase before emulsion is formed (g/ml), (θ) is the oil volume fraction (0.1, 0.2 and 0.3 for 10, 20 and 30% oil, respectively), and (Ø) is the optical path (0.01m). Measurements were performed in triplicate after the same day of preparation.

Physical Stability

Emulsion stability was measured with a vertical scan analyser Turbiscan MA 2000 (Formulaction, Toulouse, France) with an electro-luminescent diode in the near infrared (λair = 850 nm), as reported by Hebishy et al. [1]. Turbiscan is a powerful technique that allows the optical characterization of dispersions, detecting variations in droplet size (i.e.,

flocculation, coalescence) or migration phenomena (i.e., creaming, sedimentation). Turbiscan measures the backscattered light at pre-set intervals (30 min for CM emulsions, 3 days for CH and UHPH emulsions) during the experiment (5 h for CM emulsions and 18 days for CH and UHPH emulsions). In order to follow the creaming kinetics, migration velocity (V; μm/min) was also calculated by Turbisoft software.

Creaming stability was also determined by measuring droplet size (d4.3) at the top or at the bottom of the emulsions stored for 9 days at room temperature, as reported by Hebishy [18].

Emulsion Microstructure

Microstructure of emulsions was performed using confocal laser scanning microscopy, as detailed by Hebishy [18]. The oil and protein were fluorescently stained with the fluorescent dyes, fluorescein isothiocyanate (FITC; Fluka, Steinheim, Germany) for protein, and Nile red (Sigma, Steinheim, Germany) for oil droplets. To assess changes in emulsion microstructure, micrographs were also obtained by using a transmission electron microscope with a Jeol 1400 (Jeol Ltd., Tokyo, Japan) equipped with a Gatan Ultrascan ES1000 CCD Camera, preparing samples according to Hebishy et al. [1].

Oxidative Stability of Emulsions

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× g for 2 min. The organic solvent phase (200 μL) was added to 2.8 mL of methanol/1-butanol (2:1, v/v), followed by 15 μL of 3.97 M ammonium thiocyanate and 15 μL of ferrous iron solution (prepared by mixing 0.132 M $BaCl_2$ and 0.144 M $FeSO_4$). The absorbance of the solution was measured at 510 nm, 20 min after the addition of the iron [19]. The hydroperoxide

concentration was determined using a Fe^{+3} standard curve with an iron concentration varying from 1 to 20 µg, as described by Shantha and Decker [19]. The peroxide value, expressed as milliequivalents of peroxide per kilogram of oil, was calculated using Eq. (2).

$$\text{Peroxide Value (PV)} = \frac{(As-Ab) \times m}{55.84 \times m_0 \times 2} \quad \text{Eq. (2)}$$

where As = absorbance of the sample, Ab = absorbance of the blank, m = slope of the calibration curve, m0 = mass (g) of the oil contained in mass of the emulsion used, 55.84 = atomic weight of iron. The result was divided by a factor of 2 to express the peroxide value as milliequivalents of peroxide instead of milliequivalents of oxygen.

For the determination of secondary oxidation products, thiobarbituric acid-reactive substances (TBARS) were determined according to an adapted method of McDonald and Hultin [20]. 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, 0.1 g of butylated hydroxy Toluene (BHT) and 82.8 mL of H_2O) 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 and presented as (µg malondialdehyde/mL).

Statistical Analyses

Statistical analyses were performed using SAS System® v9.2 (SAS Institute Inc., Cary, NC, USA) at 5% ($p < 0.05$) significance level and multiple comparisons of means using Tukey test. A general linear model with repeated measures was performed to compare between

samples. The rheological consistency coefficient (K value) was compared separately for the CH and UHPH treatments containing 30% oil content, due to the high variation in viscosity between CH and UHPH emulsions comparing to other treatments, which made it hard to detect statistical differences. A second comparison was needed for K value excluding the CH and UHPH treatments containing 30% oil content. Due to the high variation of data, d3.2, d4.3 and SSA values were compared only between CH and UHPH emulsions, excluding CM emulsions. However, emulsifying activity index (EAI), hydroperoxides and TBARS values were compared between the CM, CH and UHPH emulsions.

Results and Discussion

Temperature elevation during UHPH treatment

Temperatures of emulsions were monitored before (T1) and at the outlet (T2) of the high-pressure valve (Table 1). Very little and non-significant variations in temperature (T1) were noticed. On the other hand, results showed an increase in temperature (T2) of emulsions with different oil concentrations (10, 20 and 30%) by the rate of 21.19, 21.5 and 23.7 °C per 100 MPa (as pressure increased from 200 to 300 MPa). Similar increase (12-18 °C per 100 MPa) has been reported by previous studies [21-24] in high-pressure homogenized emulsions. This increase in the temperature could be due to the high velocity, shear, turbulence and cavitation forces at which the fluid exits the HP-valve, which may be turned into heat.

A marked increase in temperature (T2) was shown when the oil concentration increased. T2 increased by 0.459 and 0.585 °C per 1% oil content for emulsions treated at 200 and 300 MPa, respectively. However, this increase was only significant when oil concentration increased to 30% and not to 20% ($P < 0.05$). Hayes and Kelly [22] reported that milk (0-10% fat) outlet temperature increased (0.5 °C / 1% fat) as milk fat content increased in samples

homogenized at 150 MPa. This could be a direct result of viscous dissipation or the increased number of oil droplets, which increases collision between droplets. Another explanation could be the high fluid compression in the intensifier during the pressure built up as the oil content increased from 10 to 30%. This is due to higher heat of compression for oil comparing to water [4].

Droplet size distribution

Table 2 and Figure 1 (A-C) show the mean droplet sizes (d3.2 and d4.3) and specific surface area (SSA, m^2/ml) of SC emulsions containing different oil contents.

CM treatment resulted in emulsions with largest droplet size (average of d4.3 value ~ 15 µm) comparing with CH and UHPH treatments (average of d4.3 value ~ 1.12 and 0.123 µm, respectively). In CM emulsions, droplets tend to coalesce after homogenization (Fig. 2 A (A)), as a result of high droplet sizes obtained in this type of equipment, as the energy input is not as high as pressure homogenizers (the more the energy input, the more the interfacial area that can be created) [3]. Droplets with larger sizes would cream more rapidly, coming close to each other in the cream layer, thereby promoting membranes disruption [25]. Low protein coverage (Fig. 2 A (B)) and high interfacial tension could be another reason for the high coalescence rate in CM emulsions. Droplet size (d3.2) of CM emulsions has been influenced by varying the oil content, as can be seen in Table 1.

CH emulsions containing 10% oil showed larger droplet size which was significantly decreased when oil concentration increased to 20% after which the decrease was not significant. Droplet size distribution curves (Fig. 1 A-C) show that CH emulsions with 10% oil had a bimodal distribution with a first population of droplets at ~ 0.5 µm and a second population of droplets at ~ 2 µm. On the other hand, in emulsions containing 30% oil, the

first and second population of droplets were decreased to ~ 0.1 and 1 µm, respectively. CLSM images (Fig. 3 (D-F)) have shown a high degree of flocculation in all CH emulsions. This could be attributed to poor protein coverage in these emulsions [26]. These results are not in agreement with other research studies that had been done in our lab under the same conditions of pressure levels and oil concentrations, but using isolates of whey and soy proteins [5, 8] at a lower protein concentration (4%, w/v). This increment in biopolymer concentration in the aqueous phase to 5% (w/v) in the present study might have promoted depletion flocculation where droplet aggregation is promoted by the non-adsorbed protein remaining in the aqueous phase.

UHPH emulsions slightly showed signs of flocculation and coalescence (Fig. 2 B (D-I)), which was more pronounced in emulsions containing 10% oil, which may explain the high creaming rate observed in these emulsions (Physical Stability section).

Rheological behavior

Rheological behavior of emulsions (consistency coefficient (K) value and the flow behavior index (n)) is presented in Table 3.

CM emulsions showed low viscosities and Newtonian flow behavior due to low interaction between droplets. Increasing the oil concentration from 10 to 20 and 30% had a significant effect on viscosity of CM emulsions.

CH emulsions exhibited a shear thinning behavior (viscosity decreases on shearing during the test due to deformation and breakdown of aggregates) with a flow behavior index below 1 which was accompanied by a significant increment in viscosity with increased oil concentration from 10 to 30%. Although no change was observed in the flow behavior index when the oil concentration increased from 10 to 20%, this change became significant when

the oil concentration further increased to 30%. Increasing oil concentration increased emulsion viscosity as previously reported [27, 28]. Mewis and Wagner [29] attributed this viscosity increase to the strong inter-droplet interactions.

Applying UHPH homogenization pressures (200 and 300 MPa) at 10 and 20% oil concentration resulted in emulsions with similar viscosities to the CH emulsions, however viscosity increased dramatically when the same pressure was applied to emulsions containing 30% oil with a complete change of the behavior to shear thinning. Floury et al. [13] reported a change in flow behavior of UHPH emulsions (1.5% whey protein) from highly fluid to highly thick with varying oil volume fractions (10-50%). Similar trend was found in our recent published work [8] using whey protein isolate to produce emulsions with oil concentrations between 10-50% (v/v) under homogenization pressures (100-200 MPa). It was reported that viscosity had increased and flow behavior changed from Newtonian to shear-thinning when oil content increased from 10 to 50% in emulsions treated at 200 MPa. This increase in viscosity was more pronounced in emulsions containing 50% oil than those containing 30% oil. What distinguishes the latterly mentioned study using whey proteins from the present study is that it was not possible to produce SC emulsions containing 50% oil, as the emulsions completely gelled giving a mayonnaise-like structure (data not shown). Considerable increase in viscosity and change in flow behavior has been also reported in emulsions produced by the UHPH technology [4] using 4% whey protein isolate and 15-45% oil content and [14] using micellar casein at 2-3.5% and oil content of 10-30%.

Emulsifying activity index (EAI)

Emulsifying property refers to the stable interface area per unit weight of protein, which represents the capability of proteins to adsorb at the oil-water interface. CM emulsions presented low EAI values. Applying low-pressure (CH treatment) increased significantly the

EAI; however, applying ultra high-pressures (200 and 300 MPa) resulted in lower EAI values (Table 3). Fernández-Ávila and Trujillo [6] also reported higher EAI values for emulsions treated by CH than UHPH treatment, which was attributed to the increase in surface area created during emulsification per unit mass in UHPH emulsions.

In our previous study [8] under the same conditions of CM, CH and UHPH but using whey protein isolate as emulsifier, it was reported that protein load (mg/m^2) on the surface of the oil droplets was lower than CM and CH emulsions. However, the authors reported that when 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) was much higher in UHPH emulsions (41 and 53.51 mg/mL at 100 and 200 MPa, respectively) than in CM and CH emulsions (23.30 and 25.80 mg/mL, respectively). This was attributed to the increased spreading and rearrangement of adsorbed protein molecules at the interface. What can be concluded is that, taking into consideration the SSA, UHPH treatment improved the emulsifying activity of SC.

Cha et al. [30] reported an increase in the EAI in emulsions produced using myofibrillar proteins and lecithin as emulsifiers and high pressure homogenization at pressures ranging between 40 and 120 MPa, using emulsions produced by ultraturrax as a control. The elevated EAI was attributed by the authors to exposed hydrophobic groups, which enhanced the interactions between proteins and lipids and increased solubility which promoted proteins to diffuse at oil–water interface, thus improving the emulsifying properties.

CH emulsions presented higher EAI with an increase being significant when the oil concentration increased from 10 to 20% however; this increase was not statistically significant when oil concentration further increased to 30%. The EAI results correlated with

the droplet size and SSA results, presenting same trend. The EAI results were also in line with the TEM (Fig. 2 A) images. In this sense, the emulsions containing 10% oil presented a poor surface coverage (Fig. 2 A (C)), while emulsions with 30% oil presented oil droplets with high surface protein covering the droplets (Fig. 2 A (D)).

Table 3 shows a significant increase in EAI value in UHPH emulsions with increasing the oil concentration from 10 to 20% oil ($P < 0.05$). Fernández-Ávila and Trujillo [6] also reported similar results when oil content increased from 10 to 20% in UHPH emulsions stabilized by soy proteins. However, in our study, no further significant effect on the EAI was observed when oil concentration increased to 30%. This may indicate that the amount of SC started to become limited to cover the newly created O/W interface. Increasing the oil concentration, with a fixed protein amount, reduces the protein at the interface, thus suggesting the spreading of protein at an interface to form a thinner layer [31]. A similar trend was observed in emulsions stabilized by bovine serum albumin [32] when the oil volume fraction increased from 25 to 56%.

Physical stability of emulsions

Figure 4 (A–F) shows the backscattering (BS) profiles for all emulsions containing 5% of SC prepared with CM, CH and UHPH at 200 MPa. UHPH emulsions have shown longer stability (Fig. 4 E,F) as compared to CM (Fig. 4 A,B) and CH emulsions (Fig. 4 C,D). For instance, the same extent of creaming appears about 17 days after UHPH treatment at 200 MPa vs. 2 days after conventional homogenization (CH) and 5 hours after colloid mill (CM). Backscattering results have shown a drop of BS at the bottom of all samples, due to clarification of the mixture in the following order: CM > CH > UHPH emulsions. On the other hand, there was an increase in BS at the top of samples, associated to creaming (particle migration) with a creaming rate in the following order: CM > CH > UHPH emulsions.

Physical stability was also assessed in the emulsions, measuring the d4.3 value at the top or at the bottom of the emulsion tubes stored at room temperature for 9 days and under the same conditions for comparison. Physical stability was determined in the homogenized emulsions (conventional and UHPH), but not in the CM emulsions where oily or creamy phases were clearly separated from the aqueous phases 2 hours after preparation.

CM emulsions containing the lowest oil concentration (10%) showed the highest creaming rate (Fig. 4 A). However, increasing the oil concentration improved creaming stability (Fig. 4 B). The explanation for this low creaming stability of CM emulsions containing 10% oil could be the large droplet size (Table 2) and the high probability of coalescence, as discussed before in the Particle Size Distribution section.

CH emulsions had higher creaming stability than CM emulsions; however, they were not as stable as UHPH emulsions (Table 3 and Fig. 4 C-F). Oil-phase concentration played an important role in the creaming stability of CH emulsions (higher oil concentration slowed down the creaming rate). Even d4.3 value obtained at the top or the bottom of the CH emulsions (Table 3) showed significant differences after 9 days of storage during 9 days, regardless of the oil concentration, Figure (4 D) shows clearly the slow change of backscattering in CH emulsions containing 30% oil in comparison to their counterpart of emulsions containing 10% oil (Fig. 4 C). This could be due to the increase in packing fraction of oil droplets [33], which enhanced emulsion viscosity and lowered the creaming rate. High creaming stability with increasing oil content was also reported in CH emulsions stabilized by whey protein isolate [8] when oil content increased from 10 to 30 and 50%, and in non-heated soy protein isolate (SPI) [5] when soybean oil content increased from 10 to 20%, owing to high consistency. The later study reported that these emulsions also exhibited greater thickness of SPI at the droplets surface and the absence of clusters of protein

aggregates. Higher oil content results in multiplied number of droplets [34], improving the resistance of emulsions to flow, and increasing the apparent viscosity [35].

UHPH emulsions displayed better creaming stability; the emulsions remained turbid with no visual separation during 18 days (Fig. 4 E,F) comparing to CM (Fig. 4 A,B) and CH (Fig. 4 C,D) emulsions. High-pressure homogenization reduces droplet size resulting in emulsions that are, according to Stokes law, higher stable towards creaming [36]. On the other hand, smaller size and the rigid interfacial layers, as a result strong interactions between adsorbed proteins at the interface due to the unfolding and exposure of hydrophobic sites of proteins, increase emulsion density, embedding droplets migration. San Martín-González et al. [14] observed that high-pressure homogenization (300 MPa), regardless of oil and casein concentration, reduced creaming index to zero during 10 days of storage. The authors attributed this high stability to increased availability of caseins due to extensive disruption.

Although the changes in d4.3 value between top and bottom of emulsions with 10% oil showed no significant differences, Turbiscan was able to detect such slight creaming in emulsions with 10% oil (Fig. 4 E) comparing to no creaming in those containing 30% oil (Fig. 4 F). This may be attributed to large droplet size in these emulsions due the flocculation or coalescence observed, as explained in the Droplet Size Distribution section. These results are in line with what was reported in a previous study [5], UHPH emulsions showed no creaming after more than 5 months of cold storage.

Oxidative stability

Table 4 shows the hydroperoxide and TBARS (µg malondialdehyde/mL) contents of CM, CH and UHPH emulsions stabilized by SC using different oil concentrations.

CM emulsions presented generally higher hydroperoxides and TBARS values than other

emulsions especially those containing 10% and 30% oil content. There were no significant differences for hydroperoxides at day 1 between CM, CH and UHPH emulsions. The high hydroperoxide and TBARS indicates the progression to a secondary state of oxidation in these emulsions. This high sensitivity of CM emulsions to oxidation may be attributed to exposure of oil droplets to the oxidation factors due to poor protein coverage at the interface and the high coalescence rate between oil droplets (Fig. 2 A (A)). Similar trend was also observed in our previous study [8] using whey protein isolate. Oil concentration significantly affected the oxidative stability of CM emulsions. As can be seen from Table 4, all emulsions presented similar level of hydroperoxides and TBARS contents at day 1 of storage, except for significant amount in emulsions containing 10% oil. As the storage time progressed to 10 days, emulsions containing 10% oil presented the highest hydroperoxide content. Emulsions containing 20% oil showed the lowest amount of TBARS after 10 days, contrary to emulsions containing 10 and 30 %.

CH emulsions containing 10 and 20% oil presented lower amount of hydroperoxides which has significantly increased after 10 days of storage, being higher in hydroperoxides in emulsions containing 20% oil. On the other hand, the TBARS content has been decreased or maintained the same in these samples after 10 days of storage with no significant differences (day 10 – day 1). No significant changes were found in hydroperoxide content of CH emulsions at first or last day of storage. There was an increase in TBARS levels in emulsions containing 30% oil, unlike emulsions containing 10 and 20% oil, being significant when comparing to emulsions with 10% oil. Therefore, it can be concluded that increasing the oil content in CH emulsion systems more than 20% oil may facilitate lipid oxidation and bring it from primary to secondary oxidation.

UHPH emulsions showed no differences in hydroperoxides neither at first nor last day of

storage. Lower oxidative stability was observed in emulsions containing 10% oil; however, UHPH emulsions (20% oil) showed the best oxidative stability; the increase in TBARS content was not significant after 10 days of storage, it had even decreased significantly in emulsions containing 20% oil and treated at 200 MPa. This may indicate the sensitivity of emulsions containing 10% oil to oxidation. Results obtained by Fernández Ávila and Trujillo [6] indicated more protein coverage at the interface of CH and UHPH emulsions stabilized with non-heated SPI containing 20% (v/v) oil than those containing 10% (v/v) oil. The possible reasons for the high oxidation rate in emulsions with low oil content (10%), especially those treated at 300 MPa, could be the following: 1) the creaming observed in these emulsions, which makes the lipids closer to the ambient and favors oxidation [28]; 2) the increase in the amount of free radicals as a reason of the proportional increase in the aqueous phase fraction, as well as the water soluble prooxidants [37]; 3) the low viscosity of these emulsions, in comparison to emulsions with high oil content (30%). It has been proposed that elevated viscosity can affect oxidation by reducing the diffusion of potential pro-oxidative molecules, such as ferrous ions or lipid hydroperoxides [38-40].

Improved oxidative stability was found by other researchers when oil volume fraction increased from 10 to 20% [5, 6] 5 to 40% [28], or from 5 to 30% [37]. In a recent study [8], we reported that increasing the oil content in UHPH emulsions stabilized by whey proteins from 10 to 30% oil resulted in improved oxidative stability, which is in line with what has been found in the present study. However, additional increase in oil concentration to 50% caused poor emulsion stability to oxidation.

Conclusion

Ultra high pressure homogenization technology is capable of producing submicron emulsions with up to 30% (v/v) oil content using SC (5%, w/v) as emulsifier with a high physical and

oxidative stability compared to conventional treatments. Using high oil concentrations (20 and 30%) enhanced physical and creaming stability of all emulsions. Oxidative stability is oil concentration and homogenization treatment dependent. While increasing oil concentration, especially in emulsions containing 20% oil, produced the most stable emulsions in case of CM and UHPH emulsions, increasing oil concentration to 30% adversely affected lipid oxidation of CH emulsions during storage. To sum up, findings of the present study suggest the advantages of using UHPH technology to produce submicron emulsions with high physical and oxidative stability which might be used as carriers for bioactive ingredients with high sensitivity to oxidation.

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Table 1. Mean ± SD values of temperature measured before (T1) and at the outlet (T2) of the high-pressure valve for emulsions containing different oil concentrations (10, 20 and 30%) treated by ultra high-pressure homogenization at 200 and 300 MPa (Tin = 25°C).

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

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

Pressure (MPa)	Oil content (%)	Particle size distribution		
		d3.2 (µm)	d4.3 (µm)	Specific surface area SSA (m^2/ml)
CM	10	6.358 ± 0.643^{a}	18.06 ± 4.194^{a}	0.915 ± 0.154^{a}
	20	5.410 ± 0.303^{ab}	13.40 ± 2.776^{a}	1.117 ± 0.068^{a}
	30	5.232 ± 0.417^{b}	12.73 ± 2.693^{a}	1.152 ± 0.091^{a}
CH	10	0.614 ± 0.042^{c}	1.315 ± 0.234^{b}	9.841 ± 0.617^{a}
	20	0.521 ± 0.036^{c}	0.961 ± 0.122^{c}	11.56 ± 0.825^{b}
	30	0.547 ± 0.106^{c}	1.076 ± 0.104^{b}	11.40 ± 2.376^{b}
UHPH (200MPa)	10	0.110 ± 0.007^{d}	0.131 ± 0.009^{d}	54.91 ± 3.15^{c}
	20	0.102 ± 0.004^{d}	0.126 ± 0.005^{d}	59.21 ± 1.80^{c}
	30	0.108 ± 0.008^{d}	0.130 ± 0.010^{d}	55.70 ± 4.060^{c}
UHPH (300MPa)	10	0.093 ± 0.007^{d}	0.111 ± 0.006^{d}	65.16 ± 4.10^{c}
	20	0.105 ± 0.014^{d}	0.119 ± 0.007^{d}	57.06 ± 6.991^{c}
	30	0.103 ± 0.014^{d}	0.121 ± 0.017^{d}	59.48 ± 7.992^{c}

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

Table 3. Mean ± SD of rheological characteristics (flow and consistency indices), emulsifying activity index (EAI, m^2/g) and creaming stability (d4,3 values at the top or at the bottom of samples stored at room temperature for 9 days under the same conditions for comparison) of emulsions containing sunflower and olive oils (10, 20 and 30%) and prepared by colloidal mill (CM), conventional homogenization (CH, 15 MPa) and ultra high-pressure homogenization at 200 and 300 MPa with 5% of sodium caseinate.

Treatment	Oil content (%)	Rheological behavior & emulsifying activity			Emulsions creaming stability after 9 days		
		Consistency coefficient (K, mPa × s)	Flow behavior index (n)	Emulsifying activity index (EAI,m^2/g)	D4.3 (µm) (TOP)	D4.3 (µm) (BOTTOM)	*P value*
CM	**10**	0.005 ± 0.001^{h}	0.988 ± 0.009^{a}	6.76 ± 0.37^{f}	ND	ND	ND
	20	0.012 ± 0.001^{g}	0.986 ± 0.025^{a}	15.50 ± 4.00^{e}			
	30	0.024 ± 0.002^{f}	1.003 ± 0.008^{a}	28.20 ± 6.18^{cd}			
CH	**10**	0.010 ± 0.002^{g}	0.858 ± 0.019^{ab}	55.00 ± 4.36^{b}	1.07 ± 0.10^{a}	0.41 ± 0.26^{a}	**0.0022****
	20	0.044 ± 0.012^{de}	0.754 ± 0.038^{bc}	133.14 ± 17.81^{a}	1.11 ± 0.27^{a}	0.27 ± 0.12^{a}	**0.0022****
	30	$0.209 \pm 0.104^{C*}$	0.608 ± 0.068^{c}	217.41 ± 10.00^{a}	1.14 ± 0.23^{a}	0.38 ± 0.19^{a}	**0.0022****
UHPH (200MPa)	**10**	0.005 ± 0.001^{h}	0.998 ± 0.017^{a}	8.93 ± 1.40^{f}	0.12 ± 0.01^{b}	0.12 ± 0.01^{a}	0.9654
	20	0.038 ± 0.009^{e}	0.885 ± 0.089^{ab}	26.90 ± 1.61^{cd}	0.14 ± 0.02^{b}	0.14 ± 0.02^{a}	0.9740
	30	$1.937 \pm 0.148^{B*}$	0.339 ± 0.052^{d}	38.76 ± 4.41^{bc}	0.12 ± 0.01^{b}	0.12 ± 0.01^{a}	0.8442
UHPH (300MPa)	**10**	0.005 ± 0.001^{h}	1.011 ± 0.008^{a}	6.49 ± 0.98^{f}	0.10 ± 0.01^{b}	0.11 ± 0.01^{a}	0.3745
	20	0.049 ± 0.009^{d}	0.850 ± 0.044^{ab}	20.55 ± 3.47^{de}	0.12 ± 0.02^{b}	0.12 ± 0.02^{a}	0.7338
	30	$4.283 \pm 1.022^{A*}$	0.252 ± 0.039^{d}	27.31 ± 5.42^{cd}	0.11 ± 0.01^{b}	0.11 ± 0.01^{a}	0.8593

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

*A-C Different capital letters in the same column indicate significant differences ($P < 0.05$) between CH and UHPH emulsions with 30% oil content. This group of samples were compared separately from rest of the samples due to the high variation in viscosity.

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

* ND means not determined

572 Table 4. Mean ± SD of hydroperoxides (milliequivalents /kg) and TBA reactive substances (µg malondialdehyde/mL) of O/W emulsions
573 containing sunflower and olive oils (10, 20 and 30%) and prepared by colloidal mill (CM), conventional homogenization (CH, 15 MPa) and
574 ultra high-pressure homogenization (UHPH) at 200 and 300 MPa with 5% of sodium caseinate.

Pressure (MPa)	Oil content (%)	Hydroperoxides (Milliequivalents /kg)			TBARS (µg Malondialdehyde/mL)		
		Day 1	Day 10	Diference (Day 10 – Day 1)	Day 1	Day 10	Diference (Day 10 – Day 1)
CM	10	0.482 ± 0.297^{a}	2.322 ± 0.218^{a}	1.840 ± 0.079^{a}	0.217 ± 0.054^{a}	0.239 ± 0.055^{a}	0.022 ± 0.017^{c}
	20	0.421 ± 0.305^{a}	0.833 ± 0.417^{b}	0.412 ± 0.112^{bc}	0.107 ± 0.008^{cd}	0.146 ± 0.008^{bcde}	0.039 ± 0.014^{bc}
	30	0.197 ± 0.087^{a}	0.578 ± 0.112^{bc}	0.381 ± 0.025^{bc}	0.155 ± 0.010^{abc}	0.218 ± 0.019^{ab}	0.062 ± 0.013^{ab}
CH	10	0.320 ± 0.033^{a}	0.542 ± 0.189^{bc}	0.222 ± 0.156^{bc}	0.174 ± 0.006^{abc}	0.144 ± 0.003^{bcde}	– 0.030 ± 0.005^{e}
	20	0.198 ± 0.062^{a}	0.703 ± 0.077^{b}	0.505 ± 0.016^{b}	0.065 ± 0.003^{d}	0.070 ± 0.002^{e}	0.005 ± 0.004^{cde}
	30	0.603 ± 0.399^{a}	0.493 ± 0.334^{bc}	– 0.109 ± 0.099^{bc}	0.154 ± 0.012^{abc}	0.172 ± 0.008^{abc}	0.017 ± 0.008^{cd}
UHPH 200MPa	10	0.655 ± 0.514^{a}	0.248 ± 0.065^{bc}	– 0.406 ± 0.449^{c}	0.141 ± 0.005^{bc}	0.182 ± 0.009^{abc}	0.040 ± 0.012^{bc}
	20	0.280 ± 0.212^{a}	0.181 ± 0.037^{bc}	– 0.099 ± 0.175^{bc}	0.107 ± 0.004^{cd}	0.091 ± 0.004^{de}	– 0.015 ± 0.008^{de}
	30	0.237 ± 0.075^{a}	0.106 ± 0.073^{bc}	– 0.131 ± 0.013^{bc}	0.187 ± 0.008^{ab}	0.200 ± 0.011^{ab}	0.013 ± 0.016^{cd}
UHPH 300MPa	10	0.602 ± 0.108^{a}	0.225 ± 0.099^{bc}	– 0.377 ± 0.062^{bc}	0.148 ± 0.008^{abc}	0.239 ± 0.014^{a}	0.091 ± 0.020^{a}
	20	0.032 ± 0.003^{a}	0.023 ± 0.004^{c}	– 0.008 ± 0.001^{bc}	0.108 ± 0.013^{cd}	0.114 ± 0.011^{cde}	0.005 ± 0.013^{cde}
	30	0.108 ± 0.025^{a}	0.097 ± 0.031^{bc}	– 0.011 ± 0.040^{bc}	0.132 ± 0.011^{bcd}	0.155 ± 0.010^{bcd}	0.023 ± 0.005^{c}

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

576
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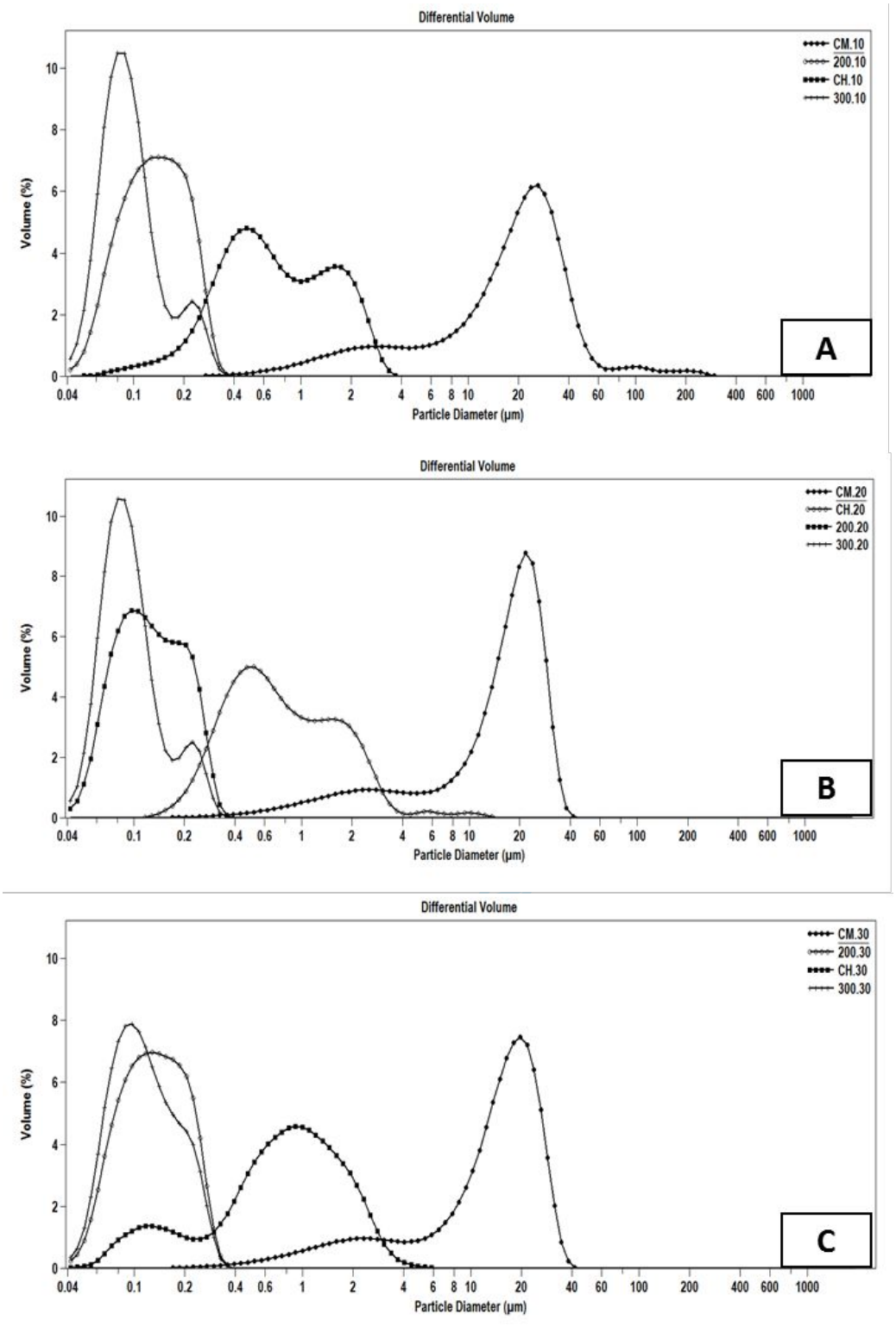
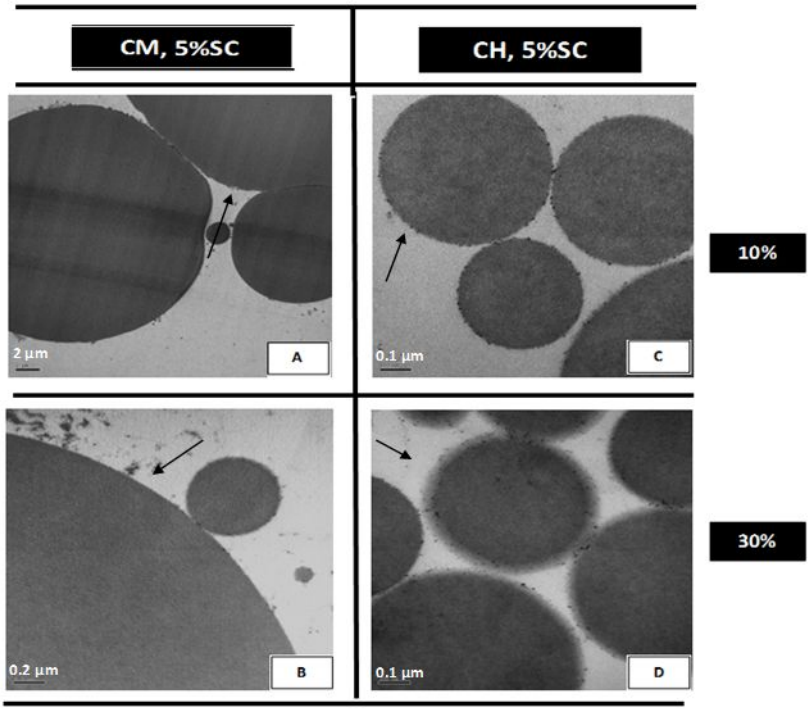


Figure 1.

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

(A)


CM, 5%SC
CH, 5%SC
10%
30%
2 µm
A
0.1 µm
C
0.2 µm
B
0.1 µm
D

(B)

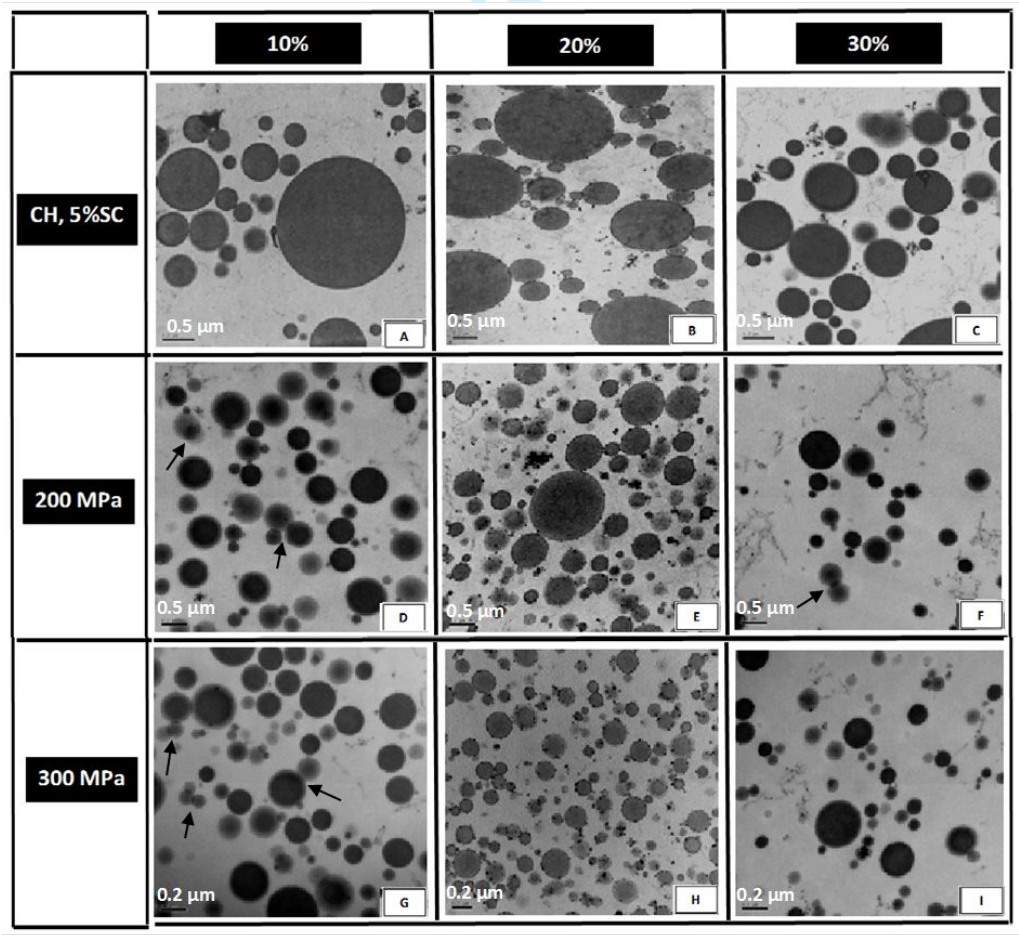

10%
20%
30%
CH, 5%SC
200 MPa
300 MPa
0.5 µm
A
0.5 µm
B
0.5 µm
C
0.5 µm
D
0.5 µm
E
0.5 µm
F
0.2 µm
G
0.2 µm
H
0.2 µm
I

Figure 2.

(A) TEM images of O/W emulsions containing sunflower and olive oils at 10% (A,C) and 30% (B,D) and prepared by (A,B; ×4000 and ×50000, respectively) colloidal mill (CM), and by (C,D; ×100000) conventional homogenization (15 MPa) with 5% of sodium caseinate. Arrows indicate the coalescence between droplets in image (A) and difference in the protein amounts on the interface of oil droplets in images (B, C and D).
(B) TEM images (×50000) of O/W emulsions containing sunflower and olive oils (10, 20 and 30%) and 5% of sodium caseinate, prepared by (A-C) conventional homogenization (15 MPa) and (D-I) by ultra high-pressure homogenization at 200 MPa (D-F) and 300 MPa (G-I). Arrows indicate flocculation and coalescence between oil droplets.

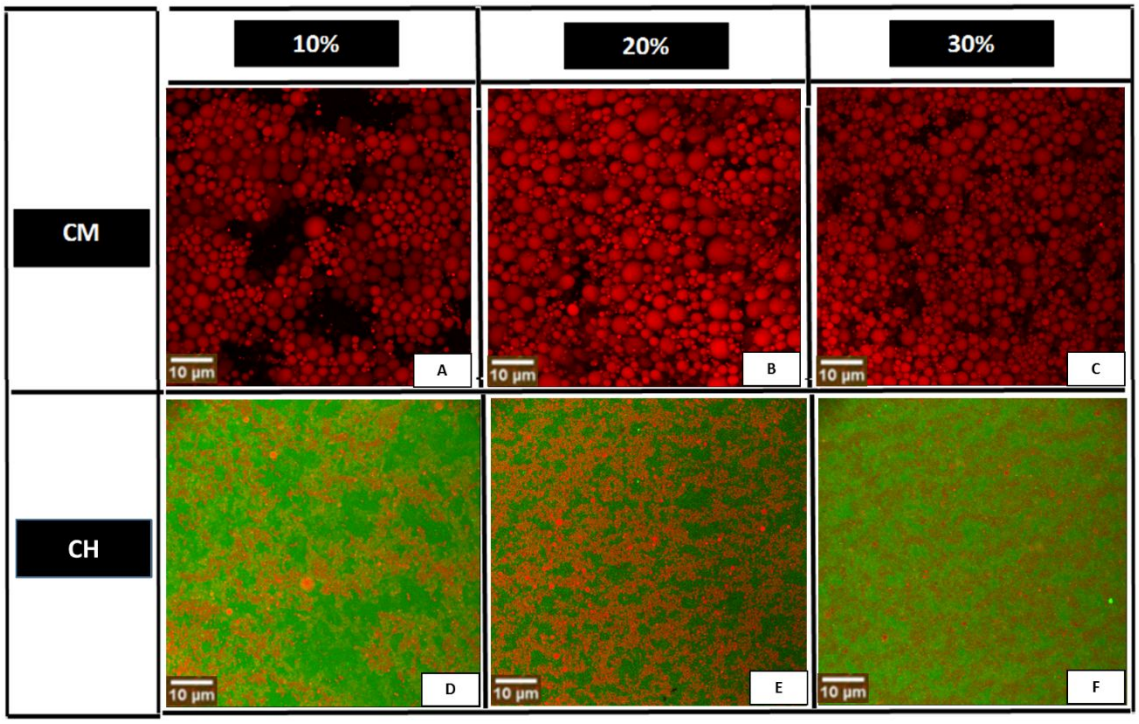


Figure 3.
Confocal laser scanning microscope images of O/W emulsions containing sunflower and olive oils (10, 20 and 30%) and 5% of sodium caseinate, and prepared by (A-C) colloidal mill (CM) and (D-F) conventional homogenization (15 MPa).

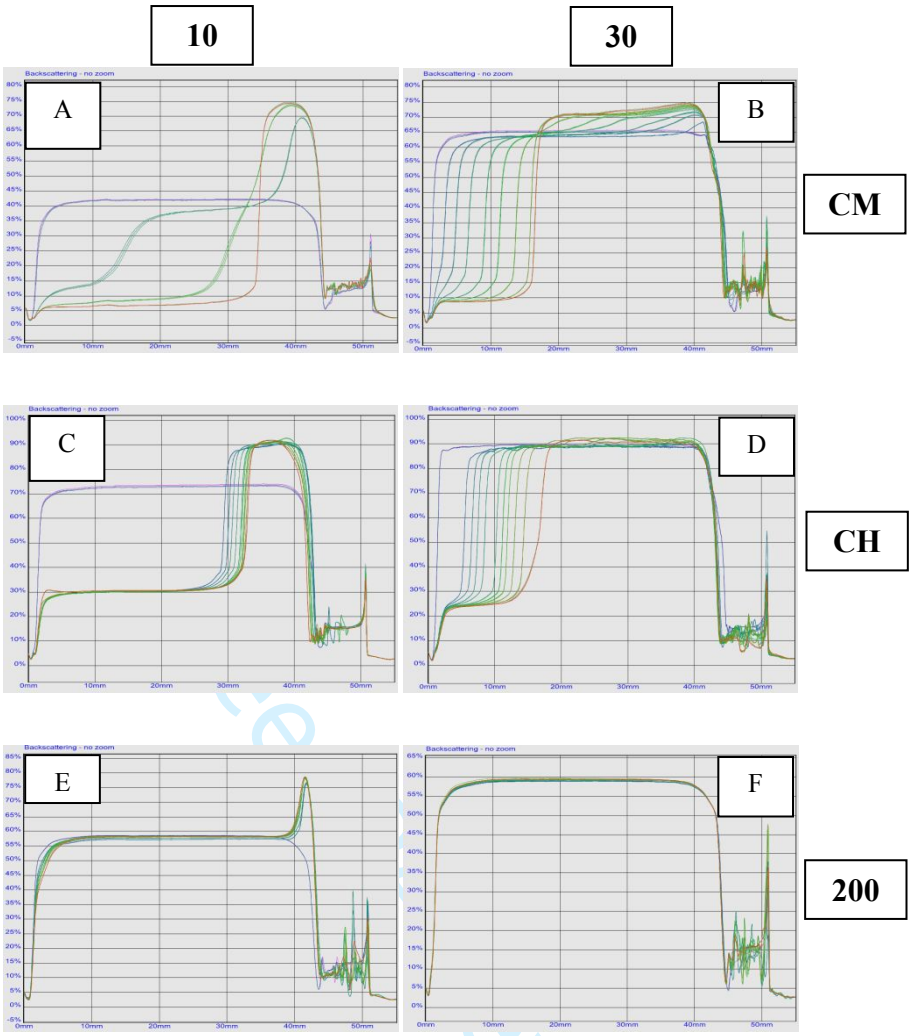
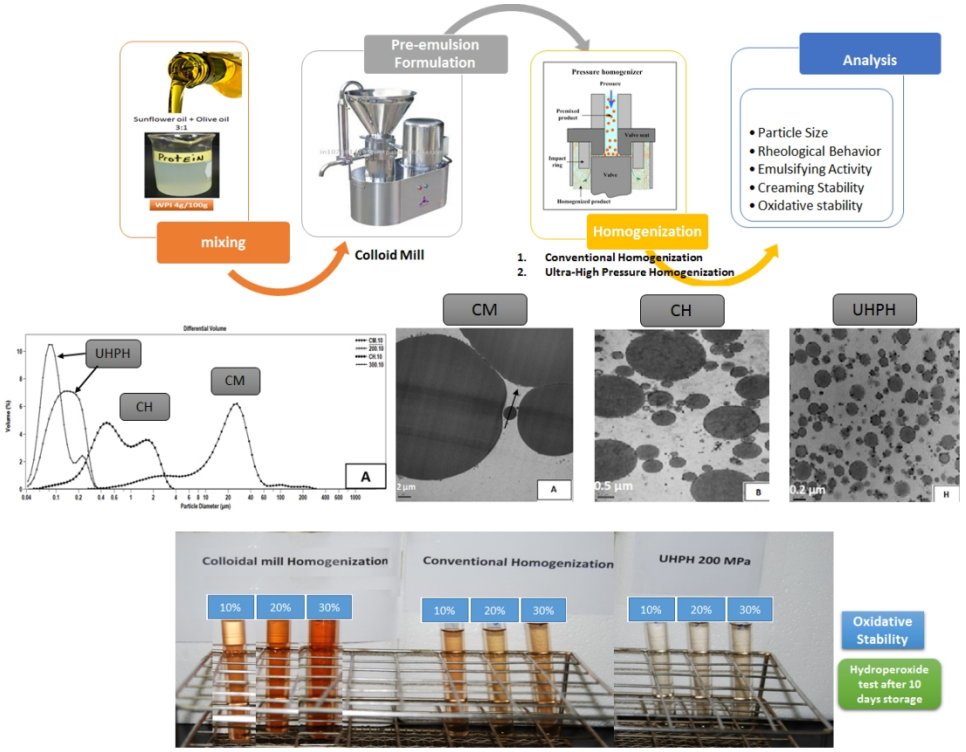


Figure 4. Changes in backscattering profiles of O/W emulsions containing sunflower and olive oils (10 and 30%) and 5% of sodium caseinate and prepared by (A,B) colloidal mill (CM), conventional homogenization (15 MPa) (C,D), and by ultra high-pressure homogenization (UHPH) at 200 (E,F), as a function of sample height with storage time (5 h for CM emulsions and 18 days for both CH and UHPH emulsions).


Sunflower oil + Olive oil
3:1
Protein
WPI 4g/100g
mixing
Pre-emulsion Formulation
Colloid Mill
Pressure homogenizer
Homogenization
1. Conventional Homogenization
2. Ultra-High Pressure Homogenization
Analysis
• Particle Size
• Rheological Behavior
• Emulsifying Activity
• Creaming Stability
• Oxidative stability
UHPH
CH
CM
A
CM
CH
UHPH
2 µm
0.5 µm
0.2 µm
Colloidal mill Homogenization
Conventional Homogenization
UHPH 200 MPa
10%
20%
30%
Oxidative Stability
Hydroperoxide test after 10 days storage