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Ultra High-Pressure Homogenized Emulsions Stabilized by Sodium Caseinate: Effects of Protein Concentration and Pressure on Emulsions Structure and Stability

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Abstract

Microstructure, physical properties and oxidative stability of emulsions treated by colloid mill (CM), conventional homogenization (CH, 15 MPa) and ultra-high-pressure homogenization (UHPH, 100-300 MPa) by using different concentrations of 1, 3 and 5 g/100 g of sodium caseinate (SC), were evaluated. The application of UHPH treatment at 200 and 300 MPa resulted in emulsions that were highly stable to creaming and oxidation, especially when the protein content increased from 1 to 3 and 5 g/100 g. Further, increasing the protein content to 3 and 5 g/100 g in UHPH emulsions tended to change the rheological behaviour from Newtonian to shear thinning. CH emulsions containing 1 g/100 g of protein exhibited Newtonian flow behaviour with lower tendencies to creaming compared to those formulated with 3 or 5 g/100 g. This study has proved that UHPH processing at pressures (200-300 MPa) and in the presence of sufficient amount of sodium caseinate (5 g/100 g), produces emulsions with oil droplets in nano-/submicron scale with a narrow size distribution and high physical and oxidative stabilities, compared to CM and CH treatments.

Keywords: Ultra High-Pressure Homogenization (UHPH), sodium caseinate, submicron emulsions, physical and oxidative stabilities.

1. Introduction

Nano/submicrom emulsions are systems with particle size between 20-500 nm (Huang, Yu, & Ru, 2010). High energy input is needed to prepare emulsions with droplet sizes in the submicron range that is generally achieved by high shear stirring, high-pressure homogenizers or by ultrasound generators (Weiss, Takhistov, & McClements, 2006). Ultra high-pressure homogenization (UHPH) is a non thermal technology that recently

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has been studied in the pharmaceutical, food and cosmetic areas to produce fine and stable emulsions. Ultra high-pressure homogenizers of piston-gap type developed by manufacturers such as Avestin™, APV™, Stansted Fluid Power™ and more recently Ypsicon™ consist of one or two piston intensifier(s) capable of creating high pressures (up to 400 MPa), and high-pressure valve rigged with ceramic needles and seat of uniquely studied design. The fluid is subjected during the homogenization process to various concurrent force-induced phenomena such as cavitation, turbulence, shear, friction, heat, compression, acceleration, rapid pressure drop, and impact (Floury, Desrumaux, & Lardieres, 2000).

Droplet-droplet collisions happen much of the time during mechanical shearing and homogenization as a result of the intensive mechanical agitation of the emulsion. To keep coalescence from occurring, it is vital an adequately thick emulsifier layer to be formed around a droplet before it has time to collide with its neighbors (McClements, 2005). Proteins are broadly utilized as emulsifiers as a reason of their amphiphilic nature and their ability to be adsorbed at the oil-in-water interface. Milk proteins, for example, sodium caseinate (SC) can protect oil droplets against coalescence through electrostatic and steric repulsion (Dickinson, 1999). Although a great deal of research has been emphasised on the physical stability and interfacial properties of protein-stabilized O/W submicron-emulsions produced by high homogenization pressures (up to 300 MPa) (Floury, Desrumaux, Axelos, & Legrand, 2003; San Martín-González, Roach, & Harte, 2009; Perrechil & Cunha, 2010), only few studies have been focused on the oxidative stability of these emulsions. However, these studies included globular proteins i.e. whey proteins (Hebishy et al., 2015) or soy proteins (Fernandez-Avila and Trujillo, 2016) as emulsifiers. Sodium caseinate has a specific nature different from the globular proteins which may make the UHPH-emulsions produced from it to behave

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differently regarding oxidation. Nevertheless, there is a lack of literature evidence regarding any association of this technology (up to 300 MPa) with oxidative stability of emulsions containing SC. Hence, the aim of the present work was to study the physical and oxidative stability of emulsions containing SC under various conditions of protein concentration and pressure using the UHPH technology in comparison with other emulsification methods such as colloid mill (CM) and conventional homogenization (CH).

2. Material and Methods

2.1.Materials

Refined sunflower and olive oils were purchased from Gustav Heess Company (Barcelona, Spain). The characteristics and composition of oils are described in Table 1. 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 g/100 g; granulometry (% < 300 µm) = 99.99; pH = 6.7; sediment at 70 ºC (%) = 0.05; minerals = 3.52 g/100 g; MAT (N × 6.38) = 90 g/100 g; fat = 1 g/100 g; density = 0.42.

2.2. Preparation of emulsions

2.2.1. Preparation of protein dispersions

Sodium caseinate dispersions containing 1, 3 and 5 g/100 g were prepared utilizing decalcified water by agitation with high speed mechanical blender (Frigomat machine,

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97 Guardamiglio, Italy) at room temperature avoiding foam formation. Protein dispersions
98 (pH ≈ 6.5-7) were stored overnight at 4 ºC to permit protein hydration.

99

100 *2.2.2. Homogenization treatments*

101 After rehydration, protein dispersions and oil (20 g/100 g) were equilibrated at 20 ºC
102 before blending. Pre-emulsions (or coarse emulsions) were prepared by blending the
103 above protein dispersions with the oil mixture (3 sunflower : 1 olive oil) using a colloid
104 mill (E. Bachiller B. S.A, Barcelona, Spain) operating at 5000 rpm for 5 min at 20 ºC
105 (CM emulsions). The secondary or final emulsions were formed by the use of the
106 coming homogenizers. A Stansted high-pressure homogenizer (Model/DRG number
107 FPG 11300:400 Hygienic Homogenizer, Stansted Fluid Power Ltd., UK) was used with
108 a flow rate of 120 l/h to form the UHPH-treated emulsions. Emulsions were UHPH-
109 treated at pressures of 100, 200 and 300 MPa (single-stage) with inlet temperature (Tin)
110 of 25 ºC (UHPH emulsions). Throughout the experiment, the Tin, the temperature after
111 the homogenization valve (T1) and the temperature of the outlet product (T2) were
112 monitored (Fig. 1). Two spiral-type heat-exchangers (Garvía, Barcelona, Spain) located
113 behind the high-pressure valve were used to minimize temperature retention after
114 treatment,. CM emulsions were also treated by conventional homogenization (CH)
115 using an APV Rannie Copenhagen Series Homogenizer (Model 40.120H, single stage
116 hydraulic valve assembly, Copenhagen, Denmark) with Tin of 60 ºC at 15 MPa (CH
117 emulsions).

118 The entire experiment was repeated on three independent occasions.

119

120 ***2.3.Emulsion analyses***

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121 *2.3.1. Particle Size Distribution*

122 The particle size distribution, and d3,2 and d4,3 were determined in the emulsion
123 samples using a Beckman Coulter laser diffraction particle size analyzer (LS 13 320
124 series, Beckman Coulter, Fullerton, CA, USA) as described by Hebishy et al. (2015).

125

126 *2.3.2. Rheological measurements*

127 Rheological behavior measurements were carried out using a controlled stress
128 rheometer (Haake Rheo Stress 1, Thermo Electron Corporation, Karlsruhe, Germany)
129 using a parallel plate (1º, 60 mm diameter) geometry probe at 25 ºC. Flow curves were
130 determined at incrementing then decreasing shear rates between 0 and 140 s^{-1}. Flow
131 curves were fitted to the Ostwald de Waele rheological model: $\tau = K \dot{\gamma}^{n}$ and the
132 consistency coefficient (K, Pa × s) and flow behavior index (n) were obtained. All
133 viscosity parameters were performed at least in triplicate.

134

135 *2.3.3. Physical stability*

136 Physical stability was measured in the emulsions by measuring the d4,3 value at the
137 top or at the bottom of the emulsion tubes kept at room temperature for 9 days.
138 Measurements were performed in triplicate using the laser diffraction particle size
139 analyzer (LS 13 320 series, Beckman Coulter, Fullerton, CA, USA) as detailed before
140 in the particle size section.

141 The stability of emulsions was also measured in triplicate using vertical scan
142 analyzer Turbiscan MA 2000 (Formulaction, Toulouse, France) in the backscattering
143 mode, as Hebishy et al. (2015) described. Emulsions were analysed at preset interims
144 (30 min for CM emulsions, 3 days for CH and UHPH emulsions) over a foreordained

timeframe (5 h for CM emulsions and 17 days for CH and UHPH emulsions). Turbisoft software (Formulaction, 2005) was likewise used to calculate the migration rate velocity V (µm/min) of the clarification front in order to follow the kinetics of the creaming phenomenon. The particle migration velocity calculated by the software is based on the general law of sedimentation (Stokes Law extended to concentrated dispersions), as shown in the following equation (B):

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

where V = particle migration velocity (µm/min), p_c = continuous phase density (kg/m^3), p_p = particle density (kg/m^3), g = gravity constant (9.81 m/s²), d = particle mean diameter (µm), ν = continuous phase dynamic viscosity (cP) and φ = volume fraction (without unit).

2.3.4. Emulsions microstructure

To examine the changes in emulsion microstructure, emulsion samples were observed by transmission electron microscopy with a Jeol 1400 (Jeol Ltd, Tokyo, Japan) equipped with a Gatan Ultrascan ES1000 CCD Camera, preparing samples as described by Cruz et al. (2007).

2.3.5. Oxidative stability

Emulsions were kept in a controlled light room (2000 lux/m^2) at 10 ºC for 10 days under light in glass transparent capped bottles, as such systems are normally stored with limited oxygen availability to prevent lipid oxidation and increase the shelf life.

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Lipid hydroperoxides, as primary oxidation products, were measured as described by Shantha & Decker (1994) and results were expressed as absorbance (A_{510}). For the determination of secondary oxidation products, thiobarbituric acid-reactive substances (TBARs) were determined according to an adapted method of McDonald & Hultin (1987). Concentrations of TBARs were calculated from a calibration curve prepared with 1, 1, 3, 3-tetraethoxypropane.

Emulsions were then tested in triplicate on the starting and the last day of storage.

2.4. Statistical analyses

Descriptive statistics, mean and standard deviation, were listed for each variable in this study. A General Lineal Model with repeated measures was performed in order to evaluate the physical and oxidative stability of emulsions among type of emulsion (CM, CH or UHPH) and concentration of protein (1, 3 and 5 g/100g),. Variables of interest related to physical and oxidative stability needed to be transformed using log-transformation in order to stabilize the variance. The statistical analysis was performed using SAS System ® v9.2 (SAS Institute Inc., Cary, NC, USA), using a nominal significance level of 5% ($P < 0.05$) and Tukey adjustment was performed for multiple comparisons of the means.

3. Results and Discussion

3.1.Rise of temperature during UHPH processing

The temperature of the emulsions increased with increasing the pressure when passed through the homogenizer (Table 2). The warming up of the emulsion is due to force-

192 induced phenomena of shear, turbulence, and cavitation, which happen simultaneously,
193 dissipating the mechanical energy as heat during emulsification (Floury et al., 2003).

194 Temperature (T2) measured after the HP-valve increased by 47.7, 51 or 47.4 °C
195 between 100 and 300 MPa for the three respective protein concentrations (1, 3 or 5
196 g/100 g, respectively). These results are similar to those of Floury et al. (2003) who
197 reported a significant temperature ascend in the emulsions, notwithstanding utilizing a
198 cooling jacket at the outlet of the HPH valve.

199

200 *3.2. Particle size distribution*

201 Droplet size index (d3,2) for emulsions containing 20 g/100 g oil and different SC
202 concentrations (1, 3 and 5 g/100 g) is shown in Table 3 and Figure 2. CM emulsions had
203 the largest particle size (d3,2) followed by CH emulsions and the minimum droplet size
204 was found in emulsions stabilized by UHPH. This decrease in the particle size was also
205 confirmed by TEM microscopy (Fig. 3 A-J). Generally, the protein concentration
206 affected the particle size (d3,2) of emulsions treated by CM. Increasing the protein
207 concentration from 1 to 3 g/100 g of SC decreased the particle size of CM in a
208 significant manner, but no more decrease in the particle size was noticed when more
209 protein was added (Table 3). This result was also confirmed by the size distribution
210 curves of CM emulsions (Fig. 2 A-C) where a shift in the particle diameter towards
211 smaller diameter was observed in CM emulsions as the protein concentration increased
212 to 5 g/100 g rather than emulsions containing 1 and 3 g/100 g.

213 CH emulsions presented much lower particle size than that of CM emulsions with a
214 wide distribution curve at all protein concentrations. The protein concentration had no
215 effect on the d3,2 value in CH emulsions (Table 3).

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Concerning UHPH emulsions, the homogenization pressure generally had an effect on the particle size only in emulsions containing 1 and 3 g/100 g SC when the pressure increased from 100 to 200 and 300 MPa. These results may be confirmed by the size distribution (Fig. 2 A-C) where the size distribution curves, only in case of emulsions containing 1 and 3 g/100 g SC, were shifted to smaller sizes as the pressure increased to 200 and 300 MPa however, no shift of the curve was observed in emulsions containing 5 g/100 g SC.

At low SC concentration (1 g/100 g), UHPH emulsions treated at 200 and 300 MPa exhibited a lower particle size (only significant in emulsions treated at 200 MPa) in comparison to emulsions treated at 100 MPa, but they presented a bimodal droplet distribution (Fig. 2 A). In this case, the increase of homogenization pressure was capable of producing smaller droplets, nonetheless, there were insufficient protein molecules to adsorb onto the newly formed surface producing the bimodal distribution. However, when protein was increased to 3 and 5 g/100 g, droplet distribution changed from bimodal to monomodal distribution (Fig. 2 B,C), indicating a sufficient protein coverage.

In respect to the effect of protein concentration on the particle size of UHPH emulsions, it seems to have a limited effect in UHPH emulsions treated at 100 MPa, only when SC content increased from 1 to 3 g/100 g. The droplet size, which determines emulsion formation and stability, is reduced when the surfactant concentration increases until a plateau is come to after which no further decline happens (Canselier, Delmas, Wilhelm, & Abismail, 2002). However, no significant impact on the particle size could be seen in UHPH emulsions treated at 200 and 300 MPa.

3.3. Rheological Behavior

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241 The consistency coefficient (K) and flow behavior index (n) values, which
242 corresponds to the viscosity when the fluid is Newtonian if n ≈ 1 are presented in Table
243 3.

244 CM emulsions demonstrated a Newtonian flow behavior with low viscosity, perhaps
245 because of the little interaction between particles in these emulsions. Despite the fact
246 that, in these emulsions the consistency increased with increasing the protein content,
247 the protein content had no noteworthy impact on CM emulsion viscosity.

248 In general, applying CH treatment brought about a noteworthy increment in the K of
249 emulsions, in contrast with their homologues CM emulsions, with a change in the flow
250 behavior from Newtonian to shear thinning when protein concentration increased from
251 1 to 3 and 5 (g/100g). In these emulsions, the increase of protein concentration had a
252 reasonable noteworthy impact on the K value of CH emulsion.Concerning the UHPH,
253 generally, emulsions with statistically comparable K values to those obtained in CM and
254 CH emulsions, according to the homogenization pressure used in the treatment, were
255 produced . UHPH-treated emulsions at 100 MPa showed similar viscosity to those
256 treated by CM; however, UHPH-treated emulsions at 200 and 300 MPa exhibited
257 similar K value to CH emulsions. As for the impact of protein concentration on the K
258 value of the UHPH-treated emulsions, increasing the protein concentration from 1 to 3
259 g/100 g in all UHPH emulsions had no impact on the emulsion K value but, further
260 increase in the protein concentration to 5 g/100 g significantly increased the K value.
261 Emulsions treated at 100 MPa exhibited a flow Newtonian behaviour, whatever the
262 protein content was. On the other hand, the Newtonian flow behavior was only observed
263 in UHPH emulsions treated at 200 and 300 MPa containing low protein concentration (1
264 g/100 g), whereas increasing the protein concentration to 3 and 5 g/100 g tended to
265 change the flow behavior towards the shear thinning behavior. The explaination behined

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266 the viscosity increase with extensively high-pressures (i.e. 300 MPa) and high protein
267 concentrations (5 g/100 g), may be the enhanced depletion flocculation due to the
268 presence of excessive protein in the continuous phase, forming casein aggregates or
269 protein gels, as can be seen in the TEM image for UHPH emulsion containing 5 g/100 g
270 of SC and treated at 300 MPa (Fig. 3 J). In the study of Hebishy et al. (2015), higher
271 viscosity was found in emulsions stabilized with high concentration of whey protein
272 isolate (4 rather that 1 and 2 g/100 g) and subjected to high-pressure homogenization at
273 200 MPa but, unlike the results of the current study, no change in the rheological
274 behavior from Newtonian to shear thinning was observed. They attributed that increase
275 to the reduced droplet size and the change in the properties of the stabilizing molecules
276 (whey protein isolate) and the simultaneous adsorption of proteins on the increased fat
277 globule surface.

278

279 *3.4. Physical stability of emulsions*

280 Figure 4 A (A-F) and B (A-D) shows the backscattering profiles for all emulsions
281 prepared by CM, CH and UHPH at 100 and 200 MPa. Simple visual examination of
282 graphics from Figure 4 shows longer stability of UHPH-made emulsions. A drop of BS
283 at the bottom of samples, due to clarification of the mixture, and an increase of BS at
284 the top of samples, associated to particle creaming, was higher in CM emulsions
285 followed by CH emulsions and the minimum creaming rate was observed in the UHPH
286 emulsions.

287 CM emulsions, at all protein concentrations, exhibited a high degree of creaming
288 (total separation at the same day of preparation) as a direct consequence of the large
289 particle size and low viscosity, which resulted in a high degree of coalescence as can be
290 observed in the TEM images (Fig. 3 A-C). CM emulsions containing 1 g/100 g SC were

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291 the most instable emulsions (Fig. 3 A), where the phase separation was completed in 30
292 min. However, increasing the protein concentration to 5 g/100 g SC (Fig. 4 C) tended to
293 slow down the creaming process, with a completed separation in approximately 4 h.

294 The CH emulsions were more stable against creaming than CM emulsions, although
295 creaming could be detected in all CH emulsions by Turbiscan Lab (Fig. 4 (A) D-F) and
296 by the d4,3 values obtained at the top or the bottom of the CH emulsions tubes (Table
297 4). The optical characteristics of CH emulsions containing 1 g/100 g of SC showed slow
298 changes in their backscattering patterns (Fig. 4 (A) D), significant differences between
299 the d4,3 values at the top or at the bottom of the emulsion (Table 4) but with no visual
300 separation during approximately 18 days of storage at room temperature. The
301 microscopic examination of these emulsions by TEM indicated the presence of bridging
302 flocculation (Fig. 3 D-F) possibly due to limited protein surface coverage (Dickinson,
303 Golding, & Povey, 1997), suggesting that this phenomenon may have a stabilizing
304 effect of the emulsion. CH emulsions made with 3 g/100 g SC showed extensive
305 creaming, with the clarification front of the Turbiscan appearing after 3 days (Fig. 4 (A)
306 E), indicating the limited shelf life of these emulsions. Additional increase in the protein
307 concentration in CH emulsions (from 3 to 5 g/100 g SC) led to a reduction in the
308 creaming rate (Fig. 4 (A) F). This fact can be attributed to the formation of a depleted
309 network structure at higher SC concentrations, as explained before (see rheological
310 section), increasing the K value, which limits the droplets movement (Table 3). These
311 results were also confirmed by calculating the migration or creaming velocity V (t) in
312 the clarification layer using the Turbiscan software. A lower creaming value was
313 observed in emulsions containing 1 g/100 g SC (207 µm/min), however, increasing the
314 protein content from 1 to 3 g/100 g increased the creaming rate (861 µm/min) while a
315 further increase to 5 g/100 g decreased the rate (272 µm/min).

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316 Emulsions processed by UHPH were surprisingly stable, because of the prominent
317 droplet size reduction, and remained completely turbid upon storage at room
318 temperature for 18 days, with no creaming being visually noticed. It has been shown
319 that when the particle sizes are ~100 nm (some particle sizes in the present study fell
320 into this range), creaming would be greatly reduced and aggregation become a
321 predominant mechanism for emulsion instability (McClements, 2005). The protein
322 concentration in combination with the homogenization pressure seemed to significantly
323 affect the creaming stability of the UHPH emulsions. In this way, the d4,3 values at the
324 top and at the bottom of UHPH emulsions (Table 4) and Turbiscan fingerprints (Fig. 4
325 (B) A-D) indicated a slight creaming effect in emulsions containing 1 and 5 g/100 g SC
326 treated at 100 MPa, and in emulsions containing 1 g/100 g SC and treated at 200 MPa,
327 but creaming was not observed in emulsions containing 5 g/100 g SC when were treated
328 at 200 and 300 MPa. Increasing flaxseed protein concentration in the emulsion would
329 encourage relatively smaller droplets adsorbing more protein at the interface of oil
330 droplet (causing a higher zeta-potential), then increasing the density of droplets,
331 consequently decreasing the creaming rate (Wang, Li, Wang, & Özkan, 2010).

332

333 3.5. *Oxidative stability*

334 Lipid oxidation may be relied upon to be speedier in emulsions with small droplets
335 (CH and UHPH), owing to the larger total interfacial area in comparison to larger
336 droplets (CM emulsions). Interestingly, considerable amounts of hydroperoxides and
337 TBARs were observed in CM emulsions (Table 5). This high concentration of oxidation
338 products found in CM emulsions could be attributed to the poor protein coverage at the
339 emulsion interface (Fig. 3 A-C) together to the fact that these emulsions are prone to
340 creaming, due to the large particle size, which causes the oil droplets to become directly

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exposed to oxygen in the headspace (Phoon et al., 2014). Similar levels of primary oxidation products, compared to CM emulsions, were formed in CH emulsions at day 1. Although a significant evolution in the TBARs after 10 days was observed in CH emulsions, these amounts were lower than those of the corresponding CM emulsions, indicating that CH emulsions were more stable against oxidation. Similar results have been reported in our previous study in emulsions produced by whey protein isolate under the same technological conditions (Hebishy et al., 2015). As it was explained in the rheological behavior section, CH emulsions were more viscous in comparison to their homologues CM emulsions. It has been proposed that viscosity can affect oxidation by reducing the diffusion of potential pro-oxidative molecules, such as ferrous ions or lipid hydroperoxides (Sims, 1994).

UHPH-treated emulsions generally exhibited lower levels of hydroperoxides, in comparison to CM and CH emulsions. Similar results were observed in the study of Hebishy et al. (2015) working on oil-in-water emulsions treated by UHPH (100 and 200 MPa) and using whey protein isolate (1, 2 and 4 g/100 g) as emulsifier. Increasing the homogenization pressure from 100 to 300 MPa resulted in high oxidative stability being those treated at 300 MPa the most stable emulsions, with lower amounts of primary oxidation products, especially when 5 g/100 g of SC was used. On the contrary to the results of the present study, Hebishy et al. (2015) working on emulsions added of whey protein isolate reported that increasing the homogenization pressure to more than 100 MPa negatively affected the oxidative stability of emulsions. They related that fact to the decrease in the efficiency of whey proteins to protect the oil droplets when the pressure was increased as a result of the over processing phenomenon caused by the increase in the product temperature at the outlet of the homogenization valve, which affects the emulsifying properties of whey proteins.

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366 In the case of secondary oxidation, UHPH emulsions presented higher values of
367 TBARs at day 1 after production, in comparison to CM and CH emulsions. Even if
368 UHPH emulsions presented higher values of TBARs at day 1, the evolution of
369 secondary oxidation products during 10 days of storage (day 10 - day 1) was generally
370 not significant comparing to CM and CH emulsions, except for some specific
371 treatments. O' Dwyer et al. (2013) observed anomalous behaviour for the caseinate
372 stabilized camelina emulsions distinguishing high levels of lipid hydroperoxides and
373 secondary oxidation products (*p*-anisidine value) promptly taking after emulsification,
374 in contrast to the bulk oil. They explained the initial increment in oxidation products
375 after emulsification by frictional effects in the microfluidizer, making increased levels
376 of oxygen, or a large surface area because of the droplet disruption and shearing amid
377 homogenization. However, as storage time proceeded, hydrophobic interactions
378 amongst caseinate and lipophilic oxidation products increased due to the exposure of
379 hydrophobic and other amino acid residues (aromatic residues), bringing about an
380 obvious antioxidant effect explaining the no significant evolution of oxidation during
381 storage.

382 A study by Phoon et al. (2014) has reported that high-pressure homogenization
383 improves the intrinsic oxidative stability of 4 g/100 mL menhaden oil-in-water
384 emulsions stabilized by 1 g/100 mL caseinate at pH 7. The authors reported that high
385 pressures increment interfacial cross-linking of sodium caseinate at the interface,
386 accordingly creating a rigid interfacial layer. This thick interfacial layer keeps the
387 transition metals in the continuous phase a way from coming near to the oil droplets,
388 thus impeding lipid oxidation during storage.

389 In the present study, and generally, increasing the protein concentration resulted in an
390 increase in the oxidative stability of emulsions. However, an exeption was noticed in

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391 UHPH emulsions treated at 100 MPa where the increase in the SC to 5 g/100 g resulted
392 in more oxidized emulsions. This may be due to the relatively high creaming rate in
393 these emulsions as indicated by the Turbiscan image (Fig. 5 (B) C) which increases the
394 oxidation rate, as explained before. In UHPH emulsions treated at 200 and 300 MPa,
395 increasing the protein content to 5 g/100 g resulted in lower primary and secondary
396 oxidation products as no significant evolution of both hydroperoxides and TBARs could
397 be noticed.

398 In concurrence with data presented in the current study, several studies with casein as
399 emulsifier have demonstrated that the rate of lipid oxidation diminishes with increasing
400 levels of casein (Faraji, McClements, & Decker, 2004; Ries, Ye, Haisman, & Singh,
401 2010). Ries et al. (2010) working with different casein concentrations (0.5-10%) to
402 stabilize a linoleic acid emulsion from oxidation, found that the degree of lipid
403 oxidation decreased as the protein concentration increased. As indicated by the authors,
404 casein can form a rigid interfacial layer (up to 10 nm), which works as an efficient
405 barrier to the diffusion of lipid oxidation initiators into the oil droplets.

406 The impact of SC on lipid oxidation in emulsions have in some studies mainly been
407 related to their effects at the interface, whereas in other studies it has mainly been
408 related to their effects in the aqueous phase (Faraji et al., 2004; Let, Jacobsen, & Meyer,
409 2007). It has been proposed (Sun & Gunasekaran, 2009) that unabsorbed protein can
410 enhance the oxidative stability of emulsions, by the interaction with metal ions, or by
411 scavenging free-radicals in the aqueous phase. O' Dwyer et al. (2013) reported that
412 lipid oxidation was 20% less in in camelina oil-in-water emulsions microfluidized at
413 138 MPa, rather than those treated at 21 MPa as the SC concentration increased from
414 0.25 to 3 g/100 mL. The authors reported that the reason behind the high oxidation in
415 emulsions stabilized using lower levels of SC probably that these emulsions did not

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have enough SC to surround the droplets and cover such a large surface area. However, in emulsions containing 3 g/100 g protein content, there was excessive emulsifier to permit maximum protein load at the interface. In the present study, it can be seen from the TEM images (Fig. 3 D-I) that excess amount of protein aggregates could be found in CH and UHPH emulsions containing 3 and 5 g/100 g of SC (Fig. 3 E,F and H,I) in comparison to those containing only 1 g/100 g of SC (Fig. 3 D,G). Therefore, SC was present in excess, and it must be assumed that protein was present both at the interface and in the aqueous phase, increasing the oxidative stability at higher protein concentration. In addition, emulsions containing high protein amounts also presented significant increases in emulsion viscosity which may slow down the oxidation rate as explained before.

4. Conclusions

This study revealed that using UHPH technology at ≥ 200 MPa could result in physically and oxidatively stable emulsions stabilized by SC when sufficient protein concentration (5 g/100 g) is used. However, using lower homogenization pressures (100 MPa) with lower amounts of SC (1 g/100 g) results in less stable to creaming and oxidation emulsions. On the contrary, in CH emulsions, a low concentration of SC (1 g/100 g) resulted in emulsions that are stable against creaming and oxidation, however, higher protein amounts (5 g/100 g), in general, increases the depletion flocculation and results in a high creaming and oxidation rate in these emulsions.

The results show the ability of the UHPH together with SC as an emulsifier to produce O/W emulsions with reduced particle size that are physically stable against

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creaming and coalescence, and also stable against oxidation. These results open up a range of possibilities in creating physical and oxidatively stable emulsions as a delivery vehicle for bioactive components of lipophilic nature with high propensity for oxidation (i.e. fat soluble vitamins, carotenoids, polyunsaturated fatty acids, conjugated linoleic acid, ...) to be applied in different functional food products with a lipid profile improved.

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Figure Captions:

Figure 1.

Schematic representation of high-pressure homogenizer. Tin, initial fluid temperature in the feeding tank; T1, temperature at the HP-valve inlet; T2, temperature at the HP-valve outlet.

Figure 2.

Droplet size distribution curves measured by light scattering of O/W emulsions containing, 1 (A), 3 (B) and 5 g/100 g (C) of sodium caseinate plus 20 g/100 g of sunflower and olive oils and prepared by: colloid mill (CM, +), conventional homogenization (CH, ○) and ultra high-pressure homogenization at 100 (●), 200 (■) and 300 (□) MPa.

Figure 3.

TEM images of emulsions containing 1, 3 and 5 g/100 g of sodium caseinate and stabilized by (A-C) colloid mill (CM) ×5000, (D-F) conventional homogenization (CH) ×25000 and by ultra high-pressure homogenization at 200 MPa (G-I) ×50000 and at 300 MPa (sodium caseinate, 5 g/100 g) ×100000.

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545 Figure 4.

546 (A) Changes in backscattering profiles of emulsions containing 20 g/100 g oil and
547 different sodium caseinate contents, 1 (A, D), 3 (B, E) and 5 g/100 g (C, F) and
548 prepared by (A-C) colloid mill (CM) and (D-F) conventional homogenization (CH),
549 and (B) emulsions containing 20 g/100 g oil and different sodium caseinate contents, 1
550 (A, B) and 5 g/100 g (C, D) and prepared by ultra high-pressure homogenization at 100
551 MPa (A, C), and 200 (B, D) MPa, as a function of storage time (5 h for CM emulsions
552 and 18 days for both CH and UHPH emulsions).

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Table 1

Chemical composition of sunflower and olive oils.

Chemical characteristics	Sunflower oil	Olive oil
Density at 20 ºC	0.921	0.913
Acid value	0.09 (mg KOH/g)	0.11 (g/100 g, oleic)
Peroxide value (meqO_2/kg)	0.02	0.5
Unsaponifiable (% m/m)	< 0.05	< 1.5
Fatty acid composition (%)		
C 16 : 0	6.34	11.97
C 18 : 0	3.97	3.30
C 18 : 1	26.65	75.23
C 18 : 2	61.02	6.75
C 18 : 3	–	0.38

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Table 2.

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

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

Data listed are the mean of three different replicates

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Table 3.

Mean ± SD of particle size distribution index (d3,2) and rheological characteristics (flow and consistency indices) of O/W emulsions containing 20 g/100 g of sunflower and olive oils plus sodium caseinate 1, 3 and 5 g/100 g and prepared by colloid mill (CM), conventional homogenization (CH) and ultra high-pressure homogenization (100, 200 and 300 MPa).

Treatments	Protein content (g/100 g)	Particle size distribution	Rheological behavior	
		d3,2 (μm)	Consistency coefficient (K) Pa × s	Flow behavior index (n)
CM	1	6.828 ± 0.310^{a}	0.0015 ± 0.0003^{e}	1.092 ± 0.017
	3	5.641 ± 0.395^{b}	0.0047 ± 0.0017^{de}	1.041 ± 0.044
	5	5.421 ± 0.362^{b}	0.0121 ± 0.0005^{cde}	1.006 ± 0.015
CH	1	0.578 ± 0.074^{c}	0.0018 ± 0.0002^{e}	0.994 ± 0.006
	3	0.597 ± 0.089^{c}	0.0201 ± 0.0094^{c}	0.776 ± 0.006
	5	0.572 ± 0.094^{c}	0.0426 ± 0.0073^{ab}	0.739 ± 0.046
100	1	0.210 ± 0.046^{d}	0.0023 ± 0.0004^{e}	0.971 ± 0.020
	3	0.151 ± 0.014^{e}	0.0068 ± 0.0026^{de}	0.977 ± 0.029
	5	0.116 ± 0.009^{ef}	0.0241 ± 0.0026^{cd}	0.911 ± 0.029
200	1	0.141 ± 0.010^{ef}	0.0033 ± 0.0020^{e}	0.930 ± 0.091
	3	0.120 ± 0.013^{ef}	0.0162 ± 0.0045^{cde}	0.850 ± 0.035
	5	0.108 ± 0.008^{ef}	0.0307 ± 0.0077^{bc}	0.840 ± 0.042
300	1	0.129 ± 0.002^{ef}	0.0028 ± 0.0005^{e}	0.966 ± 0.024
	3	0.098 ± 0.001^{f}	0.0154 ± 0.0037^{cde}	0.863 ± 0.020
	5	0.111 ± 0.009^{ef}	0.0491 ± 0.0089^{a}	0.857 ± 0.032

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

Data listed are the mean of at least three measurements from three separate productions

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Table 4.

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

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

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

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

Data listed are the mean of at least three measurements from three separate productions

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

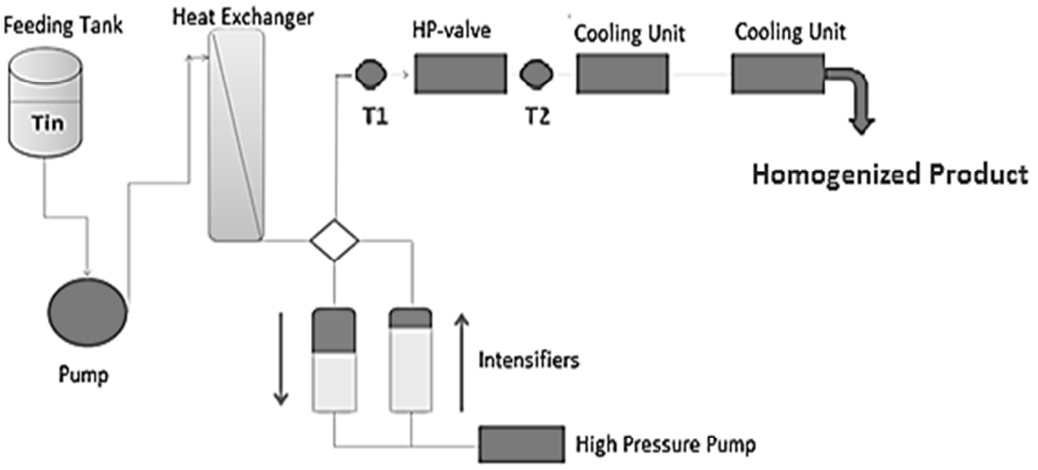
Treatments	Protein content (g/100 g)	Hydroperoxides (A_{510} nm)			TBARS (µg/ml)		
		Day 1	Day 10	Difference (Day 10 – Day 1)	Day 1	Day 10	Difference (Day 10 – Day 1)
CM	1	0.019 ± 0.005^{ab}	0.116 ± 0.050^{a}	$0.097 \pm 0.048^{a*}$	0.039 ± 0.018^{cd}	0.116 ± 0.033^{a}	$0.077 \pm 0.051^{a*}$
	3	0.022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

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

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

Data listed are the mean of at least three measurements from three separate productions

1 **Figure 1.**



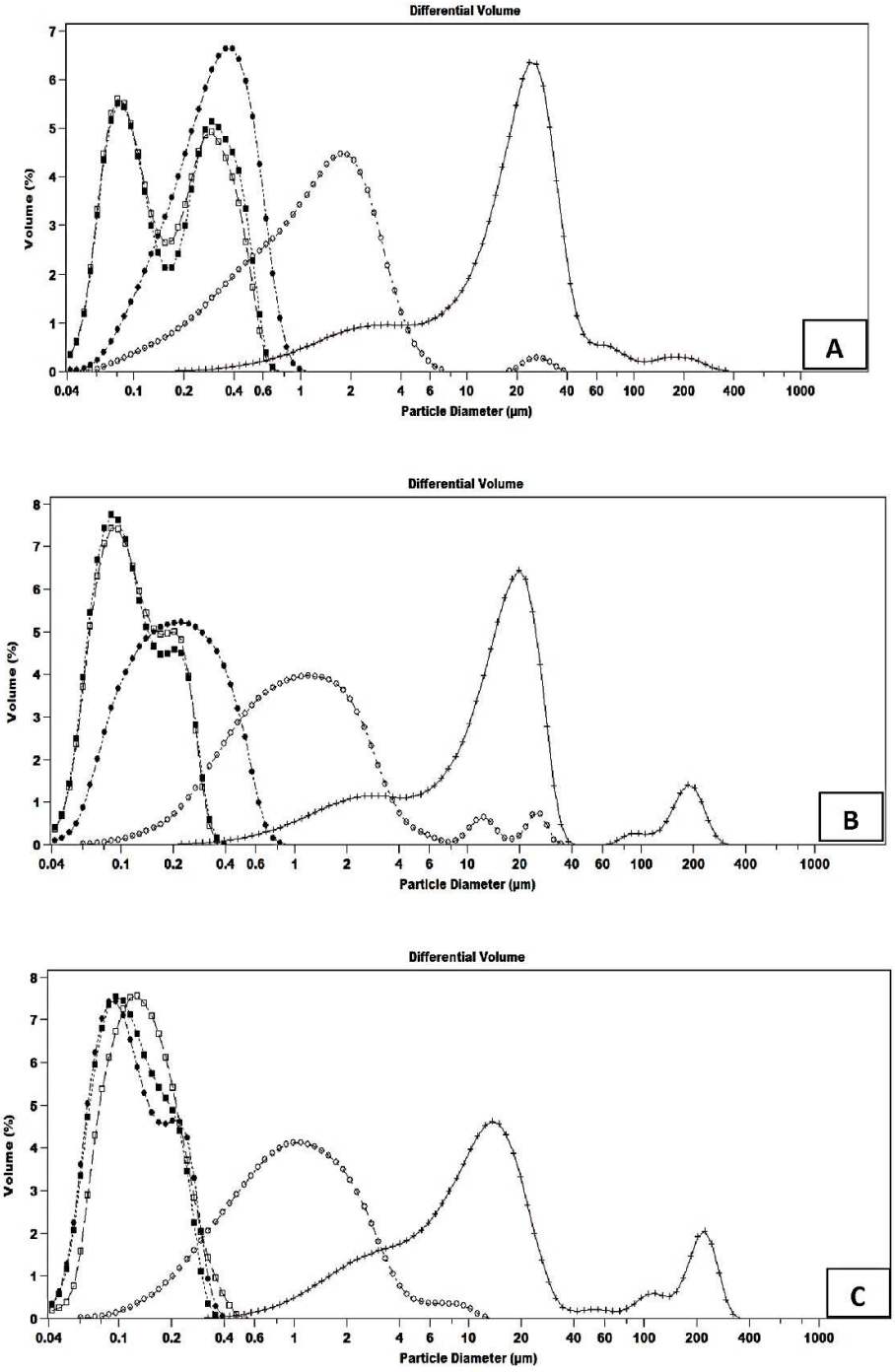
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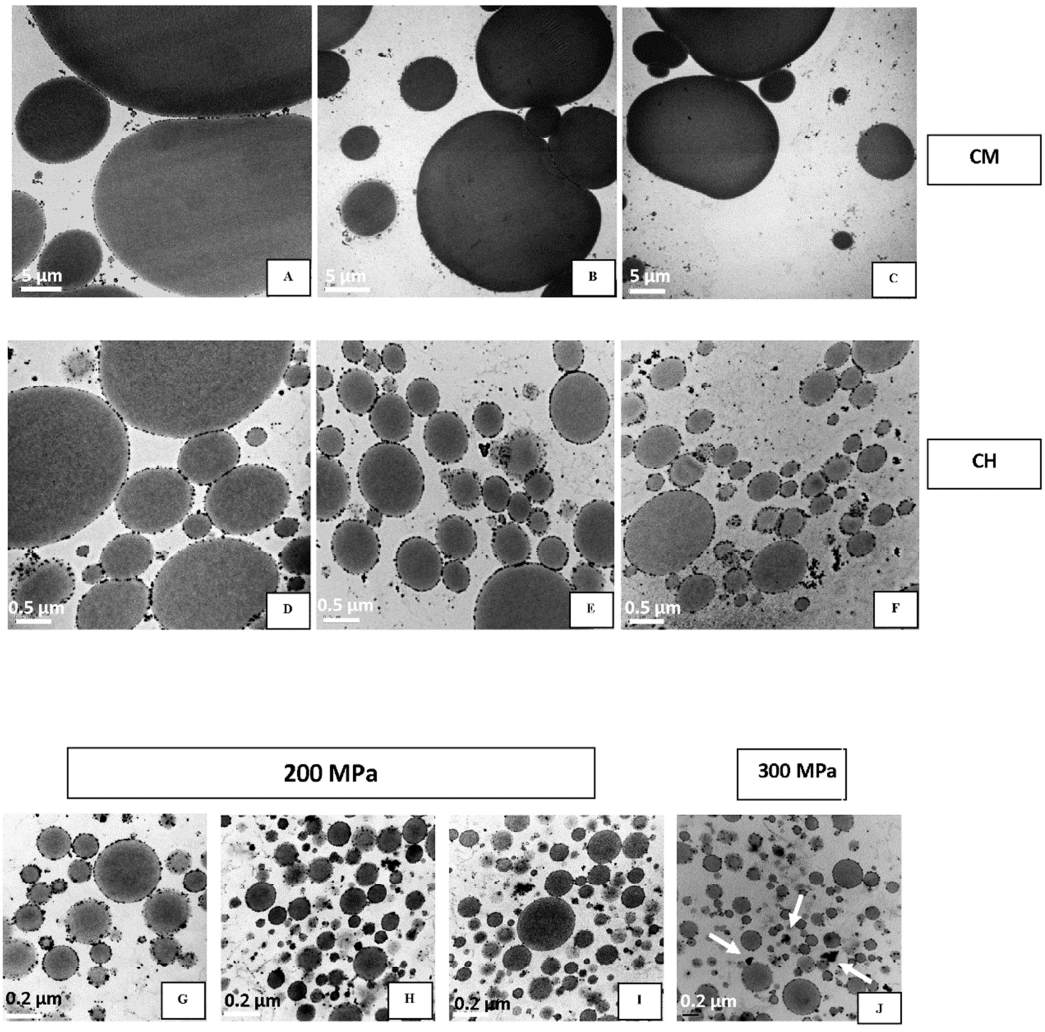
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Figure 2.


Differential Volume
Volume (%)
Particle Diameter (µm)
A
Differential Volume
Volume (%)
Particle Diameter (µm)
B
Differential Volume
Volume (%)
Particle Diameter (µm)
C

8 **Figure 3**

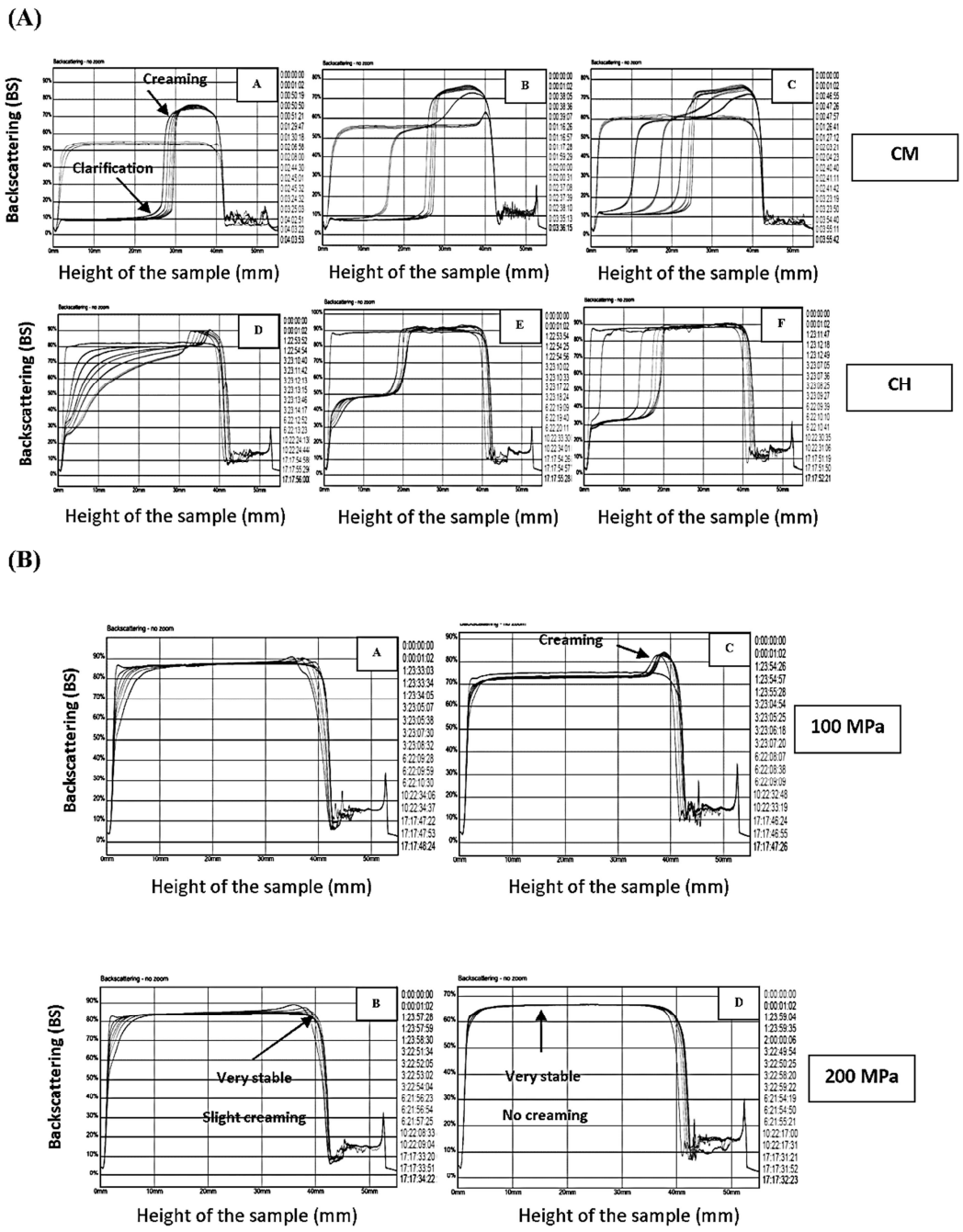


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11 **Figure 4**



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Highlights

- Sodium caseinate and pressure levels impacted the emulsion stabilities
- Conventional homogenization with 1 g/100 g sodium caseinate increased physical stablity
- Pressures (200-300 MPa) and 5 g/100 g sodium caseinate increased emulsions stabilities
- Emulsions rheology was affected by increasing sodium caseinate concentration
- The emulsion droplet size has an effect on the oxidation rate

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