

**Universitat de Lleida**

Document downloaded from:

<http://hdl.handle.net/10459.1/65712>

The final publication is available at:

<https://doi.org/10.1007/s11947-018-2221-3>

Copyright

(c) Springer Science+Business Media, LLC, part of Springer Nature, 2018

1 Formation of double ( $W_1/O/W_2$ ) emulsions  
2 as carriers of hydrophilic and lipophilic  
3 active compounds

---

4  
5 **Artiga-Artigas, M<sup>1</sup>., Molet-Rodríguez, A<sup>1</sup>., Salvia-Trujillo, L<sup>1</sup>., Martín-**  
6 **Belloso, O<sup>1</sup>\***

7  
8 <sup>1</sup> Department of Food Technology, Universidad de Lleida – Agrotecnio Center, Av.  
9 Alcalde Rovira Roure 191, 25198, Lleida, Spain

10  
11 \* Corresponding author email: [omartin@tecal.udl.cat](mailto:omartin@tecal.udl.cat).

12 Fax +34 973702596; Tel +34 973702593;

## 15 Abstract

16 This work aimed at obtaining an optimized formation procedure of water-in-oil-in-water  
17 ( $W_1/O/W_2$ ) double emulsions as potential templates to carry hydrophilic (*eg.*  
18 *chlorophyllin*; CHL) and/or hydrophobic (*eg.* lemongrass essential oil; LG-EO) active  
19 compounds. As a first step, the impact of the hydrophobic surfactant (*ie.* Span 80 or  
20 PGPR), sodium alginate or NaCl concentration as well as the homogenization method (*ie.*  
21 high-shear homogenization, ultrasonication or microfluidization) on the particle size of  
22 the primary  $W_1/O$  emulsions was evaluated. The inner phase ( $W_1/O$ ) formulated with  
23 PGPR (4% *w/w*) and sodium alginate (2% *w/w*) with NaCl (0.05M) and treated by high-  
24 shear homogenization (11,000 rpm, 5 min) presented the smallest particle size ( $d[4;3] \approx$   
25  $0.51 \mu\text{m}$ ). As a second step, the primary  $W_1/O$  emulsion was subsequently dispersed in a  
26 secondary aqueous phase ( $W_2$ ) at varying hydrophilic surfactant (*ie.* lecithin or Tween  
27 20), sodium alginate or NaCl concentrations and magnetic stirring rate (rpm and time) to  
28 obtain double emulsions ( $W_1/O/W_2$ ). The formation of stable  $W_1/O/W_2$  emulsions with  
29  $d[4;3]$  of  $7 \mu\text{m}$  was achieved with the use of lecithin (2% *w/w*), sodium alginate (2% *w/w*)  
30 with NaCl (0.05M) and treated by low-intensity UT homogenization (5,600 rpm, 2 min)  
31 followed by 24h of magnetic stirring. The incorporation of CHL and LG-EO in the inner  
32 aqueous phase and lipid phase respectively did not change the double emulsion  
33 characteristics. Overall, this study presents an effective two-step optimized procedure to  
34 form stable double emulsions as potential delivery systems for functional compounds.

35  
36 **Keywords:** double emulsion; chlorophyllin; lemongrass essential oil; PGPR; two-step  
37 procedure

38

## 39 1. Introduction

40 Water-in-oil-in-water ( $W_1/O/W_2$ ) double emulsions are emulsion-based systems in  
41 which the dispersed phase is an emulsion itself (Dickinson, 2011a). The inner aqueous  
42 phase ( $W_1$ ) is usually miscible with the final external phase ( $W_2$ ) since they have the same  
43 polarity, whereas the intermediate phase (O) is immiscible with the other two (Wang et  
44 al. 2006). Recently, several applications have been attributed to double emulsions, such  
45 as fat replacers or delivery systems of active compounds. The formation of double  
46 emulsions is generally achieved with a two-step emulsification procedure in which first a  
47 primary water-in-oil emulsion ( $W_1/O$ ) is formed, and is subsequently emulsified to form  
48 the secondary emulsion ( $W_1/O/W_2$ ) (Muschiolik & Dickinson, 2017). However, the  
49 fabrication and stabilization of double emulsions remains as a challenge as they are highly  
50 susceptible to destabilization phenomena. Several destabilization mechanisms have been  
51 described in double emulsions, being (i) the coalescence of the lipid dispersed phase; (ii)  
52 coalescence of the inner aqueous droplets between them or with the external aqueous  
53 phase and (iii) swelling or shrinkage of the inner water droplets as result of diffusive  
54 transport between both aqueous phases (Dickinson, 2011).

55 For the overall double emulsion stabilization, the stability of both the inner aqueous  
56  $W_1$  and the outer lipid  $W_1/O$  dispersed phase and their respective interfaces needs  
57 consideration. On the one hand, the presence of a lipophilic surfactant is required for the  
58 emulsification and stabilization of the inner aqueous phase. Spans are commonly used for  
59 this purpose but their efficiency in primary emulsion stabilization needs to be elucidated.  
60 Polymeric surfactants such as polyglycerol polyricinoleate (PGPR) at a 4 to 6% w/w  
61 concentration are often utilized as an alternative of spans (Su et al. 2006). However, its  
62 use in food-grade formulations is strictly regulated and it may be detected rapidly due to  
63 its unpleasant off-taste when incorporated in the required doses for double emulsion

64 stabilization (Altuntas et al. 2017). On the other hand, the outer  $W_1/O$  dispersed phase  
65 may be stabilized by using an hydrophilic surfactant, such as lecithin, Tweens or proteins  
66 (Garti and Bisperink 1998). The formation and stabilization of the secondary emulsion  
67 with the dispersed  $W_1/O$  droplets depends on two main factors. First, the osmotic balance  
68 between the two aqueous phases must be kept in order to avoid diffusive transport of  
69 water, which leads to destabilization of double emulsions (Muschiolik, 2007). For  
70 instance, the addition of salt in the  $W_1$ -droplets may be able to reduce the osmotic  
71 differences and avoid swelling of the inner aqueous dispersion (Yan and Pal 2001). And  
72 second, the homogenization method to disperse the oil phase containing the  $W_1$ -droplets  
73 determines in a high extent the retention of the aqueous inner droplets within the lipid  
74 phase. In this regard, moderate emulsification conditions should be applied to prevent  
75 intense high shearing hydrodynamic forces that may destabilize the previously formed  
76 primary emulsion (Muschiolik & Dickinson, 2017). Due to this mild emulsification  
77 conditions, the droplet size of the dispersed  $W_1/O$  phase remains relatively large, which  
78 in turn allows the retention of the inner  $W_1$  dispersed droplets. However, their large  
79 droplet size may result in significant destabilization and phase separation of the  $W_1/O$   
80 phase from the  $W_2$  aqueous phase. This effect may be diminished or avoided by the use  
81 of thickening agents in the aqueous phases, such as sodium alginate among others, in  
82 order to reduce the mobility of the  $W_1/O$  phase and as a consequence prevent phase  
83 separation. Sodium alginate has a strong defined polarity in the aqueous media since it is  
84 deprotonated, which avoids its migration to the internal oil-water interface and may  
85 enhance the achievement of aqueous phase rheological control (Artiga-Artigas, Acevedo-  
86 Fani, & Martín-Belloso, 2017; Dickinson, 2011a).

87 Therefore, the aim of this work was to study the formation and stabilization of double  
88 emulsions. First of all, the formation of the primary  $W_1/O$  emulsion was investigated with

89 regards the effect of the homogenization method (ultrasonication, microfluidization or  
90 high-shear mixing) and processing conditions on the particle size of the dispersed  $W_1$   
91 droplets in the oil phase. Moreover, the use of several lipophilic emulsifiers (PGPR or  
92 Span 80) at different concentrations was evaluated. Afterwards, the formation of double  
93 ( $W_1/O/W_2$ ) emulsions was studied by applying different low-shear homogenization  
94 conditions and emulsifiers (lecithin or Tween 20). Additionally, the salt addition in both  
95 aqueous phases in order to maintain the osmotic balance between both phases was studied  
96 as well as the incorporation of sodium alginate as thickening agent. Finally, the use of the  
97 formulated double emulsion as carriers of *chlorophyllin* (CHL) in the  $W_1$  phase and/or  
98 lemongrass essential oil (LG-EO) in the oil phase as examples of active compounds due  
99 to their high antioxidant capacity to be encapsulated and delivered in food systems was  
100 assessed (Cheel et al. 2005; Guerra-Rosas et al. 2017; López-Carballo et al. 2008; Tumolo  
101 and Lanfer-Marquez 2012).

102

103

## 104 2. Material and Methods

### 105 2.1. Materials

106 Sodium alginate (MANUCOL<sup>®</sup>DH) was obtained from FMC Biopolymer Ltd (Scotland,  
107 U.K.). CHL (coppered trisodium salt) with a molecular weight of 724.15 g/mol, copper  
108 contain of 3.5-6.5% and a purity of  $\geq 95\%$  was purchased from Alfa Aesar (Thermo Fisher  
109 Scientific, GmbH, Karlsruhe, Germany). NaCl from POCH S.A. (Gliwice, Poland) was  
110 used to improve interfacial thermodynamic stability by controlling the osmotic balance  
111 between the two aqueous phases. Corn oil (Koipesol Asua, Deoleo, Spain) and LG-EO  
112 (*Cymbopogon citratus*) from Laboratories Dicana (Spain) were used as lipid phase.  
113 Sunflower oil, which was kindly donated by Borges (Lleida, Spain), was the dispersant  
114 in particle size measurements. Span 80 (Sorbitane monooleate) obtained from Alfa Aesar  
115 (Thermo Fisher Scientific, GmbH, Karlsruhe, Germany) or Polyglycerol Polyricinoleate  
116 (PGPR 90) from castor oil (Grinsted<sup>®</sup>, DuPont Danisco NHIB Iberica S.L, Barcelona,  
117 Spain) were utilized as hydrophobic surfactants. Tween 20 (Polyoxyethylenesorbitan  
118 Monoesterate) (Lab Scharlab, Barcelona, Spain) or L- $\alpha$ -Soybean lecithin was acquired  
119 from Alfa Aesar (Thermo Fisher Scientific, GmbH, Karlsruhe, Germany) and used as  
120 food-grade non-ionic surfactants. Ultrapure water, obtained from Millipore Milli-Q  
121 filtration system (0.22  $\mu\text{m}$ ) was used for the formulation and analysis of nanoemulsions.

### 122 2.2. Water-in-oil emulsions ( $W_1/O$ ) and double emulsions ( $W_1/O/W_2$ ) 123 formation

124  $W_1/O/W_2$  nanoemulsions were prepared following a “two-step” emulsification  
125 process where first a water-in-oil ( $W_1/O$ ) emulsion was formed and this one was  
126 subsequently dispersed in a second aqueous phase to obtain the so called double emulsion  
127 ( $W_1/O/W_2$ ).

128

129 Formation of water-in-oil ( $W_1/O$ ) emulsions

130 For the formation of the  $W_1/O$  emulsions, a ratio aqueous phase ( $W_1$ )/oil phase (O) of  
131 30/70 (w/w) was used. Different parameters including hydrophobic surfactant type and  
132 concentration, emulsification mechanism as well as sodium alginate and salt  
133 concentration in the inner aqueous phase were evaluated during the formation of  $W_1/O$   
134 emulsions. In addition, the feasibility of incorporating LG-EO as a hydrophobic  
135 compound in the lipid phase of  $W_1/O$  emulsions was tested.

136 Firstly, in order to assess the type and concentration of hydrophobic surfactant, the  
137 aqueous phase of emulsions was prepared by dissolving 1% w/w sodium alginate in  
138 ultrapure water at 70 °C and stirred during 3 hours to ensure its complete hydration. After  
139 reaching room temperature, the exact amount of CHL (27 ppm) was added to the alginate  
140 solution until its dissolution. This aqueous phase was dispersed into a lipid phase  
141 containing corn oil and Span 80 (4, 6 or 10% w/w) or PGPR (4, 6 or 10% w/w), which  
142 were evaluated as hydrophobic surfactants. Both phases were mixed through three  
143 different procedures for the formation of  $W_1/O$ : A) high shear homogenization (HSH)  
144 with a T25 digital Ultra-Turrax (IKA, Staufen, Germany) at 11,000 or 22,000 rpm and  
145 during 1, 2, 3 or 5 min; B) HSH (11,000 rpm, 5 min) followed by ultrasonication (US)  
146 with a UP 400S Hielscher sonifier (Hielscher Ultrasound Technology, Teltow, Germany)  
147 at amplitudes of 30, 60 or 100  $\mu\text{m}$  and for 1, 3 or 5 min and C) HSH (11,000 rpm, 5 min)  
148 followed by microfluidization (MF) with a microfluidizer (M110P, Microfluidics,  
149 Massachusetts, USA) at 150 MPa and 1-5 cycles.

150 Secondly, for the establishment of sodium alginate and NaCl concentration  $W_1/O$   
151 emulsions containing 0-2% w/w of biopolymer and 0-0.25M in their aqueous phase were  
152 mixed by HSH at 11,000 rpm during 5 min.



153 Finally, when the optimization procedure and formulation of the double emulsions as  
154 a model of delivery system of bioactive compounds was established, 1% w/w lemongrass  
155 (as hydrophobic bioactive compound) was incorporated to the lipid phase of W<sub>1</sub>/O  
156 emulsions.

#### 157 Formation of double (W<sub>1</sub>/O/W<sub>2</sub>) emulsions

158 The previously prepared W<sub>1</sub>/O emulsion was dispersed in a secondary aqueous phase  
159 (W<sub>2</sub>) in a ratio 1/4 (primary emulsion/W<sub>2</sub>) leading to W<sub>1</sub>/O/W<sub>2</sub> emulsion formation. The  
160 W<sub>2</sub> phase contained 2% w/w sodium alginate, NaCl (0-0.25 M) and Tween 20 or lecithin  
161 (2-4% w/w) as hydrophilic surfactants. W<sub>1</sub>/O/W<sub>2</sub> emulsions were obtained by HSH with  
162 an Ultra Turrax (T25 digital Ultra-Turrax, IKA, Staufen, Germany) at 5,600 rpm and 2  
163 min followed by magnetic stirring at 750 rpm, during 3, 5, 18 and 24h.

### 164 **2.3. Characterization of water in oil emulsions (W<sub>1</sub>/O) and double** 165 **emulsions (W<sub>1</sub>/O/W<sub>2</sub>)**

166 In order to establish the most suitable processing conditions and formulation for the  
167 formation of W<sub>1</sub>/O and W<sub>1</sub>/O/W<sub>2</sub> emulsions, these were characterized in terms of mean  
168 droplet diameters (d[4:3]) and particle size distribution. Turbidity measurements were  
169 performed on selected double emulsions in order to detect possible flocculation or  
170 creaming phenomena. Once the formulation and processing conditions were optimized,  
171 apparent viscosity and color with regards to *a*\* and *b*\* parameters and whiteness index of  
172 W<sub>1</sub>/O and W<sub>1</sub>/O/W<sub>2</sub> emulsions were measured. Also, particle size and morphology of the  
173 resultant W<sub>1</sub>/O/W<sub>2</sub> emulsions were evaluated through confocal microscopy together with  
174 the assessment of their turbidity during 21 days of refrigerating storage.

175 **2.3.1. Particle size and particle size distribution**

176 The emulsion droplet size was measured by the laser diffraction technique with a  
177 Mastersizer 3000<sup>TM</sup> (Malvern Instruments Ltd., Worcestershire, UK). The measured  
178 droplet size was expressed as volume-weight and surface-weight diameter ( $d[4;3]$  and  
179  $d[3;2]$  in  $\mu\text{m}$ . Refractive indexes (RI) of corn oil and lemongrass essential oil were 1.47  
180 and 1.48, respectively. For the measurement of particle sizes of  $W_1/O$  emulsions,  
181 sunflower oil, whose RI was the same as corn oil, was used as dispersant, whereas  
182  $W_1/O/W_2$  emulsions were dispersed in distilled water (RI=1.33).

183 **2.3.2. Apparent viscosity**

184 A vibro-viscometer (SV-10, A&D Company, Tokyo, Japan) vibrating at 30 Hz  
185 was used to measure the viscosity (mPa·s) of 10 mL aliquots of the  $W_1/O$  and  $W_1/O/W_2$   
186 emulsions. Moreover, the viscosity of water, which was used as dispersant phase, was  
187 0.91 mPa·s. This value was considered with regard to DLS measurements, which were  
188 all performed at  $25 \pm 2$  °C.

189 **2.3.3. Color of  $W_1/O$  and  $W_1/O/W_2$  emulsions**

190 The color of  $W_1/O$  and  $W_1/O/W_2$  emulsions was measured with a colorimeter  
191 (Minolta CR-400, Konica Minolta Sensing, Inc., Osaka, Japan) at room temperature set  
192 up for illuminant D65 and 10° observer angle and calibrated with a standard white plate.  
193 CIE  $L^*$ ,  $a^*$  and  $b^*$  values were determined, and the whiteness index (WI) was calculated  
194 with equation 1 (Salvia-Trujillo, Rojas-Graü, Soliva-Fortuny, & Martín-Belloso, 2013a):

195

196 
$$WI = 100 - ((100 - L)^2 + (a^2 + b^2))^{0.5} \quad \text{eq.(1)}$$

197

198 **2.3.4. Turbidity over time**

199 The stability of the prepared  $W_1/O$  and  $W_1/O/W_2$  emulsions containing CHL and/or LG-  
200 EO was performed in duplicate through a turbidity study with a Turbiscan Classic  
201 (Formulation, Toulouse, France) during 21 days of refrigerated storage at 4°C. The  
202 turbidity measurement allows the detection of the most common destabilization  
203 mechanisms of emulsions such as creaming, sedimentation, flocculation or coalescence  
204 by multiple light scattering. Then, the Turbiscan software enables to interpret the obtained  
205 data easily.

206 **2.3.5. Confocal fluorescence microscopy**

207 Fresh double emulsions containing CHL and/or LG-EO were dyed with Nile red  
208 (Sigma Aldrich, Merk, Darmstadt, Germany), a fat-soluble fluorescent dye that was  
209 previously dissolved at 0.1% (w/v) in polyethylenglycol (Sigma Aldrich, Merk,  
210 Darmstadt, Germany). Afterwards, double emulsions microstructure was observed with  
211 an Olympus Spectral Confocal Microscope (Olympus FV1000, Melville, NY) with 100x  
212 oil immersion objective lens. All images were taken and processed using the instrument  
213 software program (Olympus FV10-ASW viewer, Melville, NY).

214  
215 **2.3.6. Encapsulation efficiency of double emulsions ( $W_1/O/W_2$ ) containing**  
216 ***chlorophyllin***

217 In order to calculate the CHL encapsulation efficiency (EE) of  $W_1/O/W_2$  emulsion,  
218 aliquots of 10 mL were placed inside a centrifuge tube and 20 mL of food grade methanol  
219 were added. After centrifuging (3000 g, 10 min) with a Hettich® Universal 320 centrifuge  
220 (Sigma-Aldrich, Darmstadt, Germany) the outer aqueous phase with the non-  
221 encapsulated CHL (free CHL) was filtered through a 0.22 mm Vinylidene Polyfluoride  
222 (PVDF) syringe filter and quantified by analyzing the solvent spectrophotometrically

223 with a V-670 spectrophotometer (Jasco, Tokyo, Japan) at 405 nm. The EE of the obtained  
224 W<sub>1</sub>/O/W<sub>2</sub> emulsions was calculated by equation (2) (Giroux et al. 2013):

225

$$226 \quad \%EE = \frac{C(W_1)*X(W_1) - C_S*(V+X(W_2))}{C(W_1)*X(W_1)} \times 100 \quad \text{eq.(2)}$$

227

228 where C(w<sub>1</sub>) is the initial CHL concentration in the internal aqueous phase of the emulsion  
229 (27 ppm), C<sub>S</sub> is the CHL concentration in the subphase collected after centrifugation of  
230 the diluted emulsion, X(W<sub>1</sub>) and X(W<sub>2</sub>) are, respectively, the mass fractions of the  
231 internal (0.06) and external (0.8) aqueous phases for 1Kg (V) of emulsion. All the  
232 measurements were performed in triplicate.

### 233 **2.3.7. Antioxidant capacity of double emulsions (W<sub>1</sub>/O/W<sub>2</sub>)**

234 The antioxidant capacity of CHL and lemongrass essential oil (LG-EO) both  
235 solved in methanol (used as controls), as well as, CHL- and CHL/LG-EO-loaded  
236 emulsions was determined by DPPH and FRAP assays. Although both methods are able  
237 to measure the antioxidant capacity of a sample, the main difference between them is that  
238 DDPH assay is based on the presence of radicals (DPP•), whereas FRAP consists of an  
239 electrons exchange (Thaipong et al. 2006).

240 The DPPH procedure was conducted according to the method of Brand-Williams, W.;  
241 Cuvelier, M. E. & Berset, (1995) with some modifications. The DPPH radical solution  
242 was prepared by dissolving 3.75 mg of DPPH radical in 100 mL of methanol. The  
243 absorbance of solution was adjusted to a value between 0.7-0.8 ± 0.02 (measured at 515  
244 nm). Aliquots of 10 µL of sample were placed in a microplate with 90 µL of Milli-Q water  
245 and 3900 µL of DPPH radical solution was added to each sample. Samples were incubated  
246 for 30 min in the dark and the absorbance at 405 nm was measured.

247 FRAP assay was carried out as described by Benzie & Strain (1996) with some  
248 modifications where 150  $\mu$ L of sample were placed into each tube and mixed with 2850  
249  $\mu$ L of FRAP solution. The samples were incubated at room temperature in the dark for 30  
250 min and the absorbance was measured at 630 nm after prior filtration.

251 Results were reported as mg of Trolox equivalents per mL of solution (mg TE/mL) using  
252 a standard curve of Trolox (Velderrain-Rodríguez et al., 2015). In both methods, triplicate  
253 determinations were made at each dilution of the standard.

254

## 255 **2.4. Statistics**

256 All the procedures were assessed in duplicate, and at least three measurements of each  
257 parameter were carried out. The statistical software SigmaPlot 11.0 (Systat Software Inc.,  
258 Pennsylvania, USA) was used to perform the analysis of variance. To determine  
259 differences among mean of the different procedures One Way ANOVA test was run at a  
260 5% significance level.

## 261 **3. Results and Discussion**

262 First, the formation of the primary ( $W_1/O$ ) emulsion was studied by determining the  
263 influence of the surfactant type and concentration, and homogenization method on the  
264 particle size and particle size distribution of the inner  $W_1$ -droplets dispersion. Second, the  
265 formation of the subsequent double emulsion was evaluated in terms of the surfactant  
266 type and concentration and emulsification conditions. The effect of the presence of salt  
267 and sodium alginate were assessed both in the primary and double emulsions. Finally, the  
268 incorporation of CHL in the inner  $W_1$  phase and LG-EO in the oil phase of the optimized  
269 double emulsion formulation was determined and the physicochemical stability and  
270 antioxidant capacity of the final double emulsion was determined.

### 271 **3.1. Water-in-oil emulsions (W<sub>1</sub>/O)**

272 Primary W<sub>1</sub>/O emulsions were formed at varying Span 80 or PGPR concentrations (4,  
273 6 and 10% w/w) (Figure 1A) by HSH (11,000 rpm, 5 min). Subsequently, several  
274 homogenization methods and conditions were studied, such as HSH, US or MF (Figure  
275 2). Also the influence of sodium alginate incorporation (0-2% w/w) as well as salt addition  
276 (0-0.25M) on the particle size of the primary W<sub>1</sub>/O dispersion was investigated (Figure  
277 1B and C, respectively).

#### 278 **3.1.1. Effect of type and concentration of the hydrophobic surfactant in the** 279 **particle size of W<sub>1</sub>/O emulsions**

280 The particle size of W<sub>1</sub>/O emulsions decreased at increasing the concentration of  
281 Span 80 or PGPR from 4% to 10% w/w (Figure 1A). Nevertheless, the performance in  
282 reducing the particle size of the W<sub>1</sub>/O emulsions was significantly different for both  
283 surfactants. In this regard, PGPR led to W<sub>1</sub>/O emulsions with remarkably smaller particle  
284 sizes than Span 80. In fact, particle sizes of PGPR-stabilized W<sub>1</sub>/O emulsions ranged  
285 between 0.396 and 0.802 μm with monomodal particle size distributions while those with  
286 Span 80 exhibited particle sizes above 32 μm for all the tested concentrations. Moreover,  
287 particle size distributions of W<sub>1</sub>/O emulsions containing Span 80 presented intensity  
288 peaks around 1 and 10 μm suggesting a high polydispersity.

289 Span 80 is a small molecular surfactant that is expected to rapidly adsorb to water  
290 droplets thus reducing the interfacial tension and avoiding coalescence (Wooster et al.  
291 2008). However, Surh et al. (2007) observed that although Span 80 was soluble in  
292 vegetable oils such as corn oil at room temperature, the resultant W<sub>1</sub>/O rapidly separated  
293 after the homogenization process. Therefore, in order to obtain water-in-oil emulsions  
294 with particle sizes sufficiently small to be re-encapsulated during the second step, Span  
295 80 needs to be at very high concentrations in the oil (Dickinson, 2011).

296 One alternative to avoid the use of elevated concentrations of surfactant is to  
297 increase the viscosity of the oil phase (Weiss and Muschiolik 2007). In this regard,  
298 polymeric surfactants such as PGPR are potential substitutes to stabilize multiple  
299 emulsions. Certainly, the use of PGPR at surfactant concentration of 4% w/w led to W<sub>1</sub>/O  
300 emulsions with particle size below 1 μm (Figure 1A). PGPR may be able to better interact  
301 with lipid phase due to its higher hydrophobicity compared to Span 80, thus forming a  
302 kinetic barrier protecting the emulsion against droplets coalescence (Tabibiazar and  
303 Hamishehkar 2015). Therefore, the chosen hydrophobic surfactant used for the formation  
304 of the subsequent double emulsions was PGPR (4% w/w).

305

### 306 **3.1.2. Influence of the homogenization procedure on the formation of W<sub>1</sub>/O** 307 **emulsions**

308 It has been reported that the fabrication of W<sub>1</sub> dispersed droplets of nanosized  
309 range contributes in the overall stability of the resultant W<sub>1</sub>/O/W<sub>2</sub> emulsion (Fathi et al.  
310 2012; Lamba et al. 2015). Therefore, three different high energy procedures including  
311 laboratory HSH, HSH followed by US or MF were tested in order to obtain W<sub>1</sub>/O  
312 emulsions with small particle sizes. The first HSH was performed at 11,000 or 22,000  
313 rpm during 1, 2, 3 or 5 minutes. At 11,000 rpm, the longer the processing time, the smaller  
314 the mean particle size (d[4:3]) of W<sub>1</sub>/O emulsions reaching values of 509 nm after 5 min  
315 (Figure 2A). However, as increasing the frequency of HSH processing up to 22,000 rpm,  
316 the destabilization of W<sub>1</sub>/O emulsions started after the first 2 minutes of processing  
317 leading to polydisperse distributions with particles sizes higher than 15 μm. This suggests  
318 that HSH frequency significantly affected the reduction of particle size. HSH device  
319 produces cavitation, collision and turbulence forces, which causes breakdown of the  
320 droplets and uniform dispersion of the dispersed phase (Lamba et al. 2015). However,

321 under certain conditions, these forces can cause an over-processing of the emulsions that  
322 may provoke re-coalescence due to an increase in the surface area, thus favoring  
323 polydispersion (Jafari et al. 2007). Therefore, the most suitable conditions to obtain  
324 nanoparticles and monomodal distributions were 11,000 rpm during 5 min.

325 After these conditions of HSH, US at amplitudes of 30, 60 and 100  $\mu\text{m}$  during 1,  
326 3 or 5 min; or MF (1-5 cycles at 800 bar) were applied in order to evaluate their effect in  
327  $W_1/O$  emulsions particle size. Regarding US application, the longer the processing time,  
328 the larger the particle size of  $W_1/O$  emulsions regardless the amplitude applied (Figure  
329 2B). In this regard, the smallest particle sizes were obtained after 1 min of US in all the  
330 cases being the one prepared with an amplitude of 30  $\mu\text{m}$ , which lead particle sizes below  
331 1  $\mu\text{m}$ , ( $d[4:3]= 850 \text{ nm}$ ). Moreover, all the distributions were polydisperse thus suggesting  
332 an over-processing of emulsions, which may cause droplets re-coalescence and further  
333 emulsions destabilization (Jafari et al. 2007).

334 In the case of using MF after HSH, particle size of  $W_1/O$  promptly increased  
335 immediately after the first cycle of microfluidization from 0.51 to 38.06  $\mu\text{m}$  and it was  
336 further reduced during the following cycles (Figure 2C). Despite the fact that MF is  
337 considered as the most efficient technique to form fine emulsions, the reduction in particle  
338 size it is effective only until a certain limit (Xu et al. 2014). This means that shearing the  
339 emulsion at the same pressure may not produce any additional rupturing or change in  
340 particle size (Meleson et al. 2004; Laura Salvia-Trujillo et al. 2013b). Indeed, MF reduced  
341 particle size of  $W_1/O$  emulsions with increasing cycles up to the 4<sup>th</sup> one, reaching values  
342 of 1.51  $\mu\text{m}$  (Figure 2C). Then, particle size slightly increased after the application of the  
343 last cycle (Figure 2C). In agreement with our results, Jafari et al. (2007) observed an  
344 increase in the  $d[4:3]$  of their submicron emulsions after increase the microfluidization  
345 pressure from 20 to 60 MPa. Several authors including Kolb, Viardot, Wagner, & Ulrich



346 (2001) and Schultz, Wagner, Urban, & Ulrich (2004) also observed this over-processing  
347 during high-pressure homogenization. This phenomenon has been attributed to the fact  
348 that the efficiency of each microfluidization cycle is not complete since shear flow is not  
349 equal distributed in all the emulsion being the droplets near the walls who experimented  
350 the weakest forces thus resulting in size distributions containing larger particles.

351

### 352 **3.1.3. Impact of sodium alginate and NaCl salt incorporation in the** 353 **stabilization of $W_1/O$ emulsions**

354 Sodium alginate incorporation has a critical impact in the stabilization of  $W_1/O$   
355 emulsions since its absence led to emulsions with huge particle sizes ( $>280 \mu\text{m}$ ), whereas  
356 a concentration of 1%  $w/w$  was enough to obtain monomodal distributions and particle  
357 sizes below  $1 \mu\text{m}$  (Figure 1B). Moreover, at increasing the concentration of sodium  
358 alginate up to 2%  $w/w$  water-in-oil emulsions showed twice of apparent viscosity (data  
359 not shown). It is reported that the enhancement of the viscosity of the prior  $W_1/O$  emulsion  
360 may improve the stability of the subsequent double emulsion (Muschiolik & Dickinson,  
361 2017). In addition, other authors have also observe a positive effect on  $W_1/O/W_2$   
362 stabilization after the incorporation of biopolymers to the inner aqueous phase  
363 (Dickinson, 2011b; Mezzenga, Folmer, & Hughes, 2004). It is believed that the origin of  
364 this enhancement of stability is due to the interaction between polysaccharide and  
365 lipophilic surfactant, which provides a viscoelastic barrier thus preventing droplets  
366 coalescence (Garti 1997). Indeed, some authors as Dickinson (2011b) also observed this  
367 synergistic stabilizing effect between the biopolymer (*eg.* sodium caseinate) and PGPR.

368 Also, the incorporation of electrolytes like NaCl together with biopolymers to the  
369 inner water phase of double emulsions ( $W_1$ ) can improve their thermodynamic stability  
370 by controlling the osmotic balance (Benichou et al. 2004). Figure 1C shows that salt

371 incorporation had not a significant effect in the particle size of fresh emulsions. However,  
372 Figure 3A revealed that water-in-oil emulsions prepared without NaCl, experimented an  
373 increase of particle size (zone II) and creaming (zone III) during the first 72 h. Each  
374 backscattering plot generated can be split in three zones named as I (on the left), II (in the  
375 middle) and III (on the right) corresponding to the bottom, the intermediate part and the  
376 top of the tube, respectively. In this regard, down peaks in zone I mean clarifications and  
377 are usually related with up peaks in zone III thus suggesting creaming. Likewise, up peaks  
378 in zone I may indicate sedimentation and are often accompanied by down peaks in zone  
379 III. The displacement of the horizontal lines from zone II points out variations in  
380 emulsions particle size due to flocculation or coalescence. However, those containing  
381 0.05M of NaCl salt did not experiment variation in their turbidity thus suggesting that  
382 they were stable during at least a week of refrigerated storage (Figure 3C). In the case of  
383  $W_1/O$  emulsions with concentrations of salt below or over 0.05M, phase separation  
384 occurred after 4 days of storage (Figures 3B or 3E and 3F, respectively) being this  
385 disruption even more abrupt in emulsions containing NaCl concentrations of 0.25M (zone  
386 II). Scherze, Knoth, & Muschiolik (2006) also observed that the addition of NaCl to the  
387 inner aqueous phase of  $W_1/O$  emulsions containing PGPR as surfactant was essential to  
388 prevent coalescence phenomenon. This suggests that the interaction between NaCl and  
389 PGPR may contribute to increase droplet size stability since electrolytes can increase the  
390 adsorption density of surfactant thus reducing emulsion interfacial tension (Aronson &  
391 Petko, 1993; Dickinson, 2011).

### 392 **3.2. Double emulsions ( $W_1/O/W_2$ )**

393 Initially, the prepared  $W_1/O$  emulsion containing 2% w/w of sodium alginate and  
394 NaCl 0.05M in the inner aqueous phase, was dispersed in a second aqueous phase ( $W_2$ )  
395 and the effect of the time of magnetic stirring (3-24h) on the formation of  $W_1/O/W_2$

396 emulsions was assessed (Figure 4A). Secondly,  $W_1/O/W_2$  emulsions were prepared using  
397 different concentrations (2-4% w/w) of Tween 20 or lecithin as hydrophobic surfactants  
398 (Figure 4B-D). Finally, the impact of NaCl incorporation (0-0.25 M) in the  $W_2$  on the  
399 particle size of the resultant  $W_1/O/W_2$  emulsions was also evaluated (Figure 4E).

400

### 401 **3.2.1 Effect of the homogenization procedure on the formation of $W_1/O/W_2$**

402 After dispersing  $W_1/O$  emulsion into the  $W_2$  both phases were mixed by HSH at  
403 5,600 rpm and 2 min, followed by magnetic stirring at 750 rpm during different times (3-  
404 24h) to the formation of  $W_1/O/W_2$  emulsions. Regarding Figure 4A, all the obtained  
405 double emulsions were polydisperse probably due to the application of low shear energies  
406 required to prevent the disruption of the initial water-in-oil interface (Márquez, Palazolo,  
407 & Wagner, 2007; Muschiolik & Dickinson, 2017). As it is shown in Figure 4A, the higher  
408 the time of stirring, the lower the particle size of  $W_1/O/W_2$  emulsions, reaching a value of  
409 3.6  $\mu\text{m}$  after 24h with regards the initial one of 8.6  $\mu\text{m}$ . It is well-known that lower particle  
410 sizes provide high colloidal stability and large interfacial area to volume ratio thus  
411 preventing phenomena such as coalescence (Wooster et al. 2008). Nonetheless, the final  
412 particle size of  $W_1/O/W_2$  emulsion has to be large enough to ensure the absence of external  
413 ( $W_1/O$  globule- $W_1/O$  globule) and internal ( $W_1$  droplet- $W_1$  droplet) coalescence  
414 phenomena (Bonnet et al. 2010). In their study Bonnet et al. (2010) reported that their  
415  $W_1/O/W_2$  emulsions, in which  $W_1/O$  globules were about eight times larger than  $W_1$   
416 droplets, remained invariant over 30 days. In this regard, concerning our results,  $W_1$   
417 droplets of 509 nm should be able to coexist without coalescing within those  $W_1/O$ , whose  
418 mean particle sizes were around 4  $\mu\text{m}$ . Therefore, magnetic stirring performed at 750 rpm  
419 during 24 h were considered as valid processing conditions to prepare  $W_1/O/W_2$   
420 emulsions.

### 3.2.2 Influence of the hydrophilic surfactant type and concentration on the particle size of $W_1/O/W_2$

In order to observe the influence of type and concentration of surfactant on their particle size and stability over time,  $W_1/O/W_2$  emulsions containing Tween 20 or lecithin as hydrophilic surfactant at concentrations of 2 and 4% *w/w* were prepared (Figure 4B and C-D, respectively). As increasing the concentration of surfactant from 2 to 4% *w/w*, the mean particle size of  $W_1/O/W_2$  emulsions, decreased from 7.3 to 6.8  $\mu\text{m}$  when Tween 20 was used and from 8.7 to 5.7  $\mu\text{m}$  in those lecithin-stabilized. Therefore, only in the case of lecithin the premise that the higher the concentration of surfactant, the smaller the particle size is fulfilled (Zirak and Pezeshki 2015). In spite of 4% *w/w* lecithin led to smaller particles sizes, at lower concentration of lecithin (2% *w/w*) the minor peak observed in the particle size distribution plot was minor and the major peak was more intense. This suggested a more successful  $W_1/O/W_2$  emulsion formation since the polydispersity index was lower (Figure 4B). Moreover,  $W_1/O/W_2$  emulsions containing lecithin remained stable during at least 10 days as suggested by the turbidity measurements, whereas in those containing Tween 20 destabilization occurred after the second day of storage (Figure 4C and 4D, respectively). Indeed, according to Bastida-Rodríguez (2013) lecithin and PGPR have complementary rheological properties, which allows an optimal control in the stability of the prepared systems.

### 3.2.3 Impact of sodium alginate and NaCl salt incorporation on the stabilization of $W_1/O/W_2$

The effect of NaCl incorporation on the particle size and particle distribution of  $W_1/O/W_2$  emulsions is presented in Figure 4E. All of the emulsions showed bimodal distributions regardless the concentration of salt. Moreover,  $W_1/O/W_2$  emulsions particle size increased from 6.3 to 7.9  $\mu\text{m}$  when the concentration of NaCl in the  $W_2$  phase

446 incremented from 0.01 to 0.25M. However, significant differences between particle sizes  
447 of double emulsions containing 0.05 or 0.1M of NaCl were not observed. In order to  
448 ensure the stability of the second interface, a balance between Laplace and osmotic  
449 pressures is required, which means between  $W_1$  droplets in oil and between  $W_1$  droplets  
450 and the outer aqueous phase ( $W_2$ ) (Kanouni et al. 2002). Therefore, it is recommended  
451 that the concentration of salt in both aqueous phases ( $W_1$  and  $W_2$ ) was the same. Actually,  
452 an excess or lack of salt in one of them may cause droplets migration to or from the other,  
453 respectively causing subsequent breaking of  $W_1/O/W_2$  emulsions structure (Rosano and  
454 Hidrot 1998). Likewise, sodium alginate contains functional groups such as carboxylates,  
455 which can easily dissociate in the aqueous phase and as a result, provide negative charge  
456 to the emulsions (Pereira et al. 2013). Therefore, again an excess of a lack of ions in one  
457 of the aqueous phases may disrupt the osmotic balance between them leading to  $W_1/O/W_2$   
458 emulsion destabilization. Thus, in order to avoid ions migration, which may in turn lead  
459 to water diffusion between the inner and outer aqueous phases, 2% w/w of sodium alginate  
460 and 0.05M NaCl were also added in the  $W_2$  phase.

### 461 **3.3 Physicochemical characterization of $W_1/O$ and $W_1/O/W_2$ emulsions** 462 **containing chlorophyllin and/or lemongrass essential oil**

463  $W_1/O/W_2$  emulsions loaded with CHL and/or LG-EO were prepared in order to assess  
464 the effectiveness of these systems as carriers of one or more active compounds through  
465 the study of their physicochemical properties (*ie.* particle size and distribution, turbidity,  
466 apparent viscosity and color). Also, the encapsulation efficiency of CHL and antioxidant  
467 capacity of  $W_1/O/W_2$  emulsions were evaluated.

#### 468 ***Physicochemical properties of loaded $W_1/O$ and $W_1/O/W_2$ emulsions***

469 The three types of prepared  $W_1/O$  emulsions containing (i) 27 ppm CHL, (ii) 1%  
470 w/w LG-EO or (iii) both, showed particle sizes around 560 nm ( $d[4;3]$ ), whereas their

471 subsequent  $W_1/O/W_2$  emulsions had  $d[4;3]$  about  $6-7 \pm 0.7 \mu\text{m}$  (Figure 5A). Moreover,  
472 droplet sizes of  $W_1/O$  and  $W_1/O/W_2$  emulsions loaded with CHL and/or LG-EO were not  
473 significantly different. The formation of  $W_1/O/W_2$  emulsions containing CHL and/or LG-  
474 EO and their particle size were confirmed by confocal images. As it can be observed in  
475 Figure 5B, the majority of oil droplets contained several small water droplets within them,  
476 proving the successful formation of  $W_1/O/W_2$  emulsions (Lamba et al. 2015).

477 Moreover,  $W_1/O/W_2$  emulsions containing LG-EO exhibited high stability during  
478 storage time (Figure 5C), where initiation of creaming was registered (small up peaks in  
479 zone III) after 15 or 21 days of refrigerated storage (without or with CHL, respectively).  
480 However, particle sizes of  $W_1/O/W_2$  emulsions without LG-EO changed over time as  
481 indicated by the displacement of horizontal lines in zone II (Figure 5C). Our previous  
482 research showed a synergistic effect between LG-EO and the biopolymer (Artiga-Artigas  
483 et al. 2018). Therefore, the presence of LG-EO in the oil phase of double emulsions might  
484 improve  $W_1/O/W_2$  emulsions emulsification in presence of sodium alginate thus  
485 enhancing their stability. These results are in agreement with the %EE values since double  
486 emulsions containing CHL and LG-EO exhibited higher EE than those without EO ( $91 \pm$   
487  $6 \cdot 10^{-3}$  and  $84 \pm 1 \cdot 10^{-2}$ , respectively). This suggests that concentrations of 1%  $w/w$  LG-EO  
488 were able to slowdown inner water droplets diffusion. Therefore, a small quantity of LG-  
489 EO (1%  $w/w$ ) in the oil phase of  $W_1/O/W_2$  emulsions might contribute positively to extend  
490 their stability over time by improving emulsification.

491 In general, the apparent viscosity of  $W_1/O/W_2$  emulsions ( $>250 \text{ mPa}\cdot\text{s}$ ) almost  
492 duplicated the viscosity of  $W_1/O$  emulsions ( $\approx 145 \text{ mPa}\cdot\text{s}$ ) as it is shown in Tables 2 and  
493 1, respectively. This is probably because  $W_1/O/W_2$  emulsions also contained 2%  $w/w$  of  
494 sodium alginate in the outer aqueous phase ( $W_2$ ). Yang, Jiang, He, & Xia (2012) also  
495 observed a strong influence of sodium alginate dispersed in the aqueous phase in the

496 apparent viscosity of emulsions, which can increase until sixty times when the  
497 biopolymer was not microfluidized (Artiga-Artigas et al. 2017).

498 Moreover, the color of  $W_1/O$  and  $W_1/O/W_2$  emulsions varied significantly  
499 depending on the encapsulated bioactive as it can be observed in Tables 1 and 2,  
500 respectively. In emulsions containing CHL,  $a^*$  values were negative and  $b^*$  values  
501 positive, which respectively determine green and yellow characteristics. After  $W_1/O/W_2$   
502 emulsion formation  $a^*$  parameter decreased (became less negative), whereas  $b^*$  increased  
503 (Tables 1-2). This can be explained because CHL in the  $W_1/O/W_2$  emulsion is doubly  
504 encapsulated, then if there is no migration of the pigment to the  $W_2$ , green color should  
505 be less intense. Additionally, this double encapsulation consists of dispersing the primary  
506 emulsion containing the CHL into a second water phase ( $W_2$ ) in a ratio 1/4. Therefore,  
507 the final concentration of CHL in the  $W_1/O/W_2$  emulsion is lower than in the  $W_1/O$   
508 emulsion. High values of  $b^*$  parameter were also expected because of the intense yellow  
509 color of corn oil (Ekthamasut and Akesowan 2010). In addition, WI of  $W_1/O/W_2$  emulsion  
510 was higher than of the primary emulsion (Tables 2 and 1, respectively). After  
511 homogenization corn oil as many vegetable oils may increase emulsions opacity, which  
512 translates as the increase of their WI (Artiga-Artigas et al. 2017). It is described that the  
513 light scattering of oil droplets depends on emulsions droplet size ( McClements, 2002).  
514 Therefore, large particles of  $W_1/O/W_2$  emulsion scatter the light more intensely than  
515 smaller ones ( $W_1/O$  emulsion), which may cause an increase in the lightness, opacity and  
516 whiteness index of emulsions (McClements, 2011; Salvia-Trujillo, Rojas-Graü, Soliva-  
517 Fortuny, & Martín-Belloso, 2015).

518 Likewise, in those emulsions containing LG-EO,  $b^*$  parameter was predominant  
519 since both oils (LG-EO and corn oil) are yellow-colored. Moreover, as observed in Tables  
520 1 and 2, this parameter abruptly increased after the formation of  $W_1/O/W_2$  emulsion. This

521 increase might be directly related to a decrease in WI according to equation 1. The WI of  
522  $W_1/O$  emulsions containing LG-EO was higher than that of those prepared with just corn  
523 oil. This parameter depends fundamentally on the RI of continuous and dispersed phases  
524 thus since the RI of LG-EO is 1.48 while corn oil's RI is 1.47, LG-EO contributed to  
525 increase emulsions opacity (McClements, 2002). However, the second HSH and  
526 following magnetic stirring did have a positive effect in reducing WI. Similarly to our  
527 results, Guerra-Rosas, Morales-Castro, Ochoa-Martínez, Salvia-Trujillo, & Martín-  
528 Belloso (2016) and Salvia-Trujillo et al. (2013a) also reported a decrease of WI in  
529 nanoemulsions containing LG-EO and alginate in the aqueous phase after HSH  
530 procedures.

#### 531 *Antioxidant capacity of loaded $W_1/O/W_2$ emulsions*

532 Antioxidant capacity (AC) of  $W_1/O/W_2$  emulsions and the two prepared controls  
533 expressed as mg of Trolox equivalents (TE) per g of emulsion is shown in Table 3.  
534 Regarding the solutions of pure bioactive compounds in MeOH, both assays showed that  
535 AC of LG-EO was higher than the AC of CHL. Based on the results obtained by DPPH  
536 assay, all  $W_1/O/W_2$  emulsions presented high AC and similar among them and to both  
537 control solutions ( $\approx 8,000,000$  mgTE/g). However, when FRAP assay was performed, AC  
538 of  $W_1/O/W_2$  emulsions containing LG-EO was significantly higher than the other two  
539 types of double emulsions (Table 3) being those containing both bioactive compounds  
540 which exhibited the lowest AC ( $1,696.4 \pm 643.9$  mgTE/g). It could be due to FRAP assay  
541 is based on the reducing power of double emulsions and more specifically, of bioactive  
542 compounds within them. Thus, this assay consists of detecting the reduction of ferric ion  
543 ( $Fe^{3+}$ ) into ferrous iron ( $Fe^{2+}$ ) using ferrozine as dye (Cheung et al. 2016). In this regard,  
544 if some species are reduced, others have to be oxidized being the LG-EO the most  
545 exposed bioactive compound in double emulsions. After catalytic oxidation, the essential



546 oils show stronger antioxidant effects than before oxidation, as measured by both  
547 methods, DPPH and FRAP (Jukić and Miloš 2005). Thus, the lower AC of LG-EO in  
548 presence of CHL suggests that the latter is preventing essential oil oxidation.

#### 549 **4. Conclusion**

550 Summarizing, neither US nor MF techniques combined with HSH were able to  
551 reduce  $W_1/O$  emulsion particle size as much as the HSH alone since they cause an over-  
552 processing of the emulsions. This HSH (11,000 rpm, 5 min) followed by HSH (5,600  
553 rpm, 2 min) and magnetic stirring (750 rpm, 24h) allowed stable  $W_1/O/W_2$  emulsion  
554 preparation. PGPR acted more efficiently than Span 80 as emulsifier of the first interface  
555 probably because of the higher hydrophobicity of the polymeric surfactant. Sodium  
556 alginate and NaCl (0.05M) incorporated in the  $W_1$  of  $W_1/O$  emulsions were absolutely  
557 necessary to reduce particle size and better stabilize  $W_1/O$  emulsions. In this regard, the  
558 outer aqueous phase had to contain the same concentrations of sodium alginate and salt  
559 as  $W_1$  to prevent ions migration from one phase to the other causing the disruption of  
560  $W_1/O/W_2$  emulsion.

561 Furthermore, template  $W_1/O/W_2$  emulsions allowed the successful incorporation of  
562 CHL and LG-EO without suffering destabilization. Both active compounds loaded in the  
563 prepared dual systems not only maintained their AC but showed a synergic behavior.  
564 Indeed, LG-EO incorporation increased the %EE of CHL, suggesting that the oil was able  
565 to slowdown inner water droplets diffusion and in turn, CHL delayed essential oil  
566 oxidation according to AC values, **which is of great importance from the technological and**  
567 **nutritional point of view.** Moreover, LG-EO and sodium alginate contributed positively to  
568 reduce particle size and extending the stability of  $W_1/O/W_2$  emulsions over time.

569 Therefore, this study presents an effective two-step optimized procedure to form

570 stable W<sub>1</sub>/O/W<sub>2</sub> emulsions and evidences their potential capacity as delivery system  
571 templates to encapsulate and carry two or more active compounds with different polarity  
572 and diverse functionality.

## 573 5. Acknowledgments

574 This study was funded by the Ministry of Economy, Industry and Competitiveness  
575 (MINECO/FEDER, UE) throughout project **AGL2015-65975-R**. Authors María Artiga-  
576 Artigas and Anna Molet-Rodríguez thank the University of Lleida for their pre-doctoral  
577 fellowship. Author Laura Salvia-Trujillo thanks the “Secretaria d’Universitats i Recerca  
578 del Departament d’Empresa i Coneixement de la Generalitat de Catalunya” for the  
579 Beatriu de Pinós post-doctoral grant **BdP2016 00336**.

580

581 Conflict of Interest: The authors declare that they have no conflict of interest.

## 582 6. References

583 Altuntas, O. Y., Sumnu, G., & Sahin, S. (2017). Preparation and characterization of W/O/W type  
584 double emulsion containing PGPR–lecithin mixture as lipophilic surfactant. *Journal of*  
585 *Dispersion Science and Technology*, 38(4), 486–493.

586 Aronson, Michael P.; Petko, M. F. (1993). Highly Concentrated Water-in-Oil Emulsions:  
587 Influence of Electrolyte on Their Properties and Stability. *Journal of Colloid and Interface*  
588 *Science*, 159, 134–149.

589 Artiga-Artigas, M., Acevedo-Fani, A., & Martín-Belloso, O. (2017). Effect of sodium alginate  
590 incorporation procedure on the physicochemical properties of nanoemulsions. *Food*  
591 *Hydrocolloids*, 70, 191–200.

592 Artiga-Artigas, M., Guerra-Rosas, M. I., Morales-Castro, J., Salvia-Trujillo, L., & Martín-  
593 Belloso, O. (2018). Influence of essential oils and pectin on nanoemulsion formulation: A  
594 ternary phase experimental approach. *Food Hydrocolloids*, 81, 209–219.

- 595 Bastida-Rodríguez, J. (2013). The Food Additive Polyglycerol Polyricinoleate (E-476):  
596 Structure, Applications, and Production Methods. *ISRN Chemical Engineering*, 2013, 1–  
597 21.
- 598 Benichou, A., Aserin, A., & Garti, N. (2004). Double emulsions stabilized with hybrids of  
599 natural polymers for entrapment and slow release of active matters. *Advances in Colloid  
600 and Interface Science*, 108–109, 29–41.
- 601 Benzie, I. F. F., & Strain, J. J. (1996). The Ferric Reducing Ability of Plasma (FRAP) as a  
602 Measure of “Antioxidant Power”: The FRAP Assay. *Analytical Biochemistry*, 239(1), 70–  
603 76.
- 604 Bonnet, M., Cansell, M., Placin, F., Anton, M., & Leal-Calderon, F. (2010). Impact of sodium  
605 caseinate concentration and location on magnesium release from multiple W/O/W  
606 emulsions. *Langmuir*, 26(12), 9250–9260.
- 607 Brand-Williams, W.; Cuvelier, M. E.; Berset, C. (1995). Use of a free radical method to evaluate  
608 antioxidant activity. *Lebensmittel Wissenschaft und Technologie*, 30, 25–30.
- 609 Cheel, J., Theoduloz, C., Rodríguez, J., & Schmeda-Hirschmann, G. (2005). Free radical  
610 scavengers and antioxidants from lemongrass (*Cymbopogon citratus* (DC.) Stapf.).  
611 *Journal of Agricultural and Food Chemistry*, 53(7), 2511–2517.
- 612 Cheung, T., Nigam, P., & Owusu-Apenten, R. (2016). Antioxidant Activity of Curcumin and  
613 Neem (*Azadirachta indica*) Powders: Combination Studies with ALA Using MCF-7 Breast  
614 Cancer Cells. *Journal of Applied Life Sciences International*, 4(3), 1–12.
- 615 Dickinson, E. (2011a). Double Emulsions Stabilized by Food Biopolymers. *Food Biophysics*,  
616 6(1), 1–11.
- 617 Dickinson, E. (2011b). Double Emulsions Stabilized by Food Biopolymers. *Food Biophysics*,  
618 6(1), 1–11.
- 619 Dickinson, E. (2011). Mixed biopolymers at interfaces: Competitive adsorption and multilayer

- 620 structures. *Food Hydrocolloids*, 25(8), 1966–1983.
- 621 Ekthamasut, K., & Akesowan, A. (2010). Effect of Vegetable Oils on Physical Characteristics of  
622 Edible Konjac Films. *Water*, (4).
- 623 Fathi, M., Mozafari, M. R., & Mohebbi, M. (2012). Nanoencapsulation of food ingredients  
624 using lipid based delivery systems. *Trends in Food Science & Technology*, 23(1), 13–27.
- 625 Garti, N. (1997). Progress in stabilization and transport phenomena of double emulsions in food  
626 applications. *LWT - Food Science and Technology*, 30(3), 222–235.
- 627 Garti, N., & Bisperink, C. (1998). Double emulsions: Progress and applications. *Current*  
628 *Opinion in Colloid & Interface Science*, 3(6), 657–667.
- 629 Giroux, H. J., Constantineau, S., Fustier, P., Champagne, C. P., St-Gelais, D., Lacroix, M., &  
630 Britten, M. (2013). Cheese fortification using water-in-oil-in-water double emulsions as  
631 carrier for water soluble nutrients. *International Dairy Journal*, 29(2), 107–114.
- 632 Guerra-Rosas, M. I., Morales-Castro, J., Cubero-Márquez, M. A., Salvia-Trujillo, L., & Martín-  
633 Belloso, O. (2017). Antimicrobial activity of nanoemulsions containing essential oils and  
634 high methoxyl pectin during long-term storage. *Food Control*, 77.  
635 doi:10.1016/j.foodcont.2017.02.008
- 636 Guerra-Rosas, M. I., Morales-Castro, J., Ochoa-Martínez, L. A., Salvia-Trujillo, L., & Martín-  
637 Belloso, O. (2016). Long-term stability of food-grade nanoemulsions from high methoxyl  
638 pectin containing essential oils. *Food Hydrocolloids*, 52, 438–446.
- 639 Jafari, S. M., He, Y., & Bhandari, B. (2007). Production of sub-micron emulsions by ultrasound  
640 and microfluidization techniques. *Journal of Food Engineering*, 82(4), 478–488.
- 641 Jukić, M., & Miloš, M. (2005). Catalytic Oxidation and Antioxidant Properties of Thyme  
642 Essential Oils (*Thymus vulgarae* L.). *Croatica Chemica Acta*, 78(1), 105–110.
- 643 Kanouni, M., Rosano, H. L., & Naouli, N. (2002). Preparation of a stable double emulsion  
644 (W1/O/W2): Role of the interfacial films on the stability of the system. *Advances in*

645 *Colloid and Interface Science*, 99(3), 229–254.

646 Kolb, G., Viardot, K., Wagner, G., & Ulrich, J. (2001). Evaluation of a new high-pressure  
647 dispersion unit (HPN) for emulsification. *Chemical Engineering and Technology*, 24(3),  
648 293–296.

649 López-Carballo, G., Hernández-Muñoz, P., Gavara, R., & Ocio, M. J. (2008). Photoactivated  
650 chlorophyllin-based gelatin films and coatings to prevent microbial contamination of food  
651 products. *International Journal of Food Microbiology*, 126(1–2), 65–70.

652 Lamba, H., Sathish, K., & Sabikhi, L. (2015). Double Emulsions: Emerging Delivery System  
653 for Plant Bioactives. *Food and Bioprocess Technology*, 8(4), 709–728.

654 Márquez, A. L., Palazolo, G. G., & Wagner, J. R. (2007). Water in oil (w/o) and double (w/o/w)  
655 emulsions prepared with spans: Microstructure, stability, and rheology. *Colloid and*  
656 *Polymer Science*, 285(10), 1119–1128.

657 McClements, D. J. (2002). Theoretical prediction of emulsion color. *Advances in Colloid and*  
658 *Interface Science*, 97(1–3), 63–89.

659 McClements, D. J. (2011). Edible nanoemulsions: fabrication, properties, and functional  
660 performance. *Soft Matter*, 7(6), 2297–2316.

661 Meleson, K., Graves, S., & Mason, T. G. (2004). Formation of concentrated nanoemulsions by  
662 extreme shear. *Soft Materials*, 2(2–3), 109–123.

663 Mezzenga, R., Folmer, B. M., & Hughes, E. (2004). Design of double emulsions by osmotic  
664 pressure tailoring. *Langmuir*, 20(9), 3574–3582. doi:10.1021/la036396k

665 Muschiolik, G. (2007). Multiple emulsions for food use. *Current Opinion in Colloid and*  
666 *Interface Science*, 12(4–5), 213–220.

667 Muschiolik, G., & Dickinson, E. (2017). Double Emulsions Relevant to Food Systems:  
668 Preparation, Stability, and Applications. *Comprehensive Reviews in Food Science and*  
669 *Food Safety*, 16(3), 532–555.

- 670 Muschiolik, G., & Dickinson, E. (2017). Double Emulsions Relevant to Food Systems:  
671 Preparation, Stability, and Applications. *Comprehensive Reviews in Food Science and*  
672 *Food Safety*, 16(3), 532–555.
- 673 Pereira, R., Carvalho, A., Vaz, D. C., Gil, M. H., Mendes, A., & Bártolo, P. (2013).  
674 Development of novel alginate based hydrogel films for wound healing applications.  
675 *International Journal of Biological Macromolecules*, 52, 221–230.
- 676 Rosano, H. L., & Hidrot, J. P. (1998). Stability of W /O/W multiple emulsions 1 2 In uence of  
677 ripening and interfacial interactions, 138, 109–121.
- 678 Salvia-Trujillo, L., Rojas-Graü, A., Soliva-Fortuny, R., & Martín-Belloso, O. (2013a).  
679 Physicochemical Characterization of Lemongrass Essential Oil-Alginate Nanoemulsions:  
680 Effect of Ultrasound Processing Parameters. *Food and Bioprocess Technology*, 6(9).
- 681 Salvia-Trujillo, L., Rojas-Graü, A., Soliva-Fortuny, R., & Martín-Belloso, O. (2015).  
682 Physicochemical characterization and antimicrobial activity of food-grade emulsions and  
683 nanoemulsions incorporating essential oils. *Food Hydrocolloids*, 43, 547–556.
- 684 Salvia-Trujillo, L., Rojas-Graü, M. A., Soliva-Fortuny, R., & Martín-Belloso, O. (2013b). Effect  
685 of processing parameters on physicochemical characteristics of microfluidized lemongrass  
686 essential oil-alginate nanoemulsions. *Food Hydrocolloids*, 30(1), 401–407.
- 687 Scherze, I., Knoth, A., & Muschiolik, G. (2006). Effect of emulsification method on the  
688 properties of lecithin- and PGPR-stabilized water-in-oil-emulsions. *Journal of Dispersion*  
689 *Science and Technology*, 27(4), 427–434.
- 690 Schultz, S., Wagner, G., Urban, K., & Ulrich, J. (2004). High-pressure homogenization as a  
691 process for emulsion formation. *Chemical Engineering and Technology*, 27(4), 361–368.
- 692 Su, J., Flanagan, J., Hemar, Y., & Singh, H. (2006). Synergistic effects of polyglycerol ester of  
693 polyricinoleic acid and sodium caseinate on the stabilisation of water-oil-water emulsions.  
694 *Food Hydrocolloids*, 20(2–3 SPEC. ISS.), 261–268.

695 Surh, J., Vladislavljević, G. T., Mun, S., & McClements, D. J. (2007). Preparation and  
696 characterization of water/oil and water/oil/water emulsions containing biopolymer-gelled  
697 water droplets. *Journal of Agricultural and Food Chemistry*, 55(1), 175–184.

698 Tabibiazar, M., & Hamishehkar, H. (2015). Formulation of a food grade water-in-oil  
699 nanoemulsion: Factors affecting on stability. *Pharmaceutical Sciences*, 21(4), 220–224.

700 Thaipong, K., Boonprakob, U., Crosby, K., Cisneros-Zevallos, L., & Hawkins Byrne, D. (2006).  
701 Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity  
702 from guava fruit extracts. *Journal of Food Composition and Analysis*, 19(6–7), 669–675.

703 Tumolo, T., & Lanfer-Marquez, U. M. (2012). Copper chlorophyllin: A food colorant with  
704 bioactive properties? *Food Research International*, 46(2), 451–459.

705 Velderrain-Rodríguez, G. R., Ovando-Martínez, M., Villegas-Ochoa, M., Ayala-Zavala, J. F.,  
706 Wall-Medrano, A., Álvarez-Parrilla, E., et al. (2015). Antioxidant Capacity and  
707 Bioaccessibility of Synergic Mango (cv. Ataulfo) Peel Phenolic Compounds in Edible  
708 Coatings Applied to Fresh-Cut Papaya. *Food and Nutrition Sciences*, 6(6), 365–373.

709 Wang, Y., Zhang, T., & Hu, G. (2006). Structural evolution of polymer-stabilized double  
710 emulsions. *Langmuir*, 22(1), 67–73.

711 Weiss, J., & Muschiolik, G. (2007). Factors affecting the droplet size of water-in-oil emulsions  
712 (W/O) and the oil globule size in Water-in-oil-in-water emulsions (W/O/W). *Journal of*  
713 *Dispersion Science and Technology*, 28(5), 703–716.

714 Wooster, T. J., Golding, M., & Sanguansri, P. (2008). Ripening Stability. *Langmuir : the ACS*  
715 *journal of surfaces and colloids*, 24(10), 12758–12765.

716 Xu, J.-H., Ge, X.-H., Chen, R., & Luo, G.-S. (2014). Microfluidic preparation and structure  
717 evolution of double emulsions with two-phase cores. *RSC Advances*, 4(4), 1900.

718 Yan, J., & Pal, R. (2001). Osmotic swelling behavior of globules of W/O/W emulsion liquid  
719 membranes. *Journal of Membrane Science*, 190(1), 79–91.

720 Yang, J. S., Jiang, B., He, W., & Xia, Y. M. (2012). Hydrophobically modified alginate for  
721 emulsion of oil in water. *Carbohydrate Polymers*, 87(2), 1503–1506.

722 Zirak, M. B., & Pezeshki, A. (2015). <. *International Journal of Current Microbiology and*  
723 *Applied Sciences*, 4(9), 924–932.

724

725

726

727

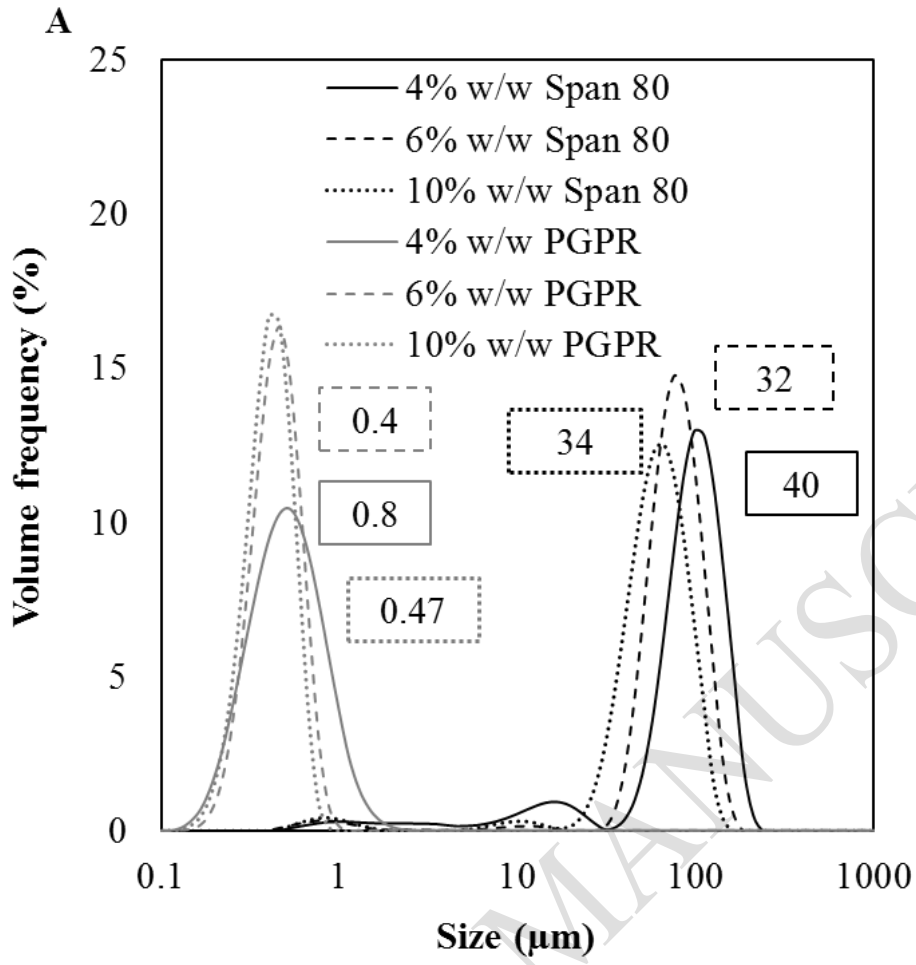
728

729

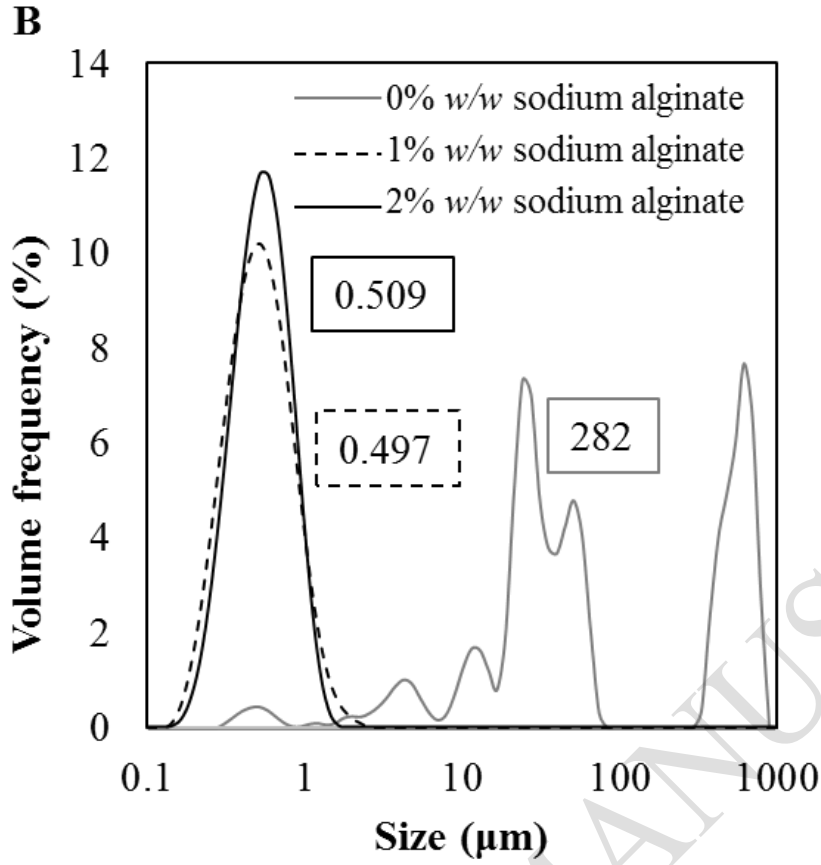
730

ACCEPTED MANUSCRIPT

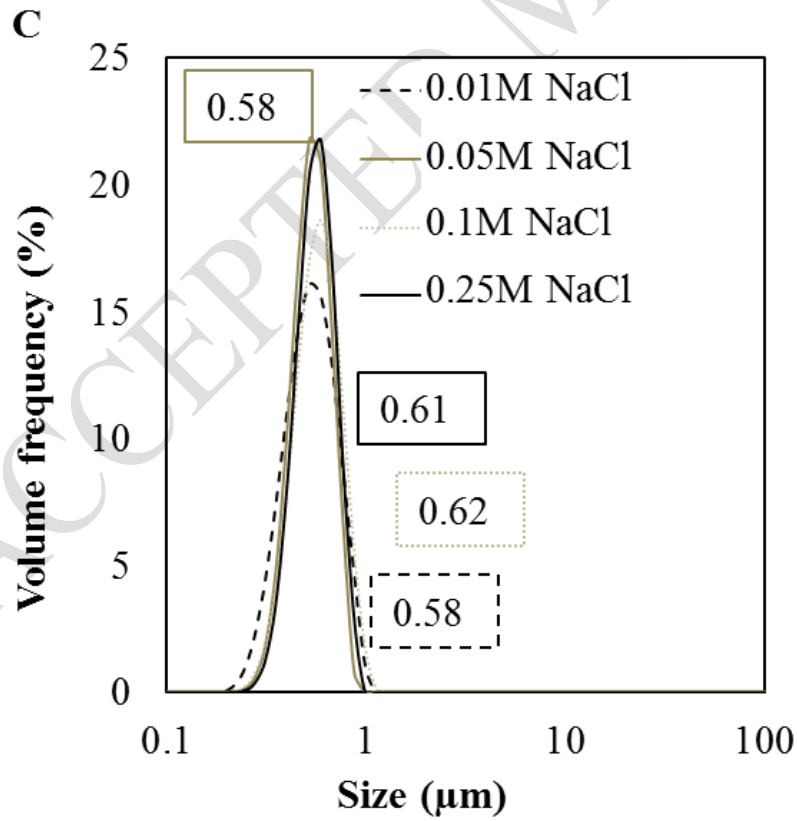




731



732  
 733



734  
 735  
 736

**Figure 1.** Effect of the type and concentration of hydrophobic surfactant in the particle

737 size of water-in-oil emulsions (W<sub>1</sub>/O) produced by high-shear homogenization (11,000  
738 rpm, 5 min) containing 27 ppm of *Chlorophyllin* and 1% w/w of sodium alginate in the  
739 aqueous phase and corn oil as dispersed phase **(A)**. Influence of sodium alginate  
740 concentration (0-2% w/w) **(B)** and NaCl concentration (0-0.25 M) **(C)** in the inner  
741 aqueous phase on the formation W<sub>1</sub>/O emulsions, containing 27 ppm of *Chlorophyllin*  
742 and 2% w/w sodium alginate in the aqueous phase and 4% w/w of PGPR in corn oil as  
743 dispersed phase. Mean volume-weighted droplet diameters (d<sub>[4;3]</sub>) of emulsions  
744 expressed in μm are specified within boxes.

745

746

747

748

749

750

751

752

753

754

755

756

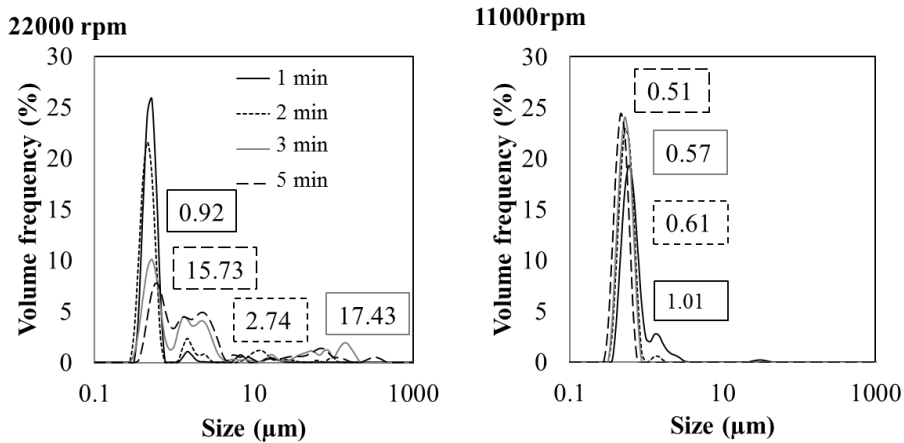
757

758

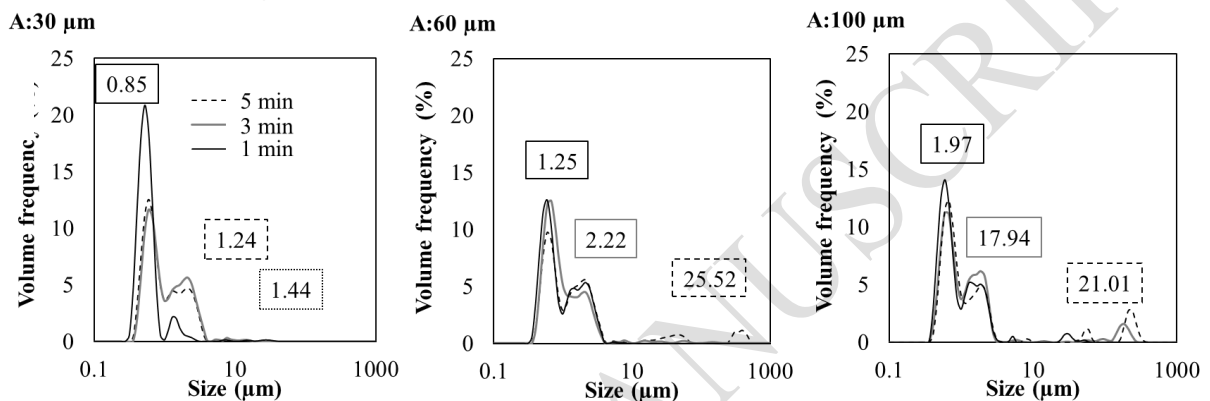
759

760

761

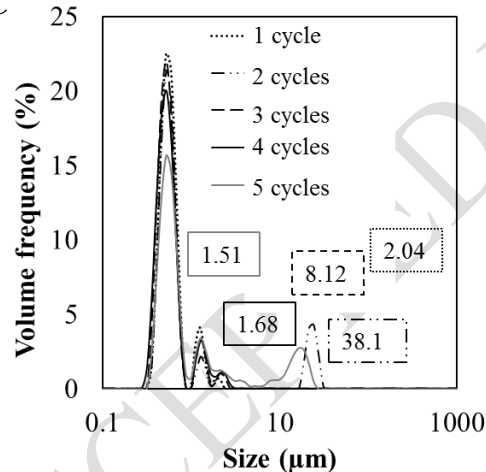
**A**

762

**B**

763

764

**C**

765

766

767

768

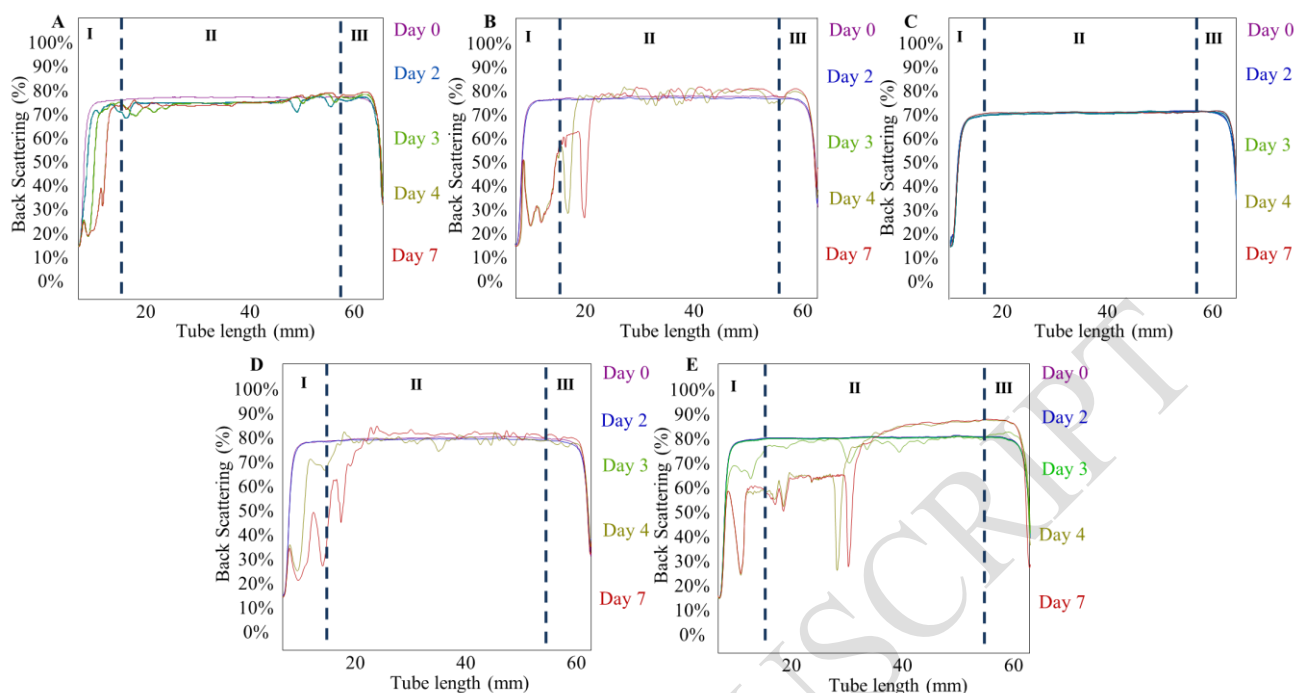
769

770

771

772

**Figure 2.** Effect of the homogenization procedure in the formation of  $W_1/O$  (3/7) emulsions containing 27 ppm of *chlorophyllin* (CHL) and 1% *w/w* sodium alginate as aqueous phase and 4% *w/w* PGPR in corn oil as lipid phase. **(A)** Variation of the high shear homogenization frequency and time. **(B)** High shear homogenization (11,000 rpm, 5 min) followed by sonication at different amplitude and times. **(C)** High shear homogenization (11,000 rpm, 5 min) followed by microfluidization (150 MPa, 1-5 cycles). Mean droplet diameters ( $d[4;3]$ ) of emulsions are specified within boxes.



773

774

775

776

777

778

779

780

781

782

783

784

785

786

787

788

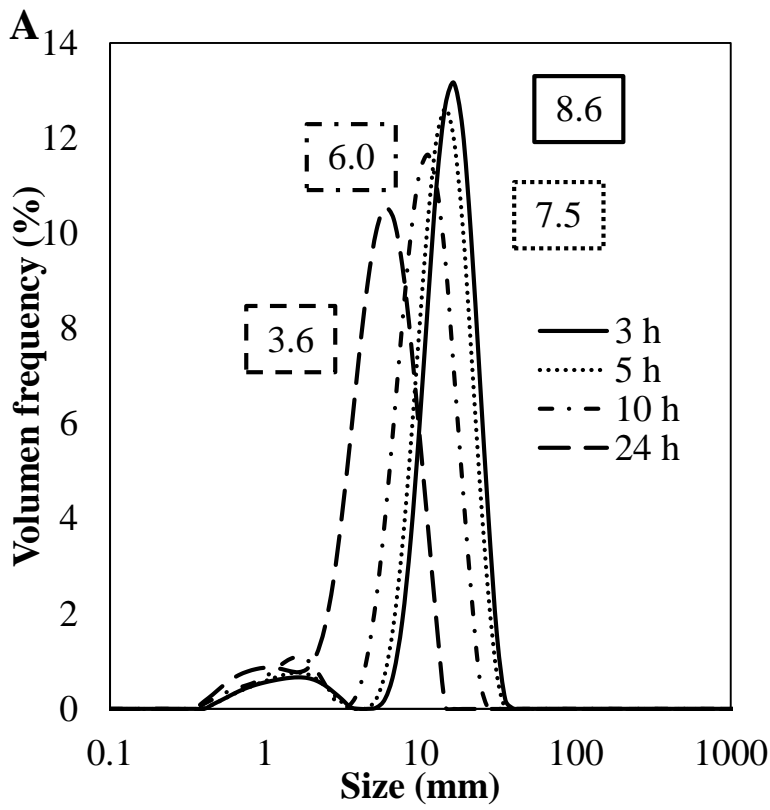
789

790

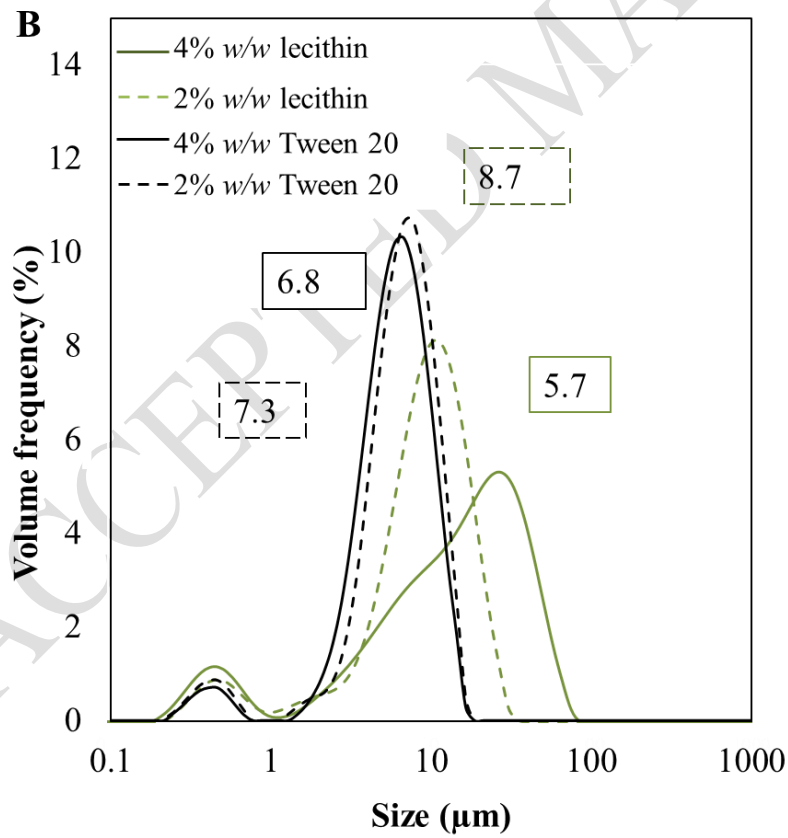
791

**Figure 3.** Turbidity assessment expressed as back scatter intensity (%) along a tube (mm) containing water-in-oil emulsions (W<sub>1</sub>/O) formulated with 27 ppm of *chlorophyllin*, 2% w/w sodium alginate and 4% w/w PGPR, after no salt addition (A) or 0.01M NaCl (B); 0.05M NaCl (C), 0.1M NaCl (D) and 0.25M NaCl (E) was added. The destabilization phenomena usually registered by the Turbiscan include clarifications (down peaks in zone I), variations in particle size (displacement of the horizontal lines from zone II: flocculation or coalescence) and creaming (up peaks in zone III).

792



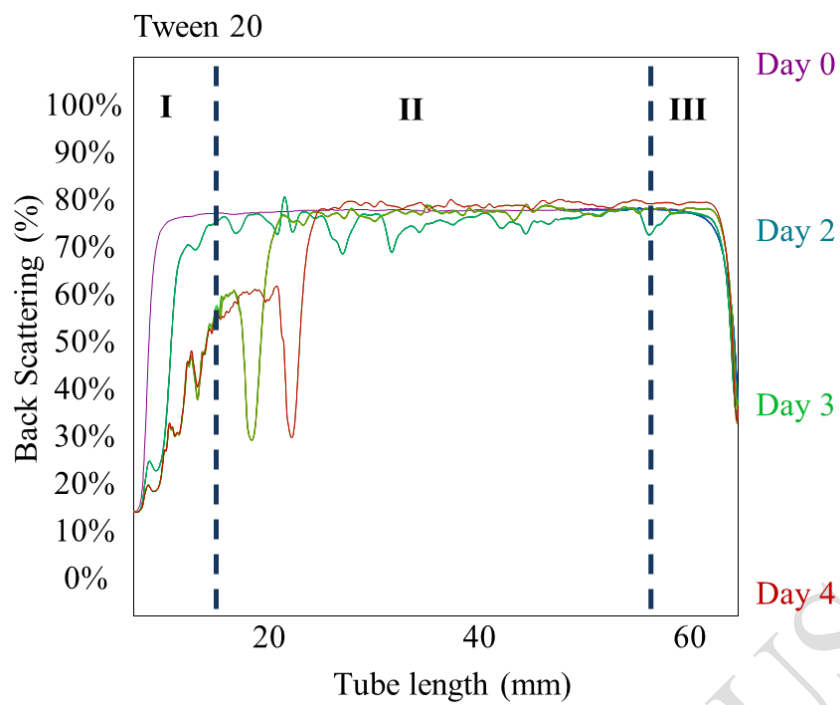
793



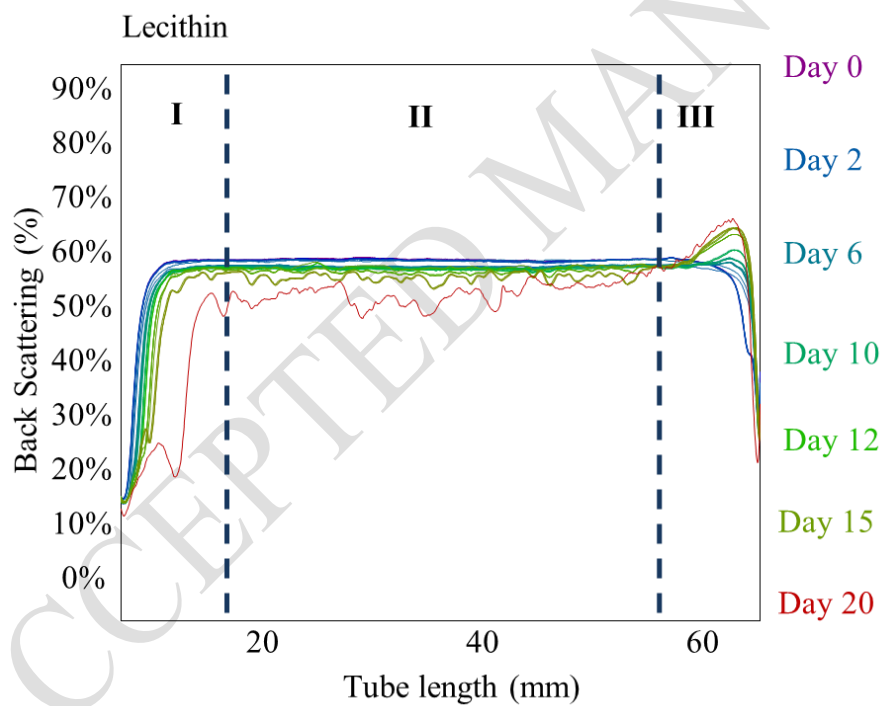
794

795

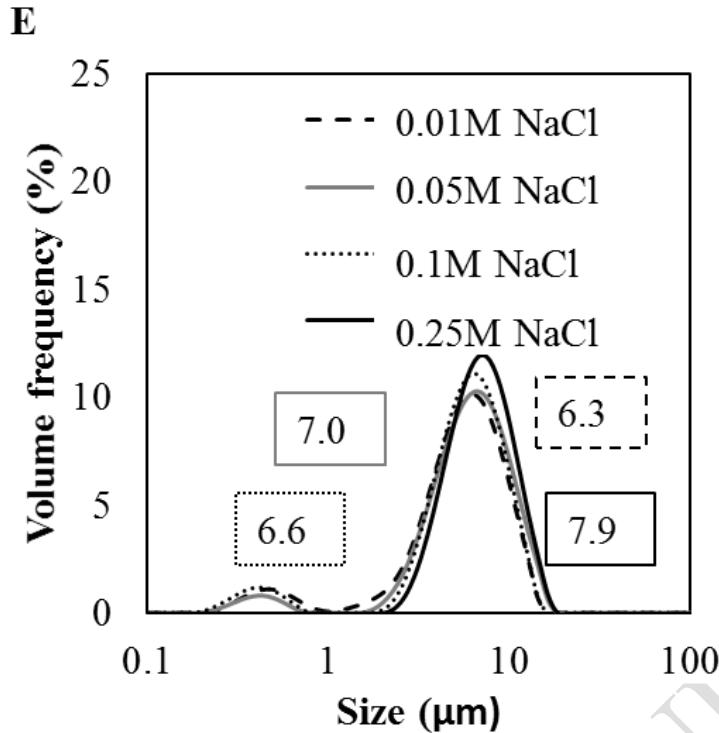
796

**C**

797  
798

**D**

799  
800



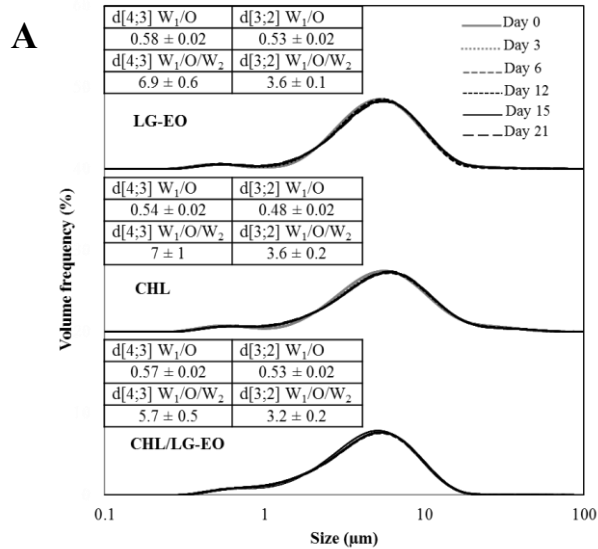
801  
802

803 Figure 4. Effect of the magnetic stirring time on the particle size distribution of double  
804 emulsions ( $W_1/O/W_2$ ) containing 2% w/w sodium alginate, 27 ppm of *chlorophyllin* and  
805 0.05M NaCl in the inner aqueous phase ( $W_1$ ) and 4% w/w PGPR and corn oil as dispersed  
806 phase (O). As a model of outer aqueous phase ( $W_2$ ), 2% w/w sodium alginate and 4% w/w  
807 Tween 20 was used as stabilizing agent and surfactant, respectively (A). Influence of  
808 Tween 20 and lecithin concentration in the (B) particle size and (C, D) turbidity during  
809 time of  $W_1/O/W_2$  emulsions at concentrations of hydrophilic surfactant of 2 and 4% w/w.  
810 (E) Impact of NaCl incorporation (0-0.25 M) in the particle size of  $W_1/O/W_2$  emulsions  
811 containing 2% w/w as hydrophilic surfactant.

812 Overall,  $W_1/O$  emulsions had been prepared by high shear homogenization (11,000 rpm,  
813 5 min) and mean volume-weighted droplet diameters ( $d[4;3]$ ) of emulsions are specified  
814 within the boxes.  $W_1/O/W_2$  were prepared by low-energy high shear homogenization at  
815 5,600 rpm during 2 min followed by magnetic stirring at 750 rpm, during 3, 5, 10 or 24 h  
816 (A) or during 24 h in the rest of plots.

817



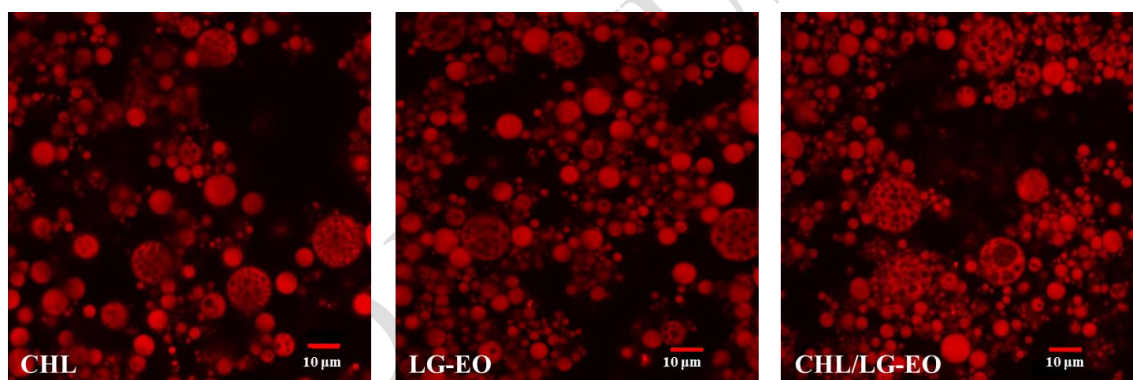


818

819

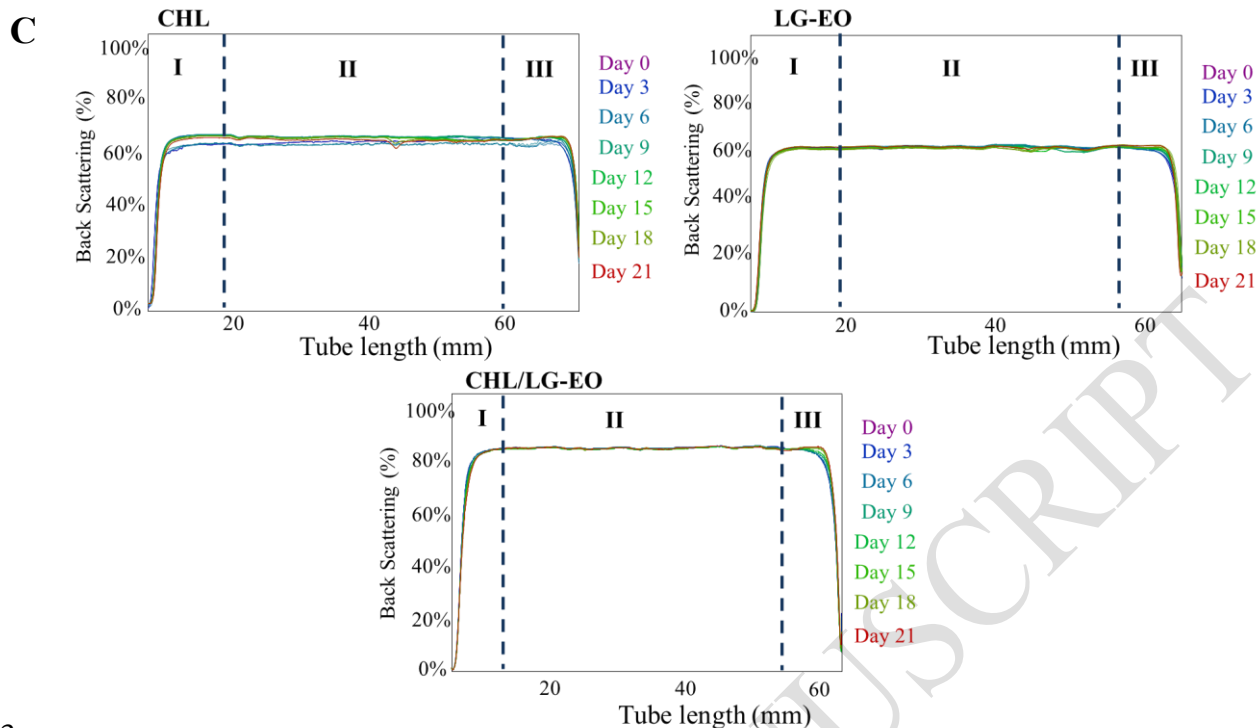
820

**B**



821

822



823  
 824 **Figure 5.** Mean droplet diameters ( $d[3;2]$  and  $d[4;3]$ ) of water-in-oil emulsions ( $W_1/O$ )  
 825 and double emulsions ( $W_1/O/W_2$ ) and particle size distributions (A), confocal images (B)  
 826 and turbidity measurements during 21 days of refrigerated storage (C) of  $W_1/O/W_2$   
 827 emulsions containing *chlorophyllin* (CHL) and/or lemongrass essential oil (LG-EO).  
 828

829 **Table 1. Physicochemical properties of water-in-oil (W<sub>1</sub>/O) carrying *chlorophyllin* and/or lemongrass essential oil.**

Bioactive compound	Viscosity (mPa·s) W <sub>1</sub> /O	<i>a</i> * W <sub>1</sub> /O	<i>b</i> * W <sub>1</sub> /O	Whiteness index (WI) W <sub>1</sub> /O
<i>Chlorophyllin</i> (CHL)	144 ± 1 <sup>a</sup>	-5.82 ± 0.05 <sup>a</sup>	3.24 ± 0.08 <sup>a</sup>	73.38 ± 0.03 <sup>a</sup>
Lemongrass EO (LG-EO)	153 ± 5 · 10 <sup>-3</sup> <sup>b</sup>	-0.87 ± 0.03 <sup>b</sup>	4.50 ± 0.04 <sup>b</sup>	79.63 ± 0.03 <sup>b</sup>
<b>CHL/LG-EO</b>	141 ± 3 · 10 <sup>-4</sup> <sup>a</sup>	-4 ± 5 · 10 <sup>-3</sup> <sup>c</sup>	4 ± 8 · 10 <sup>-3</sup> <sup>c</sup>	76.33 ± 0.09 <sup>c</sup>

830

831 <sup>a,b,c</sup> Mean in same column with different letters are significantly different at p < 0.05.

832

833 **Table 2. Physicochemical properties of double emulsions (W<sub>1</sub>/O/W<sub>2</sub>) carrying *chlorophyllin* and/or lemongrass essential oil.**

Bioactive compound	Viscosity (mPa·s) W <sub>1</sub> /O/W <sub>2</sub>	<i>a</i> * W <sub>1</sub> /O/W <sub>2</sub>	<i>b</i> * W <sub>1</sub> /O/W <sub>2</sub>	Whiteness index (WI) W <sub>1</sub> /O/W <sub>2</sub>
<i>Chlorophyllin</i> (CHL)	253 ± 23 <sup>ab</sup>	-4.72 ± 0.01 <sup>a</sup>	6.34 ± 0.04 <sup>a</sup>	74.89 ± 0.05 <sup>a</sup>
Lemongrass EO (LG-EO)	279 ± 2 <sup>a</sup>	-2.32 ± 0.07 <sup>b</sup>	13.48 ± 0.07 <sup>b</sup>	73.65 ± 0.04 <sup>b</sup>
<b>CHL/LG-EO</b>	266 ± 26 <sup>b</sup>	-4.60 ± 0.06 <sup>c</sup>	13.7 ± 0.2 <sup>b</sup>	72.3 ± 0.1 <sup>c</sup>

834

835 <sup>a,b,c</sup> Mean in same column with different letters are significantly different at p < 0.05.

836

837

838