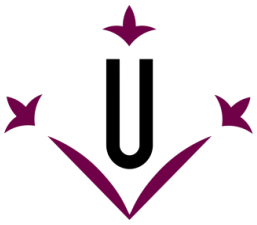

**Title:** The effect of sodium carboxymethylcellulose on the stability and bioaccessibility of anthocyanin water-in-oil-in-water emulsions

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## Abstract

Water-in-oil-in-water ($W_1/O/W_2$) emulsions provide protective encapsulation to plant bioactive compounds in food matrix and under gastrointestinal conditions. However, the stability of the emulsions during the storage is crucial for their use in the food industry. Hence, the aim of this study was to enhance the stability and bioaccessibility of $W_1/O/W_2$ emulsions containing anthocyanins with the use of sodium carboxymethylcellulose (CMCNa). The emulsions were prepared by ultrasound technology, adding polyglycerol polyricinoleate (PGPR) in the inner aqueous phase of emulsions, and lecithin and Tween 20 in the outer aqueous phase. The systems were physicochemical characterized over the time and their behaviour under simulated gastrointestinal conditions was investigated. Our results showed high encapsulation efficiencies above 90% and an increase in bioaccessibility with the use of CMCNa. Moreover, the polymer addition slowed down the free fatty acids release and increased the oil digestibility of lecithin-stabilized emulsions. These latter emulsions presented the highest bioaccessibility (31.08 ± 1.73%), the more negative values of ζ-potential and no variations on the particle size and the backscattering profile over the time, thus being the most stable emulsions. These results provide useful information for the design of anthocyanin emulsion-based delivery systems to guarantee their functionality in food matrices as well as through the gastrointestinal tract.



**Keywords**: water-in-oil-in-water emulsion, ultrasound technology, anthocyanins, sodium carboxymethylcellulose, stability, bioaccessibility

## 1. Introduction

Anthocyanins are flavonoids belonging to an important group of water-soluble blue, red, purple and orange natural pigments of different fruits and vegetables. Nutraceuticals containing anthocyanins purified from bilberry (*Vaccinium myrtillus*) and black currant (*Ribes nigrum*), have been used in clinical studies demonstrating major health benefits such as the modulation of several pro-inflammatory mediators in healthy adults (Karlsen et al., 2007), the improvement of the lipoprotein profile (HDL-cholesterol/LDL-cholesterol) in dyslipidemic patients (Qin, Xia, Ma, Hao, & Liu, 2009), the enhancement of the antioxidant capacity and the prevention of insulin resistance in subjects with type 2 diabetes (Li, Zhang, Liu, Sun, & Xia, 2015). However, the use of these nutraceuticals has not been exploited for their application in food so far. The use of anthocyanin extracts as replacers of synthetic colorants in food is limited due to their low stability during processing and storage of food products. The color and stability of anthocyanins can be affected by physical and chemical factors such temperature, pH, light, chemical structure, concentration, presence of copigments, metallic ions, enzymes, oxygen, ascorbic acid, sugar, among others (Cavalcanti, Santos, & Meireles, 2011; Sari, Wijaya, Sajuthi, & Supratman, 2012; Shipp & Abdel-Aal, 2010). Therefore, new encapsulating delivery systems for anthocyanins are needed for further food applications.

Water-in-oil-in-water ($W_1/O/W_2$) double emulsions consist of a $W_1$ phase dispersed as small water particles in a lipid phase, which separates the internal water phase from the external water phase ($W_2$) (Qi, Wang, & Zhu, 2011). Currently, $W_1/O/W_2$ emulsions are of great importance in the food industry because they allow to prepare reduced-fat emulsion products compared to

conventional oil-in-water emulsions (O/W) maintaining a similar in-mouth perceived texture and flavor. They have also the capacity to encapsulate and protect both lipophilic and hydrophilic compounds, which are isolated from the surrounding aqueous environment, and control its release during the digestion process (Aditya, Aditya, Yang, Kim, Park, & Ko, 2015; Kaimainen, Marze, Järvenpää, Anton, & Huopalahti, 2015; Matos, Gutiérrez, Coca, & Pazos, 2014; Muschiolik & Dickinson, 2017). However, the stabilization of these systems and further application in food is still a challenge.

Emulsifiers or surfactants are surface-active amphiphilic molecules often selected based on their ability to enhance physical stability of emulsions. Polyglycerol polyricinoleate (PGPR) is the most commonly used hydrophobic emulsifier in food-based $W_1/O/W_2$ emulsions to promote and stabilize the inner phase ($W_1/O$) (Aditya, Aditya, Yang, Kim, Park, & Ko, 2015; Aditya, Aditya, Yang, Kim, Park, Lee, et al., 2015; K. Frank et al., 2012; Kaimainen et al., 2015). In contrast, lecithin and Tween 20 were added to the outer phase ($O/W_2$) in order to study their influence in the stabilization of the inner phase into the outer phase. Tween 20 is a synthetic hydrophilic emulsifier used in the aqueous phase of $W_1/O/W_2$ double emulsions (K. Frank, Kohler, & Schuchmann, 2011), whereas lecithin, a mixture of different glycerophospholipids, can also be adsorbed to oil-water interface acting as a natural emulsifier (Klang & Valenta, 2011; Pichot, Watson, & Norton, 2013). The use of different biopolymers as thickening and gelling agents has shown to improve encapsulation efficiency and stability of $W_1/O/W_2$ double emulsions (Benna-Zayani, Kbir-Ariguib, Trabelsi-Ayadi, & Grossiord, 2008; Dickinson, 2011; Su, Flanagan, & Singh, 2008). Sodium carboxymethylcellulose (CMCNa) is proved to be a suitable emulsifier for

$W_1/O/W_2$ double emulsions and it is commonly chosen as a stabilizing agent for its low cost (Matos et al., 2014; A. Schuch, Helfenritter, Funck, & Schuchmann, 2015). The aim of this study was to encapsulate and stabilize anthocyanins in double emulsions for its further application in food. We compared the influence of using different stabilizers in the outer phase of the $W_1/O/W_2$ double emulsions, a natural emulsifier (lecithin) compared to a synthetic emulsifier (Tween 20) and the addition of a biopolymer (sodium carboxymethylcellulose) on size, zeta potential, encapsulation efficiency, free fatty acid release, bioaccessibility and stability of the systems during 21 days of the storage at 4 ºC.

## 2. Materials and methods

### *2.1. Materials*

Anthocyanin extract (Medox ®, Biolink Group AS, Sandnes, Norway), glycerol, polyglycerol polyricinoleate (PGPR) (Danisco, DuPont, EEUU), Tween 20 (Scharlab, Sentmenat, Spain), lecithin Alfa Aesar ThermoFisher (Kandel, Germany), sodium carboxymethylcellulose (CMCNa) (Sigma-Aldrich, Inc. St. Louis, MO), sodium acetate (Scharlab, Sentmenat, Spain), potassium chloride (Panreac AppliChem ITW Reagents, Barcelona, Spain) and corn oil was purchased from a local supermarket. Ultrapure water obtained from a Milli-Q filtration system was used to prepare emulsions and reagents of the experiment.

### *2.2. Methods*

The $W_1/O/W_2$ emulsions were prepared using a unique inner emulsifier (PGPR) at a final concentration in the emulsion of 1.25% w/w. Tween 20 and lecithin emulsions at a concentration of 1.5% w/w were formulated to compare the effect

of the two different outer surfactants. Moreover, to investigate the effect of CMCNa on the systems, Tween 20 and lecithin emulsions were stabilized with 1% w/w and without the biopolymer.

*2.2.1. Preparation of water-in-oil-in-water ($W_1/O/W_2$) double emulsions*

Firstly, the anthocyanins extract was diluted 1:20 in a NaCl 0.1M solution, by agitation (10 min), centrifugation (15 min at 14000 rpm) and filtration.

For the formulation of the double emulsions, a two-step emulsification method described by Aditya et al. (2015) with some modifications was used. To obtain the primary $W_1/O$ emulsion, the two phases were prepared separately. The $W_1$ phase, comprising of 22 % of the extract solution and 3 % of glycerol, and the oil phase, consisting of 70 % of corn oil and 5 % of PGPR, were heated at 60 ºC for 15 min, separately. Subsequently, the two phases were mixed and homogenized using a high-speed homogenizer (Ultra-Turrax, Janke & Kundel, Staufen, Germany) at 6000 rpm for 8 min. Lastly, a sonication of the primary $W_1/O$ emulsion was performed using a P400S Hielscher sonicer (Hielscher Ultrasound Technology, Teltow, Germany) for 180 s at a frequency of 24 kHz and amplitude of 40%.

The secondary water phase ($W_2$) was prepared adding the surfactant (lecithin or Tween 20) on the NaCl 0.1M solution and heated at 60 ºC for 30 min. In the emulsions with biopolymer, CMCNa was also added into the $W_2$ phase. Finally, $W_1/O$ and $W_2$ were mixed at a ratio of 25:75, homogenized using the high-speed homogenizer at 6000 rpm for 4 minutes and sonicated for 90 s at a frequency of 24 kHz and amplitude of 30%.

*2.2.2. Quantification of anthocyanins in emulsions*

The anthocyanin content was determined by the spectrophotometric pH-differential method in which two buffer systems were used, potassium chloride (0.025 M pH 1) and sodium acetate (0.4 M pH 4.5) following Turfan et al. (2011) method. Briefly, samples were diluted 1:3 with each buffer and the absorbance of the mixtures at pH 1 and 4.5 was then measured with a UV-visible-NIR spectrophotometer (V-670, Jasco Corporation, Tokio, Japan) at 515 and 700 nm against a blank of methanol. The anthocyanin content (mg/L) was calculated according to equation (1) and expressed as equivalents of cyanidin-3-glucoside.

$$TA = \frac{\left[(A_{515} - A_{700})_{pH\ 1.0} - (A_{515} - A_{700})_{pH\ 4.5}\right] \times MW \times DF \times 1000}{\varepsilon \times L} \quad (1)$$

where MW is the molecular weight of cyanidin-3-glucoside (449.2 g/mol), DF is the dilution factor, L is the pathlength in cm and ε is the molar extinction coefficient for cyanidin-3-glucoside (26900 L $\cdot mol^{-1} \cdot cm^{-1}$).

*2.2.3. Determination of encapsulation efficiency (EE)*

To determine the encapsulation efficiency of the anthocyanins, a previously described method by Aditya et al. (2015) with some slight modifications was used. A portion of 10 mL of the emulsions were centrifuged at 4500 rpm for 10 min at 4 ºC. Then, the $W_2$ phase at the bottom of the tube, containing free anthocyanins, was collected, and centrifuged at 7500 rpm for 15 min at 4 ºC, prior a dilution at 1:4 with methanol. Finally, the amount of free anthocyanins was determined using the differential pH method described in the previous section (2.2.1.).

Based on the content of free anthocyanins obtained according to the equation (1), the encapsulation efficiency was calculated according to the equation (2):

$$EE\ (\%) = \frac{Total\ anthocyanins - Free\ anthocyanins}{Total\ anthocyanins} \cdot 100 \quad (2)$$

where the total anthocyanins is the amount of the compound added to the inner aqueous phase in the preparation of the emulsions. The total anthocyanins value was determined by reference to a calibration curve of the extract diluted in NaCl 0.1 M.

*2.2.4. Emulsion characterization*

The mean droplet diameter (nm) and the particle size distribution were measured using static light scattering technique (Mastersizer 2000, Malvern Instruments Ltd, Worcestershire, UK). Emulsions were previously diluted in ultrapure water and stirred at 1800 rpm. The particle size diameter (μm) was reported as the volume mean diameter ($d_{43}$).

The electrical charge (ζ-potential) was measured by phase-analysis light scattering (PALS) using a Zetasizer NanoZS laser diffractometer (Malvern Instruments Ltd Worcerstershire, UK) to determine the surface charge (mV) at the droplet interface. Emulsions were previously diluted in ultrapure water at a dilution ratio of 1/2000 sample-solvent and placed in a capillary cell equipped with two electrodes to assess the electrophoretic mobility of the particles. The results were expressed in millivolts (mV).

Apparent viscosity of emulsions was determined using a SV-10 vibro-viscometer (A&D Company, Tokyo, Japan), which produces a vibration of 30 Hz and constant amplitude of 0.4 mm at a controlled room temperature. The results were expressed in mPa·s.

The optical properties of the emulsions were measured using a colorimeter (Konica Minolta CR-400, Konica Minolta Sensing, Inc, Osaka, Japan). The

equipment was set up for an illuminant D65 and 10º observer angle. CIE L* (lightness), a* (green- red) and b* (blue- yellow) values were reported.

*2.2.5. Confocal scanning laser microscopy*

Micrographs of the $W_1/O/W_2$ double emulsions were obtained with a confocal scanning laser microscopy (CSLM) (Olympus FV1000 Spectral Confocal Microscope Olympus, Melville, NY). Samples of $W_1/O/W_2$ emulsions were stained with Nile red (fluorescent lipid dye) and examined with a 100X magnifications lens and a laser with an excitation line of 559 nm.

*2.2.6. In vitro digestion*

The *in vitro* digestion method was performed according to Minekus et al. (2014). The protocol included both gastric and small intestinal phases. Briefly, 20 mL of the sample were mixed with 18.2 mL of simulated gastric fluid (SGF) containing pepsin (2000 U/mL), 0.4 mL HCl solution (1 M) and 10 µL of a $CaCl_2$ solution (0.3 M). Finally, 1.39 mL of Milli Q water was added to reach a final volume of 40 mL. The mixture was placed into an incubator at 37 °C for 2 h while shaking gently. In order to carry out the intestinal phase, a pH-stat device was used. Once the gastric phase was completed, an aliquot of 30 mL of gastric sample were placed in a 37 °C water bath. Then, 3.5 mL of bile solution (54 mg/mL) and 1.5 mL of salt solution (NaCl 0.150 mM and $CaCl_2$ 0.01 mM) were added and the pH was adjusted to 7 with NaOH (1 M). Finally, 2.5 mL of pancreatin solution (75 mg/mL) was incorporated to the mixture. The pH of the sample was maintained to 7 by adding NaOH (0.25 M) constantly for 2 h. The final volume of NaOH (0.25 M) was recorded and used to calculate the amount of free fatty acids (FFA) released

during the intestinal phase. The % FFA was determined according to equation (3):

$$\text{FFA (\%)} = \frac{V_{NaOH} \times M_{NaOH} \times M_{oil}}{m_{oil} \times 2} \times 100 \quad (3)$$

where $V_{NaOH}$ was the final volume of NaOH consumed during the *in vitro* digestion, $M_{NaOH}$ was the molarity of NaOH (0.25 M), $M_{oil}$ was the molarity of the oil used in the emulsion (800 g $mol^{-1}$) and $m_{oil}$ was the mass of oil that was digested during the intestinal phase of the digestion (2.63 g).

*2.2.7. Anthocyanin bioaccessibility*

Anthocyanin extraction was carried out following the method proposed by Gómez-Plaza et al. (2008) with some modifications. An aliquot of 10 mL of digested sample was centrifuged at 11000 rpm for 15 min at 4 °C. The micelle phase was discarded and the aqueous phase was recovered. Then, 1 mL of the aqueous phase was mixed with 3 mL of methanol. The mixture was vortexed and centrifuged at 9000 rpm for 15 min at 4 °C. Aqueous phase was recovered and pellet was discarded.

The anthocyanin content (mg/L) was determined by the spectrophotometric pH-differential method described by Turfan et al. (2011), as it was described previously (section 2.2.1.). The results were expressed as equivalents of cyanidin-3-glucoside.

The bioaccessibility was obtained according the equation (4):

$$\text{Bioaccessibility (\%)} = \frac{C_{digestion}}{C_{emulsion}} \times 100 \quad (4)$$

where $C_{digestion}$ is the anthocyanin content of digested samples and $C_{emulsion}$ is the anthocyanin content in the initial emulsion.

### *2.2.8. Stability by Turbiscan*

Stability of emulsions was studied using a vertical scan analyzer Turbiscan MA 2000 (Formulaction, Toulouse, France) during 21 days of storage at 4 ºC. A sample of 7 mL were introduced into a glass cylindrical cell and analyzed by a light beam emitted in near infrared (800 nm) wavelength which scanned vertically, from bottom to top, the sample cell. Two synchronous optical sensors receive respectively light transmitted through the sample (180° from the incident light), and light backscattered by the sample (45° from the incident radiation). In this study, the variation of backscattering (BS) during 21 days at 4 ºC was studied to assess the stability of emulsions over the time.

### *2.2.9. Statistical analyses*

All the experiments were assayed in duplicate and three analyses were carried for each sample. The results were analyzed using the Statgraphics Plus v.5.1 Windows package (Statistical Graphics Co., Rockville, Md), being the confidence interval set at 0.95. Analysis of the variance (ANOVA) was performed to compare treatments. In addition, multiple rang tests were carried out to determine significant differences between the obtained averages. Least significant difference (LSD) test was employed to determine differences between means.

## 3. Results and Discussion

### *3.1. Characterization of initial $W_1/O/W_2$ emulsions*

*Encapsulation efficiency.* High values of encapsulation efficiency ranged from 74.84 ± 1.53% in Tween 20-stabilized $W_1/O/W_2$ double emulsions to 92.57 ± 0.20% in the same emulsions stabilized with CMCNa (Table 1). Aditya et al.

(2015) managed to encapsulate catechin and curcumin in double emulsions with similar encapsulation efficiencies, around 90-95%. In our study, a significant increase of 15% and 25% in lecithin- and Tween 20-stabilized $W_1/O/W_2$ double emulsions, respectively, was observed with the addition of CMCNa. Similar behaviour was observed in the study of Matos et al. (2014), who reported higher encapsulation efficiency as the concentration of CMCNa increased (0 - 0.5% w/v) in the $W_2$ outer phase of Tween 20-stabilized $W_1/O/W_2$ double emulsions. CMCNa may affect the interactions between Tween 20 or lecithin molecules adsorbed at the outer interface. Due to the low interfacial activity of CMCNa, its presence in the $W_2$ outer phase could provide a steric effect, physical barrier for particle interactions, playing a significant role in the stabilization of the outer interface (Matos et al., 2014; A. Schuch et al., 2015). Different hydrocolloids added to the $W_2$ outer phase have increased encapsulation efficiency. Values higher than 90% were observed with the addition of 10% of a modified gum arabic (Acacia (sen) SUPER GUM$^{TM}$) to the $W_2$ outer phase of $W_1/O/W_2$ double emulsions (Su et al., 2008). The encapsulation efficiencies of $W_1/O/W_2$ double emulsions stabilized by CMCNa have shown much higher encapsulation efficiencies than the emulsions stabilized by beet pectin or gum Arabic (Schuch et al., 2015). In literature, a greater retention of active material is related to a reduction of emulsion droplet size, which generally represents an increased stability (A. Schuch et al., 2015; Anna Schuch, Deiters, Henne, Köhler, & Schuchmann, 2013). However, our results obtained for encapsulation efficiency could not be related to the droplet size. In accordance with our results, Carneiro et al. (2013) attributed the differences observed in the encapsulation efficiency of

300 flaxseed oil microencapsulated by spray drying to the wall materials used, with
301 different interfacial properties, rather than the droplet size.
302 *Particle size and size distribution*. The particle size of the $W_1/O/W_2$ double
303 emulsions are shown in Table 1. The values were in the 3.36 ± 0.69 and 8.39 ±
304 0.83 μm range. Similar particle sizes (5.05 ± 0.16 μm to 8.28 ± 0.33 μm) were
305 reported in CMCNa Tween 20-stabilized $W_1/O/W_2$ double emulsions containing
306 *trans*-resveratrol at different biopolymer concentrations, from 0 to 0.5% w/v
307 (Matos et al., 2014). In our study, emulsions formulated with Tween 20 presented
308 lower particle size compared to those formulated with lecithin ($p < 0.05$), which
309 could be explained by the hydrophilic-lipophilic balance (HLB) of the emulsifiers.
310 According to literature, the higher the HLB values the smaller the particles in an
311 O/W emulsion (Yuan, Gao, Zhao, & Mao, 2008). The HLB values of Tween 20
312 are 16.7 but the HLB of lecithin are rarely higher than 9, commonly ranging
313 between 2 and 7. Thus, the lowest droplet size observed in Tween 20 emulsions
314 could be explained because of its high HLB value.
315 Particle size was significantly affected by the addition of CMCNa in $W_1/O/W_2$
316 emulsions, although the effect varied depending on the surfactant. These
317 differences between Tween 20 and lecithin could be attributed to changes in the
318 initial pH before the addition of CMCNa. In the case of lecithin emulsions (pH ~
319 5), the addition of the biopolymer increased the particle size. In contrast, in the
320 case of Tween 20 (pH ~ 3), the addition of CMCNa decreased the particle size.
321 It has been also reported a particle size increase of casein micelles in a mixture
322 with carboxymethylcellulose at pH 5 in comparison with that in absence of the
323 hydrocolloid. The increase was attributed to the adsorption of
324 carboxymethylcellulose onto casein micelles, which led to effectively larger

particles (Du et al., 2009). Based on this latter study, at low pH, the decrease of particle size with the addition of CMCNa in Tween 20-stabilized $W_1/O/W_2$ double emulsions could be due to the presence of adsorbed surfactant that might hinder the adsorption of arriving CMCNa chains due to the electrostatic repulsion. Therefore, interactions between CMCNa and the emulsifiers onto the oil surface are suggested to be pH dependent. Apart from these interactions, Matos et al. (2014) proposed that the addition of CMCNa in the outer $W_2$ phase of Tween 20-stabilized $W_1/O/W_2$ double emulsions could be associated to its capacity to reduce the interfacial tension, which would also explain the decrease of the mean diameters values in our study.

Figure 1 shows the droplet size distribution of $W_1/O/W_2$ emulsions. Lecithin-stabilized double emulsions presented a well-defined monomodal particle size distribution, ranging from 1 to 20 μm. In contrast, Tween 20- stabilized $W_1/O/W_2$ double emulsions showed a bimodal distribution, with small particles in the range from 0.2 to 12 μm and large particles from 12 to 80 μm. The polydispersity of Tween 20-stabilized double emulsions decreased with the addition of CMCNa and small particles from 0.2 to 12 μm were shown. However, the values increased with the addition of the biopolymer in lecithin emulsions and large particles up to 50 μm were detected. A bimodal distribution has been typically observed in $W_1/O/W_2$ emulsions formulated with PGPR as lipophilic emulsifier (Hemar, Cheng, Oliver, Sanguansri, & Augustin, 2010; Su, Flanagan, Hemar, & Singh, 2006), but a monomodal distribution was observed by Cofrades et al. (2013) when sodium caseinate and whey protein concentrate were used as hydrophilic emulsifiers. In other studies, $W_1/O/W_2$ emulsions stabilized with different hydrocolloids, such as propylene glycol alginate, gellan, carragenan, pectin

methylcellulose, gum Arabic, xanthan and others, have shown to exhibit high polydispersity, similar to our double emulsions with CMCNa (Huang, Kakuda, & Cui, 2001).

*Confocal scanning laser microscopy (CSLM)*. Oil fat globules containing small droplets inside (inner phase) can be clearly identified in Figure 2. CSLM images have proved the formation of double emulsions in other studies (Aditya, Aditya, Yang, Kim, Park, Lee, et al., 2015). These authors confirmed that a secondary emulsification does not result in the destruction of the primary water-in oil emulsion. The addition of CMCNa increased the oil droplet size in lecithin-stabilized emulsions (Figure 2A and C), whereas, smaller droplets were observed for emulsions with the addition of CMCNa in Tween 20-stabilized $W_1/O/W_2$ double emulsions (Figure 2B and D). These results are confirmed by the particle size measurements (Table 1). The confocal images also presented some aggregation of the oil droplets of Tween 20 emulsions, not detected in lecithin emulsions.

*ζ-potential*. In Table 1 are shown the electrical charges of the $W_1/O/W_2$ double emulsions. The values were negative and oscillated between -54.83 ± 2.36 mV and -88.06 ± 4.51 mV. Initially, ζ-potential values of lecithin-stabilized emulsions were more negative than those of Tween 20 emulsions ($p < 0.05$) (Table 1). Although Tween 20 is a non-ionic emulsifier, negative charges were observed in this study. It could be explained because of the presence of free fatty acid impurities in the surfactant or oil, or due to the preferential absorption of $OH^-$ from water to the droplet surfaces (Hu, Feng, & Cao, 2012; Uluata, McClements, & Decker, 2015). The droplet surface charge of lecithin-stabilized emulsions is usually in the negative range due the presence of negatively charged phospholipids. This natural emulsifier, mainly consisted of phosphatidyl choline,

375 is a zwitterionic polar lipid that has previously been reported to give emulsion
376 droplets a negative charge (Chang & McClements, 2016; Hu et al., 2012; Uluata
377 et al., 2015). The addition of CMCNa in lecithin-stabilized emulsions could have
378 increased significantly the electrostatic repulsion between oil droplets, causing a
379 decrease in ζ-potential from -65.58 ± 4.81 to -88.06 ± 4.51. Similar effect was
380 observed in acidified milk drinks, where the addition of carboxymethylcellulose to
381 casein micelles also increased the absolute magnitude of ζ-potential, contributing
382 to the stability of the system by electrostatic repulsion (Due et al., 2009). In
383 comparison with other biopolymers, CMCNa or gum arabic hydrocolloids
384 provided more negative charges than whey protein isolate in O/W emulsions
385 (Berendsen, Güell, Henry, & Ferrando, 2014).
386 *Viscosity*. Apparent viscosity values of $W_1/O/W_2$ double emulsions ranged from
387 2.02 ± 0.06 to 277.67 ± 38.41 mPa·s. No differences on viscosity were observed
388 between surfactants, but as it can be shown in Table 1, there was a significant
389 increase of the viscosity in the emulsions when the biopolymer was added. The
390 addition of CMCNa, as well as other polymers, in $W_1/O/W_2$ emulsions causes a
391 considerably increase of viscosity (Benna-Zayani et al., 2008; Dickinson, 2011;
392 Matos et al., 2014), and permit to achieve a rheological control of the continuous
393 phase acting as a thickening agents (Dickinson, 2009). Such effect may improve
394 the stability of the $W_1/O/W_2$ emulsion. Moreover, an increase of the viscosity has
395 been reported to help reducing the particle size of double emulsions (Li et al.,
396 2012), which has also been observed in this study in Tween 20-stabilized
397 emulsions (Table 1).
398 *Color and pH*. Color intensity was measured by a* values, which ranged from
399 5.24 ± 1.52 in CMCNa Tween 20-stabilized $W_1/O/W_2$ double emulsions to 14.50

400 ± 0.65 in lecithin-stabilized $W_1/O/W_2$ double emulsions (Table 1). The a* values
401 in emulsions stabilized with lecithin were higher than those of Tween 20-stabilized
402 emulsions ($p < 0.05$), which indicated that lecithin-stabilized emulsions were more
403 red than those with Tween 20. When CMCNa was added, a* values decreased
404 and thus, emulsions became less red. It is generally accepted that the color,
405 intensity and stability of anthocyanins changed significantly in the pH 1 - 12 range.
406 pH values affected their stability, being highly stable at very acidic pH < 3 and low
407 stable at the slightly acid to neutral pH values of most foods (Cabrita, Fossen, &
408 Andersen, 2000). In the present study, pH values ranged from 3.37 ± 0.37 in
409 Tween 20-stabilized emulsions to 5.49 ± 0.06 in CMCNa lecithin-stabilized
410 $W_1/O/W_2$ double emulsions. Although CMCNa addition increased significantly the
411 emulsion pH, especially in lecithin-stabilized $W_1/O/W_2$ double emulsions, the
412 compound encapsulation could overcome the stability-related limitations of
413 anthocyanin utilization in food.
414
415 *3.2. Oil digestibility and anthocyanin bioaccessibility*
416 The lipid digestion profile was significantly different among lecithin- and Tween
417 20-stabilized emulsions. The rate and extent of lipid digestion of emulsions
418 stabilized with lecithin was significantly lower than those where Tween 20 was
419 used as surfactant (Figure 3). A rapid FFA release up to 30% was observed after
420 30 min of the *in vitro* duodenal digestion in Tween 20-stabilized $W_1/O/W_2$ double
421 emulsions, followed by a more gradual increase up to 42.68% at the end of the 2
422 h duodenal digestion. In contrast, in lecithin-stabilized $W_1/O/W_2$ double emulsions
423 the initial increase was much smaller, and the final total FFA released was the
424 lowest (23.44%), probably due to the difficulty for lipase to act. The low lipolysis

425 that extends up to 30% in some studies could be explained either by a possible
426 accumulation of fatty acids in the O/W-interfaces or by an inhibitory effect of the
427 emulsifiers (Frank et al., 2012). In addition, the *in vitro* digestibility of lipid droplets
428 by pancreatic lipase can vary as a function of the type and the amount of the
429 emulsifier used (Mun, Decker, & McClements, 2007; Nik, Wright, & Corredig,
430 2011; Yao et al., 2013). Mun et al. (2007) reported an improved digestibility of
431 whey protein isolate (WPI)-stabilized emulsions compared to lecithin- and Tween
432 20-stabilized emulsions. Chang and McClements (2016) also showed a faster
433 rate of lipid digestion for Tween 80 than for lecithin. Several studies indeed
434 reported an increase of the droplet size during the digestion, usually due to
435 droplet aggregation observed by optical microscopy (Mun et al., 2007; Nik et al.,
436 2011; Salvia-Trujillo, Qian, Martín-Belloso, & McClements, 2013). Chang and
437 McClements (2016) related the initial lipid digestion rate of lecithin-stabilized fish
438 oil-in-water emulsions to the moderate amount of droplet aggregation observed
439 in these emulsions prior to the addition of lipase. Although they also observed
440 large dense aggregates in Tween 80-stabilized emulsions at the beginning of the
441 small intestine phase, in this case, they suggested that these aggregates
442 consisted of lipid droplets that were only weakly held together by attractive forces,
443 and were therefore easily disrupted during lipid digestion. Kaimainen et al. (2015)
444 suggested that not only the aggregation of the outer droplets may inhibit the
445 lipase activity, but also the high concentration of lecithin, with phospholipids,
446 could compete with bile salts and lipase at the oil-water interface, thus inhibiting
447 the digestion of lipids.
448 A different lipid digestion profile was observed with the addition of CMCNa in
449 either lecithin- or Tween 20-stabilized $W_1/O/W_2$ double emulsions. In Tween 20

emulsions, the addition of CMCNa slowed down the release of FFA, but, at the end of the intestinal digestion phase, no differences were observed in the total amount released. In the lecithin-stabilized $W_1/O/W_2$ double emulsions, the addition of CMCNa had no effect initially, but a rapid increase was observed after 50 min, being the final amount of FFA released significantly higher than in the emulsions without polymer. CMCNa has been reported to inhibit aggregation of the outer droplets (Pays, Giermanska-Kahn, Pouligny, Bibette, & Leal-Calderon, 2002; A. Schuch et al., 2015). Therefore, although the addition of CMCNa slowed down the initial digestion rate, a remarkable FFA release is observed at the end of the lipid digestion, being the total FFA release of ~ 40%. This digestion behavior may be related to the time take for surface-active components from the bile extract to displace the biopolymer from the oil droplet surface and thereby facilitate lipase adsorption and activity (Mun et al., 2007).

The bioaccessibility of an aqueous solution of the anthocyanin extract used in this study was about 2% (data not shown). It has been reported a low bioaccessibility of anthocyanin extracts, which may be due to their notoriously low stability into simulated intestinal fluids. This is one of the reasons because anthocyanins isolated from plant cells are easily degraded during their passage throughout the human digestive system and they are poorly absorbed and rapidly excreted (Frank et al., 2012). Hence, the systemic bioavailability of anthocyanins is estimated to be 0.26–1.8% in animal studies (Borges et al., 2007; Ichiyanagi, Shida, Rahman, Hatano, & Konishi, 2006; Koli et al., 2010). In our study, the bioaccessibility of anthocyanin-loaded emulsions increased substantially in comparison with the aqueous solution of the anthocyanin extract. The values ranged from 22.52 ± 0.21% in lecithin-stabilized $W_1/O/W_2$ double emulsions and

475 31.08 ± 1.73% CMCNa lecithin-stabilized $W_1/O/W_2$ double emulsions (Figure 4).
476 No differences between surfactants were observed, but the addition of CMCNa
477 showed a positive effect on the bioaccessibility, only significant in lecithin
478 emulsions, probably because of the stabilization effect of the biopolymer, as it
479 has been previously mentioned. The highest bioaccessibility observed in CMCNa
480 lecithin emulsions could be attributed to the fact that the oil droplets are more
481 stable in the simulated gastrointestinal fluids and the release of FFA in these
482 samples is more progressive, extending the anthocyanin protection and avoiding
483 its degradation (Figure 3).
484
485 *3.3. Changes of $W_1/O/W_2$ emulsion properties over time*
486 The emulsions were stored at 4 ºC in darkness and changes in the emulsion
487 properties were monitored for 21 days. Table 2 shows the encapsulation
488 efficiencies over the time for all the emulsions studied. The amount of
489 anthocyanins enclosed of the total compound initially encapsulated in lecithin and
490 Tween 20-stabilized double emulsions was ~ 70-75% after 2 weeks and ~ 65%
491 after 21 days. Similar retention has been reported in $W_1/O/W_2$ emulsions
492 containing *trans*-resveratrol over the time (Hemar et al., 2010; Matos et al., 2014).
493 Initial encapsulation efficiency was maintained during the storage with the
494 addition of CMCNa, which could be attributed to the capacity of biopolymers to
495 stabilize the outer droplets of double emulsions, preventing creaming and
496 coalescence phenomena (Benna-Zayani et al., 2008; Dickinson, 2011; Matos et
497 al., 2014). An emulsion has a good stability when the initial encapsulation
498 efficiency is around 90-95%, and about 70-80% after a few weeks of storage

499 (Dickinson, 2011; O'Regan & Mulvihill, 2010). Hence, the emulsions formulated
500 in this study present a good stability.
501 The droplet size of double emulsions stabilized with Tween 20 did not change
502 during 21 days of storage. Contrary, the particle size increased in the emulsions
503 formulated with lecithin after 14 days of storage, which may be due to droplet
504 coalescence, as it was confirmed by Turbiscan measurements (Figures 5 and 7).
505 Chang and McClements (2016) suggested that the interfacial coating formed by
506 lecithin molecules was less resistance to coalescence than that formed by the
507 other emulsifiers. With the addition of CMCNa in double emulsions, no important
508 droplet size variations were shown over the time. Similar observations have been
509 reported by Schuch et al. (2015), who suggested CMCNa and gum arabic as a
510 suitable alternative for stabilizing the outer drops in double emulsions by
511 hydrophilic emulsifiers.
512 No noteworthy variation of ζ-potential values in emulsions was observed over the
513 21 days of storage at 4 ºC (Figure 6). Values greater than 25 mV (absolute
514 values) are considered generally stable, and the higher the ζ-potential, the more
515 stable the emulsion is, since the predominance of repulsion forces will avoid the
516 flocculation phenomenon (Lamba, Sathish, & Sabikhi, 2015). Considering that
517 the values obtained in this study are relatively high in absolute magnitude, we
518 can assume that stable $W_1/O/W_2$ emulsions have been formulated, especially in
519 the case of emulsions formulated with lecithin and CMCNa, with very negative
520 values lower than -80 mV. The stability is suggested to be attributed to the
521 interfacial composition of emulsions and the electrostatic repulsion between oil
522 droplets over the time. In comparison with other biopolymers, it has been reported

that CMCNa provided more negative charge in O/W emulsions than whey protein isolate (Berendsen et al. 2014).

Viscosity values remained stable in Tween 20-stabilized double emulsions over the time. In contrast, an increase of viscosity was observed in the emulsions formulated with lecithin without biopolymer after 2 days and throughout the storage time (Table 3). According to our results, the addition of biopolymer prevented a viscosity increase. The rise of viscosity during the storage time could be associated with the droplet aggregation or flocculation (Regan & Mulvihill, 2009). Thus, the CMCNa addition could prevent these instability issues. The use of polysaccharides in the outer phase of double emulsions, as thickening and gelling agents, could prevent creaming and coalescence. Scleroglucan as well as mixtures of xanthan and locust bean solutions exhibited relatively low values of yield stress and plastic viscosity, which could better maintain the large droplets suspended in the external aqueous phase and avoid the breakdown of multiple droplets (Benna-Zayani et al., 2008).

### *3.4. Stability of $W_1/O/W_2$ emulsions by Turbiscan*

The backscattering (BS) profiles obtained in a Turbiscan apparatus of $W_1/O/W_2$ emulsions were shown in Figure 7. The profiles were analysed in three different zones of the test tube (bottom, middle and top) during 21 days of storage in order to detect different instability phenomena in the emulsions. The oil droplets are susceptible to creaming, flocculation, coalescence, and Ostwald ripening, meanwhile the inner droplets are susceptible to flocculation, coalescence and Ostwald ripening process (Mcclements, Decker, Park, & Weiss, 2009).

No important variations in the sample mean that the formulation is stable. A variation greater than 10% either as a positive or as a negative of BS value could be an indicator of instability (Celia, Trapasso, Cosco, Paolino, & Fresta, 2009). Maximum variations of BS were detected in lecithin and Tween 20-stabilized $W_1/O/W_2$ double emulsions at the bottom of the test tube (Figure 7A and B). In both cases, the destabilization process involves creaming, since the BS of the emulsions decreased in the bottom of the test tube because droplet concentration is reduced (clarification), whereas it increased on the top due to an increase of droplet concentration (creaming), as it was reported by Wulff-Pérez et al. (2009) and Wang et al. (2017). In addition, lecithin-stabilized emulsions presented a decrease of BS at the middle of the test tube (Figure 7A), suggesting that coalescence of the oil droplets could have led to an increase of particle size over time, as it was mentioned.

In emulsions stabilized with CMCNa, low backscattering variation was observed (Figure 7C and D), especially in those formulated with lecithin. In CMCNa lecithin-stabilized emulsions, backscattering variation over time was very low in all the sample (<10%), indicating no significant changes in droplet size and high stability for 21 days. Such as long-term stability has been related to the biopolymer capacity to increase the viscosity of the external aqueous phase (Dickinson, 2011). Nevertheless, the addition of CMCNa in Tween 20-stabilized double emulsions extended the stability of the systems, although a remarkable decline of the BS was observed after 14 days (Figure 7D), suggesting flocculation of droplets as the particle size did not change (section 3.3). It could be explained because of the tendency of small particles to aggregate when they are numerous at a given phase ratio and more susceptible to the influence of Brownian motion,

572 which would result in a greater probability of collision (McClements, 2016). The
573 presence of biopolymer acting as a thickening agent reduced creaming by
574 increasing the viscosity of the continuous phase.
575
576 **4. Conclusions**
577 CMCNa-stabilized $W_1/O/W_2$ emulsions were formulated using a two-step
578 emulsification method. The addition of CMCNa with low interfacial activity
579 increased the encapsulation efficiency and provided higher protection to
580 anthocyanins throughout the simulated digestion. CMCNa lecithin-stabilized
581 emulsions showed the highest anthocyanin bioaccessibility, probably because
582 the compound remained more time encapsulated during the lipid digestion, and
583 were the most stable systems over the time. The combination of lecithin and
584 CMCNa in the external aqueous phase inhibited aggregation of the outer droplets
585 during the storage. Therefore, our results have shown that double emulsions are
586 appropriate systems for the encapsulation of anthocyanins and for their controlled
587 release through the gastrointestinal tract. Moreover, the effect of CMCNa in our
588 study seems to be more beneficial with the use of lecithin rather than Tween 20.
589
590 **Conflict of interest**
591 The authors declare no conflict of interest.
592
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821

822 **Figures**

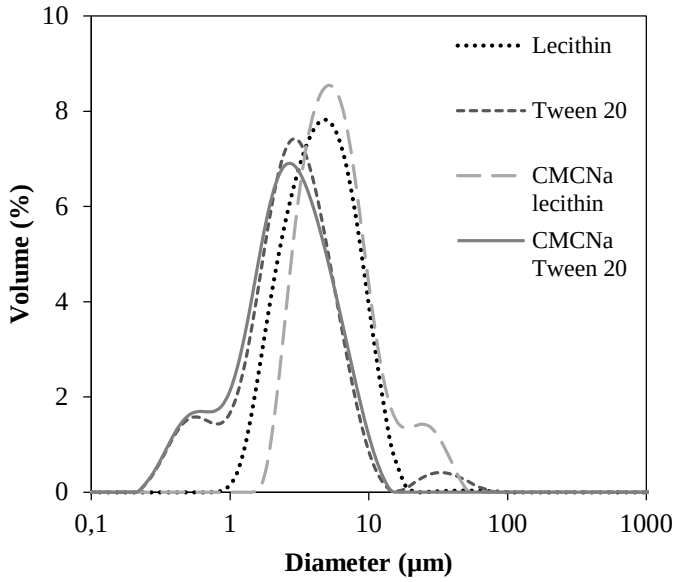


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**Figure 1.-** Droplet size distribution of the different $W_1/O/W_2$ double emulsions. Lecithin: lecithin-stabilized emulsions, Tween 20: Tween 20-stabilized emulsions, CMCNa lecithin: sodium carboxymethylcellulose and lecithin-stabilized emulsions, CMCNa Tween 20: sodium carboxymethylcellulose and Tween 20-stabilized emulsions.

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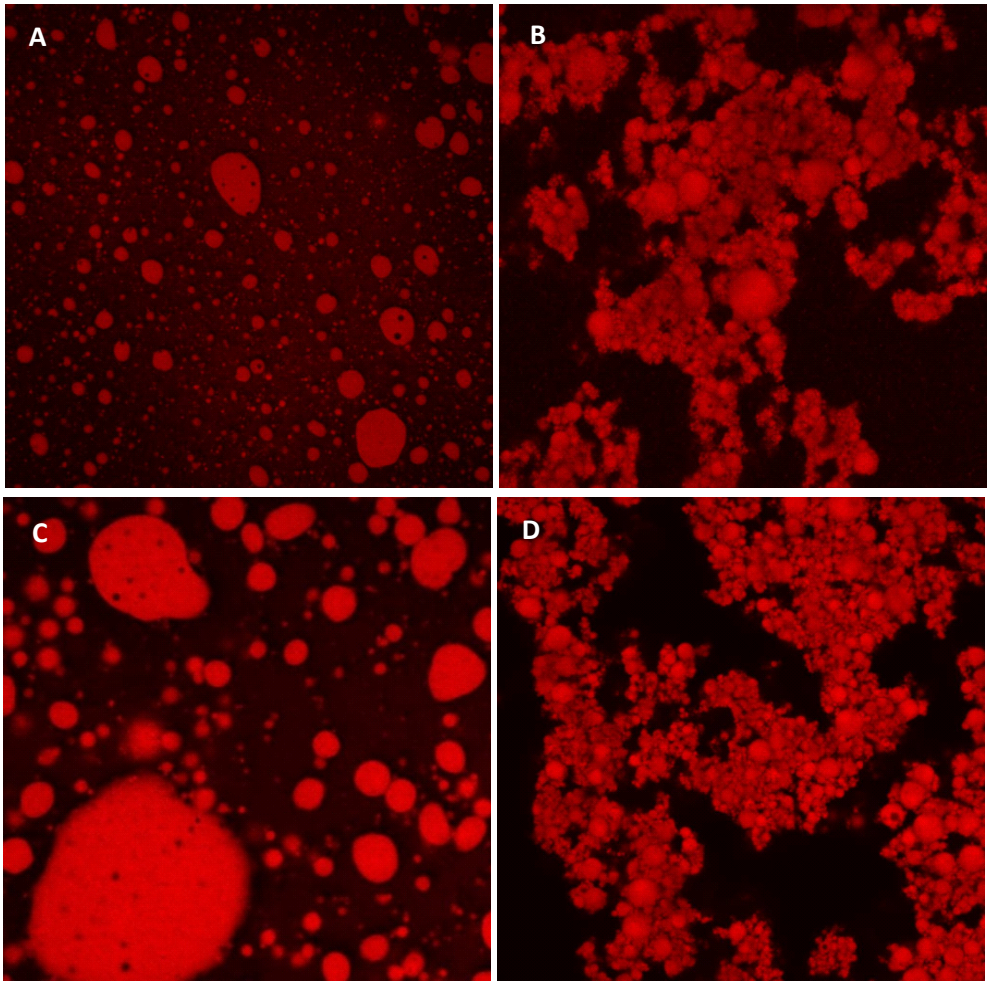


**Figure 2.-** Confocal laser scanning microscopy images of the different $W_1/O/W_2$ double emulsions. Lecithin: lecithin-stabilized emulsions (A), Tween 20: Tween 20-stabilized emulsions (B), CMCNa lecithin: sodium carboxymethylcellulose and lecithin-stabilized emulsions (C), CMCNa Tween 20: sodium carboxymethylcellulose and Tween 20-stabilized emulsions (D)**.**

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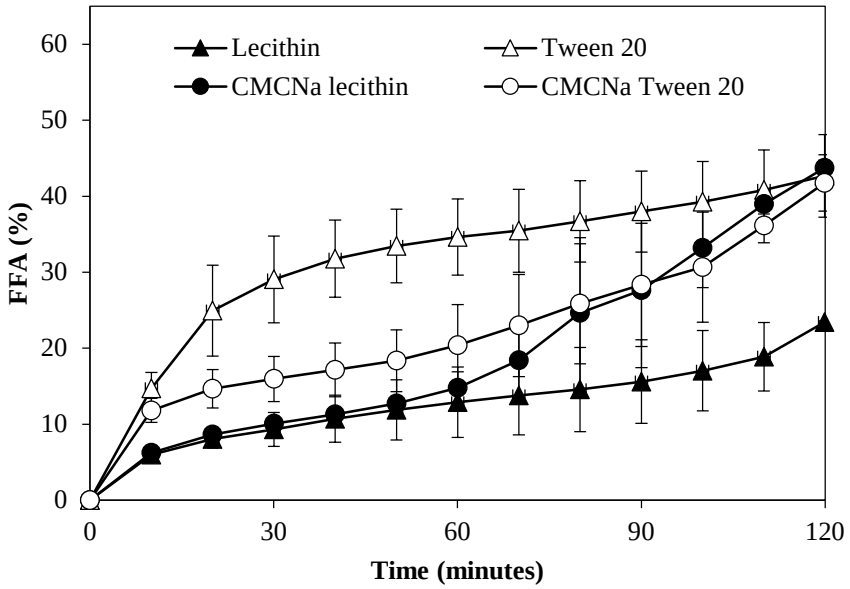


**Figure 3.-** Free fatty acid (FFA%) release during the intestinal phase of the different $W_1/O/W_2$ double emulsions. Lecithin: lecithin-stabilized emulsions, Tween 20: Tween 20-stabilized emulsions, CMCNa lecithin: sodium carboxymethylcellulose and lecithin-stabilized emulsions, CMCNa Tween 20: sodium carboxymethylcellulose and Tween 20-stabilized emulsions**.**

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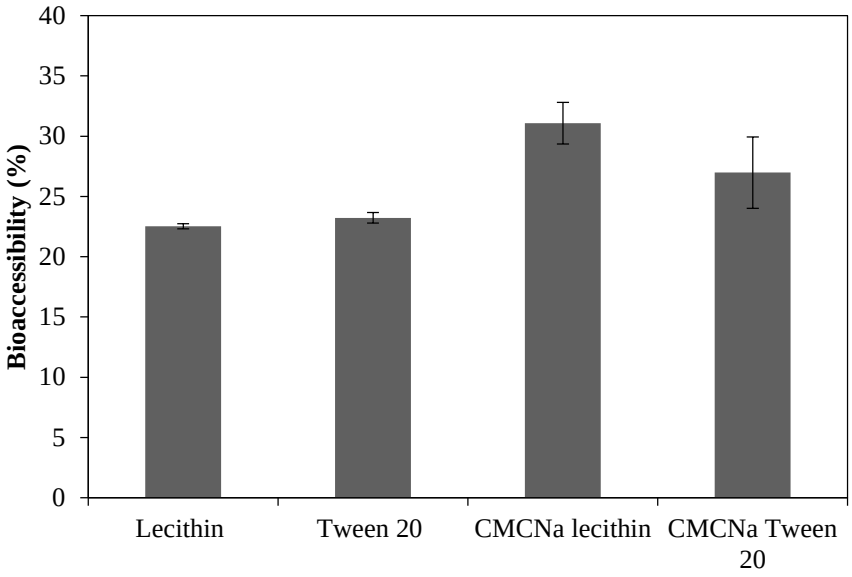


**Figure 4.-** Anthocyanins bioaccessibility (%) of the different $W_1/O/W_2$ double emulsions. Lecithin: lecithin-stabilized emulsions, Tween 20: Tween 20-stabilized emulsions, CMCNa lecithin: sodium carboxymethylcellulose and lecithin-stabilized emulsions, CMCNa Tween 20: sodium carboxymethylcellulose and Tween 20-stabilized emulsions**.** Different capital letters indicate significant differences ($p < 0.05$) among emulsions.

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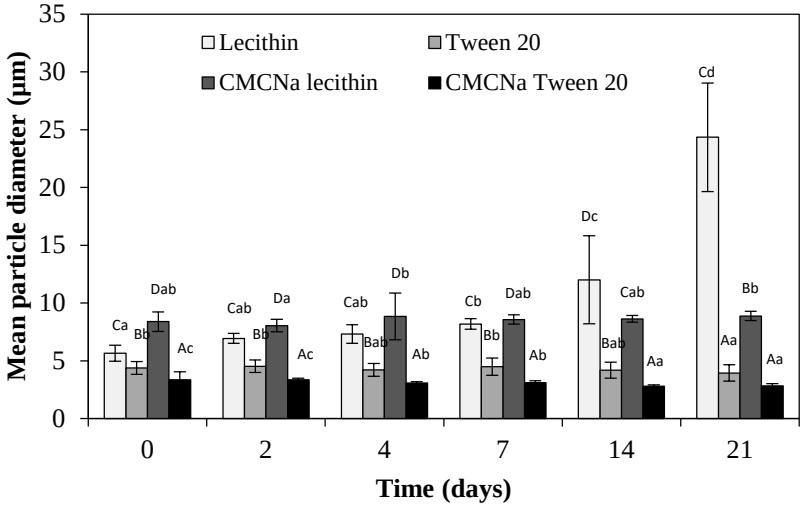


**Figure 5.-** Mean particle size of the different $W_1/O/W_2$ double emulsions during 21 days at 4 ºC. Lecithin: lecithin-stabilized emulsions, Tween 20: Tween 20-stabilized emulsions, CMCNa lecithin: sodium carboxymethylcellulose and lecithin-stabilized emulsions, CMCNa Tween 20: sodium carboxymethylcellulose and Tween 20-stabilized emulsions**.** Different capital letters indicate significant differences ($p < 0.05$) among emulsions. Different lowercase letters indicate significant differences ($p < 0.05$) in an emulsion during the storage time.

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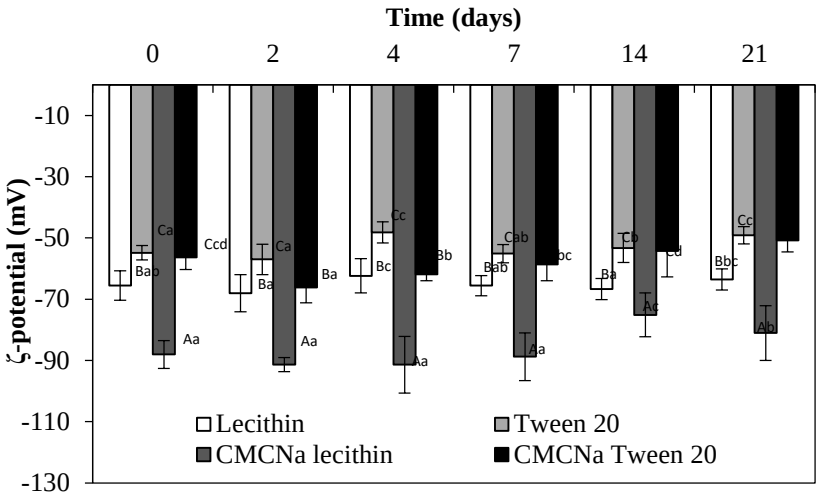


**Figure 6.-** ζ-potential of the different $W_1/O/W_2$ double emulsions during 21 days at 4 ºC. Lecithin: lecithin-stabilized emulsions, Tween 20: Tween 20-stabilized emulsions, CMCNa lecithin: sodium carboxymethylcellulose and lecithin-stabilized emulsions, CMCNa Tween 20: sodium carboxymethylcellulose and Tween 20-stabilized emulsions**.** Different capital letters indicate significant differences ($p$ < 0.05) among emulsions. Different lowercase letters indicate significant differences ($p$ < 0.05) in an emulsion during the storage time.

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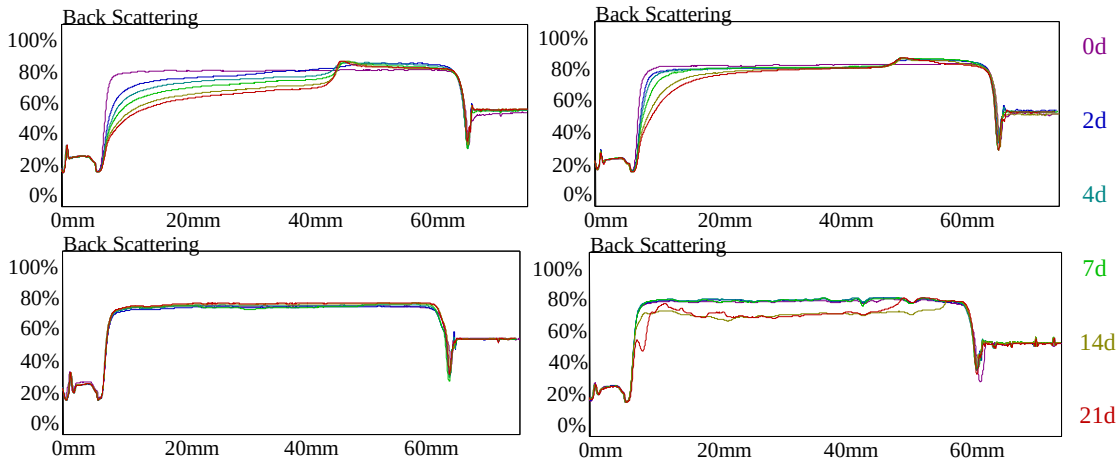


**Figure 7.-** Variation of backscattering (BS) of the different $W_1/O/W_2$ double emulsions during 21 days at 4 ºC. Lecithin: lecithin-stabilized emulsions (A), Tween 20: Tween 20-stabilized emulsions (B), CMCNa lecithin: sodium carboxymethylcellulose and lecithin-stabilized emulsions (C), CMCNa Tween 20: sodium carboxymethylcellulose and Tween 20-stabilized emulsions (D)**.**

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## Tables

**Table 1.** Initial encapsulation efficiency (EE), particle size, ζ-potential, viscosity, color and pH of $W_1/O/W_2$ double emulsions

| | Lecithin [1] | Tween 20 [2] | CMCNa Lecithin [3] | CMCNa Tween 20 [4] |
|---|---|---|---|---|
| **EE (%)** | 78.38 ± 3.87 A | 74.84 ± 1.53 A | 90.14 ± 1.57 B | 92.57 ± 0.20 B |
| **Particle size (μm)** | 5.66 ± 0.70 C | 4.39 ± 0.55 B | 8.39 ± 0.83 D | 3.36 ± 0.69 A |
| **ζ-potential (mV)** | -65.58 ± 4.81 B | -54.83 ± 2.36 C | -88.06 ± 4.51 A | -56.3 ± 4.00 C |
| **Viscosity (mPa·s)** | 2.16 ± 0.08 A | 2.02 ± 0.06 A | 277.67 ± 38.41 B | 257.50 ± 27.69 B |
| **Color (a*)** | 14.50 ± 0.65 C | 11.57 ± 0.59 B | 12.71 ± 0.75 B | 5.24 ± 1.52 A |
| **pH** | 4.83 ± 0.07 B | 3.37 ± 0.37 A | 5.49 ± 0.06 C | 5.38 ± 0.03 C |

Values are expressed as mean ± standard deviation

[1] Lecithin: lecithin-stabilized emulsions

[2] Tween 20: Tween 20-stabilized emulsions

[3] CMCNa lecithin: sodium carboxymethylcellulose and lecithin-stabilized emulsions

[4] CMCNa Tween 20: sodium carboxymethylcellulose and Tween 20-stabilized emulsions

[ABC] Different letters within the same line indicate significant differences ($p < 0.05$) among emulsions

**Table 2.** Encapsulation efficiency of the different $W_1/O/W_2$ double emulsions during 21 days at 4 ºC

| Time (days) | Emulsions | | | |
|---|---|---|---|---|
| | Lecithin [1] | Tween 20 [2] | CMCNa lecithin [3] | CMCNa Tween 20 [4] |
| 0 | 78.38 ± 3.87 Ab | 74.84 ± 1.53 Ab | 90.14 ± 1.57 Ba | 92.57 ± 0.20 Bab |
| 2 | 72.87 ± 2.77 Aab | 74.2 ± 0.91 Ab | 90.14 ± 0.59 Ba | 90.14 ± 0.59 Ba |
| 4 | 76.54 ± 1.77 Ab | 74.41 ± 0.26 Ab | 90.84 ± 0.25 Ba | 91.84 ± 2.30 Bab |
| 7 | 74.31 ± 2.98 Ab | 75.53 ± 0.45 Ab | 89.16 ± 2.40 Ba | 95.49 ± 0 Bb |
| 14 | 76.17 ± 3.20 Ab | 69.71 ± 4.79 Aab | 89.6 ±0.22 Ba | 92.55 ± 1.75 Bab |
| 21 | 66.11 ± 1.12 Aa | 66.26 ± 3.56 Aa | 91.29 ± 2.86 Ba | 92.29 ± 2.57 Bab |

Values are expressed as mean ± standard deviation

[1] Lecithin: lecithin-stabilized emulsions

[2] Tween 20: Tween 20-stabilized emulsions

[3] CMCNa lecithin: sodium carboxymethylcellulose and lecithin-stabilized emulsions

[4] CMCNa Tween 20: sodium carboxymethylcellulose and Tween 20-stabilized emulsions

[ABC] Different letters within the same line indicate significant differences ($p < 0.05$) among emulsions in a day

[abc] Different letters within the same column indicate significant differences ($p < 0.05$) among emulsions throughout the storage time

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**Table 3.** Apparent viscosity of the different $W_1/O/W_2$ double emulsions during 21 days at 4 ºC

| Time (days) | Emulsions | | | |
|---|---|---|---|---|
| | Lecithin [1] | Tween 20 [2] | CMCNa lecithin [3] | CMCNa Tween 20 [4] |
| 0 | 2.16 ± 0.08 Aa | 2.02 ± 0.06 Aa | 277.67 ± 38.41 Bab | 257.50 ± 27.69 Bab |
| 2 | 2.87 ± 0.07 Ab | 2.32 ± 0.20 Ab | 236.50 ± 8.92 Ba | 248.17 ± 19.23 Bab |
| 4 | 2.95 ± 0.17 Ab | 2.67 ± 0.45 Ac | 286.50 ± 36.64 Bab | 273.50 ± 13.37 Bab |
| 7 | 3.27 ± 0.48 Abc | 2.17 ± 0.10 Aab | 301.50 ± 36.77 Bb | 277.83 ± 29.79 Bb |
| 14 | 3.58 ± 0.41 Ac | 2.64 ± 0.31 Ac | 304.67 ± 69.92 Cb | 234.17 ± 21.63 Ba |
| 21 | 5.78 ± 0.67 Ad | 2.05 ± 0.09 Aab | 309.33 ± 82.58 Bb | 250.83 ± 63.68 Bab |

Values are expressed as mean ± standard deviation

[1] Lecithin: lecithin-stabilized emulsions

[2] Tween 20: Tween 20-stabilized emulsions

[3] CMCNa lecithin: sodium carboxymethylcellulose and lecithin-stabilized emulsions

[4] CMCNa Tween 20: sodium carboxymethylcellulose and Tween 20-stabilized emulsions

[ABC] Different letters within the same line indicate significant differences ($p < 0.05$) among emulsions in a day

[abc] Different letters within the same column indicate significant differences ($p < 0.05$) among emulsions throughout the storage time

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