**Potential of microencapsulation through emulsion-electrospraying to
improve the bioaccessibility of β -carotene**

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ABSTRACT

The development of carotenoid-enriched functional foods is limited by the low bioaccessibility of these bioactive compounds. The aim of this work was to improve the bioaccessibility of β -carotene after *in-vitro* digestion through its encapsulation within electrosprayed protein microparticles. Two different protein matrices (zein and a whey protein concentrate, WPC) and two emulsification procedures (high-speed homogenization and ultrasonication) were used to prepare the microcapsules through emulsion-electrospraying, using a soy bean oil as lipid carrier, and the impact of the emulsion properties on the microencapsulation efficiency (MEE) and the bioaccessibility of β -carotene was studied. Results showed that the stability of the prepared emulsions was the main factor affecting the microencapsulation efficiency. The application of an ultrasonic treatment was necessary to stabilize the WPC emulsions and increase the MEE of the WPC microcapsules, but had a slight negative impact on the total β -carotene content of the zein particles, due to thermal degradation of β -carotene, without significantly affecting their MEE. The highest MEE was achieved for the capsules obtained from zein emulsions ($34\pm 7\%$). All the encapsulation structures, except those obtained from WPC emulsions prepared by high-speed homogenization, increased the bioaccessibility of β -carotene after *in-vitro* digestion, which was negligible in its free form.

KEYWORDS

Electrospraying; emulsion; encapsulation; β -carotene; bioaccessibility.

Chemical compounds studied in this article: β -carotene (PubChem CID: 5280489).

1. Introduction

Carotenoids are a group of natural pigments with many attributed health benefits when consumed in sufficient levels (Maiani, et al., 2009; Qian, Decker, Xiao, & McClements, 2012). Especially, β -carotene has been described to exert protection against a number of severe health disorders, including cancer, cardiovascular diseases or macular degeneration (Albanes, 1999; Rock, 1997). Hence, there is increasing interest in the incorporation of β -carotene as a functional ingredient in food formulations. However, the poor solubility of this compound in aqueous media complicates its application in the food industry and causes its bioavailability to be extremely low (Deng, Chen, Huang, Fu, & Tang, 2014).

In order for β -carotene to be uptaken by the enterocytes in the small intestine, it has to be previously incorporated into mixed micelles during digestion (Kaulmann, André, Schneider, Hoffmann, & Bohn, 2016). Its simultaneous consumption with digestible lipids has shown to enhance its bioavailability (Borel, 2003; Thakkar, Maziya-Dixon, Dixon, & Failla, 2007; van het Hof, West, Weststrate, & Hautvast, 2000), since they can solubilize β -carotene and transport it to the mixed micelles via the free fatty acids which are released upon lipid digestion, thanks to the biliary salts and pancreatic lipase (Tyssandier, Lyan, & Borel, 2001). Hence, emulsion-based systems have been proposed as vehicles for the incorporation of β -carotene into aqueous food products in order to increase its bioaccessibility (Qian, et al., 2012). However, the microbiological stability of oil-in-water emulsions is generally poor, especially when proteins are used as emulsifiers, since they favour the intensive growth of microorganisms (Glibowski, Kordowska-Wiater, & Glibowska, 2011). In order to improve their storage stability, these emulsions can then be dried to obtain microencapsulation structures (Deng, et al., 2014) obtaining easy-to-handle powdery ingredients.

Spray-drying is the most frequently employed technique in the food industry to produce dry microencapsulation structures from emulsions (Gharsallaoui, Roudaut, Chambin, Voilley, & Saurel, 2007). It has been previously employed for the microencapsulation of carotenoids, including β -carotene, within a number of biopolymeric matrices, being the most commonly used arabic gum, maltodextrins and modified starches (Janiszewska-Turak, 2017; Loksuan, 2007; Mahfoudhi & Hamdi, 2014; Przybysz, 2012). However, this technology involves the use of high temperatures, which may have a negative impact on thermosensitive bioactives like carotenoids. Alternatively, electrospraying is emerging as a promising technology for the microencapsulation of labile bioactive compounds, as it allows processing at room temperature (Gómez-Mascaraque & López-Rubio, 2016). It is a technique based on the electrohydrodynamic processing of polymeric fluids, which consists of subjecting the feed solution or emulsion to a high voltage electric field in such a way that a charged polymer jet is ejected from a conductive capillary through which it is pumped, to a grounded collector. Due to the electrostatic forces generated within the fluid, the jet breaks into small droplets and the solvent evaporates before the material is deposited on the collector in the form of dry micro- or nanoparticles (Gómez-Mascaraque, Lagarón, & López-Rubio, 2015).

Recently, Pérez-Masiá et al. (2015) used the emulsion-electrospraying technique to microencapsulate lycopene, another prominent carotenoid, and compared the performance of the obtained structures with those obtained by spray-drying. Their results showed that much higher encapsulation efficiencies were obtained through electrospraying, due to the thermal degradation of lycopene upon spray-drying (Pérez-Masiá, Lagaron, & Lopez-Rubio, 2015). Similar results were also obtained for other thermosensitive bioactive compounds, such as α -linolenic acid (Gómez-Mascaraque &

López-Rubio, 2016). Furthermore, the encapsulation of these and other labile bioactive ingredients (e.g. polyphenols, vitamins and probiotics) within electrosprayed microparticles has proven to successfully increase their stability in detrimental pH conditions (Gómez-Mascaraque, et al., 2015), high relative humidity (Gómez-Mascaraque, Morfin, Pérez-Masiá, Sanchez, & Lopez-Rubio, 2016) or when subjected to a thermal treatment (R. Pérez-Masiá, et al., 2015). β -Carotene has been previously incorporated within protein fibers by electrospinning, increasing its light stability (Fernandez, Torres-Giner, & Lagaron, 2009). However, the potential of electrosprayed microencapsulation structures, which are easier to disperse into food products than fiber matts (Gómez-Mascaraque, et al., 2015), to increase the bioaccessibility of carotenoids has not been explored yet.

In the present work, β -carotene was microencapsulated through emulsion-electrospraying for the first time, and the potential of the proposed systems to increase its bioaccessibility after *in-vitro* digestion was assessed. For this purpose, two different proteins were used as encapsulation matrices, i.e. zein and a whey protein concentrate (WPC). Proteins are particularly interesting matrices for emulsion electrospraying, since their amphiphilic structures allow their use as effective emulsifiers (McClements, 2004), and both electrospun/sprayed zein and WPC had previously shown to enhance the stability of β -carotene (Fernandez, et al., 2009; López-Rubio & Lagaron, 2012). A soy bean oil (SBO) was used as a carrier oil to dissolve β -carotene and oil-in-water emulsions were prepared through two different procedures, the first one consisting of a single high-speed homogenization step and the second one including a subsequent ultrasonication treatment. The emulsions and the encapsulation structures obtained thereof were characterized and the microencapsulation efficiency of the different

systems was estimated. Moreover, the recovery of β -carotene from the mixed micelles fraction of the digestas was assessed after an *in-vitro* digestion simulation.

2. MATERIALS AND METHODS

2.1. Materials

Whey protein concentrate (WPC) was kindly donated by ARLA (ARLA Food Ingredients, Viby, Denmark). Under the commercial name of Lacprodan® DI-8090, the composition per 100 g of product consisted of ~80 g of protein, ~9 g of lactose, and ~8 g of lipids, being the rest water and minerals like sodium and potassium. Zein from corn (grade Z3625, 22–24 kDa), β -carotene, soybean oil (SBO), Tween20®, pepsin from porcine gastric mucosa, bile extract porcine, pancreatin from porcine pancreas and dimethylformamide (DMF) were supplied by Sigma-Aldrich (Spain). 96% (v/v) ethanol was purchased from Panreac (Spain). All inorganic salts used for the *in-vitro* digestion assays were used as received. Methanol and tert-butyl methyl ether (HPLC grade quality) were provided by Sigma-Aldrich (Spain).

2.2. Preparation of the emulsions

The emulsions were prepared based on the procedures described in Gómez-Mascaraque and López-Rubio (2016), with some modifications. The aqueous phase of each emulsion consisted of a protein solution/dispersion in water or in a water-ethanol mixture. Two different proteins were used to prepare the emulsions: whey protein concentrate (WPC) and zein. Soy bean oil (SBO) was used as the oily phase. When β -carotene was incorporated into the emulsions, it was previously dissolved in SBO at a

concentration of 5% (w/w). For this purpose, brief heating (4 to 5 min on a plate at 90 °C) was necessary (Qian, et al., 2012). Tween20® was used as a surfactant to help stabilizing the emulsions and decreasing the surface tension to improve the subsequent electrospraying process.

2.2.1 Preparation of the protein solutions/dispersions

Each protein was dissolved/dispersed in an adequate solvent at the optimal concentration determined in previous works to allow subsequent processing by electrospraying. Thus, WPC (20% w/v) was dispersed in distilled water (Gómez-Mascaraque & López-Rubio, 2016), and zein (12% w/v) was dissolved in a water-ethanol mixture (20:80 v/v) (Gómez-Mascaraque, et al., 2017).

2.2.2 Preparation of the premix

Tween20® (5% w/v) was added to the protein dispersions and the mixtures were stirred until homogenization. Then, SBO (or β -carotene-containing SBO) was added in a proportion of 50% (w/w) with respect to the mass of protein, to achieve a final content of β -carotene of 2.5% (w/w) with respect to the mass of protein in the final bioactive-containing capsules.

2.2.3 Emulsification

The premixes were emulsified using two different procedures, based on the protocols described in (Gómez-Mascaraque & López-Rubio, 2016). The first one consisted of a one-step high-speed homogenization process conducted using an IKA T-25 Digital ULTRA-TURRAX® (Germany) equipped with a S 25N-25F dispersing element (stator diameter of 25 mm) at 6000 rpm during 5 min. The other approach included a second step of ultrasonication aimed at reducing the droplet size (Leong, Wooster, Kentish, &

Ashokkumar, 2009), which was performed using a ultrasonic probe (Bandelin electronic, Germany) at an amplitude of 10% and a frequency of 20 kHz for 2 min in pulse mode (50% active cycle), using an ice bath to avoid excessive heating of the samples.

2.3. Characterization of the emulsions

The surface tension of the emulsions was measured using the Wilhemy plate method in an EasyDyne K20 tensiometer (Krüss GmbH, Hamburg, Germany) at room temperature.

The electrical conductivity of the emulsions was measured using a conductivity meter XS Con6 (Labbox, Barcelona, Spain) at room temperature.

The rheological behaviour of the emulsions was studied using a rheometer model AR-G2 (TA Instruments, USA), with a parallel plate geometry, and the method described in (Lopez-Rubio, et al., 2016). Briefly, continuous shear rate ramps were performed from 1 to 200 s⁻¹ during 20 min at 25°C ± 0.1°C using a stainless steel plate with a diameter of 60 mm and a gap of 1 mm. All measurements were made at least in triplicate.

Optical and fluorescence microscopy images were also taken using a digital microscopy system (Nikon Eclipse 90i) fitted with a 12 V, 100 W halogen lamp and equipped with a digital imaging head which integrates an epifluorescence illuminator. The autofluorescence of β-carotene in the emulsions was captured using a 480/15 excitation filter and 535/20 emission filter. A digital camera head (Nikon DS-5Mc) was attached to the microscope. Nis Elements software was used for image capturing.

2.4. Stability of the emulsions

The creaming index method (Surh, Decker, & McClements, 2006) was used to assess the stability of the emulsions. The emulsions were introduced in sealed tubes and stored for different time periods (i.e. 5, 24 and 48 h) at room temperature. When phase separation occurred, the height of the cream layer (H_c , phase rich in oil) and the total height of each emulsion in the tube (H_E) were measured, and the creaming index (CI) was calculated according to Eq. (1):

$$CI = 100 [(H_E - H_c)/H_E] \quad \text{Eq. (1)}$$

2.5. Encapsulation of β -carotene through electrospraying

The emulsions were processed using an electrospinning/electrospraying apparatus assembled in house, equipped with a variable high-voltage 0-30 kV power supply and a Fluidnatek® L-10 syringe pump. Emulsions were introduced in a 5 mL plastic syringe and pumped at a steady flow-rate of 0.15 mL/h through a stainless-steel needle, which was connected to the syringe through a PTFE tube and placed perpendicularly to the stainless-steel plate used as collector, at a distance of 10 cm. The applied voltage was 13kV for zein emulsions and 15-16 kV for WPC emulsions, based on preliminary trials. Samples were processed through electrospraying during 5 hours after preparation of the emulsions.

2.6. Morphological characterization of the particles

Scanning electron microscopy (SEM) was conducted on a Hitachi microscope (Hitachi S-4800) at an accelerating voltage of 10 kV and a working distance of ~10 mm. Samples were sputter-coated with a gold-palladium mixture under vacuum prior to examination. Particle diameters were measured from the SEM micrographs in their original magnification using the FIJI software (Schindelin, et al., 2012). Size distributions were obtained from a minimum of 200 measurements.

2.7. Fourier transform infrared (FT-IR) analysis of the particles

Free and microencapsulated β -carotene (ca. 1-2 mg) was grounded and dispersed in about 130 mg of spectroscopic grade potassium bromide (KBr). A pellet was then formed by compressing the samples at ca. 150 MPa. FT-IR spectra were collected in transmission mode using a Bruker (Rheinstetten, Germany) FT-IR Tensor 37 equipment. The spectra were obtained by averaging 10 scans at 1 cm^{-1} resolution.

2.8. Microencapsulation efficiency

The total amount of β -carotene incorporated within the electrosprayed materials was calculated as the percentage of the initial β -carotene content added to the emulsions that was present in the electrosprayed materials, according to Eq. (2).

$$\text{Total } \beta\text{-carotene (\%)} = \frac{\text{total } \beta\text{-carotene content in electrosprayed materials}}{\text{theoretical } \beta\text{-carotene content in feed emulsions}} \times 100 \quad \text{Eq. (2)}$$

The microencapsulation efficiency (MEE) of the β -carotene loaded capsules was calculated as the percentage of the initial β -carotene content added to the emulsions that was effectively incorporated within the cores of the electrosprayed microparticles, according to Eq. (3).

$$\text{MEE (\%)} = \frac{\text{total } \beta\text{-carotene content in electrosprayed materials} - \beta\text{-carotene content on the surface}}{\text{theoretical } \beta\text{-carotene content in feed emulsions}} \times 100 \quad \text{Eq. (3)}$$

The total content of the carotenoid present within the electrosprayed materials was estimated by UV-vis measurements after extraction of the bioactive compound from the electrosprayed structures. For this purpose, WPC particles (ca. 10 mg) were disrupted in distilled water at 2 mg/mL, releasing their contents. The reconstituted emulsions were subsequently diluted 2-fold with DMF to dissolve the β -carotene content and the WPC was removed by centrifugation. On the other hand, zein particles (ca. 10 mg) were directly dissolved in DMF at 2 mg/mL, as the protein is soluble in this solvent. In both cases, the absorbance of the resulting solutions was measured at 451 nm using a V-1200 spectrophotometer (VWR, Barcelona, Spain). Calibration curves for β -carotene quantification were previously obtained for the carotenoid in DMF/H₂O (50:50) and in DMF in the presence of 1 mg/mL of zein ($R^2_{\text{DMF/H}_2\text{O}} = 0.9998$, $R^2_{\text{DMF/zein}} = 0.9991$).

The content of β -carotene on the surface of the capsules was estimated according to a method adapted from Umesha, Monahar, and Naidu (2013). Briefly, the capsules (ca. 10 mg) were re-suspended in hexane (1 mg/mL), vortexed for 5 s and immediately filtered using WhatmanTM filter devices with 0.2 μm PTFE membranes (GE Healthcare Life Sciences, UK). The absorbance of the filtrate at 451 nm was also measured as explained above, previous calibration ($R^2_{\text{hexane}} = 0.9994$).

2.9. *In-vitro* digestion

Suspensions of pure (ca. 2.5 mg) and microencapsulated β -carotene (ca. 200 mg) in 2.5 mL distilled water, i.e. a theoretical β -carotene concentration of 1 mg/mL in all cases, were subjected to *in-vitro* digestion following the protocol described in (Gómez-Mascaraque, Soler, & Lopez-Rubio, 2016). Solutions of simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) were prepared according to the compositions described in (Minekus, et al., 2014). In the oral phase, the suspensions were mixed with SSF (50:50 v/v) and incubated at 37 °C for 2 min under agitation in a thermostatic bath. In the gastric phase, the oral digesta was mixed with SGF (50:50 v/v) and porcine pepsin (3.8 mg/mL), and incubated at 37 °C for 2 h under agitation. In the duodenal phase, the gastric digesta was mixed with SIF (50:50 v/v), porcine bile extract (37.8 mg/mL) and porcine pancreatin (16.25 mg/mL), and incubated at 37 °C for 2 h under agitation. The pH was adjusted to 7, 3, and 7 in the oral, gastric and duodenal phases, respectively, using 1M HCl or 1M NaOH solutions. After the duodenal phase, the protease inhibitor Pefabloc® (1 mM) was added.

2.10. HPLC analysis of bioaccessible β -carotene after *in-vitro* digestion

The samples obtained from the *in-vitro* digestion assays after the duodenal phase (the ‘digestas’) were centrifuged at 1795 g and 4°C for 20 min using an Eppendorf 5804R Centrifuge (Eppendorf, Germany), and aliquots of the clear micelle phase, i.e. disregarding the sediment phase at the bottom and the oily phase at the top when present (Qian, et al., 2012), were collected. One aliquot of each sample was then filtered using 0.45 μ m, nylon, Whatman® Syringe filters in order to remove any residual large particles or droplets, as described in (Qian, et al., 2012). The micelle phase of the

digestas was then extracted as follows: 5 mL of methanol/tetrahydrofuran (1/1, v/v) was added to 2 mL of sample, mixed by vortexing during 1 min and evaporated using a speed-vacuum concentrator (Savant™ SPD131DDA SpeedVac™ Concentrator, Thermo Scientific, UK). The residue was resuspended in 0.5 mL of tert-butyl methyl ether and MeOH (50:50, v/v), centrifuged at 20.817g during 10 min at room temperature, and analyzed by HPLC-DAD. Chromatography separation was performed using a C30 column 250 x 4.6 mm, 5 µm i.d. (Trentec, Gerlingen, Germany) maintained at 17 °C, and using tert-butyl methyl ether (A) and MeOH (B) as mobile phases at a flow of 1 mL/min. The gradient started with 2% A in B, to reach 35% A at 35 min, 60% A at 45 min, 60% at 55 min, followed by washing and then a return to the initial conditions. β -carotene (all-*trans*- β -carotene) was identified in the chromatograms according to their UV spectra and retention times, by comparisons with the standard, and the chromatographic peak area, recorded at 450 nm, was quantified as β -carotene.

2.11. Statistical analysis

A statistical analysis of experimental data was performed using IBM SPSS Statistics software (v.23) (IBM Corp., USA). Significant differences between homogeneous sample groups were obtained through two-sided t-tests (means test of equality) at the 95% significance level ($p < 0.05$). For multiple comparisons, the p-values were adjusted using the Bonferroni correction.

3. RESULTS AND DISCUSSION

3.1. Characterization of the emulsions SBO-protein emulsions

The properties of emulsions, which depend both on their composition and on the size and aggregation state of the dispersed phase, determine factors such as their stability or the bioavailability of the active ingredients they may contain (McClements, 2011, 2012). Moreover, the successful production of microcapsules through electrospraying is strongly dependent on the feed solution/emulsion properties. For instance, the viscosity should be enough to lead to the necessary polymer entanglements to form the encapsulation structures, the surface tension should be low enough for the Taylor cone to form and yield a stable electrospraying process, and the electrical conductivity should not be too high as to not destabilize the electrospraying jet (Bock, Dargaville, & Woodruff, 2012; Ding, Lee, & Wang, 2005; Shenoy, Bates, Frisch, & Wnek, 2005). Therefore, the SBO/protein emulsions prepared through both emulsification procedures were thoroughly characterized before incorporation of β -carotene to produce the encapsulation structures. Table 1 shows the viscosity (at 200 s⁻¹), surface tension and electrical conductivity of all the different formulations assayed. The complete rheological profiles of the emulsions are also shown in Figure 1.

Table 1. Properties of the SBO/protein emulsions. Different letters (a-b) within the same column indicate significant differences at $p < 0.05$ among the samples

Emulsion		Surface tension (mN/m)	Electrical conductivity (μ S/cm)	Viscosity at 200 s ⁻¹ (mPa·s)
Protein	Emulsion procedure			
Zein	HSH	25.9 $\pm$ 0.1 ^a	386 $\pm$ 1 ^a	19.7 $\pm$ 0.9 ^a (Newtonian)
Zein	HSH+US	26.1 $\pm$ 0.1 ^a	373 $\pm$ 5 ^a	19.6 $\pm$ 0.9 ^a (Newtonian)
WPC	HSH	36.5 $\pm$ 0.2 ^b	1102 $\pm$ 51 ^b	21.3 $\pm$ 0.1 ^a (Pseudo-plastic)
WPC	HSH+US	36.5 $\pm$ 0.2 ^b	1149 $\pm$ 9 ^b	25.7 $\pm$ 0.3 ^b (Pseudo-plastic)

HSH = High-speed homogenization; US = Ultrasonication

Figure 1. Rheological behaviour of the emulsions: variation of the shear stress (left) and viscosity (right) as a function of the shear rate.

The surface tension of all the emulsions was low enough to allow the success of the electro spraying process, as they did not surpass the limit of 50 mN/m above which, according to previous studies, a liquid cannot be electro sprayed (Jaworek, 2007). The lower values achieved for the zein emulsions can be related to the presence of ethanol in the continuous phase, whose surface tension is lower than that of water. Moreover, no significant differences were observed before and after the application of the ultrasound treatment for a given composition. Neither did the electrical conductivity significantly change upon ultrasonication which, as expected, was considerably higher for the WPC emulsions, due to the greater water content of their continuous phase and higher protein concentration. The rheological properties of the emulsions, however, did change upon ultrasonication for the WPC-stabilized emulsions. These exhibited a shear-thinning behaviour, which is generally indicative of a certain degree of flocculation (Surh, et al., 2006). In this case, the viscosity significantly increased upon ultrasonication, which generally suggests a decrease in the droplet size since the average distance of separation

between the droplets decreases (Youce Ettoumi, Zouambia, & Moulai-Mostefa, 2017). Moreover, ultrasound treatments have been previously reported to alter the structural characteristics of whey proteins (Chandrapala, Zisu, Kentish, & Ashokkumar, 2012; Chandrapala, Zisu, Palmer, Kentish, & Ashokkumar, 2011). Unfolding of globular proteins and subsequent increased exposure of their non-polar amino acids, which would facilitate the interaction among different protein chains, could also contribute to the observed change in the rheological properties. Conversely, zein emulsions exhibited a Newtonian behaviour, indicative of well dispersed and non-flocculated droplets (Surh, et al., 2006), and their viscosity was not altered after the ultrasound treatment. Apparently, the non-globular structure of this protein, with a dominant α -helical conformation in ethanolic solution (Tatham, et al., 1993), was less affected by the ultrasound treatment.

3.2. Morphology and creaming stability of the β -carotene loaded emulsions

Once the emulsions had been characterized, β -carotene was incorporated in their oil phase by dissolving it in the SBO before emulsification. Figure 2 shows the appearance of the obtained emulsions. Due to the extremely high turbidity of the WPC emulsions, their accurate observation was not possible. However, it can be inferred from the pictures that some big-sized droplets were present in the WPC emulsion prepared using only high-speed homogenization (Figure 2a), while their size was reduced upon ultrasonication (Figure 2b), as previously observed for similar emulsions (Gómez-Mascaraque & López-Rubio, 2016). In contrast, due to the good solubility of zein in ethanol and the subsequent transparency of the continuous phase of its emulsions, the droplets in that case could be clearly observed. Again, some bigger droplets were

observed in the emulsions prepared using only high-speed homogenization (Figures 2c, e), and the size was slightly reduced upon ultrasonication (Figures 2d, f). It is also noticeable that, although β -carotene has a low solubility in ethanol, this solubility is so low that the carotenoid is preferentially located in the disperse phase of the zein emulsions (Figures 2e, f, where fluorescence from β -carotene can be observed).

Figure 2. Optical micrographs of the β -carotene loaded emulsions prepared using WPC (a, b) and zein (c, d), and fluorescence micrographs of zein emulsions containing β -carotene (e, f). Emulsions a, c and e were prepared by high-speed homogenization. Emulsions b, d and f were prepared applying an additional ultrasonication step. Scale bars represent 20 μ m.

The creaming index of the different emulsions was also calculated after different storage periods as a measure of their stability to gravitational separation. It is worth mentioning that the cream layer was placed at the top in the WPC emulsions, as the density of the oil droplets is lower than the aqueous WPC dispersions, but was placed at the bottom in

the zein emulsions, due to the lower density of the continuous phase consisting of an ethanolic zein solution. Results are shown in Figure 3.

Figure 3. Images of the emulsions after storage for 0, 5, 24 and 48 h, together with the values of their creaming index (CI).

From the analysis of the creaming index of the different emulsions it could be concluded that the most unstable formulation was the WPC emulsion prepared with a single high-speed homogenization step, as it experienced creaming even during the first 5 hours of storage. Consequently, phase separation of this emulsion occurred during the electrospraying process. Conversely, when the WPC emulsion was subjected to an additional ultrasonic treatment, it became very stable, not experiencing phase separation even after 48 hours of storage. This was due to both the reduction in the droplet size of

the emulsions, as inferred from Figure 2, and the unfolding/reassembly of the proteins upon ultrasonication, which might have enhanced the emulsifying properties of WPC through an increase in the exposure of their non-polar amino acids and thus faster adsorption of the protein to the oil-water interface (O'Sullivan, Arellano, Pichot, & Norton, 2014). Regarding the zein emulsions, little differences in their creaming stability were observed depending on the emulsification procedure used. The droplet deposition at the bottom of the tubes after long storage periods was only slightly lower when the ultrasonication treatment was applied, presumably due to the presence of slightly smaller droplets (cf. Figure 2). Nevertheless, both zein emulsions were stable during the whole processing time through electrospraying (i.e. 5 h).

3.3. Morphology of the β -carotene-containing microcapsules

Figure 4 shows the SEM images of the β -carotene-containing encapsulation structures prepared with the different feed emulsions, and their particle size distributions are shown in Figure 5. The WPC particles obtained from the emulsion prepared in a single high-speed homogenization step were quasi-spherical and very similar in size to those obtained in a previous work for SBO/WPC emulsions (Gómez-Mascaraque & López-Rubio, 2016). However, hollow holes were observed in some of the particles, which suggest that some oil droplets were not successfully trapped within the encapsulation structures, probably due to their big size. Therefore, a low encapsulation efficiency was anticipated for this sample. These holes were not observed when the ultrasound treatment had been applied. Also, a significant reduction in the particle size was observed for the WPC samples upon ultrasonication. This was attributed to the reduction of the droplet size of the emulsion and the conformational changes of the

protein, which had an impact on the rheological properties of the emulsions, as discussed above. On the other hand, zein yielded smaller particles than WPC when the emulsion was prepared by high-speed homogenization, and exhibited a rougher surface with a greater content of nanofibrils. This morphology, which is frequently observed in electrospayed zein microstructures (Gómez-Mascaraque, et al., 2017), was very similar for both emulsification methods, as the ultrasound did not cause significant changes in the emulsion properties in this case (cf. Sections 3.1 and 3.2).

Figure 4. SEM images of the β -carotene loaded electrospayed capsules prepared using zein (a, b) and WPC (c, d). Feed emulsions for samples in b and d were prepared by high-speed homogenization. Feed emulsions for samples in a and c were prepared applying an additional ultrasonication step. Scale bars represent 10 μ m.

Figure 5. Particle size distributions of the β -carotene loaded electrospayed capsules. Different letters in red (a-c) placed above the legends indicate significant differences at $p < 0.05$ among the samples.

3.4. Analysis of the particles through infrared spectroscopy

FTIR spectroscopy was used to chemically analyze the obtained capsules. Also, the spectra of the unloaded electrospayed proteins (in the absence of β -carotene and its carrier oil) was obtained for comparison purposes. Figure 6 shows the obtained spectra.

Figure 6. FT-IR spectra of the electrosprayed capsules.

The IR spectrum of the β -carotene-loaded capsules showed contributions from both the protein wall material and the carrier oil containing the carotenoid. The characteristic bands of proteins, i.e. amide A or N-H stretching (3294 cm^{-1} for WPC and 3299 cm^{-1} for

zein), amide B or asymmetric stretching vibration of $=C-H$ and $-NH_3^+$ (3072 cm^{-1} for WPC and 3065 cm^{-1} for zein), amide I or $C=O$ stretching (1648 cm^{-1} for WPC and 1660 cm^{-1} for zein), amide II or N-H bending and stretching (1546 cm^{-1} for WPC and 1541 cm^{-1} for zein), and amide III or C-N stretching (1241 cm^{-1} for WPC and 1245 cm^{-1} for zein) (Aewsiri, Benjakul, Visessanguan, Wierenga, & Gruppen, 2010; Nagarajan, Benjakul, Prodpran, Songtipya, & Nuthong, 2013) were present in all the encapsulation structures. The β -carotene-loaded capsules showed a considerable increase in the absorbance of the bands between 3000 cm^{-1} and 2800 cm^{-1} with respect to their unloaded counterparts, due to the contribution of the SBO (Rocio Pérez-Masiá, et al., 2015). In addition, a band centred at 1748 cm^{-1} corresponding to the $C=O$ groups of the triglycerides from the SBO (Vlachos, et al., 2006) appeared in the loaded structures. A band at 950 cm^{-1} was also found in the loaded structures. This band might be ascribed to the contribution of $C=C$ bonds found in carotenoids (Rubio-Diaz, Francis, & Rodriguez-Saona, 2011), as reported previously for lycopene-loaded capsules (Rocio Pérez-Masiá, et al., 2015). However, the spectra of the electrosprayed capsules produced from the SBO emulsions in the absence of β -carotene also showed this band (results not shown), hindering its sole attribution to the presence of β -carotene. This, together with the low mass ratio of β -carotene in the capsules, hampered the unequivocal identification of β -carotene in the FTIR spectra of the electrosprayed materials, as well as the evaluation of its possible interactions with the proteins. Nevertheless, the presence of β -carotene in the capsules can be indirectly inferred from the presence of the carrier oil in which it was dissolved.

3.5. Microencapsulation efficiency

Table 2 summarizes the total and surface β -carotene contents, and the microencapsulation efficiencies of the different materials. The total β -carotene contents in the electrosprayed materials were reasonably high except for the samples obtained from the WPC emulsions prepared by high-speed homogenization. In that case, the great instability of the emulsions and subsequent phase separation during the electrospraying process explained the great loss of β -carotene via dripping, as the creaming of the emulsions caused the gravitational separation of the oil droplets from the aqueous polymeric dispersion in the syringe. In contrast, the rest of the emulsions were stable throughout their processing by electrospraying (5 h, cf. Figure 3), and consequently the incorporation of β -carotene within the resulting materials was significantly higher. Amongst them, the highest β -carotene content was obtained for the zein emulsions prepared in a single high-speed homogenization step. Ultrasonication of emulsions containing thermosensitive bioactives has been previously reported to decrease the amount of ingredient incorporated within electrosprayed capsules despite increasing emulsion stability (Gómez-Mascaraque & López-Rubio, 2016), which has been ascribed to an increase in the temperature of the emulsions caused by the ultrasonication treatment. This seemed to be also applicable to the systems developed in this work.

On the other hand, results showed that a considerable amount of the β -carotene present in the electrosprayed materials was located at the surface of the capsules, especially for the WPC emulsions prepared by high-speed homogenization, for which most of their β -carotene content was located on the surface. Consequently, the amount of β -carotene located in the core of these capsules and, thus, their MEE, was almost negligible. The ultrasonication treatment, however, managed to significantly increase the MEE of the samples prepared from WPC emulsions, due to an increase in their gravitational

stability and a reduction of their droplet size distribution (cf. Section 3.2). Zein showed higher MEE values than WPC, although a considerable amount of superficial β -carotene was also found in these samples. The amount of superficial β -carotene significantly decreased upon ultrasonication of the zein emulsions, presumably due to the reduction in their droplet size. Hence, although ultrasonication reduced the total amount of β -carotene in the zein capsules, the remaining β -carotene was more efficiently incorporated within the cores of the capsules, resulting in similar MEE values for the electrosprayed zein microstructures obtained from both emulsification procedures.

Table 2. Total and surface β -carotene contents, and microencapsulation efficiencies of the electrosprayed microcapsules (MEE). Different letters (a-c) within the same column indicate significant differences at $p < 0.05$ among the samples

Sample		Total β -carotene (%)	Surface β -carotene (%)	MEE (%)
Protein	Emulsion procedure			
Zein	HSH	74 ± 3^a	41 ± 6^a	33 ± 9^a
Zein	HSH+US	61 ± 3^b	27 ± 4^b	34 ± 7^a
WPC	HSH	26 ± 4^c	21 ± 4^b	6 ± 8^b
WPC	HSH+US	57 ± 1^b	$35 \pm 2^{a,b}$	$22 \pm 3^{a,b}$

3.6. Bioaccessibility of β -carotene

Free and microencapsulated β -carotene was subjected to *in-vitro* digestion in order to study the impact of microencapsulation on the bioaccessibility of the carotenoid. The clear micelle phase (i.e. disregarding the sediment phase at the bottom and the oily phase at the top when present) of each digesta was then filtered as suggested by Quian et al. (2012) to remove large particles (> 450 nm) that would not be expected to be directly absorbed by epithelium cells (Qian, et al., 2012). Figure 7 shows the HPLC chromatograms of the filtered clear micelle phase of the obtained digestas.

515

516 **Figure 7.** Chromatograms of clear micelle phase of the digestas from free and517 microencapsulated β -carotene.

516

No peaks attributable to β -carotene were detected in the chromatograms of the digesta obtained from free β -carotene, so its bioaccessibility was considered negligible. Certainly, in the absence of a carrier oil to dissolve it, the crystalline, insoluble carotenoid in its free form could not be incorporated into bioaccessible micelles. Huo, Ferruzzi, Schwartz, and Failla (2007) reported that relatively low amounts of triolein and canola oil (0.5-1%) were needed to achieve maximum micellization of carotenoids, while higher amounts were required ($\sim 2.5\%$) when the triglycerides of the oil were mainly constituted by medium-chain saturated fatty acids. For this reason, crystals were collected in the digestas from the pure β -carotene, both in the sediment phase after centrifugation and retained on the filter, which both showed the presence of red precipitates.

Conversely, the presence of β -carotene was detected in the digestas of the microencapsulated carotenoid, except for the sample obtained from the WPC emulsion prepared in a single high-speed homogenization step, probably due to the negligible MEE of this system. In fact, only 26% of the initial β -carotene added was present in these capsules (cf. Table 2), so the content of β -carotene before digestion was considerably low. In the rest of the chromatograms, the peak at a retention time of 39 min was identified as all-trans β -carotene. Other peaks appeared at 27, 31 and 41 min, which showed similar spectra but different retention times as all-trans β -carotene. These were ascribed to β -carotene cis isomers. According to the elution order, the isomers were tentatively identified as 15-cis, 13-cis and 9-cis β -carotene (Colmán-Martínez, Martínez-Huélamo, Miralles, Estruch, & Lamuela-Raventós, 2015). Although all-trans- β -carotene was used in the encapsulation process, during *in-vitro* digestion the low pH of the gastric phase could have increased the content of *cis*-isomers β -carotene,

previously described for other carotenoids (Periago, Bravo, García-Alonso, & Rincón, 2013). The pH driven isomerization is considered responsible, at least partially, for the high *cis*-isomers proportion of carotenoids found in the human body after their consumption (Moraru & Lee, 2005; Re, Fraser, Long, Bramley, & Rice-Evans, 2001).

These results showed that the microencapsulation of β -carotene within protein matrices by emulsion-electrospraying was a successful approach to improve the bioaccessibility of β -carotene. Emulsification had already been described as an effective approach to increase the bioaccessibility of β -carotene (Qian, et al., 2012), but the combined emulsion-electrospraying encapsulation technique adds the advantage of obtaining a dry, easy-to-handle powdery ingredient. However, the obtained bioaccessibilities were still quite low. Only 0.01-0.02 % of the encapsulated all-*trans* β -carotene was detected in the bioaccessible fraction of the digestas (0.03-0.05 % of total carotenoids, taking into account the *cis*-isomers). Previous works demonstrated that factors such as the chain length and saturation degree of the fatty acids have an impact on the incorporation of carotenoids into mixed micelles, and consequently on their bioaccessibility and bioavailability (Qian, et al., 2012). For instance, carotenoids have shown higher micellarization extent in the presence of triolein and canola oil than in trioctanoin and coconut oil (Dong, et al., 2013). The main fatty acid in canola oil and triolein is oleic acid, which only represents a 25-30% of total fatty acids in soya oils (Vicente, Castillo, & Cenzano, 1997) like the one used in this work. Given the decisive impact that the lipid profile of the carrier oil has on the bioaccessibility of carotenoids, future works should study the impact of different carrier oils on the microencapsulation of β -carotene through emulsion-electrospraying and its subsequent bioaccessibility in order to improve the potential of this technique.

567 **4. Conclusions**

568 β -Carotene was encapsulated within zein and WPC microstructures by emulsion-
569 electrospraying, using SBO as carrier oil and two different emulsification procedures,
570 one consisting of a single high-speed homogenization step and the other including a
571 subsequent ultrasonication treatment. The results showed that the properties of the zein
572 emulsions did not significantly change upon ultrasonication, while the rheological
573 profile, the droplet size and the stability of the WPC emulsions were noticeably affected
574 by the emulsification procedure, due to the unfolding and reassembling of the globular
575 proteins caused by the ultrasonication treatment. Although all the formulations could be
576 electrosprayed, the WPC emulsions prepared by high-speed homogenization were
577 unstable to gravitational separation, resulting in a negligible encapsulation efficiency.
578 The rest of the systems exhibited reasonably high β -carotene contents, up to $74 \pm 3\%$
579 for the zein particles obtained from the emulsions prepared by high-speed
580 homogenization. However, only around half of the total β -carotene content in the
581 microcapsules was incorporated in their cores, the rest being located on their surface.
582 Although the ultrasonication treatment was necessary for the stabilization of the WPC
583 emulsions and increase in the microencapsulation efficiency of electrosprayed WPC
584 capsules, it reduced the amount of total β -carotene incorporated within the zein
585 microstructures due to thermal degradation of β -carotene, but did not affect their overall
586 MEE. Nevertheless, all the microencapsulation structures except the WPC particles
587 obtained from unstable emulsions increased the bioaccessibility of β -carotene after *in-*
588 *vitro* digestion, which was negligible in its free form. Although the bioaccessibility was
589 still considerably low, the present study shows the potential of the emulsion-

electrospraying technique to enhance the bioaccessibility of carotenoids, prior optimization of the feed formulations.

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