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Influence of O/W emulsion interfacial ionic membranes on the encapsulation efficiency and storage stability of powder microencapsulated astaxanthin

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ABSTRACT

Astaxanthin is a powerful antioxidant with several benefits for human health. Unfortunately, its utilization is limited owing to its poor water solubility and chemical instability during food processing. The aim of this study was to develop a high-efficiency encapsulation system through the assembly of O/W emulsions ionic interfacial membranes to enhance the stability of astaxanthin during the spray-drying process and storage. The emulsions were prepared by the sequential adding of ι-carrageenan and chitosan on the surfaces of the lupin protein isolate (LPI)-coated oil droplets containing astaxanthin. The Taguchi method was used to determine the optimal spray-drying process conditions. The results showed that highly stable emulsions were prepared with 0.20% (w/v) of ι-carrageenan and 0.15% (w/v) of chitosan on LPI-stabilized (1% w/v) oil droplets. Optimal conditions for producing the powdered emulsion with the highest encapsulation efficiency (96.3%) were: 160 °C of inlet temperature, 4 mL/min of feeding rate, 20% (w/w) of total solids content and tertiary emulsion. The multilayer powdered emulsions obtained resulted in high astaxanthin retention during storage compared to a primary powdered emulsion. The powders obtained showed high water solubility (>90%). The results obtained showed that this astaxanthin powdered emulsions have a great potential for its application as a functional ingredient in foods.

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1. Introduction

In recent decades, there has been an increasing interest in the development of healthy foods that contain natural bioactive compounds (Bustamante et al., 2016). In this context, astaxanthin is a valuable antioxidant with potential applications in the nutraceutical and functional food industries. Astaxanthin is a xanthophyll carotenoid found mainly in algae and marine animals, a carotenoid that exhibits a stronger antioxidant activity than all other carotenoids, vitamin E and many other antioxidants (Ambati et al., 2014). Several studies have shown that astaxanthin has various health benefits, including antidiabetic and antioxidative activities, anti-inflammatory action, and immune system-improving and anticancer effects (Guerin et al., 2003). However, this valuable carotenoid can be easily degraded by thermal and oxidative processes during its processing or storage because this molecule presents a high unsaturation degree, which can cause the loss of its biological properties (Fakhri et al., 2018). Moreover, astaxanthin has a low solubility in water, limiting its dispersion in aqueous food matrices, low bioaccessibility and bioavailability, which are generally related to its water solubility (Anarjan et al., 2012). These factors limit its application as a functional ingredient in food applications. Based on previous considerations, microencapsulation is an effective and suitable technology to protect this valuable carotenoid against adverse conditions and thereby help to overcome problems associated with its instability. It should be noted that the success of the encapsulation system used depends mainly on the encapsulation technique employed, optimization process conditions, and wall materials used as encapsulating agents. All these parameters can affect both the preparation and encapsulation efficiency (Gharsallaoui et al., 2007).

Oil-in-water (O/W) emulsions based on encapsulation systems of bioactive lipophilic compounds can be dehydrated by spray-drying process in the food industry for increasing the shelf life of oil (and encapsulated components) against degradation and oxidation during storage (Burgos-Díaz et al., 2020). Spray-drying is a unitary operation in which an o/w emulsion (containing an active ingredient) is atomized in heated air, thus allowing a fast removal of the solvent (e.g., water). This technology is widely used in the food industry because of its relatively low cost and short drying time (Gharsallaoui et al., 2007). Nevertheless, dehydration of o/w emulsions by high temperatures may also alter the interfacial properties and lead to disruption of emulsion droplets and oil leakage (Hu et al., 2016). Therefore, there is a need to develop novel, simple and scalable strategies to maintain the stability of emulsions during drying and subsequent storage and thus reduce the loss of encapsulated bioactive compounds. In this context, some studies have been conducted on the use of conventional O/W emulsions as a system to encapsulate astaxanthin (Shen & Quek, 2014; Bustamante et al., 2016); and Pickering O/W emulsions (Burgos-Díaz et al., 2020) for protecting and improving the chemical stability of carotenoid during the spray-drying process.

In recent years, the investigation and use of multilayer emulsions have attracted significant interest in the research field of food and pharmaceuticals (Burgos-Díaz et al., 2016). This type of system consists of the preparation of multiple interfacial membranes of emulsifiers (biopolymers or surfactant) and/or polyelectrolytes around oil droplets using the layer-by-layer (LbL) technique (Ozturk and McClements, 2016). According to Burgos-Díaz et al. (2018), these types of colloidal systems (emulsions) seem to be much more advantageous and promising to develop an encapsulation and retention system of bioactive compounds during spray-drying in contrast to conventional emulsions. As stated above, the multilayer emulsions could be a good alternative to encapsulate the astaxanthin and overcome the issues related to its instability.

Consequently, a novel relevant aspect of this work was to use the multilayer emulsion as a template for encapsulating astaxanthin before to the spray-drying process. It should be noted that this type of colloidal system has not been previously used as an encapsulation model for making powder emulsion-based products using astaxanthin. Therefore, it is of interest to evaluate the effectiveness of this encapsulation model in the protection of astaxanthin during the spray-drying process and storage. In addition, another novel aspect of this study was the utilization of lupin protein isolate from Lupinus Luteus (LPI) to stabilize the first membrane of primary emulsion owing to its remarkable emulsifying properties (Burgos-Díaz et al., 2016). This non-genetically modified cultivar contains a large amount of proteins (~60%) in its dehulled seeds compared with other legumes commonly used in the food industry (Piornos et al., 2015). Subsequently, additional coating membranes of ι-carrageenan (CA) and chitosan (CHI) were deposited on the first membrane to prepare the secondary and tertiary emulsions, respectively. These ionic biopolymers were selected coatings due to their opposite electrostatic charges, and for their biodegradable and non-toxic properties (Pinheiro et al., 2012).

In this study, the Taguchi method was used as a tool to determine the optimal process conditions by spray-drying to prepare powder emulsions containing astaxanthin. According to Rao et al. (2008), traditional process optimization involves studying one variable at a time, which requires a number of combinations of experiments that are time, cost and labor-intensive, whereas the Taguchi experiment design is a simple statistical tool involving a system of tabulated designs (arrays) that allows a maximum number of main effects to be estimated in an unbiased (orthogonal) fashion with a minimum number of experimental runs. This statistical tool has been applied to predict the significant contribution of the design variable(s) and the optimum combination of each variable by conducting experiments on a real-time basis. For instance, previous studies conducted in our laboratory showed that it is possible to develop an encapsulation system (capsules and microcapsules) as a highly efficient encapsulation system in the oil phase using the Taguchi method (Morales et al., 2017; Rubilar et al., 2012).

According to the above considerations, the main aim of this study was to develop a high- efficiency encapsulation system using O/W emulsions stabilized by ionic interfacial membranes to enhance the stability of astaxanthin during the spray-drying process and storage. The multilayer emulsions were prepared by the sequential adding of CA and CHI on the surfaces of LPI-coated oil droplets. The optimal spray-drying process conditions to prepare powder emulsions containing astaxanthin with high encapsulation efficiency (EE) in the oil phase were determined by the Taguchi method. Also, the stability of astaxanthin in the powdered multilayer emulsions during storage at different temperatures was evaluated. Finally, the results obtained from this research proposal will provide a better understanding of applications of powder systems obtained from multilayer emulsions in the protection of astaxanthin, for its application in the food powder industry.

2. Materials and methods

2.1. Materials

Lupin protein isolate from Lupinus luteus (95% of protein content in dry basis, <5% of moisture) (Burgos-Díaz et al., 2019) was provided by CGNA. ι-carrageenan was purchased from Sigma Aldrich (St. Louis, MO). Chitosan was acquired from Xi'an Surnature Biological Technology Co. Ltd (China). Sunflower oil was purchased from a local market (Santiago, Chile) and astaxanthin oleoresin (Supreme Asta oil 5.0%) was supplied by Atacama Bio Natural Products S.A. (Iquique, Chile).

2.2. Preparation of o/w multilayer emulsions with microencapsulated astaxanthin

The emulsions were prepared following the protocol described by Burgos-Díaz et al. (2018) with some modifications. The primary emulsion was prepared by mixing a 10% (w/w) of the oil phase with 90% (w/w) of the aqueous phase (containing 1% w/v of LPI in sodium citrate buffer at pH 3). The oil phase of the emulsions was obtained by mixing 10% (w/w) astaxanthin with 90% (w/w) sunflower oil. The mixture was homogenized at 10,000 rpm for 5 min using a high-speed blender (Benchtop homogenizer-ProScientific, USA) and subsequently passed through the high-pressure homogenizer (HPH) (PandaPlus 2000, GEA Niro Soavi, Parma, Italy). The HPH process conditions of primary emulsion were 50 MPa of homogenization pressures and 3 cycles. It is important to emphasize that the optimal concentration of LPI used for the preparation to stabilize the primary emulsion was established in a previous study performed in our laboratory (data not shown).

The multilayer emulsions were prepared as follows: the secondary emulsion (Se-E) was prepared by mixing the primary emulsion with solutions of CA at different concentrations (0.05–0.4% w/v, in sodium citrate buffer at pH 3) in a proportion of 70/30% (w/w) (70% of CA solution and 30% of primary emulsion). Then, the tertiary emulsion (Te-E) was prepared by mixing the secondary emulsion with CHI solutions (0.05–0.4% w/v, in sodium citrate buffer at pH 3) in a ratio of 50/50% (w/w). Se-E and Te-E were homogenized at 5000 rpm for 2 min each, followed by 2 cycles at 15 MPa through an HPH to disrupt any flocs formed. All emulsions were stirred for 1 h using a magnetic stirrer at room temperature.

2.3. Characterization of o/w emulsions

2.3.1. ζ-potential measurements

ζ-Potential analysis is a technique for determining the surface charge of particles in solution. The ζ-potential analysis was carried out according to Burgos-Díaz et al. (2018). Thus, the samples were diluted with milli-Q water prior to analysis at a ratio of 1:100 (v/v), by placing them in plastic zeta cells (DTS 1061, Malvern, UK). Afterwards, a Malvern Zetasizer Nano ZS series HT instrument (Malvern Instruments, UK) was used to evaluate ζ-potential at 25 °C at different conditions. Analyses were performed in triplicate.

2.3.2. Physical stability of multilayer emulsions

The physical stability of the emulsions was determined following the protocol described by Sotomayor-Gerding et al. (2016) using a Turbiscan TMA 2000 (Formulaction, Toulouse, France). The changes in monochromatic light (k = 850 nm) backscattered by the emulsion droplets were measured as a function of time. For the measurements, a volume of 8 mL of emulsion was added to a glass tube of 14 cm high and 1.5 cm in diameter. The backscattering (BS) profiles of emulsions were determined during storage for 8 days at 20 °C. The primary and secondary emulsions were diluted with a solution of sodium citrate buffer at pH 3 in order to equal the final percentage of the dispersed phase of the tertiary emulsion. Analyses were performed in triplicate. The difference in backscattering (ΔBS) profiles was obtained using the following Eq. (1)

$$\Delta BS = BS_t - BS_0 \quad (1)$$

where: BS_t represents the BS profile at time (t) = 1–8 days, BS_0 is the BS profile at t = 0 h.

2.4. Spray-drying of multilayer emulsions containing astaxanthin

Drying processes for each emulsion were fed into a B-290 mini spray-dryer (Büchi, Flawil, Switzerland). Before the spray-drying process, the emulsions were mixed with maltodextrin to obtain a total solids content between 15 and 25% and then the mixtures were agitated for 1 h. The spray-dryer was operated at an inlet air temperature from 120 to 160 °C, feeding rate ranging from 4 to 8 mL/min, and 85% of aspiration rate. Powdered emulsions were collected and stored in a dark environment at −20 °C in sealed plastic bags until their characterization.

2.5. Experimental design

To optimize the powder emulsions containing astaxanthin with high oil phase encapsulation efficiency (EE), an experimental Taguchi design was applied. Thus, a matrix L_9 (3^4) with four independent variables and three levels of work were used. For this, the criterion 'the bigger the better' was adopted. In the preparation of powder emulsions, the effect of four independent variables such as inlet air temperature (120, 140 and 160 °C), type of emulsion (primary, secondary and tertiary), feeding rate (4, 6 and 8 mL/min) and total solids (15, 20 and 25% w/w) on EE were evaluated.

The predictive value of the Taguchi method was determined considering the average for the level of each variable using the Minitab 19 software. Table 1 shows the orthogonal matrix used with the design variables.

2.6. Characterization of powdered emulsions containing microencapsulated astaxanthin

2.6.1. Determination of encapsulation efficiency (EE)

For the determination of EE, it was necessary to quantify the total oil phase and the surface oil phase from powdered emulsions. The surface oil of the microcapsules was determined according to Alves et al. (2019). Thus, 1 g of powders were dispersed in 10 mL of hexane and stirred for 2 min in a vortex, and then centrifuged for 15 min at 5000 rpm. The supernatant was collected, and the remaining powder was washed three times with 10 mL of hexane. Then the supernatants collected from washes were mixed and subjected to evaporation at 60 °C in a vacuum evaporator (Büchi, Switzerland). After evaporating the solvent, the extracted oil phase was dried (105 °C) until constant weight.

The total oil in the powders was determined according to Rubilar et al. (2012). The powder (1 g) was washed with 150

Table 1 – Encapsulation efficiency (EE) of oil phase using the orthogonal matrix L_9 (3^4).

Design point	Temperature (°C)	Emulsion	Feeding rate (mL/min)	[a]Total Solids (% w/w)			EE
				Emulsion solids	Maltodextrin	Total	(%)
1	120	Primary	4	1.41	13.6	15	83.0 ± 0.19
2	120	Secondary	6	1.39	18.6	20	84.7 ± 0.83
3	120	Tertiary	8	1.36	23.6	25	86.5 ± 1.22
4	140	Primary	6	1.25	23.8	25	87.2 ± 1.72
5	140	Secondary	8	1.47	13.5	15	86.0 ± 1.02
6	140	Tertiary	4	1.45	18.5	20	92.8 ± 0.30
7	160	Primary	8	1.33	18.7	20	90.1 ± 1.51
8	160	Secondary	4	1.30	23.7	25	93.4 ± 0.49
9	160	Tertiary	6	1.54	13.4	15	96.1 ± 0.33

[a] Total solids: Ionic biopolymer (LPI, CA and CHI), maltodextrin, and oil phase in the final O/W emulsions.

mL petroleum ether for 5 h by Soxhlet extraction. Then, the filtrate solution containing the extracted oil was transferred to a round-bottomed flask, which was subsequently placed in a vacuum evaporator (Büchi, Switzerland). After evaporating the solvent, the extracted oil phase was dried (105 °C) until constant weight. The EE was calculated, in triplicate, according to Eq. (2):

$$EE\ (\%) = \frac{Total\ oil\ phase - Surface\ oil\ phase}{Total\ oil\ phase} \times 100\% \quad (2)$$

The amount of encapsulated oil droplets was calculated in relation with the difference between total oil and surface oil amounts.

2.6.2. *Yield of spray-drying process*

The yield in the spray-drying process was determined using a method described by Bustos-Garza et al. (2013), with some modifications. The spray-drying yield was evaluated considering the recovered powder product and it is given as a percentage by the ratio between the recovered powdered emulsion mass (W_2) and the solids of the O/W emulsion (W_1) fed into the spray-drying. The yield was calculated, in triplicate, according to Eq. (3):

$$Yield\ (\%) = \frac{W_2}{W_1} \times 100 \quad (3)$$

2.6.3. *Moisture content*

The determination of moisture of the powders was performed according to the AOAC 93406 method (AOAC, 2007). Thus, 1 g of powder was dried using a forced air-drying oven (BOV-T50F, Fremont, CA, USA) at 105 °C for 2 h. Afterwards, the sample was weighed in an analytical balance (Sartorius Entris 224i-1S, Göttingen, Germany). The moisture content was calculated, in triplicate, using the following Eq. (4):

$$M\ (\%) = \frac{M_1 - M_0}{M_1 - M_2} \times 100\% \quad (4)$$

where M_0 is the mass in grams of the melting pot, M_1 is the mass in grams of the powder before drying, and M_2 is the mass in grams of the melting pot and the powder after drying.

2.6.4. *Water solubility*

The solubility of the powdered emulsions was determined by a gravimetric method as described by Gomez-Estaca et al. (2016). The method consisted of adding 0.5 g of the powder to a conical flask containing 50 mL of distilled water and homogenizing the mixture using an orbital shaker at 100 rpm for 30 min at room temperature. The solution was then centrifuged at 3500 rpm for 5 min to induce fast sedimentation of large non-dispersed aggregates. Then, a 25 mL aliquot of the supernatant was transferred to a previously weighed porcelain dish and maintained in an oven at 105 °C for 2 h until complete evaporation of the water. The solubility was calculated, in triplicate, according to Eq. (5):

$$Solubility\ (\%) = \frac{M_2 \times 2}{M_1} \times 100\% \quad (5)$$

where, M_2 is the final mass in grams and M_1 is the initial mass in grams.

2.6.5. *Particle size*

The mean particle size was determined by laser diffraction, using the Shimadzu SALD-3101 (Shimadzu, Kyoto, Japan). For this, the powder was suspended in 2-propanol (refractive index 1.35) to avoid multiple scattering effects during measurement. Analyses were performed in triplicate.

2.6.6. *Morphology*

The microstructure or surface morphology of powders containing astaxanthin was observed by scanning electronic microscopy (SEM) using a JEOL JSM–6380 LV scanning electronic microscope (ESEM, Phenom Pro, Eindhoven, Netherlands). On the other hand, the inside of the microcapsules containing microencapsulated astaxanthin was examined by confocal laser scanning microscopy (CLSM, Zeiss LSM780, Jena, Germany) using a 488 nm argon laser, 40X objectives and the FV-ASW software v. 1.7.

2.7. *Storage stability of powdered emulsions*

2.7.1. *Determination of astaxanthin content*

The astaxanthin content in powdered emulsions was evaluated under storage conditions for 35 days, following the protocol described by Burgos-Díaz et al. (2020). For the analysis, 0.5 g of emulsion powder was dissolved in 5 mL of distilled water and vortexed for 40 min. Then, 5 mL of this extract was mixed with 20 mL of hexane-2-propanal (2:1 v/v) and vortexed for 1 min. The organic phase was separated by centrifugation at 1000 × g for 5 min. The astaxanthin was determined by UV–vis spectrophotometry (Synergy HT, BioTek Instruments Inc., Winooski, VT, USA) at 478 nm in a microplate reader. For all determinations, pure hexane was used as a blank. The astaxanthin oleoresin content of powder emulsions was

obtained, in triplicate, by applying a calibration curve using the following Eq. (6):

$$Astx\ (mg/g\ sample) = \frac{((A_{sm} - 0.045)/4.085) \times V}{W_{sm}} \quad (6)$$

In which: A_{sm} represents the absorbance of the sample, W_{sm} is the sample mass in grams and V is the dilution volume (mL).

2.7.2. Astaxanthin retention percentage and kinetic analysis

The retention percentage of the encapsulated astaxanthin was studied at temperatures of 25 and 45 °C in the storage stove, and samples were taken out every seven days for accelerated shelf-life studies. The retention percentage of astaxanthin was obtained, in triplicate, using the following Eq. (7) (Bustos-Garza et al., 2013):

$$AX\ retention\ (\%) = C_t/C_0 \times 100\% \quad (7)$$

Where C_0 and C_t was the content of astaxanthin in microcapsules initially and with storage time t, respectively.

The data of astaxanthin degradation with different stored conditions was fitted according to the following Eq. (8) (Bustos-Garza et al., 2013):

$$ln\ (C_t/C_0) = -kt \quad (8)$$

Where k (day^{-1}) is the degradation rate constant obtained by regression of experimental data, and t is the stored time. The degradation half-life ($t_{1/2}$) was calculated using the Eq. (9) (Bustos-Garza et al., 2013):

$$t_{1/2} = ln2/k \quad (9)$$

2.8. Statistical analysis

The results presented are the average and standard deviation calculated from these replicate measurements. A one-way analysis of variance (ANOVA) was carried out with a significance level set at 0.05. In the case of significant differences detected with the ANOVA, Duncan's test was performed. Statistical analyses were performed with InfoStat software 2014.

3. Results and discussion

3.1. Effect of ι-carrageenan (CA) and chitosan (CHI) on the emulsion stability

First, the required concentration of ionic biopolymers (CA and CHI) to completely cover the LPI-coated oil droplets of primary emulsion was determined. In other words, the concentration at which oil droplets of the emulsion are surrounded by polyelectrolyte was determined (Burgos-Díaz et al., 2016). Fig. 1(A) shows the effect of the addition of CA on the ζ-potential value of the LPI-coated oil droplets to obtain the secondary emulsion. The results show that in the absence of CA, the ζ-potential value of primary emulsion was 42.0 ± 1.85 mV, which is attributed to the high positive electrical charge of the oil droplets stabilized by an LPI membrane. However, the ζ-potential value on oil droplets changed from a positive to a negative electrical charge when the CA concentration increased from 0 to 0.4% (w/v) and reached a constant value of ζ-potential (−51.5 mV) at 0.2% (w/v). This means that oil droplet surfaces were fully saturated by the CA molecules. According to Burgos-Díaz et al. (2018), the stabilization of ζ-potential values (constant value) implies that ionic biopolymer molecules saturated the surfaces of lupin protein-coated oil droplets.

After determining the CA saturation concentration required to form secondary emulsion containing CA/LPI-coated oil droplets, the effect of adding CHI to form tertiary emulsion was tested (Fig. 1B). In this case, the ζ-potential value of oil droplets in emulsions changed from strongly negative in the absence of CHI to strongly positive in its presence, reaching a constant value (50.8 mV) at 0.15% (w/v). This effect is associated with the adsorption of cationic CHI molecules onto the surfaces of CA/LPI-coated oil droplets as the result of a strong electrostatic attraction between them. As stated by several authors, colloidal systems which present high ζ-potential values, greater than +25 mV or less than −25 mV, are very stable due to strong repulsive electrostatic forces between the oil droplets (Burgos-Díaz et al., 2018; Julio et al., 2018; Fang et al., 2019). In this sense, high stability against aggregation by electrostatic repulsion arising from their electrical charge would be expected.

On the other hand, the physical stability of emulsions was evaluated by analyzing the backscattering (ΔBS) profiles through Turbiscan measurements. Fig. 2 shows the presence of two peaks in ΔBS profiles, which is indicative of the destabilization mechanism of clarification (zone I: 0–20 mm) and also the formation of a cream layer (zone II: 70–80 mm) in the sample tube containing the emulsion (Arancibia et al., 2017). The physical stability behavior of the primary emulsion was similar to the secondary and tertiary emulsions until day 1 of storage. However, from day 2 there was a great increase in the ΔBS (%) profiles of the primary emulsion due to the movement of oil droplets by the action of gravitational force between the bottom (I) and top (II) zones of the sample tube. The primary emulsion showed a destabilization process by creaming evidenced for a high peak in the zone II of 18.6 ± 0.40% after 8 days of storage (Fig. 2A), while the secondary and tertiary emulsions showed a lower peak in zone II of 5.3 ± 0.93 and 3.3 ± 0.56 %, respectively (Fig. 2B and C). Therefore, stabilized emulsion with two and three interfacial membranes of ionic polysaccharides (CA and CHI) presented greater physical stability than the stabilized emulsion only with a one interfacial membrane of LPI. In addition, the CA and CHI deposition on LPI-oil interfaces could stabilize these multilayer emulsion systems protecting them against creaming as a result of combined effects of the repulsion forces between oil droplets and the viscosity enhancement in the emulsion (Julio et al., 2018). This type of behavior has also been reported by Fang et al. (2019), who found a relevant improvement in the physical stability of β-carotene bilayer emulsions with the addition of chitosan and octenyl succinic anhydride starch (OSA starch) because of electrostatic repulsion forces between oil droplets and the viscosity in the emulsion. Finally, the results confirm that the stability of primary emulsion can be improved by preparing secondary and tertiary emulsions.

3.2. Optimization and characterization of powder emulsions containing astaxanthin

The spray-drying process was used to prepare the powder emulsions containing astaxanthin. The spray-drying process was optimized through the Taguchi method using an orthogo-

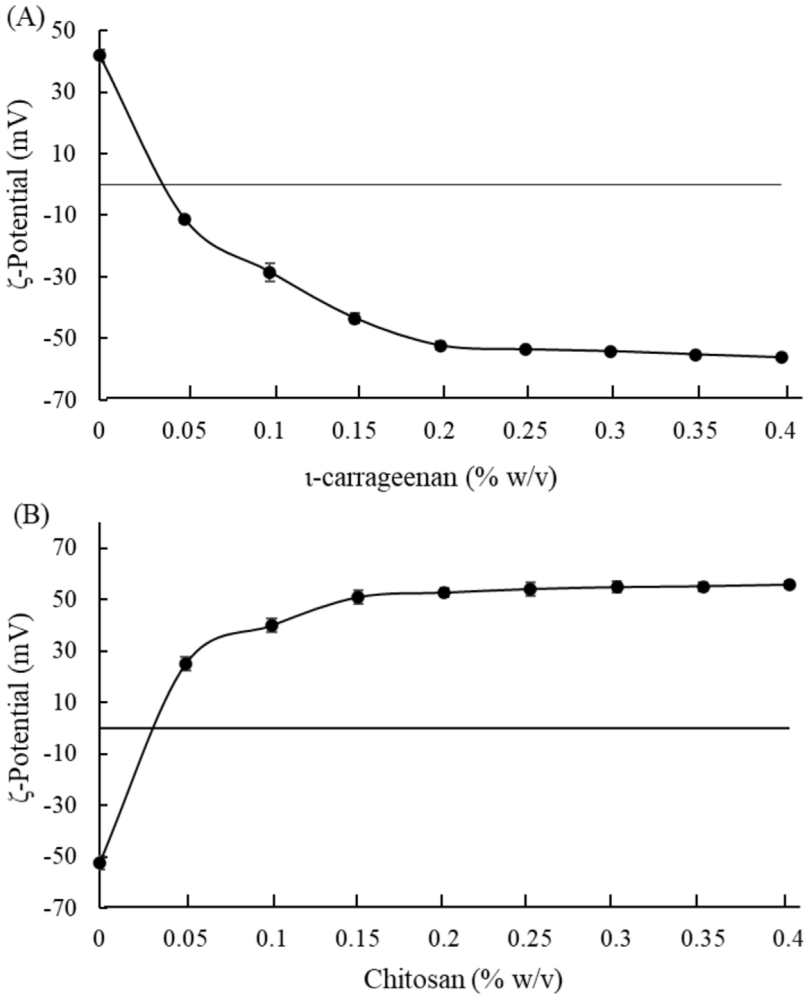


Fig. 1 – Effect of (A) CA and (B) CHI concentrations on ζ-potential of the secondary and tertiary emulsions, respectively.

nal array L_9 (3^4). Thus, the effect of four independent variables: inlet air temperature (120, 140 and 160 °C), type of emulsion (primary, secondary and tertiary), feeding rate (4, 6 and 8 mL/min) and total solids (15, 20 and 25 %w/v) on encapsulation efficiency (EE) were evaluated. After evaluating each variable, the EE values obtained varied between 83.0 and 96.1% (Table 1).

In this study, before converting the liquid O/W multilayer emulsions to powder, the samples were mixed with maltodextrin for increasing the total solid content in the emulsions. The percentage of "total solids" evaluated in the experimental design fluctuated between 15 and 25%, of which the main ingredient was maltodextrin. Normally, the incorporation of this polymer in the emulsion is carried out to increase the content of "total solids" of the sample and to increase the percentage of powder recovery (yield) during the spray drying process. In several studies, it has been reported that in order to achieve an efficient spray drying process of an emulsion (O/W), it is necessary for the sample to contain between 10 and 30% of total solids (Tonon et al., 2008; Frascareli et al., 2012).

Fig. 3 shows the degree of inclination of the slope as a response to EE, indicating that the greater the difference between levels for a variable, the higher the change magnitude on the EE. The inlet air temperature showed the highest influence on the EE with an average by the type of emulsion and feeding rate with a difference of 5.04 and 2.23 units between the working levels, respectively. Finally, the total solids content showed the lowest influence on the EE with a difference of 0.84 units between the working levels. Then, the information on the EE was subjected to an ANOVA (Supplementary material: Table S) and evaluation of the model fitting to determine the degree to which the control variables influenced the response. The coefficients of determination (R^2) for the independent variables inlet air temperature, type of emulsion, feeding rate and total solids were 68.1, 25.9, 5.24 and 0.74%, respectively. In addition, the variable "total solids" of the emulsion had a lower contribution in the EE when compared to the other variables studied. This lower contribution can be attributed to the fact that the concentration of maltodextrin used in this study (between 13% and 23% approx.) is within the optimum values for a drying process. For this reason, it was expected that no considerable variations in the EE would be observed with the concentrations of maltodextrin evaluated.

Finally, the R^2 for these four variables was significant ($p < 0.05$) with 100% indicating a high affinity or association of independent variables with the EE. Therefore, the optimal conditions needed to maximize the EE were inlet air temperature at 160 °C (outlet air temperature of 90 °C), feeding rate of 4 mL/min, total solids content of 20% (w/w) and type of tertiary emulsion. This highlights that, under optimized conditions, the encapsulation of oil containing astaxanthin showed 96.3% of EE, evidencing a high coincidence with the predicted value (97.3%). These results confirmed that the combination between control variables and working levels were sufficient to increase the EE of oil phase.

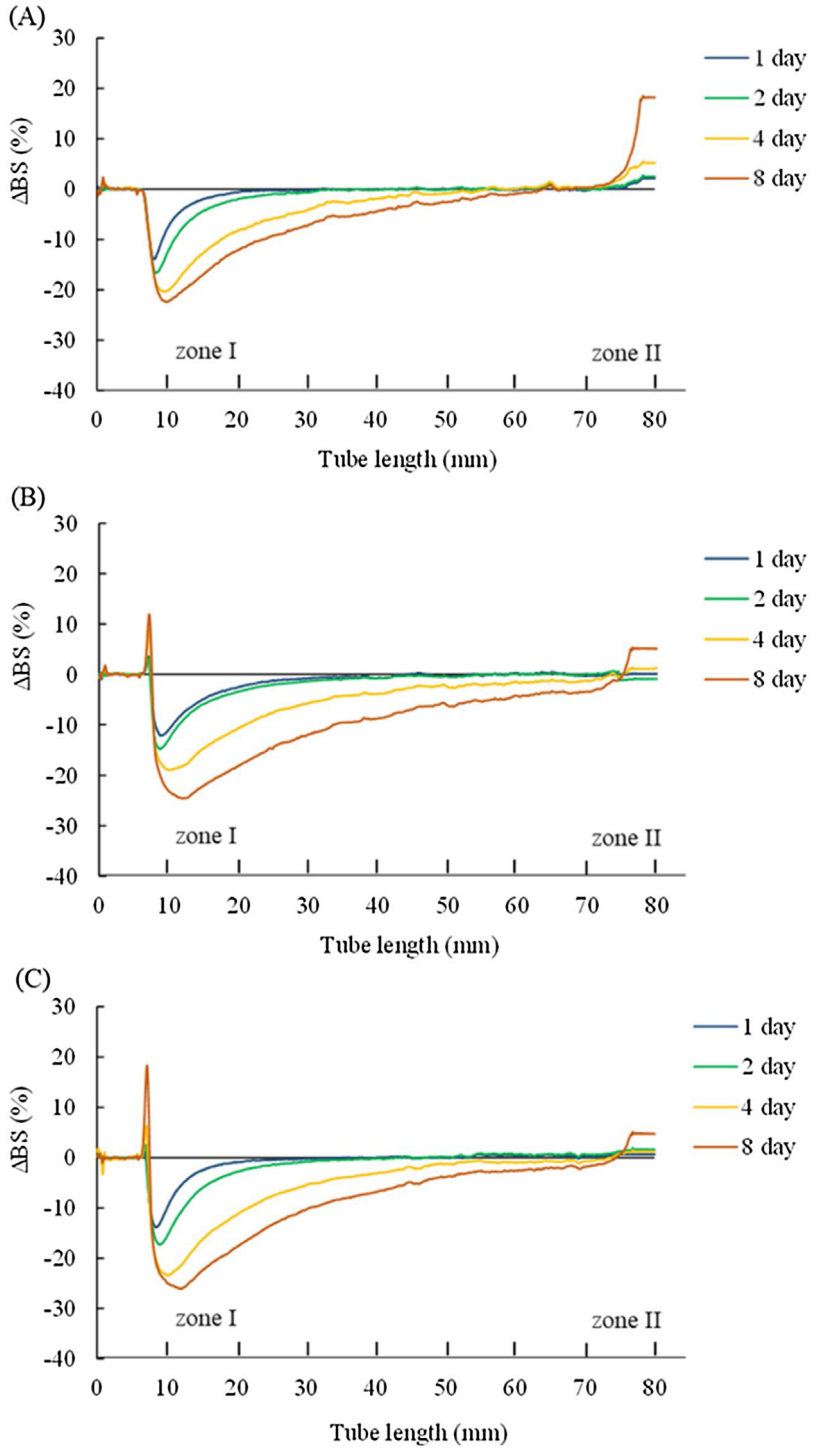


Fig. 2 – ΔBS profiles as a function of the tube length at 20 °C for 8 days, with: (A) Primary, (B) Secondary and (C) Tertiary emulsions.

Table 2 – Characterization of astaxanthin in powdered emulsions obtained under optimal spray-drying process conditions.

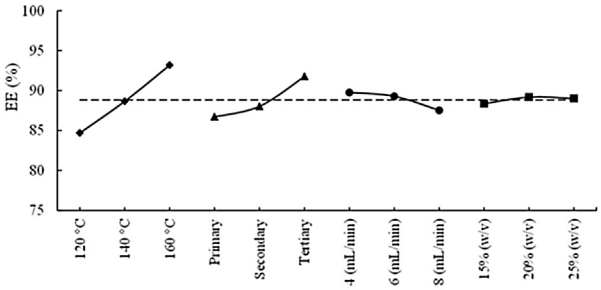
Powders	Solubility (%)	Particle size (μm)	Moisture (%)	Yield (%)	EE (%)
Primary	90.2 ± 2.16[a]	4.33 ± 0.42[a]	2.2 ± 0.10[a]	41.1 ± 5.50[a]	91.6 ± 1.35[a]
Secondary	91.1 ± 1.41[a]	10.3 ± 0.36[b]	2.2 ± 0.10[a]	42.2 ± 4.26[a]	94.0 ± 1.42[b]
Tertiary	90.7 ± 1.16[a]	13.1 ± 0.35[c]	2.2 ± 0.10[a]	40.2 ± 4.33[a]	96.3 ± 0.90[b]

[a, b, c]: Different letters indicate statistical differences between powder emulsions containing astaxanthin ($p < 0.05$). Spray-drying conditions were: 160 °C of inlet temperature, 4 mL/min of feeding rate, and 20% (w/w) of total solids.

Table 2 shows the characterization of powdered emulsions obtained under optimal process conditions. Furthermore, the primary and secondary emulsions were subjected to the same process conditions as the tertiary (optimized conditions) emulsion, so that the effect of the spray-drying process on the encapsulation efficiency, moisture, particle size and solubility of the emulsion powders could be compared. The powders prepared from the tertiary emulsion showed the highest EE (96.3%) in comparison with powders obtained from primary (91.6%) and secondary (94%) emulsions. A significantly higher ($p < 0.05$) EE observed with multilayer emulsions (two and three interfacial membranes) confers a higher protection on the encapsulated core material (oil phase containing astaxanthin) by preventing the release of oil to the surface. Also, these results could be explained by the CA and CHI deposition on LPI-oil membranes in the feed emulsion, because the viscosity of the emulsion increases and the circulation movement inside the droplets decreases, resulting in the faster formation of the crust during spray-drying, which in turn improves EE of oil phase. These results agree with those discussed by Fioramonti et al. (2019), who suggested that the EE could be improved by increasing interfacial membranes in emulsions, since they become part of the wall structure during dehydration processes acting as a supporting matrix surrounding oil droplet. Previous studies reported that the high EE of oil depends on the combination between spray-drying process variables (feeding rate, inlet and outlet air temperature of the dryer) and emulsion properties (type of wall material, total solid content, and oil concentration in the emulsion) (Frascareli et al., 2012; Bustamante et al., 2016). In addition, the yield values of the spray-drying process were around 40%. This value is relatively low for an industrial drying process and may be attributed to the oil content and viscosity of the sample. Samples containing oil (e.g., emulsions) tend to accumulate in the dryer chamber since these are aggregated during the atomization of the product. This leads to a decrease in the powder recovery in the cyclone of the dryer and thus a decrease in the yield.

On the other hand, the powdered astaxanthin emulsions obtained in this study showed a low moisture value (around of 2%). Also, between powdered emulsion there were no significant differences ($p > 0.05$) on the moisture value. This value could be attributed to the good combination between high inlet air temperature in the spray-drying (160 °C) and a lower feeding rate (4 mL/min), which results in increased evaporation of water during the drying process. Similar results were found in powdered Pickering emulsions with encapsulated astaxanthin with moisture values below 5% using inlet air temperatures of the spray-drying of 140 and 160 °C (Burgos-Díaz et al., 2020).

In addition, the mean particle size of powders reached values between 4.33 and 13.1 μm for all samples studied. These results showed that particle size was significantly higher ($p < 0.05$) when interfacial membranes increased on the emulsions. Also, changes in particle size and morphology during spray-drying are related to moisture content and drying temperature (Alamilla-Beltrán et al., 2005). Similar results were reported in previous works with mean particle sizes ranging from 5 to 6.8 μm (Montero et al., 2016) and from 3.4 to 4.5 μm (Bustamante et al., 2016) in powdered conventional emulsions containing astaxanthin. Regarding water solubility, all the powder emulsions were highly soluble (>90%) with no significant differences ($p > 0.05$) among them. Similar results were found by Montero et al. (2016), who produced astaxanthin-powdered emulsions with high water solubility



Working Levels	Temperature	Emulsion	Feeding Rate	Total Solids
1	84.7	86.7	89.8	88.4
2	88.7	88.0	89.3	89.2
3	93.2	91.8	87.5	89.0
Difference Δ(1,2,3)	8.49	5.04	2.23	0.84

Fig. 3 – Effect of the working levels of each variable on the EE. The greater the difference between working levels for a variable, the higher the change magnitude on the EE.

(92%). According to the same author, the water solubility in the powdered emulsions is an important parameter to improve bioaccessibility of astaxanthin (Ribeiro et al., 2005).

3.3. *Morphological analysis of astaxanthin in powder emulsions*

The morphology of the astaxanthin powder emulsions was observed by scanning electron microscope (Fig. 4A). The powders were mostly spherical in shape with some surface dents, non-agglomerated with a particle size from 2 to 20 μm. The powders prepared with tertiary and secondary emulsions showed spherical shapes with some dented surfaces, while powder prepared with primary emulsion showed some irregular shapes, concavities, and surface dents. The appearance of concavities on the surface is attributed to the fast water evaporation and consequent contraction of particles during the spray-drying process (Bustos-Garza et al., 2013; Bustamante et al., 2016). Therefore, different shapes of astaxanthin powders could be influenced by interfacial membranes and drying process. The astaxanthin microencapsulated in the multilayer emulsions was analyzed by confocal microscopy. The microscopic analysis showed autofluorescence of astaxanthin into the spherical structures confirming the presence of this carotenoid in the powder microcapsules (Fig. 4B). Through confocal microscopy images, it is possible to identify the distribution and uniformity of fluorescent compounds in samples (Acevedo et al., 2014). Similar distribution was reported by Montero et al. (2016), where a moderate fluorescence intensity was generated in microcapsules containing astaxanthin obtained after spray drying of a conventional emulsion.

3.4. *Stability of astaxanthin-powdered emulsions during storage*

The stability during the storage of powder emulsions was assessed at 25 and 45 °C for 35 days by measuring the astaxanthin content over time. Fig. 5 shows the effects of storage on the content of astaxanthin in a dry emulsion obtained under optimal conditions. Results showed that the astaxanthin content of secondary and tertiary powders stored at both 25 and 45 °C were significantly higher ($p < 0.05$) than with the powder prepared with primary emulsion (Fig. 5A and B). Also, between secondary and tertiary powdered emulsion there were no significant differences ($p > 0.05$) on the astaxanthin content. In addition, after 14 days of storage, the

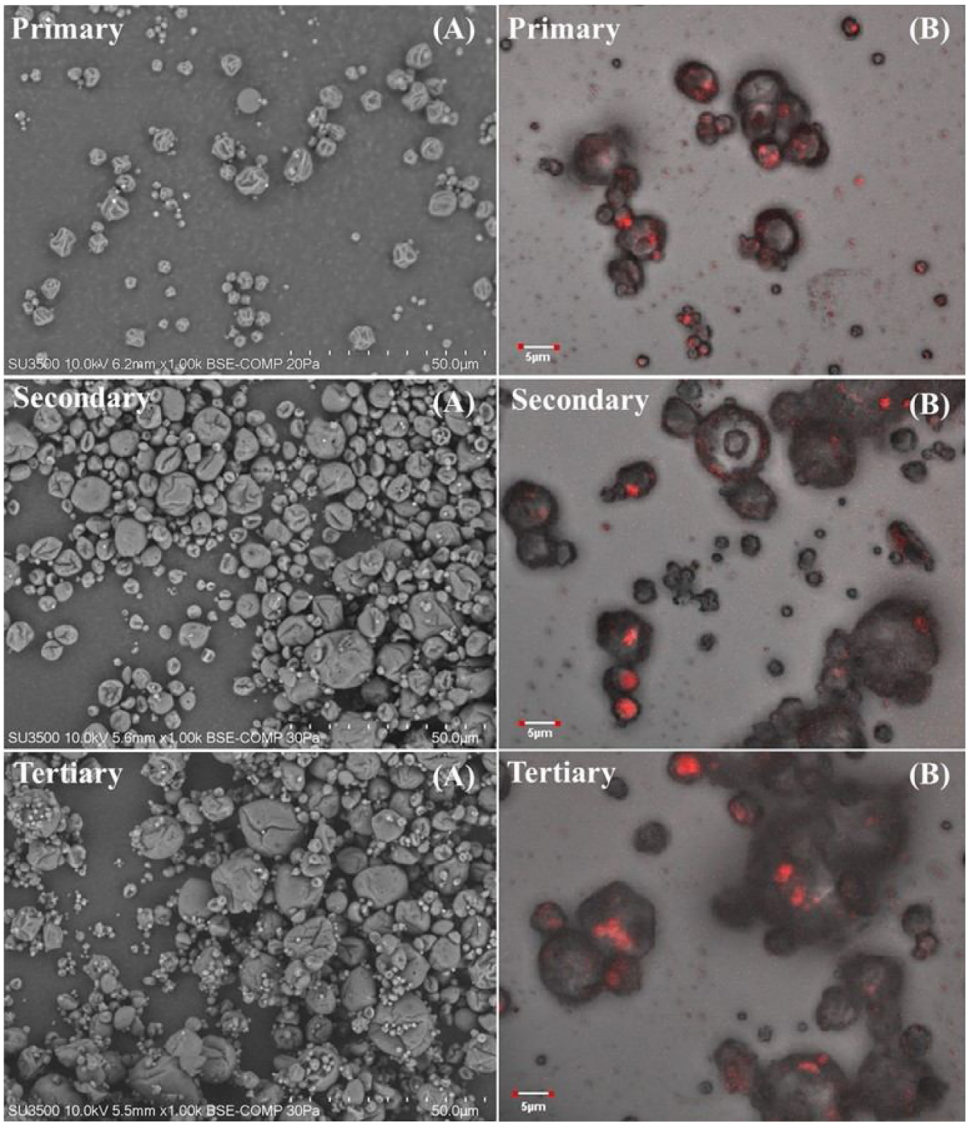


Fig. 4 – Morphology of powdered emulsions by (A) SEM. The presence of astaxanthin was detected in the powdered by fluorescence emitted using (B) confocal microscopy.

powders obtained with primary, secondary and tertiary emulsions stored at 25 °C showed a retention percentage of 71.8, 79.3, and 76.7% with respect to the initial values (0 day), while the powders stored at 45 °C showed a retention percentage of 37.8, 53.0 and 46.4%, respectively. The astaxanthin retention values of powdered multilayer emulsions were higher than those reported by Shen and Quek (2014) (45% of retention for 10 days at 45 °C for encapsulation of astaxanthin using milk proteins and soluble corn fibers as wall material by emulsion and spray-drying). Finally, after 35 days of storage, the primary secondary and tertiary emulsion powders showed an astaxanthin retention value of 49.3, 58.6, and 56.8%, respectively (25 °C), while the powders stored under accelerated conditions (45 °C) showed a retention of 5.1%, 7.6, and 6.8 %, respectively. It should also be mentioned that, although the astaxanthin amount decreased over time due to its oxidation, the powdered emulsions stabilized with two and three interfacial membranes of biopolymers obtained by spray-drying presented the highest values of astaxanthin retention after 35 days of storage. Similar behavior was reported by Fang et al. (2019), who demonstrated that carotenoid encapsulation by bilayer emulsions using spray drying improved the retention of β-carotene against the degradation during 30 days of storage.

Table 3 shows the degradation rate constants (*k*) of astaxanthin in powdered emulsions at 25 and 45 °C. Also, the coefficient of determination (R^2) was used to determine the order of reaction. Thus, a high R^2 indicated that astaxanthin degradation could be well described by first-order reaction kinetics for the temperatures under study (R^2: 0.94–0.98). It was observed that degradation rate constants for microencapsulated astaxanthin significantly decreased ($p < 0.05$) with two and three interfacial membranes on the powdered emulsions stored at 25 °C. This indicates that astaxanthin powders obtained with secondary and tertiary emulsions are more stable than primary emulsion. Similar behavior was reported by Fang et al. (2019), who demonstrated that degradation rate constants of β-carotene were lower during storage when the number of interfacial membranes on powdered emulsions increased. Also, an increase in the astaxanthin degradation rate constants was observed when the storage temperature was increased (45 °C). Similar behavior was reported by Bustamante et al. (2016) in powder emulsions containing astaxanthin. As expected, astaxanthin-powdered multilayer

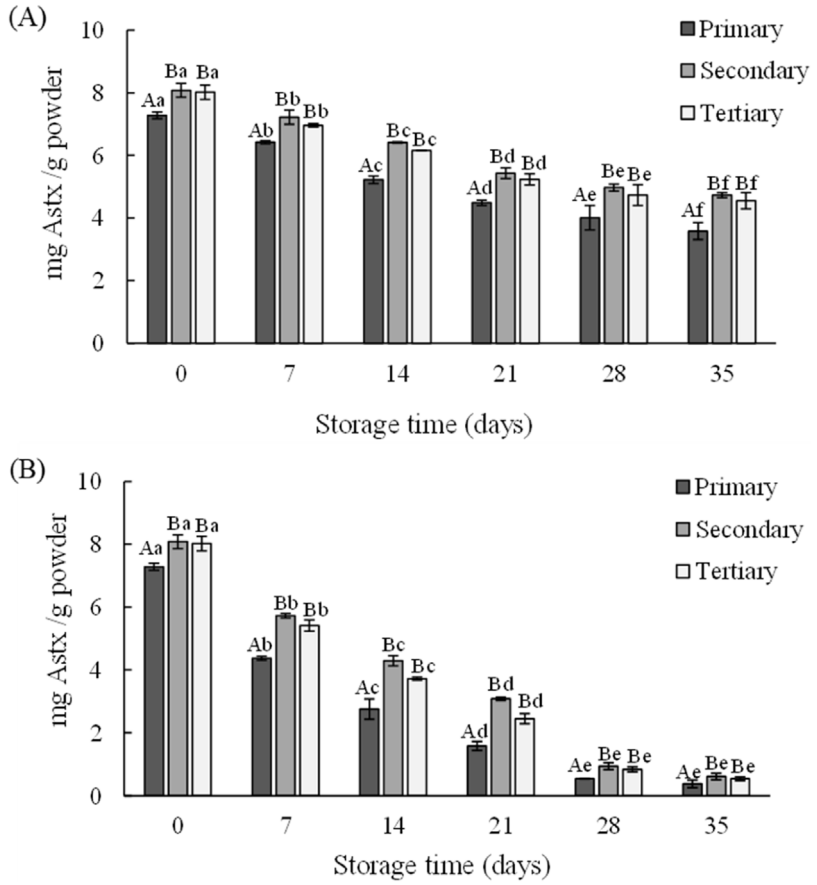


Fig. 5 – Effect of storage temperature at 25 °C (A) and 45 °C (B) on the astaxanthin content in powdered emulsions. Different capital letters indicate significant differences ($p < 0.05$) on the number of layers in the emulsion. Different lowercase letters indicate significant differences ($p < 0.05$) on the storage period for 35 days.

Table 3 – Degradation rate constants (*k*), coefficient of determination (R^2) and half-life ($t_{1/2}$) of astaxanthin in powdered emulsions under storage at 25 and 45 °C.

Temperature	Emulsion	$k \times 10^{-3} \pm SD \times 10^{-3}$ (day^{-1})	R^2	$t_{1/2}$ (days)
25 °C	Primary	20.9 ± 2.28^a	0.98	33.4
	Secondary	16.1 ± 0.05^b	0.97	42.9
	Tertiary	16.9 ± 0.40^b	0.96	40.9
45 °C	Primary	88.0 ± 5.90^a	0.97	7.82
	Secondary	76.5 ± 6.10^a	0.94	9.09
	Tertiary	80.6 ± 11.5^a	0.96	8.71

SD: Standard Deviation. ^{a,b,c}: Different letters indicate statistical differences in the astaxanthin degradation rate constants and half-life between different powder emulsions at the same temperature ($p < 0.05$).

emulsions showed a protective function against the oxidative damage of astaxanthin, decreasing the astaxanthin degradation rate constants with respect to powdered one interfacial membranes emulsion. Finally, the highest half-life ($t_{1/2}$) was observed in secondary and tertiary powdered emulsions of astaxanthin in comparison with primary powdered emulsions, which demonstrates again the advantage of increasing the number of interfacial membranes on powdered emulsions.

4. Conclusions

This study showed that stable multilayer O/W emulsions containing microencapsulated astaxanthin can be obtained using the LPI, CA, and CHI as ionic interfacial membranes. O/W emulsions stabilized with two and three interfacial membranes (secondary and tertiary emulsion) presented greater physical stability than the stabilized emulsion with only one-membrane (primary emulsion). The optimal conditions for oil phase encapsulation were obtained using inlet air temperature of 160 °C, feeding rate of 4 mL/min, total solids content of 20% (w/w) and type of tertiary emulsion. The EE under optimized conditions reached 96.3% of EE, showing a high coincidence with the predicted value (97.3%). The powders were high solubility, low moisture, and high EE, which were significantly influenced by optimal process conditions. The astaxanthin retention in dry emulsions under storage conditions was higher in dry tertiary and secondary emulsions than powder prepared with primary emulsion. Finally, the results obtained showed that this astaxanthin powdered emulsions have a great potential for its application as a functional ingredient in foods.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.fbp.2020.12.014.

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