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Development and Characterization of Poly-ε-caprolactone Nanocapsules Containing β-carotene Using the Nanoprecipitation Method and Optimized by Response Surface Methodology

Renata Calegari Lino [1]
https://orcid.org/0000-0001-9971-8060

Sabrina Matos de Carvalho [1]
https://orcid.org/0000-0002-7100-7803

Carolina Montanheiro Noronha [1]
https://orcid.org/0000-0003-2068-7439

William Gustavo Sganzerla [2]
https://orcid.org/0000-0002-1780-2160

Cleonice Gonçalves da Rosa [1,3*]
https://orcid.org/0000-0003-0894-8464

Michael Ramos Nunes [4]
https://orcid.org/0000-0003-1432-7840

Pedro Luiz Manique Barreto [1]
https://orcid.org/0000-0001-8716-8962

[1]Federal University of Santa Catarina (UFSC), Post-Graduate Program in Food Science, Florianopolis, Santa Catarina, Brazil. [2]University of Campinas (UNICAMP), School of Food Engineering (FEA), Post-Graduate Program in Food Engineering, Campinas, São Paulo, Brazil. [3]University of Planalto Catarinense (UNIPLAC), Post-Graduation Program in Environment and Health, Lages, Santa Catarina, Brazil. [4] Federal Institute of Santa Catarina (IFSC), Lages, Santa Catarina, Brazil

*Correspondence: cleorosaqm@yahoo.com.br; Tel.: +55-48-37215368; (C.G. da Rosa);


HIGHLIGHTS

- PCL/β-carotene nanocapsules were produced by the nanoprecipitation method and optimized by RSM.

- Three lipophilic surfactants and three carrier agents were evaluated in the nanoparticles formulation.

- Optimized nanocapsules presented above 95% of encapsulation efficiency and good colloidal stability.

- The optimum conditions to prepare PCL nanocapsules were 0.216 mg/mL of β-carotene, 232.42 µL of CCT and 2.59 mg/mL of soy lecithin.

Abstract: Nanoparticles demonstrate an important role in the protection of bioactive compounds from external factors such as temperature, oxygen and light. In this study, poly-ε-caprolactone (PCL) nanoparticles entrapped β-carotene was produced using the nanoprecipitation method. Firstly, was evaluated the lipophilic surfactant effect and carrier agent of the active compound in the nanocapsules formulation. After choosing the most stable formulation, the nanocapsules production was optimized using β-carotene, caprylic/capric triglycerides (CCT) and soybean lecithin. Response surface methodology (RSM) was adopted to evaluate the influence of soy lecithin concentration, volume of CCT and β-carotene concentration in the particle size, zeta potential, polydispersity index (PDI), encapsulation efficiency and recovery. Formulations containing soy

lecithin and CCT demonstrated better stability comparing to the other formulations tested. The nanoparticle formulations presented an optimized particle size below 200 nm, PDI lower than 0.1 and encapsulation efficiency above 95%. Based on the results obtained, the optimum conditions to prepare PCL nanocapsules were 0.2160 mg/mL of β-carotene, 232.42 µL of CCT and 2.59 mg/mL of soy lecithin, suggesting an applicability to promote controlled released of β-carotene in food system.

Keywords: nanoparticles; nanoencapsulation; bioactive compounds; carotenoids; optimization

GRAPHICAL ABSTRACT
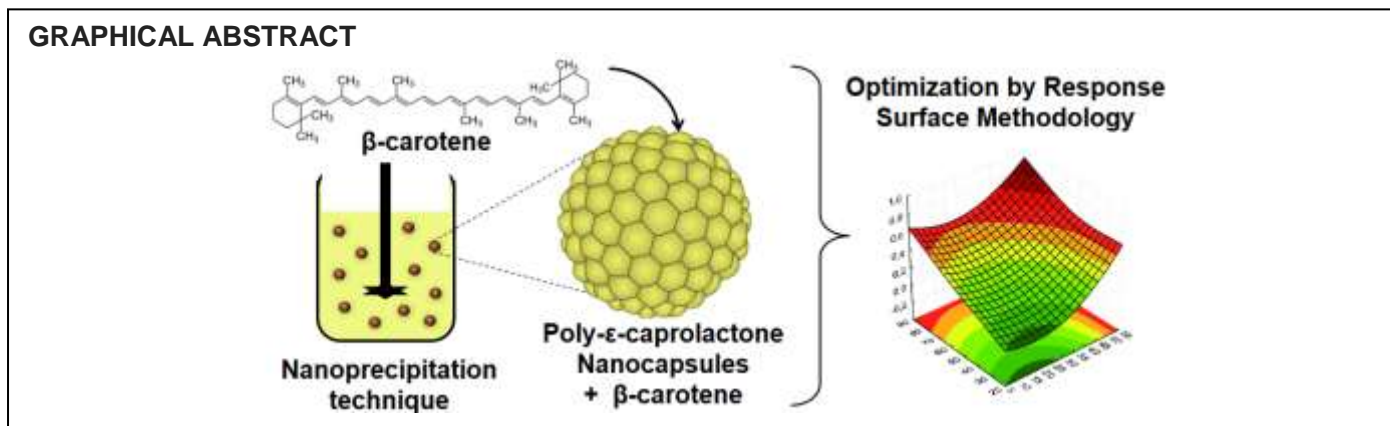
INTRODUCTION

The use of β-carotene has been received considerable attention, because is the most common carotenoid found in foods, and its importance is attributed to high pro-vitamin A content and antioxidant activity. The antioxidant property of carotenoids is related to the ability to disable singlet oxygen and free radicals, because the molecules of carotenoids present double bond conjugated system [1-3]. The highly unsaturated carotenoids are prone to isomerization and oxidation. Heat, light and acids promote the *trans* isomerization of carotenoids, whereas their usual configuration is the *cis* form. These facts promote a small loss in the color and biological activity of pro-vitamin A. Oxidative degradation is the main cause of the extensive losses of carotenoids, and can be stimulated by light, enzymes and metals [1,2]. β-carotene is non-soluble in water and only slightly soluble in oil at room temperature, making difficult the incorporation into food products [3-6]. Moreover, there is not a standard use of β-carotene in the industry, due to the heat sensitivity, pH, oxygen and light. To improve the stability, bioavailability and water solubility, carotenoids can be dissolved in the oil phase of oil-in-water nanoemulsions and then can be easily incorporated into food products [3,5,7,8]. In this context, nanotechnology arises as a tool to improve the solubility of active ingredients and increase its bioavailability.

Among the methods for nanoparticles formulation, the nanoprecipitation technique has been widely used, because it is a simple, rapid, reproducible and capable method for forming both nanospheres and nanocapsules [9]. To develop nanoparticles through the nanoprecipitation method, three basic components are required: i) polymer (synthetic, semi-synthetic or natural); ii) polymer solvent; and iii) the non-solvent of the polymer [10,11]. The polymers commonly used to the nanoparticles development by nanoprecipitation methods are biodegradable polyesters, among which polycaprolactone (PCL) particularly stands out by its biocompatible. Biodegradable polymers are also able to control the release of the compounds due to its high permeability [10]. The characteristics of the polymeric nanoparticles are influenced by the nature and concentration of the components. This point is interesting, because changes in the physicochemical properties of the β-carotene nanodispersions can affect the use of this active ingredient in food formulations [7].

Thus, the aim of this study was to apply the nanoprecipitation method to produce PCL nanocapsules entrapped β-carotene using the response surface methodology (RSM) to optimize the formulation, evaluating the effect of three types of lipophilic surfactants and three carrier agents of the active compound in the nanoparticles properties

MATERIAL AND METHODS

Chemicals

Trans-β-carotene (MM 536.87 g/mol, 93% purity) and poly-ε-caprolactone (MM 70.000-90.000 g/mol) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). The aqueous surfactant Pluronic F68®, from BASF Corporation, was kindly donated by Foscher Solutions Provider (London, GB). The oils employed as carriers of the active compound were sunflower oil (Bunge Foods, Santa Catarina, Brazil), caprylic/capric triglycerides (Palm-oil - Malasya) and glycerol (CRQ - Chromate Chemicals Ltda. São Paulo - Brazil). Fat-free, food-grade soybean lecithin with 70% phosphatidylcholine (Lipoid S75) from Lipoid GmbH (Ludwigshafen, Germany) was obtained from Lipid Ingredients & Technologies (São Paulo, Brazil). The surfactant polyethylene glycol sorbitan monolaurate, Tween® 20 was obtained from Lafan. The lipophilic surfactant sorbitan monooleate, Span™ 80, was obtained from Sigma-Aldrich (St. Louis, MO, USA). Ethyl acetate, acetonitrile, and methanol, all HPLC grade, were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Production of PCL nanocapsules entrapped β-carotene

PLC nanocapsules entrapped β-carotene was prepared through the nanoprecipitation method, following the procedure developed by Noronha and coauthors [12] and using the concentrations suggested by Mora-Huertas and Fessi Elaissari [13]. The polymer poly-ε-caprolactone (60 mg) and the active compound β-carotene (500 mg) were previously dissolved in acetone (14.5 mL) using an ultrasonic bath (Maxi-Clean A, Unique 1650, São Paulo - Brazil). After solubilization, all components of the organic phase were slowly mixed using a magnetic stirrer. The resulting organic solution was then added drop-wise into the aqueous phase containing the hydrophilic surfactant (Pluronic® F68, 290 mg) under moderate magnetic stirring at 25 °C. With the addition of the organic phase, the aqueous phase immediately became milky with an orange opalescence, as a result of the nanocapsules formation. The acetone, which quickly diffuses into the aqueous phase, was then completely removed by stirring in the dark, at room temperature (25 °C) and overnight. After the solvent be completely evaporated, the final volume was adjusted to 25 mL, and the solutions were filtered using qualitative filter paper (Whatman #1) to remove larger particles and agglomerates. After filtering, the formulations were stored in amber bottles at 5 °C for use and subsequent analysis. The final pH of the nanocapsules suspensions was 5.60.

Effect of the carrier agent of active compound and the nature of the lipophilic surfactant on the nanocapsules properties

Three types of carrier agents were tested: sunflower oil, caprylic/capric triglycerides (CCT) and glycerol. The surfactants used were Span™80, Tween®20 and Soybean Lecithin Lipoid S75. It is known that the type of carrier agent and lipophilic surfactant should be chosen according to the active compound that will be encapsulated. The oil acts as the carrier of the active compound (β-carotene), and the highest solubility of the active compound in the oil ensures the formation of nanocapsules and not nanospheres [13]. The nanoparticles solutions were prepared with each type of carrier, using the same volume for each formulation. The formulations containing the various carriers were combined with each type of lipophilic surfactant. The formulations using the surfactants Span™80 and Tween®20 did not require prior solubilization, but the addition of acetone was required in such formulations to maintain a constant organic phase volume, which in this study was maintained at 50% (v/v) of the aqueous phase volume. However, for formulations using soybean lecithin (2.5 mg mL^{-1}) was required a prior dissolution in an acetone:ethanol (60:40), using ultrasonic bath and then added to the other components of the organic phase. All of the other components were unchanged: 60 mg of PCL, 500 mg of β-carotene, 25 mg of Lecithin, 30 mg of Span™80 or Tween®20, 250 mL of carrier agent, 14.5 of mL acetone, 29 mL of Milli-Q® water and 290 mg of Pluronic F68®. The optimization of the nanocapsules formulations was determined by measuring the mean size, polydispersity index, distribution of size and zeta potential.

Optimization of the PCL/β-carotene nanocapsules Using RSM

After selecting the best carrier agent and surfactant, was evaluated the effect of the amount of β-carotene, carrier agent and lipophilic surfactant. Box-Behnken design (Table 1 and Table 2) was statistically used to optimize the nanocapsules formulation and evaluate the effect of the independent variables in the particle mean size (PS, nm), polydispersity index (PDI), zeta potential (ZP, mV), encapsulation efficiency (%) and recovery (%). The independent variables were: β-carotene concentration (mg/mL, X_1), caprylic/capric

triglycerides volume (µL, X_2) and soy lecithin concentration (mg/mL, X_3). A three-factor, three-level design was suitable to explore the quadratic response surfaces and construct the second-order polynomial models. The design consisted of replicated center points and the set of points lying at the midpoints of each edge of the multidimensional cube that defined the region of interest, thus requiring fewer runs than a central composite design. A second-order polynomial equation was defined as follows:

$$Y_I = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 \tag{1}$$

Where: Y represents the response associated with each factor level combination, b_0 is an intercept; b_1, b_2 and b_3 are the linear coefficients; b_{12}, b_{13} and b_{23} are the interactive coefficients; b_{11}, b_{22} and b_{33} are the quadratic coefficients; and X_1, X_2 and X_3 are the coded levels of independent variables. To fit the second-order polynomial equation to all of the independent variables, the experimental data were analyzed using multiple regressions, and significant differences between independent variables were determined by Analysis of Variance (ANOVA). The relationships between the responses and the independent variables were visualized using the surface response and contour plots of the fitted polynomial regression equations generated using the software Statistica 7.0 (StatSoft, Inc., EUA, 2004).

Table 1. Box-Behnken design optimization to the nanocapsules formulation.

Variables	Code	Level -1	Level 0	Level +1
β-carotene (mg/mL)	X_1	0.1	0.2	0.3
Oil volume (µL)	X_2	150	250	350
Soybean lecithin (mg/mL)	X_3	2	2.5	3

Table 2. Coded and uncoded independent variables used in the design optimization to the nanocapsules formulation.

Test	Coded variables X_1	X_2	X_3	X_1, β-carotene (mg/mL)	X_2, Oil volume (µL)	X_3, Soybean lecithin (mg/mL)
1	-1	-1	0	0.1	150	2.5
2	+1	-1	0	0.3	150	2.5
3	-1	+1	0	0.1	350	2.5
4	+1	+1	0	0.3	350	2.5
5	-1	0	-1	0.1	250	2.0
6	+1	0	-1	0.3	250	2.0
7	-1	0	+1	0.1	250	3.0
8	+1	0	+1	0.3	250	3.0
9	0	-1	-1	0.2	150	2.0
10	0	+1	-1	0.2	350	2.0
11	0	-1	+1	0.2	150	3.0
12	0	+1	+1	0.2	350	3.0
13	0	0	0	0.2	250	2.5
14	0	0	0	0.2	250	2.5
15	0	0	0	0.2	250	2.5

Nanocapsules characterization

Particle Mean Size, Polidispersity Index and Zeta Potential

The particles mean size, size distribution and zeta potential of the β-carotene loaded nanocapsules were determined via Photon Correlation Spectroscopy (PCS) using a Zetasizer Nano-series (Malvern Instruments, Worcestershire, UK). This method allows determining the mean diameter of the particle and the polydispersity index (PDI), which is a dimensionless measure of the broadness of the particle size distribution. The measurements were made at a fixed angle (173°) and temperature of 25 °C. The samples were diluted (1:100) in MilliQ® water and filtered with a 0.8 µm filter (Millex AA, Millipore, France). The zeta potential was measured using Smoluchowski's Equation for the electrophoretic mobility of the nanocapsules. Each

measurement was performed in triplicate, and the results were processed using the DTS software version 6.3.

Encapsulation Efficiency and β-carotene Recovery in the Nanocapsules Suspensions

The encapsulation efficiency of β-carotene was calculated using the difference between the total activity of the lipophilic complex in the colloidal suspension and the total activity of the free β-carotene found in the external aqueous phase [14], with some modifications. The total β-carotene concentration (C_t) was determined after the dissolution of 1 mL of β-carotene nanocapsules in 10 mL of acetone. The free β-carotene concentration (C_s) was obtained from the ultrafiltration/centrifugation of 0.5 mL of the nanocapsules suspension using an Amicon device (Amicon Ultra 0.5/30 kDa, Millipore, Irlanda). The samples were centrifuged at 4500 RPM for 30 minutes at 20 °C. The free β-carotene was then obtained in the supernatant. HPLC analyses were performed with a Shimadzu Liquid chromatographer (LC-20AT, Shimadzu, Kyoto, Japan) equipped with a SPD-M20A UV-visible detector (LC-20AT pump system, SIL-10A auto sampler, and CTO-20A oven). The β-carotene was separated in a RP-18 CLC-ODS column (5 µm x 4.6 mm x 150 mm) with octadecyl as the stationary phase and a guard column CLC-GODS surface with octadecyl as the stationary phase, where both were placed in oven at 25 °C (Waters Spherisorb®, Wilmington, EUA). The quantitative measurement of the β-carotene content was performed at 450 nm. Quantification was performed using a gradient elution of methanol, acetonitrile and ethyl acetate at a flow rate of 1.0 mL/min. The column temperature was a minimum of 25 °C and a maximum of 35 °C, the injection volume was 25 µL and the run time was 40 minutes. The encapsulation efficiency and the recovery were calculated as shown in Equations 2 and 3, respectively.

$$\text{Encapsulation efficiency (\%)} = \frac{C_t - C_s}{C_t} \times 100 \qquad (2)$$

$$\text{Recovery (\%)} = \frac{C_i - C_t}{C_i} \times 100 \qquad (3)$$

Where C_t is the total concentration of β-carotene in the formulation, C_s is the concentration of β-carotene in the supernatant and C_i is the initial concentration of β-carotene in the formulation.

Nanoparticles Morphology

PCL/β-carotene nanocapsules were characterized using a Transmission Electron Microscope (TEM). The TEM pictures were taken with a JEOL (JEM-1011) microscope operating at 80 kV acceleration and magnification of 20,000 times. The sample preparation was realized according to the studies by Kayata and coauthors [12] for the TEM analysis.

Statistical analysis

Analyses of Variance (ANOVA) were performed to verify the significant differences among the independent variables. Significant differences ($p < 0.05$) between the means were determined using the Tukey's test. All the experiments and measurements were performed at least in triplicate (n=3). To visualize the relationship between the response variable and the independent variables, the experimental design and response surface contour plots of the adjusted polynomial regression equations were generated using Statistica 7.0.

RESULTS AND DISCUSSION

Determination of the particle size, polydispersity index and zeta potential to select the best lipophilic surfactant and the best carrier agent

The average diameter of the nanocapsules was determined via Photon Correlation Spectroscopy (PCS). This technique relies on the fact that the particles move randomly under the impact of the solvent molecules onto its surface. The frequency and amplitude of this Brownian movement is dependent on the particle size and the viscosity of the solvent, i.e., the smaller the particle, the frequency of the Brownian motion is significantly higher to the relative amplitude. The results of the particle size analysis (Table 3) indicate that the mean particle size obtained for all of the developed systems are between 177.0 and 358.0 nm. ANOVA indicated that nanoparticles made with soy lecithin had particle sizes that were significantly lower ($p < 0.05$) than those of particles formulated with Tween 20 and Span 80, regardless of the type of carrier used. This

confirms the results found by Tan and Nakajima [7] and Yuan and coauthors [3] who attributed the variations in the size of particles made with different surfactants to the differences in the hydrophilicity of the emulsifiers used. The increase in the hydrophilicity of the emulsifier increases the stabilized surface area, due to its ability to engage and stabilize the particles in oil-in-water emulsions, resulting in smaller particles.

Table 3. Effect of the surfactant type (soy lecithin, Span 80 and Tween 20) and carrier agent (Caprylic/Capric triglycerides, sunflower oil and glycerin) in the particle size, polydispersity index, and zeta potential of the β-carotene nanocapsules.

Carrier Agent	Properties	Surfactants		
		Soy Lecithin	Span 80	Tween 20
Caprylic/Capric triglycerides (CCT)	Particle size (nm)	214.06±2.16[a, B]	290.92±19.90[b, A]	332.14±13.65[c, B]
	Polidispersity index	0.141±0.010[a, A]	0.293±0.063[b, A]	0.298±0.072[b, A]
	Zeta potential (mV)	−26.40±1.73[ab, A]	−29.02±2.48[a, A]	−25.06±2.97[b, A]
Sunflower oil	Particle size (nm)	233.72±4.41[a, C]	357.3±21.27[b, B]	329.52±21.33[b, B]
	Polidispersity index	0.166±0.024[a, A]	0.281±0.092[ab, A]	0.320±0.091[b, A]
	Zeta potential (mV)	−33.42±2.82[b, B]	−30.82±2.48[ab, A]	−26.66±2.35[a, A]
Glycerin	Particle size (nm)	177.58±4.94[a, A]	302.2±31.69[c, A]	200.88±7.74[b, A]
	Polidispersity index	0.165±0.033[a, A]	0.282±0.072[b, A]	0.205±0.042[ab, A]
	Zeta potential (mV)	−29.24±2.55[a, A]	−29.26±2.83[a, A]	−25.10±1.85[a, A]

Results expressed as mean ± standard deviation. Different lowercase superscript letters on the same line indicates significantly different values ($p<0.05$). Different uppercase superscript letters in the same column indicate significantly different values ($p<0.05$).

A comparison of the different formulations indicated that the type of oil used as the active compound carrier agent had significant influence ($p<0.05$) on the size of the nanoparticles. This influence in the size can be attributed to the differences in the hydrophobicity, viscosity and interfacial tension of the substances employed [15]. The particles made with glycerin had smaller sizes than the particles made with CCT and sunflower oil as the bioactive molecule carrier agents.

The PDI values indicated a narrow and unimodal particle size distribution for all of the formulated nanoparticles (PDI<0.320). The polydispersity index, which ranges from 0 to 1, indicates the uniformity of size of the nanoparticles in the nanocapsule suspension; as this value approaches 0, the size distribution of the nanocapsules in the nanoparticles suspensions becomes more homogeneous [14]. According to Byun and coauthors [16], PDI values of less than 0.5 are considered with a good size distribution. The values found for the PDI using the formulations containing Tween 20 and Span 80 were higher than the values for the formulations containing soy lecithin. The formulations containing soy lecithin as surfactant presented PDI values close to zero, being lower than 0.166 and significantly ($p<0.05$) different comparing to the other formulations. However, the influence of the carrier agent type on the polydispersity index was not significant ($p<0.05$) regardless of the type of surfactant used. The zeta potential also showed no significant differences ($p<0.05$) with the type of oil used in the formulations. This result confirmed the data obtained by Mosqueira and coauthors [17], who studied the effect of the composition on the characteristics of the poly(d,l-lactide) nanocapsules and found no significant differences in the zeta potential values when the nature of the oil was changed. They attributed this behavior to the total encapsulation of the oil by the polymer, as was not located on the outside of the nanocapsules.

The zeta potential results (Table 3) showed that the nanocapsule formulations exhibited negative zeta potential values, ranged between −25.06 and −33.42 mV. The negative zeta potential values obtained can be attributed to the composition of the nanocapsule formulations. The formulations using soybean lecithin showed the highest absolute values of the zeta potential due to the presence of negatively charged phospholipids in the molecule, which contribute to a strong negative charge at the interface. The negative charges were also attributed to the hydrophobic nature of PCL, which may cause the ionization of the carboxylic groups on the surface of this polyester and result in a negative potential to the interface, corroborating the negative zeta potential determined for all of the prepared nanocapsules regardless of the type of surfactant used. These results are consistent with studies described in the literature, which argue that negative zeta potentials are typically observed in the types of systems composed by polyesters [15,17]. According Schaffazick and coauthors [15], high absolute values of the zeta potential are important to achieve

stable colloidal suspensions because the large repulsive forces tend to prevent the aggregation of the particles due to the incidental collisions of adjacent nanoparticles.

The particles formulated using the combination of soy lecithin and CCT had average sizes of 214.0 nm and unimodal size distribution with the lower values of the PDI (0.141) significantly different ($p<0.05$) from the PDI values found for the other nanoparticle systems tested. Furthermore, over the whole period, these formulations showed fewer variations in the values of particle size, zeta potential and PDI. Moreover, the suspensions of nanoparticles showed the same appearance, without phase separation or presence of precipitates. Thus, from the average particle size, polydispersity index and zeta potential data, the formulation containing CCT and soybean lecithin was selected for further studies.

Response surface analyses

After selecting the best carrier agent for the active compound and the lipophilic surfactant, RSM was used to determine the optimal formulations for the development of PCL/β-carotene nanoparticles. Box-Behnken planning was employed with three independent variables. The independent variables were the β-carotene concentration (X1; 0.1 – 0.3 mg/mL), the volume of CCTs (X2; 150 – 350 µL) and the surfactant concentration (X3; 2.0 – 3.0 mg/mL). The values of the response variables (zeta potential, particle size, polydispersity index, encapsulation efficiency and recovery) obtained for all of the experiments are given in Table 4. The experimental data were used to calculate the coefficients of the quadratic polynomial equation, which were used to predict the values of the particle size, zeta potential, PDI, encapsulation efficiency and recovery. The predicted values were in agreement with the experimental values obtained from the RSM. Moreover, ANOVA showed that the quadratic polynomial model adequately represented the experimental data, with values of R2 for the responses of particle size, zeta potential, PDI, encapsulation efficiency and recovery as 0.959, 0.747, 0.846, 0.868 and 0.802, respectively.

Particle Size

Statistical analysis indicated that the particle size was affected significantly ($p<0.05$) only by the oil volume, showing both linear and quadratic effects. Figure 1(a) was generated by varying the β-carotene concentration and the oil volume while maintaining a constant concentration of soy lecithin at 2.5 mg/mL. As observed in the response surface graph, the particle size increased with the increasing amount of oil. The same behavior was observed in Figure 1(b), when the soy lecithin concentration and CCT volume were varied and the β-carotene concentration was constant at 0.2 mg/mL. The data agree with those obtained by Yuan and coauthors [3], who dissolved β-carotene in oil to form nanoemulsions and found that increasing the concentration of β-carotene resulted in an increase in the size of the formed nanoparticles. In the present study, the results indicate that increasing the oil volume also increases the particle size. The linear and quadratic effects of the other factors, soy lecithin and β-carotene, and the interactions between factors had no effect on the particle size in this system at the evaluated levels. The second-order model (Equation (4)) for this variable was as follows:

$$PS = 199.95 - 0.188X_{CCT} + 0.00064X_{CCT}^2 \qquad (4)$$

This is in contrast to the data obtained by Yuan and coauthors [3], who studied oil-in-water nanoemulsions of β-carotene (30% in sunflower oil) using different types of surfactants, such as Tween, and found that the surfactant concentration had a significant effect on the particle size and increasing the concentration of the surfactant generally resulted in reduced particle sizes. This is because smaller particles have larger surface areas, which requires more emulsifiers to cover them. In another study, Hentschel and coauthors [18] also found that the particle size is strongly affected by the surfactant concentration for nanocapsules of β-carotene; in this case, the behavior of the size distribution was not unimodal and the method used to determine the particle size was laser diffraction.

Table 4. Particle size (PS), polydispersity index (PDI), zeta potential, encapsulation efficiency (%) and recovery (%) for different amounts of β-carotene (X_1), caprylic/capric triglycerides (CCT) (X_2) and soybean lecithin (SL) (X_3).

Test	Coded and uncoded values*			PS (nm)	PDI	Zeta Potential (mV)	Encapsulation efficiency (%)	Recovery (%)
	X_1	X_2	X_3					
1	0.1(-1)	150(-1)	2.5(0)	195.3	0.099	-38.9	99.03	45.0
2	0.3(+1)	150(-1)	2.5(0)	177.7	0.121	-38.7	100	32.0
3	0.1(-1)	350(+1)	2.5(0)	211.2	0.08	-41.9	100	41.4
4	0.3(+1)	350(+1)	2,5(0)	211.0	0.099	-39.1	100	26.9
5	0.1(-1)	250(0)	2.0(-1)	194.8	0.046	-39.2	95.71	28.0
6	0.3(+1)	250(0)	2.0(-1)	194.7	0.094	-39.1	98.89	23.0
7	0.1(-1)	250(0)	3.0(+1)	193.2	0.097	-41.1	100	36.8
8	0.3(+1)	250(0)	3.0(+1)	189.2	0.087	-40.0	98.18	28.2
9	0.2(0)	150(-1)	2.0(-1)	185.9	0.118	-39.3	100	28.8
10	0.2(0)	350(+1)	2.0(-1)	213.8	0.099	-33.6	100	26.9
11	0.2(0)	150(-1)	3.0(+1)	185.1	0.094	-39.7	99.09	21.1
12	0.2(0)	350(+1)	3.0(+1)	212.0	0.113	-41.5	100	28.2
13	0.2(0)	250(0)	2.5(0)	187.0	0.082	-38.0	100	23.0
14	0.2(0)	250(0)	2.5(0)	194.5	0.082	-39.7	100	30.0
15	0.2(0)	250(0)	2.5(0)	195.0	0.096	-39.7	100	24.6

*Coded and uncoded independent variables used in the Response Surface Methodology (RSM) design. X_1 = β-carotene concentration (mg/mL), X_2 = oil volume (μL); X_3 = soybean lecithin concentration (mg/mL).

Polydispersity Index

The polydispersity index (PDI) of the suspensions was significantly influenced by the quadratic term of the oil ($p < 0.05$), whereas the other terms did not present a significant effect ($p > 0.05$). This behavior can be explained by a comparison to the particle size data, which are also influenced by the type of oil because when the particle size is altered in suspensions of nanocapsules, the size distribution is also changed. The RSM plot shows (Figures 1(c) and (d)) that lower values of PDI were found when an oil volume between 230 and 290 μL was used. All of the formulations developed in this work had a unimodal and particle size distribution between 30 and 300 nm. The second order equation, ignoring the non-significant effects for the dependent variable PDI, is as follows:

$$PDI = 0.218 - 0.01X_{CCT} + 0.0000019X_{CCT}^2 \qquad (5)$$

The average diameter of the particles in the nanoemulsion varied between 177.7 and 213.8 nm, with polydispersity indices (PDI) varying from 0.046 to 0.121. Similar data were found by Yuan and coauthors [3] for β-carotene nanoemulsions using sunflower oil and Tween 20, wherein the average particle size ranged from 132 to 184 nm, with the range of the PDI of approximately 0.181 – 0.360.
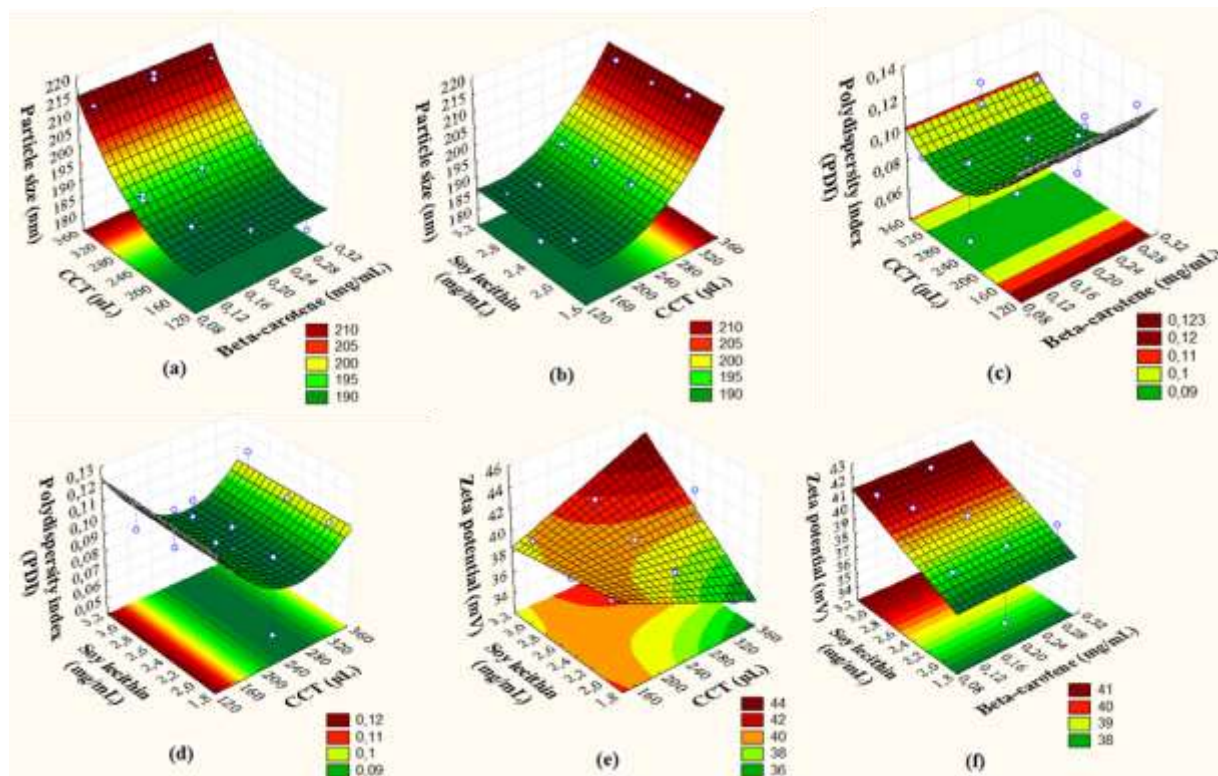
Figure 1. The response surface plots of the dependent variable: (a) particle size for the nanoparticles formulated with different β-carotene concentrations/oil volumes and (b) soy lecithin concentrations/oil volumes. (c) PDI for the nanoparticles formulated with different oil volumes/β-carotene concentrations and (d) oil volumes/soy lecithin concentrations. (e) Zeta potential for the nanoparticles formulated with different soy lecithin concentrations/oil volumes and (f) soy lecithin/β-carotene concentrations.

Zeta Potential

For the zeta potential, ANOVA revealed that the soy lecithin variable and the linear interaction of the soy lecithin concentration and oil volume appeared to be marginally significant ($p = 0.0572$ and 0.065, respectively). Using the marginally significant effects and ignoring the other effects, the model equation that describes the zeta potential is:

$$ZP = 55.96 - 0.944X_{CCT} - 6.6X_{SL} + 0.0375X_{CCT}X_{SL} \qquad (6)$$

Increasing the soy lecithin concentration resulted in higher values of the zeta potential, as shown in the red region of Figure 1(f). This behavior can be attributed to the location of the surfactant on the interface. Zeta potential increase can be explained by the higher amount of soybean lecithin in the system, which resulted in a higher concentration of negatively charged phospholipids, due to the strength of the repulsive forces. As observed in Figure 1(e), higher values of the zeta potential were obtained for both combinations of CCT and soybean lecithin. This behavior can be related to the nanoparticles surface area, which proportionally increased due to the particle size, requiring greater amounts of surfactant to exert the same repulsive force compared to smaller particles. The β-carotene concentration had non-significant effect ($p > 0.05$) on the zeta potential, concluding that β-carotene is fully encapsulated by the polymer, which is in agreement with the data found by Mosqueira and coauthors [17].

Encapsulation Efficiency

All of the tested formulations showed high encapsulation efficiencies (above 95%), as observed in Table 2. The data obtained in this study agree with the literature, where encapsulation efficiencies above 80% are expected for nanoparticles developed by the nanoprecipitation method [13]. The effect of the soy lecithin and β-carotene interaction was significant ($p = 0.017$) in the encapsulation efficiency, as observed in Figure 2(a). Higher encapsulation efficiencies were obtained closer to the center points of soybean lecithin (2.5 mg/mL) and β-carotene (0.2 mg/mL). Paz and coauthors [6] also found that higher concentrations of surfactant (100 g/L) were required to obtain a high percentage of encapsulated β-carotene, using modified starch as the surfactant. However, the oil concentration had non-significant effect in the encapsulation efficiency. Thus, the model equation for %EE, ignoring the non-significant terms ($p > 0.05$), is:

$$\%EE = 58.19 + 103.74X_{BC} - 96.05X_{BC}^2 + 24.58X_{SL} - 3.78X_{SL}^2 - 24.96X_{BC}X_{SL} \tag{7}$$

Recovery

For the recovery dependent variable, both the oil and soybean lecithin were not significant, whereas the quadratic interaction of β-carotene was marginally significant (p=0.053). Thus, the model remains linear with the quadratic effects of β-carotene, according Equation (8).

$$\%R = 62.60 - 313.66X_{BC} + 655.78X_{BC}^2 \tag{8}$$

Figure 2(d) was generated by varying the β-carotene concentration and oil volume (CCT), while maintaining a constant surfactant concentration. Similar behaviors were observed when varying the concentration of the β-carotene and soy lecithin but keeping the oil volume constant. Low levels of β-carotene resulted in higher recoveries of this compound from the nanocapsule suspensions. To choose the best formulation, we considered the formulations that showed higher recoveries, encapsulation efficiencies and zeta potentials and low PDI. Thus the optimal conditions obtained for the β-carotene concentration, caprylic/capric triglycerides volume and soybean lecithin concentration were 0.2160 mg/mL, 232.42 μL and 2.59 mg/mL, respectively.
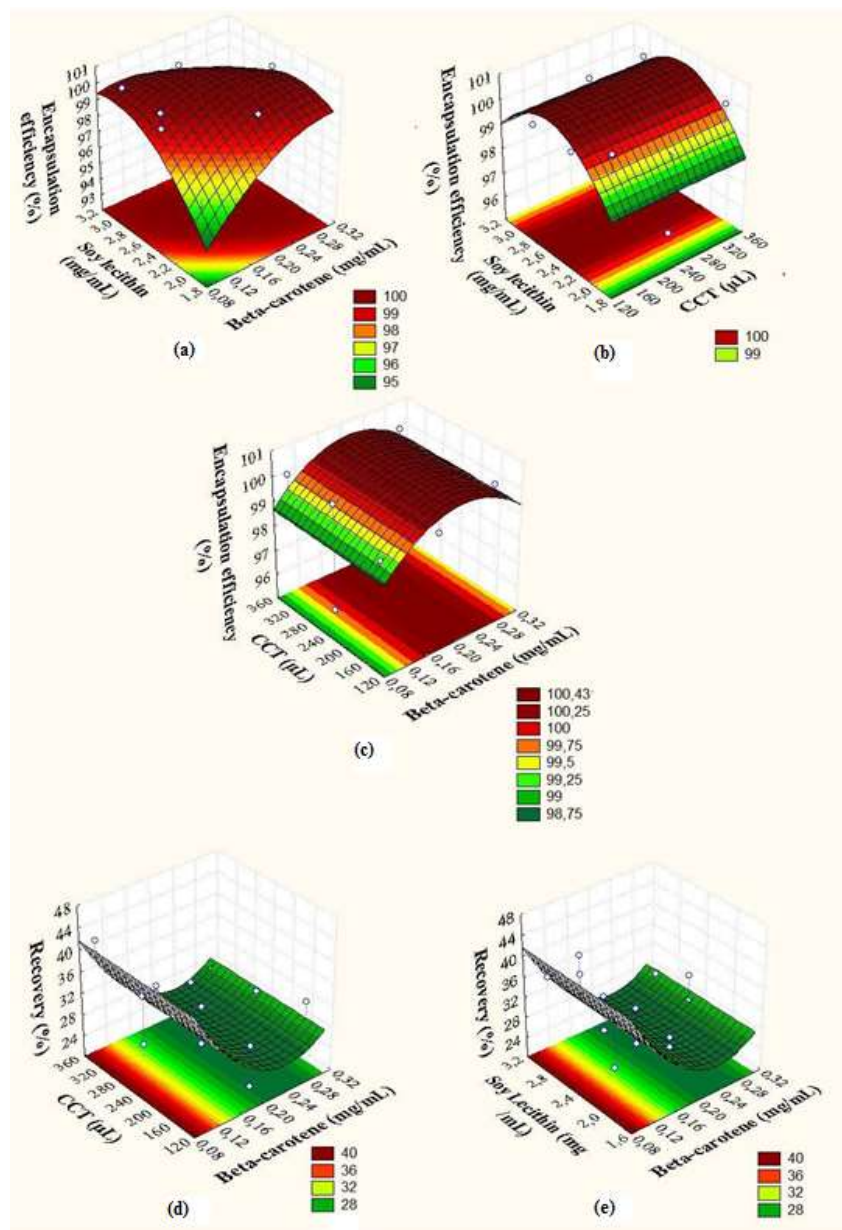

Figure 2. The response surface plots of the dependent variable: (a) encapsulation efficiency for the nanoparticles formulated with different soy lecithin/β-carotene concentrations, (b) oil volumes/soy lecithin concentrations and (c) oil volumes/β-carotene concentrations. (d) Recovery (% R) for the nanoparticles formulated with different β-carotene concentrations/oil volume and (e) soy lecithin/β-carotene concentrations.

Morphological Structure

Transmission Electron Microscopy (TEM) images show that the optimal formulation was capable of forming nanocapsules and not nanospheres. The presence of spherical nanoparticles with a well-defined oily core surrounded by a polymeric wall of poly-ε-caprolactone can be clearly observed in Figure 3. The nanoparticle distribution in the suspension of nanocapsules, without formation of agglomerates, also can be observed. The images obtained in this study were very similar to the images of nanocapsules of α-tocopherol/PCL obtained by Khayata and coauthors [14]. Moreover, it was possible to confirm that the size distribution of the nanocapsules was not homogeneous, as different sizes of the nanocapsules can be verified, but all of the particles were in the nanometric range (below 1 μm).
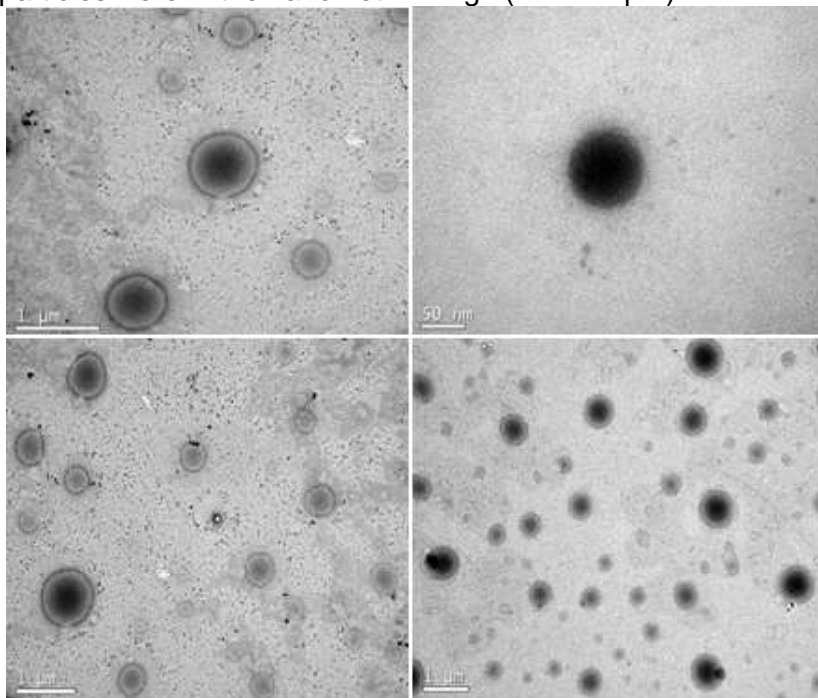

Figure 3. Typical TEM micrographs obtained for β-carotene nanocapsules produced using the nanoprecipitation method.

CONCLUSION

Formulations containing soy lecithin and caprylic/capric triglycerides were more suitable to the production of nanocapsules. Moreover, the best nanoparticle formulation was obtained by RSM, and this result was positive to obtain formulations with high encapsulation efficiency, low particle mean size, low PDI and high values of zeta potential and recovery. Thus, the chosen optimal formulation was a β-carotene concentration of 0.2160 mg/mL, CCT volume of 232.42 μL and soy lecithin concentration of 2.59 mg/mL. In addition, TEM micrographs revealed the capsular structure of the nanoparticles. Thus, the results obtained suggest that poly-ε-caprolactone nanocapsules entrapped β-carotene present wide application in food system as antioxidant food ingredients or in active packaging.

Conflicts of Interest: The authors declare no conflict of interest.

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