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STABILITY EVALUATION OF β-CAROTENE NANOEMULSIONS PREPARED BY HOMOGENIZATION-EMULSIFICATION PROCESS USING STEARIC ACID AS OIL PHASE

EVALUACIÓN DE LA ESTABILIDAD DE NANOEMULSIONES DE β-CAROTENO PREPARADAS POR UN MÉTODO DE HOMOGENEIZACIÓN-EMULSIFICACIÓN EMPLEANDO ÁCIDO ESTEÁRICO COMO FASE OLEOSA

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Abstract

The effect of processing parameters (homogenization time, stirring speed and oil:water ratio) of β-carotene nanoemulsions (stearic acid as oil phase) was studied via a central composite design (CCD) by response surface methodology (RSM). Particle size and nanoemulsions stability (β-carotene concentration, color and antioxidant activity) stored for 21 days at 25 and 4 °C were selected as response variables. Maximum particle size obtained was 1689.0 nm and minimum particle size was 418.8 nm, which a second order model were adjusted with R^2 values of 0.766 and 0.933 at 25 and 4 °C, respectively. The particle size was affected directly by the homogenization time and inversily proportional to stirring speed and oil:water ratio. Parameters as β-carotene concentration and antioxidant activity showed a gradual decrease during storage, showing a great stability those that were stored at 4 ºC. The optimal conditions to produce β-carotene nanoemulsions with minimum particle size were found at 25 °C for homogenization time 5.99 min, 5287 rpm and an oil: water ratio of 0.8:99.2; at 4 °C homogenization time 5.99 min, 8002 rpm and 0.62:99 oil:water ratio.

Keywords: β-carotene, stearic acid, central composite design, emulsification-homogenization, nanoemulsion.

Resumen

Se evaluó el efecto de los parámetros de procesamiento (tiempo de homogeneización, velocidad de agitación y relacion aceite:agua) de nanoemulsiones de β-caroteno (ácido estéarico como fase oleosa) por medio de un diseño central compuesto (DCC), empleando la metodología de superficie de respuesta (MSR). El tamaño de partícula y la estabilidad de las nanoemulsiones (concentración de β-caroteno, color y actividad antioxidante) almacenadas durante 21 días a 25 y 4 ºC fueron seleccionadas como variables respuesta. El tamaño de partícula máximo obtenido fue de 1689.0 nm y mínimo de 418.8 nm, los cuales se ajustaron a un modelo de segundo orden con valores de R^2 de 0.766 y 0.933 para 25 y 4 °C, respectivamente. El tamaño de partícula fue afectado directamente por el tiempo de homogeneización e inversamente proporcional a la velocidad de agitación y la relación aceite:agua, mientras que los parámetros de estabilidad como color y actividad antioxidante presentaron una disminución gradual durante el almacenamiento, mostrando una mayor estabilidad aquellas que fueron almacenadas a 4 ºC. Las condiciones óptimas estimadas para elaborar nanoemulsiones de β-caroteno minimizando el tamaño de partícula fueron para 25 °C tiempo homogeneización de 5.99 min, 5287 rpm y una relación aceite:agua de 0.8:99.2; para 4 ºC el tiempo de homogeneización de 5.99 min, 8002 rpm y una relación aceite:agua de 0.62: 99.38.

Palabras clave: β-caroteno, ácido esteárico, diseño central compuesto, emulsificación-homogenización, nanoemulsión.

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1 Introduction

The health benefits associated with functional lipids (carotenoids, phytosteroles and ω-3 fatty acids) have been studied for many decades and are widely used as active ingredients in some food products. However, most of these bioactive compounds are almost insoluble in water. In addition, they show a very low water solubility resulting in a low bioavailability and their applications are limited in food formulations (Deming and Erdman, 1999; Tan and Nakajima, 2005a; Yin *et al*., 2009). Nowadays, nanotechnology is focusing on development of new prospects and tools that allow the characterization of nanostructures. Moreover, new methods for preparation of these materials are searched in order to be used for technological innovation in food science. Nanostructures offer the potential to significantly improve physical properties as solubility and bioavailability of many functional ingredients such as carotenoids, polyunsaturated fatty acids and some other compounds (Moraru *et al*., 2003).

β-Carotene is a natural colorant from carotenoids family widely used in food industry. Recently, some researches have been demonstrated that β-carotene shows antioxidant activity, it also provides important health human benefits and may provide protection against serious sickness such as cardiovascular diseases, certain types of cancer (Burri, 1997; Gaziano *et al*., 1992; Nishino, 1997), colorectal adenomas (Omen *et al*., 1996), and so on. For these reasons, there is a strong interest in using β-carotene as an important functional material in food products. However, β-carotene is insoluble in water, this makes its difficult to be incorporated in food formulations (Ribeiro and Cruz, 2005). Additionally, due to its electron rich conjugated double bond structure, β-carotene is very sensitive to be attacked by oxygen, heat and light, which further limits its applications in food, nutraceutical and pharmaceutical products (Orset, 1999; Rodriguez *et al*., 2004). Consequently, emerge the need for edible delivery systems to encapsulate, protect and realese bioactive and functional lipophilic and hydrophilic constituents within the food and pharmaceutical industries (Dzu *et al*., 2013). In this way, oil in water nanoemulsions could be used as delivery systems capable of improve solubility and bioavailability properties of β-carotene. Nanodispersions systems with a particle size in the nanometer ranges are more stable compared to conventional dispersions (Mosqueira *et al*., 2000) and they can be produced using several nanoencapsulation techniques. The used methods to produce nanoemulsions might be divided into the mechanical and non-mechanical methodologies. In the mechanical (high-energy) methods use mechanical devices that generate intense breakdown forces that can mix oily and aqueous phases with or without the addition of surfactants (Sarmiento *et al*., 2014; Fathi *et al*., 2012); many techniques such as homogenization-emulsification, high-pressure homogenization, microfluidization and ultrasonication have been applied. Specifically, to develop fine stable emulsions using homogenization-emulsification processes, the lipid is melted at temperature of 5-10 ºC higher than its melting point; at the same time, the bioactive compound is dissolved or dispersed in the melted lipid. In addition, a hot aqueous surfactant solution (preheated at the same temperature) is added to the first mix and homogeneously dispersed by a high shear-mixing device and a nanoemulsion takes place (Das and Chaudry 2011). These systems have a particle size ranging from 50 to 1,000 nm and they offer great potential to encapsulate many active agents into a wide range of foodstuffs (Bhosale, *et al*., 2014; Ezhilarasi *et al*., 2013). The use of nanoemulsion technology for delivering food components has been comprehensively reviewed by Tan and Nakajima (2005a); McClements and Rao (2011); Silva *et al*. (2011), and Salvia-Trujillo *et al*. (2013). The application of experimental designs allows obtaining benefits such as increased yields, reduced variability, reduction of processing time, reduce the cost in product development and decreasing the experimental runs (Rodríguez *et al*., 2014). The response surface methodology (RSM) allows to study the effect of several process parameters on one or more responses of the design. Thus, a central composite design (CCD) is an experimental approach, it can be useful in RSM for building a second order model to obtain a variable response using a minimal trials (Xynos *et al*., 2014).

The objective of this study was to apply the homogenization-emulsification technique for preparing β-carotene nanoemulsions able to protect and provide a high stability of interested compound as well as evaluate during its storage stability. Finally, the interaction between processing parameters on particle size of β-carotene nanoemulsions using a CCD coupled to RSM methodology is analyzed and discussed.

2 Materials and methods

2.1 Materials

β-carotene (30% in sunflower oil) was purchased from Roche. Stearic acid and nonionic surfactant (Tween 80) were purchased from Sigma-Aldrich, USA. Milli-Q water (Millipore Corporation) was used for all experiments throughout the study.

2.2 Central composite design (CCD)

RSM was used to study the effects of the independent variables: homogenization time (X_1), stirring speed (X_2) and oil:water ratio (X_3) on particle size (Y) of β-carotene nanoemulsions. According to the CCD generated by Design-Expert software (Version 7.1.6, Stat-Ease Inc., MN) produced 13 experimental runs with three central points were obtained. For each factor, the lower and higher levels were represented by (-1) and (+1) signs, respectively (Table 1). The quadratic equation model for predicting the optimal point is expressed according to Eq. (1).

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1} \sum_{j=i+1}^{k} \beta_{ij} X_i X_j + \varepsilon \quad (1)$$

where Y is the response (dependent variable), β_0 is constant coefficient, β_i, β_{ii} and β_{ij} are the coefficients for the linear, quadratic and interaction effect, X_i and X_j are the factors (independent variables) while ε is the error.

Table 1. CCD provided with the lower (-1) and higher (+1) level values for each independent variable

Independent variables	Levels	
	Low level (-1)	High level (+)
Homogenization time (min)	2.0	6.0
Stirring speed (rpm)	5000	15000
Oil:Water phases ratio (%)	0.100 : 99.90	1.000 : 99.00

Table 2. CCD experimental design matrix

Sample	Homogenization time (min)	Stirring speed (rpm)	Oil phase (%)	Water phase (%)
1	4.00	1000	0.086	99.91
2	6.38	1000	0.555	99.45
3	6.00	15000	0.100	99.90
4	4.00	10000	1.186	98.81
5	6.00	5000	1.000	99.00
6	4.00	10000	0.550	99.45
7	2.00	15000	1.000	99.00
8	2.00	5000	0.100	99.90
9	4.00	2929	0.555	99.45
10	1.17	10000	0.555	99.45
11	4.00	17071	0.555	99.45
12	4.00	10000	0.555	99.45
13	4.00	10000	0.555	99.45

Homogenization time ($X_1, \Delta=0.1$), stirring speed ($X_2, \Delta=250$) and oil:water phases ratio (X_3, $\Delta=0.023$).

2.3 Preparation of β-carotene nanoemulsions

Oil in water (O/W) nanoemulsions were prepared using stearic acid containing β-carotene as the dispersed phase and Milli-Q water as the continuous phase. Tween 80 was applied as surfactant due to its high hydrophilic-lipophilic balance (HLB) value, which is favorable for formulating O/W nanoemulsions. First off, the two phases were prepared separately, first β-carotene (30% in sunflower oil) was mixed with an equal volume of stearic acid at 80 ºC, and the aqueous phase was prepared with Milli-Q water with a concentration of 2% of Tween 80. The oil:aqueous ratio was different for each formulation. The mixture of both phases was homogenized using a high-speed homogenizer (Ultra-Turrax T25, IKA, Germany) at different times and stirring speeds (Table 2). The nanoemulsions were then transferred to amber vials and stored at 25 and 4 ºC for 21 days.

2.4 Physicochemical characterization

2.4.1 pH, electrical conductivity and surface tension

The pH of dispersions was measured using a pH meter (510 pH Oakton, Malaysia). Electrical conductivity was measured using the same equipment with a conductive plastic electrode calibrated with 0.01 M KCl solution being the specific conductivity (κ) of 1413 μS cm^{-1} at room temperature (Ghosh *et al*., 2013). The surface tension was analyzed by the ring method with a platinum ring circumference of 5.983 cm using a Fisher Surface Tensiomat tensiometer (Model 21, Iowa), calibrated with distilled water (Jafari *et al*., 2012). The samples were analyzed putting 30 mL into the measuring vessel, the ring was immersed 3 to 6 mm below the sample surface, the mechanism system was activated and subsequently reading (dyne cm^{-1}). All experiments were carried out in triplicates. Each nanoemulsion was divided into two groups, which were stored at 25 and 4 ºC for 21 days. At the end of each week the nanoemulsion stability was determined.

2.4.2 Particle size analysis

Particle size of samples was measured by dynamic light scattering (DLS) technique employing a Zetasizer Nano-ZS (Malvern instruments, UK). The instrument uses a backscattering configuration where detection is done at a scattering angle of 173°. The hydrodynamic diameter was calculated using the Stokes-Einstein equation:

$$D = kT/6\eta R \tag{2}$$

where D is the translational diffusion coefficient, k is the Boltzmann´s constant, T is absolute temperature and η is the viscosity of the medium. Samples were first diluted 1:100 to avoid multiple scattering effects. Measurements were performed at 25 ºC. The particle size of the nanoemulsions was expressed by the mean particle diameter (z-average) (Yuan *et al*., 2008a; Zainol *et al*., 2012; Rebolleda *et al*., 2015).

2.4.3 Color measurement

The stability of β-carotene was monitored by measuring the color changes over storage time. Changes in color parameters (L*, a* and b*) were used as an indication of the amount of β-carotene present in the samples. L* indicates lightness, a* indicates the degree of redness to greenness, and b* indicates the degree of yellowness to blueness (Qian *et al*., 2012). A colorimeter (Hunter-Lab, MS/B, USA) with a 10º observer was used to measure the color parameters for 21 days at 25 and 4 ºC. The hue angle (h^0) values were calculated according to Eq. 3 (Mapari *et al*., 2006).

$$h^0 = \tan^{-1}\left(\frac{b^*}{a^*}\right) \tag{3}$$

2.5 Determination of β-carotene concentration

The β-carotene concentration in the emulsions was determined following the modified method of Yuan *et al*. (2008b). First, 0.5 mL of sample was extracted with a mixture of 1.0 mL ethanol and 1.5 mL of *n*-hexane. After that, the mixture was well shaken, the hexane phase was removed and the extraction was repeated a second time; then, the hexane phases were combined. Absorbance was measured with a Lambda XLS spectrophotometer (Perkin Elmer, USA) at 450 nm. The β-carotene concentration was obtained by referring to a standard curve of β-carotene prepared under the same conditions. Each nanoemulsion was divided into two groups, which were stored at 25 and 4 ºC for 21 days. At the end of each week β-carotene concentration was determined.

2.6 Antioxidant activity determination of β-carotene nanoemulsions

The ABTS (2,2-Azinobis-3-ethylbenzotiazoline-6-sulfonic acid) assay was determined using modified

method of Bustos-Garza *et al*., (2013). $ABTS^+$ cation was generated through the interaction of 20 mg of ABTS dissolved in 5 mL of Milli-Q water with a solution of potassium persulfate ($K_2S_2O_8$) (3 mg in 1 mL of Milli-Q water). The cation was incubated in the dark at room temperature for 16 h. The ABTS activated radical was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm. After, 20 μL of sample were added to 2 mL of diluted ABTS solution, the absorbance was recorded with a Lambda XLS spectrophotometer (Perkin Elmer, USA) 6 min after mixing. The ABTS percentage of radical scavenging (% inhibition) was calculated according to Eq. 4.

$$\%Inhibition = \left(\frac{Abs_{734}control - Abs_{734}sample}{Abs_{734}control}\right)100 \quad (4)$$

2.7 Statistical analysis

All measurements were performed in triplicate and were expressed as mean value ± standard deviation (SD). The data were analyzed by the analysis of variance (ANOVA) using the SAS (Statistical Analysis System Version 9.0) software. Duncan´s multiple range test was used to determine the significant differences of the mean values ($p < 0.05$).

3 Results and discussion

3.1 Physicochemical characterization

Physicochemical characterization of all formulated nanoemulsions was done evaluating the effect of storage temperature. pH, electrical conductivity and surface tension results are reported in Table 3. Values obtained for pH ranging from 5.14 to 6.74 for room and cooling storage temperature were significantly different ($p < 0.05$). Thus high concentrations of stearic acid decreased pH values. By other hand, the electrical conductivity is an important parameter for determining the stability of the nanoemulsions. In this work, the conductivity values were from 23.03 to 36.07 μS/cm and 21.93 to 33.67 μS/cm for room and cooling storage temperature, respectively. This parameter was inversely affected by the oil:water ratio, because higher conductivity values of nanoemulsion is attributed to a larger percentage of water, which allows more freedom for ions mobility (Bhosale *et al*., 2014; Talegaonkar *et al*., 2011). Moreover, the Tween 80 as well known is a non-ionic surfactant does not ionized in aqueous solution; then, it can be no considered as an important conductive medium (Reyes and Di Scipio, 2012). The statistical differences between the central points (Table 3) can be attributed to other factors such as changes in the behavior of the system, human error, instrumentation error, or simply through natural deviation from a standard situation (Rahman and Amri, 2011). In O/W nanoemulsions, the surface tension is mainly affected for lipidic phase and surfactant concentration, in this work the surfactant amount was kept constant (2%). Therefore, formulations with higher levels of stearic acid showed a decreasing on surface tension values.

3.1.1 Particle size analysis

The particle size values of β-carotene nanoemulsions prepared by emulsification-homogenization technique under different process and formulation conditions were determined. The nanoemulsions presented particle sizes from 419.05 up to 1443.50 nm and 418.80 to 1689.0 nm for samples at room temperature and cooling storage temperature, respectively. In both cases, the smaller particle size (419.05 and 418.80 nm) was obtained with the following operating conditions: 1:99 oil:water ratio, 5000 rpm during 6 min.

The RSM gave the following regression equations for the particle size (PZ) as a function of homogenization time (A), stirring speed (B) and oil:water ratio (C). Final equations in terms of actual values are shown in Eq. 5 for 25 ºC and Eq. 6 for 4 ºC. The term (AB) is the interaction between parameters above described, (A^2) and (B^2) are the polynomial terms. The main effects (A and B) postulate the average result of changing one factor at time from their low to high values. The interaction (AB) shows how the response will change when two factors are changed, accordingly. The polynomial terms (A^2 and B^2) symbolize nonlinearity (Chakraborty *et al*., 2013). A positive value in the regression equation indicates direct relationship while a negative value an inverse relationship between the factor and response (Hao *et al*., 2012; Verna *et al*., 2009). The response surface graphs were generated based on the following second order equations:

$$PZ = 1577.33 - 264.24time - 0.1504stirring\ \ speed + 523.24oil : water\ \ ratio + 0.03139time * stirring\ \ speed + 260.867time * oil : water\ \ ratio - 0.01523stirring\ \ speed * oil : water\ \ ratio - 26.0785time^2 + 4.42E^{-006}stirring\ \ speed^2 - 914.004oil : water\ \ ratio^2 \quad (5)$$

$$PZ = 1874.5061 - 292.188time - 0.1941stirring\ \ speed + 431.30oil : water\ \ ratio + 0.02545time * stirring\ \ speed + 407.938time * oil : water\ \ ratio - 9.4888E^{-008}stirring\ \ speed * oil : water\ \ ratio - 24.024time^2 + 8.727E^{-006}stirring\ \ speed^2 - 758.936oil : water\ \ ratio^2 \quad (6)$$

Table 3. Physicochemical characterization of β-carotene nanoemulsions storage at room and cooling temperature

	Storage temperature at 25 ºC			Storage temperature at 4 ºC		
Sample	**pH**	**Electrical Conductivity (µS/cm)**	**Surface tension (Dinas/cm)**	**pH**	**Electrical Conductivity (µS/cm)**	**Surface tension (Dinas/cm)**
1	6.74 ± 0.06^{a}	33.33 ± 0.58^{b}	43.67 ± 0.15^{b}	5.93 ± 0.06^{a}	33.67 ± 0.58^{a}	43.03 ± 0.61^{d}
2	5.75 ± 0.06^{b}	27.03 ± 0.06^{de}	40.67 ± 0.21^{c}	5.56 ± 0.08^{c}	29.33 ± 0.31^{c}	40.80 ± 0.10^{e}
3	5.55 ± 0.04^{c}	36.07 ± 0.12^{a}	43.33 ± 0.57^{b}	5.55 ± 0.04^{c}	26.60 ± 0.95^{d}	46.53 ± 0.35^{a}
4	5.46 ± 0.04^{d}	27.67 ± 0.58^{d}	39.70 ± 0.25^{de}	5.37 ± 0.03^{d}	23.77 ± 0.12^{ef}	42.60 ± 0.53^{d}
5	5.40 ± 0.03^{e}	26.67 ± 0.58^{ef}	38.10 ± 0.36^{fg}	5.41 ± 0.01^{d}	23.20 ± 0.56^{fg}	39.23 ± 0.21^{g}
6	5.23 ± 0.01^{g}	27.17 ± 0.29^{de}	39.77 ± 0.40^{de}	5.58 ± 0.03^{c}	23.90 ± 0.10^{ef}	39.63 ± 0.12^{fg}
7	5.22 ± 0.02^{g}	26.27 ± 0.51^{f}	38.33 ± 0.15^{f}	5.56 ± 0.04^{c}	32.77 ± 0.25^{b}	39.77 ± 0.40^{fg}
8	5.50 ± 0.02^{cd}	28.67 ± 0.32^{c}	45.17 ± 0.29^{a}	5.60 ± 0.02^{c}	24.13 ± 0.50^{e}	44.90 ± 0.10^{b}
9	5.22 ± 0.01^{g}	23.03 ± 0.32^{i}	37.80 ± 0.26^{g}	5.72 ± 0.02^{b}	23.67 ± 0.29^{ef}	38.50 ± 0.50^{h}
10	5.35 ± 0.02^{f}	25.43 ± 0.32^{g}	40.03 ± 0.06^{d}	5.59 ± 0.01^{c}	22.63 ± 0.21^{gh}	40.07 ± 0.12^{f}
11	5.14 ± 0.02^{h}	24.00 ± 0.26^{h}	39.30 ± 0.30^{e}	5.62 ± 0.03^{c}	21.93 ± 0.12^{h}	43.67 ± 0.30^{c}
12	5.24 ± 0.03^{g}	27.13 ± 0.23^{de}	39.63 ± 0.12^{de}	5.56 ± 0.04^{c}	22.63 ± 0.06^{gh}	39.40 ± 0.20^{g}
13	5.21 ± 0.02^{g}	27.20 ± 0.20^{de}	39.47 ± 0.15^{e}	5.56 ± 0.02^{c}	22.63 ± 0.25^{gh}	39.33 ± 0.15^{g}

Actual value represent means ± standard deviation of each sample (n = 3). Means with the same letter in the same column are not significantly different, Duncan ($p < 0.05$).

The interactions between homogenization time and stirring speed, homogenization time and oil:water ratio and stirring speed with oil:water ratio were evaluated trough 3D surface response graphs (Fig. 1 and 2). For both cases, an increase in the homogenization time resulted in a decrease in the particle size of the emulsions. Moreover, higher particle sizes were observed when increased the stirring speed. Triplett and Rathman (2009) mentioned that higher stirring rates reduced the polydispersity, but did not significantly reduce particle size. The increasing in oil:water ratio permit to obtain an increasing in particle size values. Several authors reported similar behavior with an increasing in the oil phase for similar O/W nanoemulsions systems. This effect is attributed to an increasing in the oil phase seems to be more difficult in the droplet disruption process, which is due to an increase in the viscosity of the dispersed phase which leads to an increase in the flow resistance and hence the droplet break-up rate would be severely restricted (Jumma *et al*., 1998; Tang *et al*., 2012; Zainol *et al*., 2012). Base on experimental results that emulsification-homogenization method could be useful to produce β-carotene nanoemulsions with particle sizes in the nanometer range.

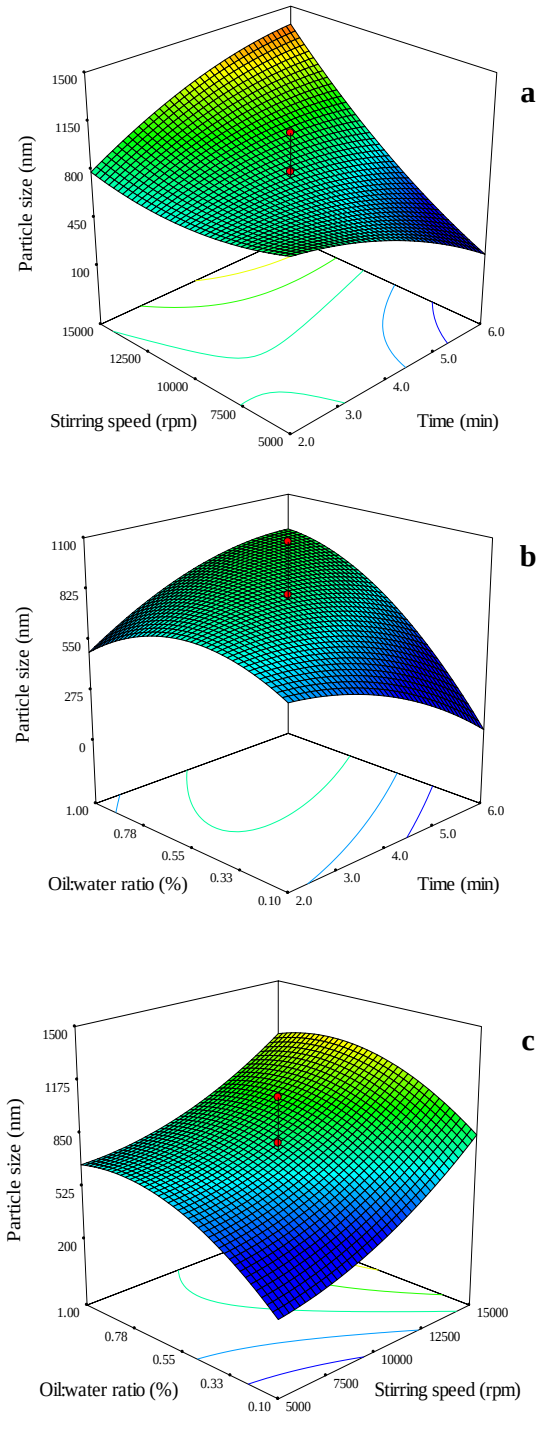

Fig. 1. Three-dimensional (3D) response surface plots showing the effect of the independent variables: a) time vs stirring speed, b) time vs oil:water ratio and c) stirring speed vs oil:water ratio on particle size of β-carotene nanoemulsions storage at room temperature (25 ºC).

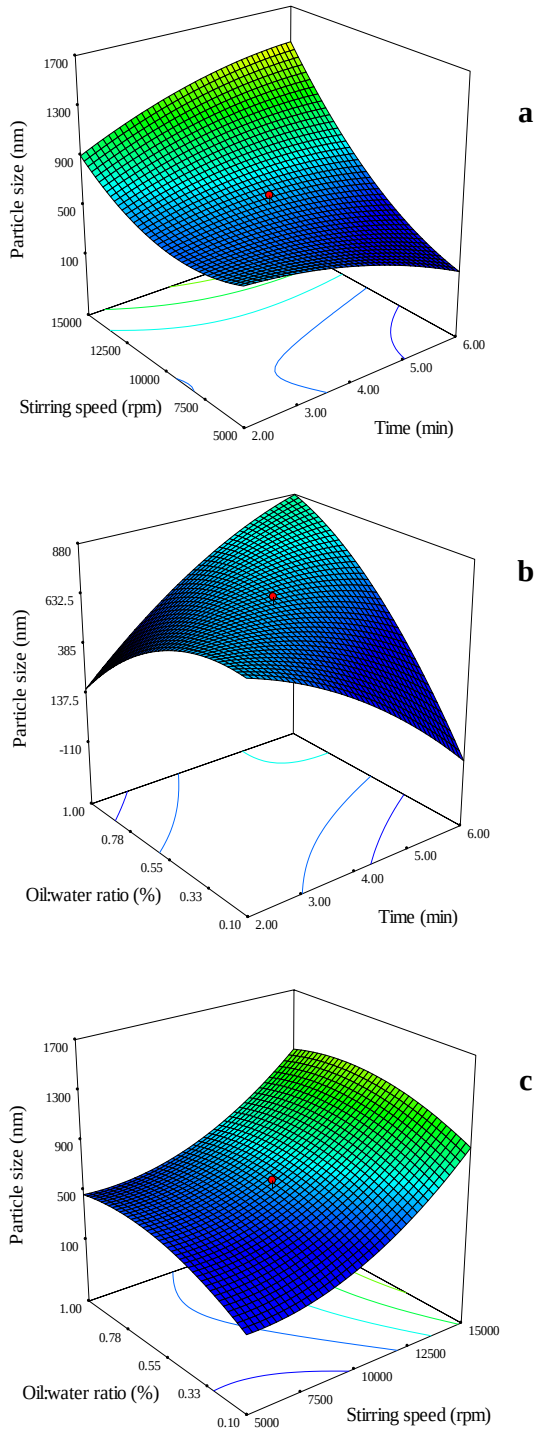

Fig. 2. Three-dimensional (3D) response surface plots showing the effect of the independent variables: a) time vs stirring speed, b) time vs oil:water ratio and c) stirring speed vs oil:water ratio on particle size of β-carotene nanoemulsions storage at cooling temperature (4 ºC).

The analysis of variance (ANOVA) of the quadratic regression model demonstrated that the model and the lack of fit value were not significant ($p < 0.05$) for both storage conditions (Table 4). The model F-value was 1.09 and 5.21 for room and cooling storage temperature, respectively. The good fit of the model was checked by coefficient of determination (R^2). R^2 values were 0.7661 and 0.9398 for room temperature and cooling temperature, respectively. It can be expressed in percentage also and interpreted as the percent variability in the response in the given model. According to the model, sample variation of 76.61 % for room temperature and 93.98 % for cooling temperature was attributed to the independent variables and the model cannot be explained only 23.39 and 6.02 %, respectively. For room temperature, correlation coefficient (R) was equal to 0.8752 and for cooling temperature 0.9694. A higher value of correlation coefficient (R) indicated a good agreement between experimental and predicted values for both conditions (Huang *et al*., 2006).

For RSM the perturbation plots show how the response changes as each factor moves from the chosen reference point, with all other factors held constant at the reference value. The software sets the reference point default at the middle of the design space. The Fig. 3 shows that changes in stirring speed has a greater effect on particle size than the homogenization time and oil:water ratio for both cases. Speeds greater than 10,000 rpm generate a negative effect on the response, resulting in larger particle sizes.

After generating the polynomial equations related to the factors and response, a further numerical optimization process was undertaken with desirable characteristics to find the optimal formulation and conditions for minimizing the particle size. The software combines individual desirabilities into a single number and then searches for the greatest overall desirability. A value of 1 represents the ideal case (Montgomery and Cook, 2009). Desirability plots are showed in Fig. 4. The optimal parameters for room temperature were 5.99 min, stirring speed of 5,287.92 rpm and a desirability of 1 with a value of particle size estimated of 399.88 nm using 0.8 oil:water ratio.

Table 4. Analysis of variance (ANOVA) for the fitted quadratic model developed for the particle size of β-carotene nanoemulsions storage at 25 ºC and 4 ºC.

	Storage temperature at 25 ºC					Storage temperature at 4 ºC				
Source	**Sum of squares**	**d*f***	**Mean square**	***F*-value**	***P*-value**	**Sum of squares**	**d*f***	**Mean square**	***F*-value**	***P*-value**
Model	7.499E+005	9	83,320.29	1.09	0.5288	1.226E+005	9	1.362E+005	5.21	0.1008
A-Time	3,828.13	1	3,828.13	0.050	0.8372	477.41	1	477.41	0.018	0.9011
B-Stirring speed	3.057E+005	1	3.057E+005	4.01	0.1391	5.930E+005	1	5.930E+005	22.67	0.0176
C-Oil/Water ratio	88,186.51	1	88,186.51	1.16	0.3612	38,642.59	1	38,642.59	1.48	0.3111
AB	1.554E+005	1	1.554E+005	2.04	0.2488	1.022E+005	1	1.022E+005	3.91	0.1425
AC	1.102E+005	1	1.102E+005	1.44	0.3156	2.696E+005	1	2.696E+005	10.31	0.0489
BC	2,349.33	1	2,349.33	0.031	0.8719	911.63	1	911.63	0.035	0.8638
A2	75,281.91	1	75,281.91	0.99	0.3938	63,888.82	1	63,888.82	2.44	0.2160
B2	84,798.91	1	84,798.26	1.11	0.3692	3.293E+005	1	3.293E+005	12.59	0.0381
C2	1.545E+005	1	1.545E+005	2.02	0.2500	1.065E+005	1	1.065E+005	4.07	0.1369
Residual	2.290E+005	3	76,321.33			78,469.64	3	26,156.55		
Lack of fit	15,265.34	1	15,265.34	0.14	0.7418	49,668.51	1	49,668.51	3.45	0.2044
Pure error	2.137E+005	2	1.068E+005			28,801.13	2	14,400.56		
Cor total	9.788E+005	12				1.304E+006	12			

d*f*: degree of freedom; 25 ºC storage temprature**: CV** = 39.05%, $\mathbf{R^2}$ = 0.7661, **R** = 0.8752; 4 ºC storage temprature **CV** = 25.09%, $\mathbf{R^2}$ = 0.9398, **R** = 0.9694.

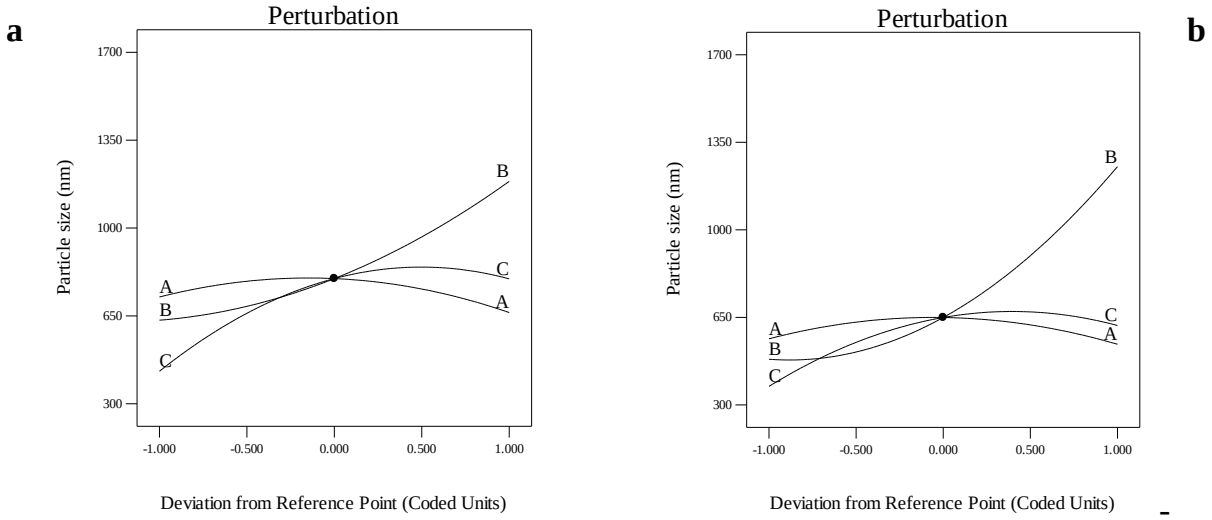

Fig. 3. Perturbation plots showing the effect of the variables: (*A*) time, (*B*) stirring speed and (*C*) oil:water ratio on the particle size of β-carotene nanoemulsions at 25 ºC (a) and 4 ºC (b).

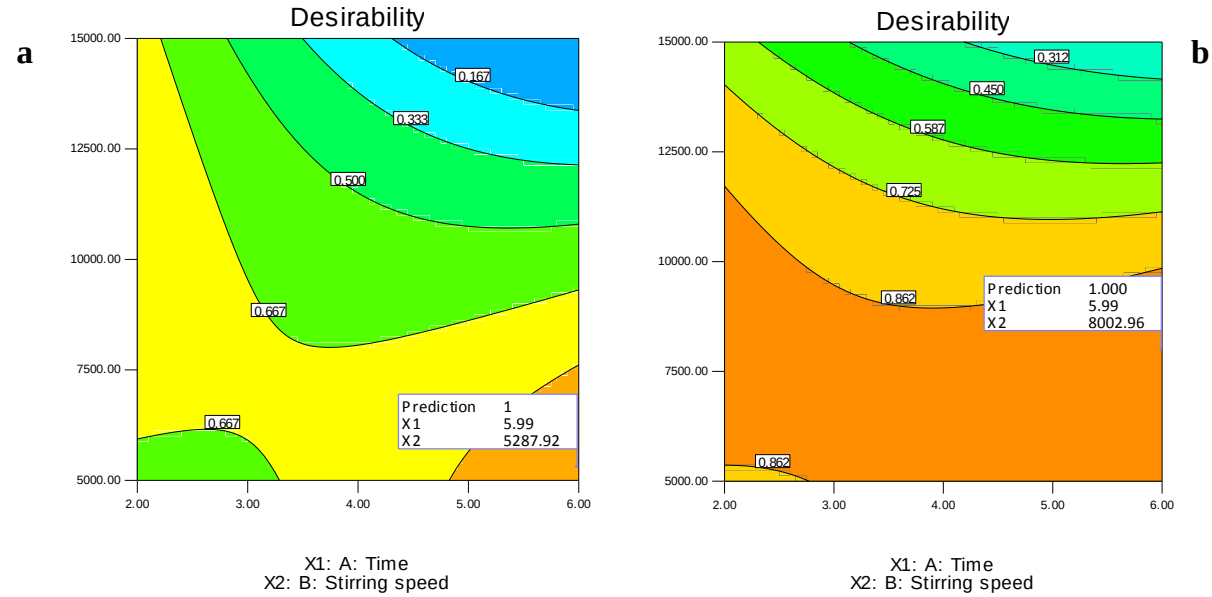

Fig. 4. Contour plots representing the predicted time and stirring speed and desirability generated by Design Expert. a) Blue to orange contours indicate minimum to maximum desirability of β-carotene nanoemulsions storage at 25 ºC. b) Green to orange contours indicate minimum to maximum desirability of β-carotene nanoemulsions storage at 4 ºC. The numbers by the circles represent the desirability for each condition. In the box prediction refers desirability value, X_1 = homogenization time and X_2 = stirring speed predicted optimal processing parameters.

Whereas that cooling temperature data were 5.99 min with a stirring speed of 8,002.96 rpm, and a desirability of 1, with a value of particle size estimated of 393.79 nm using 0.62 oil:water ratio. Finally, process conditions permitted to obtain a minimal particle size of β-carotene nanodispersions by a homogenization-emulsification technique.

3.1.2 Color measurement

The influence of time and storage temperature on stability of β-carotene nanoemulsions was examined by a colorimetric method. The hue angle values are presented in Table 5, the values were ranged from 52.94 to 85.05 for nanoemulsions analyzed immediately after preparation and after 21 days of

storage ranged from 36.56 to 73.3 at 25 ºC. For the formulations storaged at 4 ºC and 0 days the hue angle values were from 53.98 to 85.66, whereas that after 21 days storage the values were between 36.69 to 72.61. The data obtained were located in the first quadrant of the CIELAB color chart. Fig. 5 shows that all formulations exhibited an intense orange-red color distinctive of carotenoids indicating that the pigment is well embedded in the lipid matrix used as support for the generation of nanoemulsions (Qian *et al*., 2012; Bustos-Garza *et al*., 2013). Nevertheless, this color tends to degradation when it undergoes chemical degradation during storage time. At the end of this period, around 11.62 to 25.19% of the β-carotene pigment was lost for room temperature, and around 9.95-18.13% for cooling temperature showing a slightly greater loss occurred at 25 ºC than 4 ºC.

Table 5. The hue angle (h^0) of the β-carotene nanoemulsions during storage at room and cooling temperature

	Storage temperature at 25 ºC		**Storage temperature at 4 ºC**	
Sample	**0 days (hº)**	**21 days (hº)**	**0 days (hº)**	**21 days (hº)**
1	80.83 ± 0.90^{b}	73.30 ± 1.30^{a}	77.34 ± 1.24^{b,c}	72.61 ± 0.18^{a}
2	63.25 ± 2.36^{e}	36.56 ± 0.39^{h}	78.76 ± 1.79^{b}	36.69 ± 0.19^{i}
3	79.14 ± 1.80^{b}	60.13 ± 0.11^{d}	85.28 ± 0.67^{a}	69.82 ± 0.38^{b}
4	52.94 ± 1.61^{g}	46.79 ± 0.86^{f}	53.98 ± 1.50^{g}	48.61 ± 0.11^{e}
5	74.29 ± 0.12^{c}	69.65 ± 1.02^{b}	75.48 ± 0.60^{c}	64.84 ± 0.92^{c}
6	67.41 ± 0.84^{d}	43.67 ± 0.59^{g}	64.46 ± 0.28^{d,e}	41.87 ± 0.68^{h}
7	58.70 ± 1.47^{f}	42.96 ± 1.08^{g}	63.06 ± 1.29^{e}	42.95 ± 0.36^{g}
8	85.05 ± 1.40^{a}	63.63 ± 1.95^{c}	85.66 ± 2.57^{a}	63.61 ± 0.80^{d}
9	61.62 ± 1.57^{e}	52.18 ± 0.26^{e}	59.67 ± 1.07^{f}	47.35 ± 0.02^{f}
10	74.47 ± 0.27^{c}	45.31 ± 1.16^{f}	64.86 ± 1.30^{d,e}	43.67 ± 0.36^{g}
11	61.51 ± 1.82^{e}	43.02 ± 0.36^{g}	59.69 ± 1.03^{f}	43.04 ± 0.12^{g}
12	67.92 ± 0.37^{d}	43.33 ± 0.92^{g}	65.84 ± 1.43^{d}	43.93 ± 1.42^{g}
13	67.27 ± 0.36^{d}	43.40 ± 0.32^{g}	64.20 ± 0.22^{d,e}	43.59 ± 0.14^{g}

Actual value represent means ± standard deviation of each sample (n = 3). Means with the same letter in the same column are not significantly different, Duncan ($p < 0.05$).

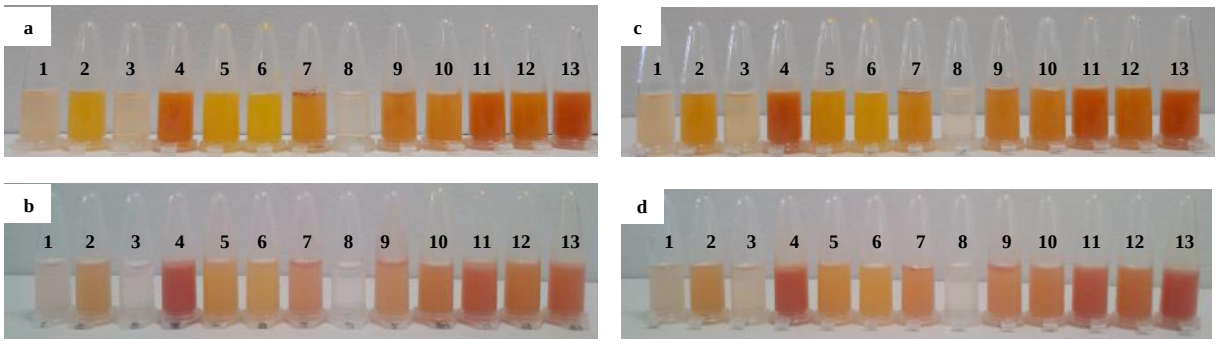

Fig. 5. Visual appearance of β-carotene nanoemulsions at 25 ºC analyzed on the day of production (a) and after 21 days of storage (b); at 4 ºC analyzed on the day of production (c) and after 21 days storage (d).

In agreement with this results Qian *et al*., (2012) evaluated the influence of storage temperature on the color of β-carotene nanoemulsions using a colorimeter, showing that in a range between 5 to 55 ºC over 14 day the carotenoid was highly unstable to degradation when stored at high temperatures in nanoemulsions. These results indicate that color measurement is presumably a good complementary method to evaluate the stability of nanoemulsions and highlight the importance of preparing, transporting and storing β-carotene nanoemulsions under relatively cool conditions to avoid color degradation and potential loss of bioactivity.

3.2 Antioxidant activity of β-carotene nanoemulsions

Due to the antioxidant property that has the β-carotene, the evaluation of this parameter is important for determining the best formulation that meets the objective to protect and preserve the functional characteristics of the molecule for longer time. Fig. 6 shows the percent inhibition for each formulation evaluated for a period of 21 days at 25 and 4 ºC, this percentage represents the amount of ABTS radical that being in contact with a substance that have antioxidant activity producing a color change of the radical from blue to uncolored. The highest values of percent inhibition were observed when the used relationship of 1.18:98.81 oil:water ratio for both storage conditions. The lowest oil:water ratio employed in the formulations caused the minimum percent inhibition. The antioxidant activity is intrinsically related to the β-carotene concentration incorporated into the nanoemulsion and storage time, similar effect was reported by Gupta *et al*. (2012) in β-carotene lipidic nanoencapsulation. Furthermore, the room storage temperature caused a significant ($p < 0.05$) decreasing in percent inhibition with respect to cooling storage temperature at 21 days.

3.3 Determination of β-carotene concentration

The β-carotene concentration changes of nanoemulsions over 21 days of storage at 25 and 4 ºC are showed in Fig. 7. The pigment concentration decreased slowly during storage. At the end of this period, a 32-80 % and 27-98 % of the β-carotene was lost for room and cooling storage temperature, respectively. The highest stability was achieved with formulations 2 and 7 for 25 and 4 ºC, respectively.

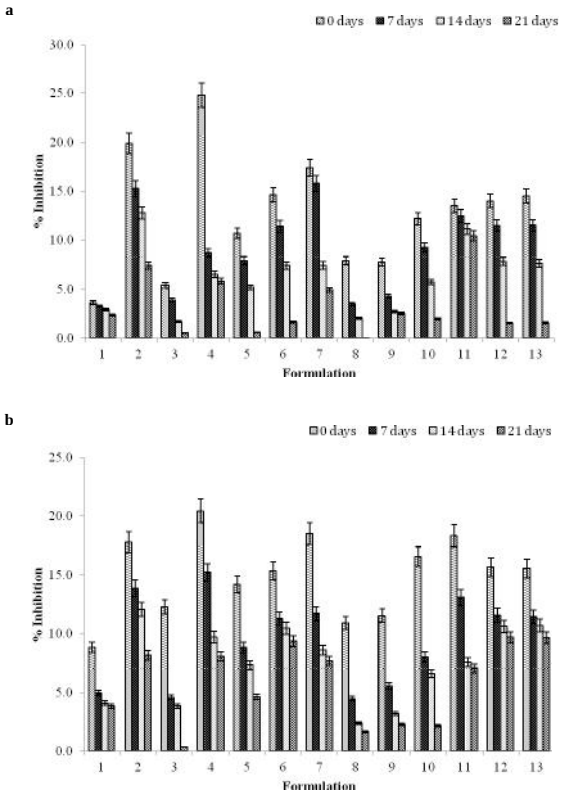

Fig. 6. Effect of storage time on the antioxidant activity of β-carotene nanoemulsions at a) 25 ºC and b) 4 ºC.

On the contrary, the lowest stability was for the formulation 1 in both cases. Tan and Nakajima (2005b) identified two main factors that could be responsible for the degradation of β-carotene in nanodispersions during storage: large surface area of the emulsion particles as a result of their size reduction to the nanometer range, and possible formation of free radicals during the homogenization process. The results above mentioned disagree with those reported by Yuan *et al*., (2008) who reported only a lost of β-carotene 14-25% for nanodispersions using a medium chain triglyceride (MCT) oil and Tween 80 produced by high pressure homogenization. A possible explanation for the discrepancy of the two studies could be the difference in the nanoemulsion production process.

Conclusion

The current study confirmed that homogenization-emulsification is a relatively simple and effective method for producing β-carotene oil in water nanoemulsions.

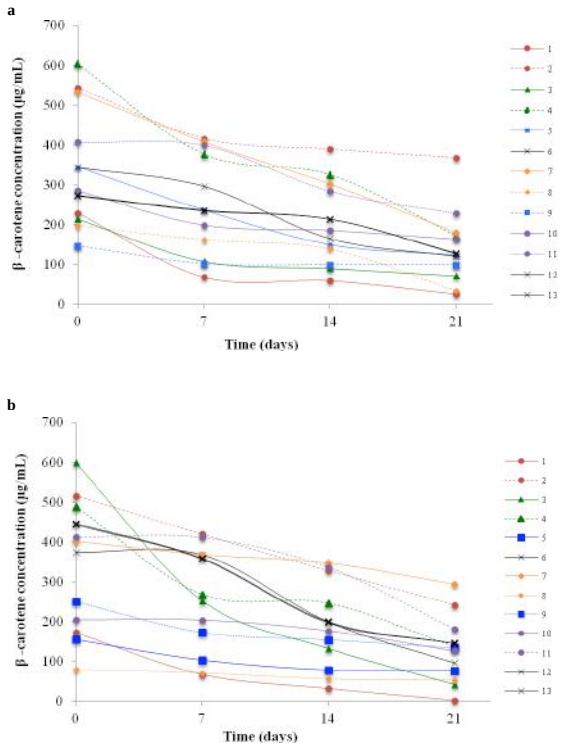

Fig. 7. Effect of storage time on the β-carotene concentration on nanoemulsions at a) 25 ºC and b) 4 ºC.

The particle size was influenced by the processing parameters such as homogenization time, stirring speed and oil:water ratio. An oil:water ratio 1:99, 5000 rpm stirring speed during 6 min permitted to obtain the smallest particle size. β-carotene was successfully incorporated into nanoemulsions, showing good physical stabilities. However, an important amount of this bioactive component was degraded during storage. RSM methodology provided the ability to design particles of desired size through minimal experimental runs formulation and process changes. Finally, these results may facilitate the design and optimization of nanoencapsulation systems for lipophilic bioactive compounds in food or pharmaceutical applications.

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