Food Chemistry 141 (2013) 3906–3912

Contents lists available at SciVerse ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Characterisation and stability evaluation of bixin nanocapsules

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ARTICLE INFO

Article history:
Received 17 January 2013
Received in revised form 23 April 2013
Accepted 30 April 2013
Available online 19 June 2013

Keywords:
Carotenoid
Bioactive compounds
Lipid-core nanocapsules
Nanoencapsulation

ABSTRACT

The aim of this study was to produce bixin nanocapsules by the interfacial deposition of preformed poly-ε-caprolactone (PCL). PCL (250 mg), capric/caprylic triglyceride (400 μL), sorbitan monostearate (95 mg) and bixin were dissolved in a mixture of acetone (60 mL) and ethanol (7.5 mL) under stirring (40 °C). This organic solution was added to the aqueous solution (130 mL) containing Tween 80 (195 mg). The size distributions in the formulations with bixin concentration from 11 to 100 μg/mL were evaluated periodically during 3 weeks of storage at ambient temperature. The optimal formulation (bixin concentration of 16.92 ± 0.16 μg/mL) was characterised in terms of particle size distribution, zeta potential, bixin content and encapsulation efficiency, and showed a volume-weighted mean diameter ($D_{4,3}$) of 195 ± 27 nm, around 100% of encapsulation efficiency and the nanocapsules were considered physically stable during 119 days of storage at ambient temperature.

1. Introduction

Annatto is a natural colourant that is mostly used in food products because of its low cost and high-quality sensorial characteristics, such as cheeses, ice creams, butters and meats (Cardarelli, Benassi, & Mercadante, 2008). The primary colouring component found in annatto seeds is bixin, a carotenoid formed by nine conjugated double bonds and two carboxylic groups (Fig. 1). The structure of bixin is responsible not only for its light absorption and antioxidant activity but also for its poor water-solubility, which impairs its use in low-fat foods (Rodriguez-Amaya, 2001).

Like other carotenoids, bixin is an efficient quencher of singlet oxygen and a scavenger of reactive species of oxygen and nitrogen (Chisté et al., 2011; Rios, Antunes, & Bianchi, 2009; Rios, Mercadante, & Borsarelli, 2007). Bixin is considered to be unstable in the presence of oxygen, heat and light. However, some studies showed that the techniques of complexation and encapsulation decrease the degradation rate of bixin caused by light, air, ozone, oxygen and high temperature (Barbosa, Borsarelli, & Mercadante, 2005; Lyng, Passos, & Fontana, 2005; Marcolino, Zanin, Durrant, Benassi, & Matioli, 2011; Parize et al., 2008).

In general, encapsulation improves the stability, solubility and bioavailability of encapsulated species and promotes its controlled release (Paese et al., 2009; Shaikh, Ankola, Beniwal, Singh, & Ravi Kumar, 2009; Zuidam & Shimoni, 2010). Nanoencapsulation is a process by which one compound is covered by another, producing particulate dispersions or solid particles, with sizes ranging from 10 nm to 1 μm. Depending upon the method of preparation of nanoparticles, nanospheres or nanocapsules can be obtained. Nanocapsules are systems in which the bioactive compound is soluble in the core, confined to a cavity surrounded by a polymer membrane, while nanospheres are matrix systems in which the drug is physically and uniformly dispersed (Mohanraj & Chen, 2006).

Nanocapsule systems are used for the delivery of drugs, peptides, proteins, genes, etc., and several compounds have been encapsulated (Couvreur, Barratt, Fattal, Legrand, & Vauthier, 2002). In the literature a number of methos are cited; most nanoparticles have been mainly prepared by dispersion of preformed polymers, polymerisation of monomers and ionic gelation or coacervation of hydrophilic polymers (Mohanraj & Chen, 2006).

For carotenoids, most research has been dedicated primarily to the encapsulation of β-carotene. Qian, Decker, Xiao, and McClements (2012) studied the effects of adding ascorbic acid, vitamin E acetate, coenzyme Q10 and ethylenediametetraacetic acid (EDTA) on the inhibition of β-carotene degradation in oil-in-water nanoemulsions. Silva et al. (2011) produced nanoemulsions of β-carotene using a high-energy emulsification-evaporation technique, studied the effect of processing variables (homogenisation time, shear rate and number of cycles), and evaluated the stability during storage.

The bixin encapsulation has been studied by Parize et al. (2008) and Barbosa et al. (2005). Parize et al. produced, characterised and evaluated the thermal stability of the urucum pigment (containing

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http://dx.doi.org/10.1016/j.foodchem.2013.04.135

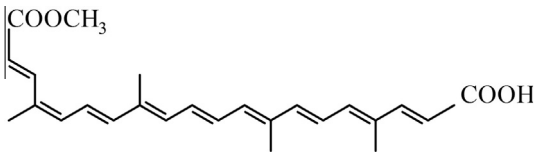

Fig. 1. Structure of *cis*-bixin (methyl hydrogen 9′-*cis*-6,6′-diapocarotene-6,6′-dioate).

bixin) microcapsules prepared by the technique of spray drying using chitosan as encapsulating agent in different solutions (acetic acid 5%, lactic acid 5% and citric acid 5%), and Barbosa, Borsarelli, and Mercadante (2005) evaluated the light stability of spray-dried bixin encapsulated with gum arabic or maltodextrin. However, to date, no studies have been published on the production and stability evaluation of bixin nanocapsules.

Indications that bixin may be important to human health and bixin's prevalence in the food industry as a colourant and antioxidant motivates the study of nanoencapsulation as a suitable technique for increasing the solubility of bixin in aqueous media. Therefore, the aim of this work was to prepare and characterise bixin nanocapsules and to evaluate their stability during storage.

2. Materials and methods

2.1. Materials

The polymer poly-ε-caprolactone (PCL) (Mw = 80,000) and sorbitan monostearate (Span 60) were obtained from Sigma (St. Louis, MO, USA). The capric/caprylic triglycerides (CCT) and polysorbate 80 (Tween 80) were purchased from Delaware (Porto Alegre, Brazil). Annatto seeds were obtained from the local market in Porto Alegre, Brazil. All other chemicals and solvents were of analytical or pharmaceutical grade.

2.2. Bixin standard

A bixin standard was prepared in triplicate according to the method of Rios and Mercadante (2004). This method consisted in the production of a bixin standard extracted from annatto seeds. Annatto seeds (25 g) were twice washed with hexane (100 mL). The solvent was discarded and the seeds were washed twice with methanol (100 mL). Methanol was also discarded and bixin was extracted from the seeds with ethyl acetate (100 mL). Each wash or extraction was carried out under magnetic stirring during 15 min. The extract was filtered and concentrated under reduced pressure in a rotary evaporator (Fisatom, model 801/802, São Paulo, SP, Brazil). After concentration, the recipient containing the extract was placed in a cold bath and dichloromethane (5 mL) was added slowly to this extract. After the addition of dichloromethane, ethanol (99.7%) was added slowly (20 mL). This solution was held at −18 °C during 12 h for crystallisation. The crystals formed in the bottom of the recipient were filtered, washed with 50 mL of ethanol (99.7%) and dried under reduced pressure ($T < 30$ °C). The purity of the standard was evaluated by high performance liquid chromatography (HPLC).

2.3. Bixin nanocapsules

Bixin nanocapsules were prepared by the technique of interfacial deposition of preformed polymers according to the method of Venturini et al. (2011). The polymer (PCL) (250 mg), triglycerides (CCT) (400 μL), span 60 (95 mg) and bixin were dissolved in a mixture of acetone (60 mL) and ethanol (7.5 mL) under magnetic stirring at 40 °C. After the solubilisation of PCL, CCT and Span 60, the standard of bixin (98.7%) was added and remained under magnetic stirring for 10 min (40 °C). This organic phase was added into an aqueous phase (130 mL) containing Tween 80 (195 mg) and remained under stirring for 10 min. The dispersion was concentrated under reduced pressure until it reached a final volume of 25 mL. In this method, acetone and ethanol (a water-miscible solvent) were used to solubilise PCL and Span 60. As the solvents migrate to the aqueous phase, an interfacial turbulence is created due to the spontaneous diffusion between the phases leading to the spontaneous formation of nanocapsules. As the concentration of water miscible solvent increases, a decrease in the size of particle can be achieved (Mohanraj & Chen, 2006).

In preliminary tests, the bixin concentrations tested in the bixin nanocapsule formulations were 100, 58, 37, 16 and 11 μg/mL; these were stored under ambient conditions (25 ± 1 °C) in amber glasses, and the parameter of size distribution was evaluated periodically during three weeks.

Based on the nanocapsules stability, an optimal formulation was prepared in triplicate and was characterised in terms of viscosity, bixin content, encapsulation efficiency, pH, diameter, zeta-potential and colour. Moreover, the stability of the optimum formulation was studied during storage at ambient temperature. The pH, diameter and bixin concentration were evaluated weekly for 9 weeks; after this period, the evaluation was performed every 2 weeks up to 119 days of storage.

2.4. Viscosity

The viscosity of the bixin nanocapsule suspension was measured immediately after preparation using a Brookfield rotational viscometer (model DV-II + Pro, spindle LV2, Brookfield Engineering, USA) at 25 °C. Data were analysed using Brookfield Rheocalc 32 software.

2.5. Colorimetric analysis

The bixin nanocapsule suspension (optimal formulation) (10 mL) and a free bixin solution (10 mL) were analysed using a portable colorimeter (Konica Minolta model CR 400, Singapore). Both samples were prepared in triplicate in the same bixin concentration (16.92 μg/mL). The free bixin was solubilised in ethanol:water (2:8) due to the low solubility of bixin in pure water. The colorimetric parameters were obtained according to the Comission Internationale de l'Eclairage (CIELAB system); the coordinates were L^* (lightness), and the colour coordinates a^* (red-green component) and b^* (yellow-blue component), which were measured using the illuminant D_{65} and an angle of viewing of 0°.

2.6. Encapsulation efficiency

The total content of bixin was determined through the extraction of bixin from the bixin nanocapsule suspension. This method consisted of the extraction from an aliquot of 250 μL of formulation with acetonitrile (4.75 mL). This extract was sonicated by ultrasound (30 min) and centrifuged (15 min at 2820×*g*). The supernatant was injected in the HPLC. The bixin content in the aqueous phase of the bixin nanocapsule suspension was determined through the injection of the filtrate in the HPLC. The filtrate was obtained after the ultrafiltration/centrifugation of an aliquot of bixin nanocapsule suspension (400 μL) using a Ultrafree-MC® (10,000 MW, Millipore, Bedford, USA) in a centrifuge (15 min at 1690×*g*). The encapsulation efficiency was determined according to the method of Venturini et al. (2011), by dividing the difference between the total concentration of bixin and the concentration of bixin in the aqueous phase by the total concentration and multiplying the results by 100.

2.7. High performance liquid chromatography (HPLC)

Analyses were carried out using an HPLC system (Agilent series 1100, Santa Clara, CA, USA) equipped with an online degasser, a quaternary pump and an automatic injector and that was coupled to a C18 Spherisorb ODS-2 column (150 × 4.6 mm i.d.; 3 μm particle size), adjusted at 25 °C. Data acquisition and processing were performed using the CHEMSTATION® software programme. Bixin was eluted isocratically at a flow rate of 1 mL/min using acetonitrile/2% v/v acetic acid/dichloromethane (63:35:2 v/v) as the mobile phase. The chromatograms were processed at the maximum absorption wavelength of bixin (470 nm).

All of the solvents used in the HPLC separation were of chromatographic grade and previously filtered through a Millipore vacuum filtration system using a 0.22 μm membrane for organic solvents (Millipore, Barueri, SP, Brazil). The injections were performed in duplicate.

Before being injected, the bixin standard was diluted in acetonitrile and the content of bixin in the nanosuspension (250 μL) was extracted with acetonitrile (4.75 mL), homogenised by ultrasonication (30 min) and centrifuged (15 min at 2820×*g*). The content of bixin present in the aqueous phase was separated from the bixin nanocapsule suspension after ultrafiltration-centrifugation (15 min at 1690×*g*). The aqueous phase was directly injected in the HPLC without dilution. All samples were filtered before the injections (0.45 μm, Millex with modified PTFE membrane for aqueous and organic solvents, Millipore, Barueri, São Paulo, Brazil).

For the quantification of bixin, a standard curve with a determination coefficient (R^2) greater than 0.99 was used. This standard curve was obtained plotting the peak areas (from the HPLC) of five solutions containing different concentrations of bixin (from 1.37 to 80.16 μg/mL) quantified previously by a spectrophotometer (UV–Visible Agilent 8453, Santa Clara, CA, USA) at 470 nm with an absorptivity coefficient of 2,826 in chloroform. The limits of detection (LOD) and quantification (LOQ) were 0.231 and 0.235 μg/mL, respectively, and were determined according to the method described by Long and Winefordner (1983).

2.8. pH

The pH of the bixin nanocapsule suspension was measured at 25 °C using a DM-22 potentiometer (Digimed, Brazil).

2.9. Determination of diameter and zeta potential

The nanocapsules mean diameter (*z*-average) and polydispersity index (PDI) were measured at 25 °C by Dynamic Light Scattering (DLS) and the zeta potential was measured by electrophoretic mobility (Zetasizer® nano-ZS ZEN mod. 3600, Nanoseries, Malvern, UK). The samples were appropriately diluted with a pre-filtered (0.45 μm) 10 mM NaCl aqueous solution or with MilliQ® water to determine the zeta potential and mean diameter (*z*-average), respectively. Data analysis was performed using Dispersion Technology Software (version 4.0, 2002, Malvern Instruments ltd).

The mean diameter of the bixin nanocapsules was also measured by laser diffraction (LD) (Mastersizer 2000® 5.54, Malvern Instruments, UK), using water as dispersant. The refractive indexes used for the polymer and for water were of 1.590 and 1.330, respectively. The data were analysed by Mastersizer 2000 5.54 software programme. The span values were determined by dividing the difference between $D_{0,1}$ and $D_{0,9}$ by $D_{0,5}$, as provided by the software. The use of DLS and LD was applied as a good means to evaluate changes during storage because Zetasizer nano ZS® and Mastersizer 2000® are able to determine particle sizes ranging from 0.003 to 10 μm and from 0.02 to 2000 μm, respectively.

2.10. Statistical analysis

The results were evaluated by a one-way analysis of variance (ANOVA) and the mean values analysed by Tukey's test using the STATISTICA® 8.0 software programme.

3. Results and discussion

3.1. Bixin standard

The purity of the bixin standard was 98.7 ± 0.20%. This value is similar to the values reported by Rios and Mercadante (2004), Rios et al. (2009) and Barbosa et al. (2005) who observed purity levels of 98%, 96% and 94%, respectively. These values indicate that the type of solvent and the characteristics of extraction, such as crystallisation and temperature, affect the final purity in terms of bixin. The bixin standard was produced with a yield of 0.86 ± 0.03% as a result of the washing procedures performed to increase the purity.

3.2. Preliminary tests of bixin encapsulation

The standard formulation of nanocapsules applied in this study was chosen because as the polymer PCL is biocompatible, biodegradable, and does not generate toxic compounds; moreover, it is approved by FDA (Food and Drugs Administration) for specific studies and has similar costs compared to other synthetic polymers. Moreover, this formulation was studied in various pharmaceutical experiments of drug delivery (Jäger et al., 2009; Paese et al., 2009; Pohlmann et al., 2002). This formulation was optimised by Venturini et al. (2011) in a study in which aqueous suspensions composed exclusively of lipid-core nanocapsules were formulated, and allows the controlled release of its core content in the gastrointestinal tract (Frozza et al., 2010).

Preliminary tests were conducted to produce suspensions composed only of bixin nanocapsules with diameters smaller than 1 μm and exhibiting a monomodal size distribution. The formulations were analysed by laser diffraction over a period of 3 weeks. In the study, five formulations denoted 1–5 and containing bixin concentrations of 100, 58, 37, 16 and 11 μg/mL, respectively, were produced.

Immediately after the preparation, formulation 1 (100 μg/mL) showed bixin crystals in suspension. The crystallisation process was induced by high-purity bixin, which resulted from the high concentration of the bixin standard. Formulation 2 (58 μg/mL) showed a bimodal size distribution, with particle sizes ranging from the nanometre to the micrometre scale (Fig. 2a). Differently, formulation 3 (37 μg/mL) showed a good monomodal distribution profile, a volume-weighted mean diameter ($D_{4,3}$) of 151 nm and a span value of 1.284; moreover, 90% of the nanocapsules had diameters ($D_{0,9}$) smaller than 115 nm. However, after 5 days of storage, this formulation showed an unstable behaviour, showing a bimodal size distribution and size larger than 1 μm (Fig. 2b).

Immediately after being produced and over 3 weeks of storage, both formulations 4 (16 μg/mL) and 5 (11 μg/mL) presented a monomodal distribution (in terms of volume and number of particles), with a mean diameter less than 1 μm (Fig. 3a and b). The volume-weighted mean diameters ($D_{4,3}$) observed in formulations 4 and 5 were 208 and 163 nm, with span values of 1.397 and 1.271, respectively.

Span values are related to the particle distributions. Low span values indicate a narrowed particles size distribution (more homogeneous sizes). Thus, formulation 5 may be considered to be more homogeneous because it presented a narrower particle size distribution than that of formulation 4. The results of the cumulative distribution show that 90% of the nanocapsules in formulations 4

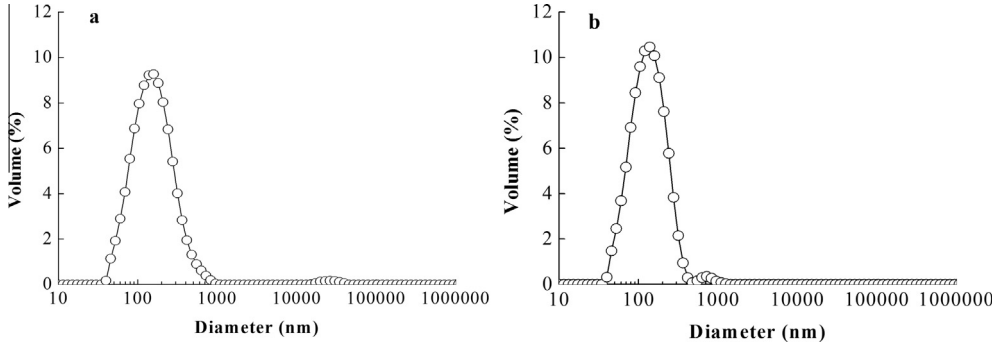

Fig. 2. Bixin nanocapsules size distribution of formulation 2 immediately after preparation (a) and of formulation 3 after 5 days of storage at ambient temperature (b).

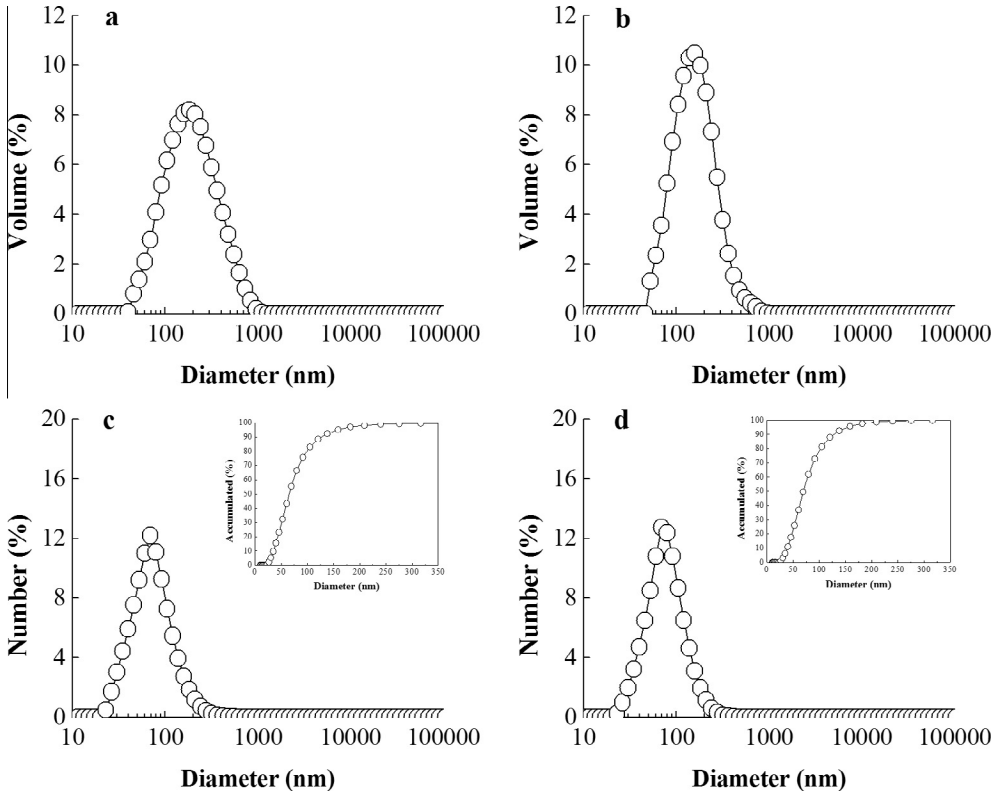

Fig. 3. Bixin nanocapsules size distribution obtained by laser diffraction of formulation 4 by volume (a) and number (c); and bixin nanocapsules size distribution of formulation 5 by volume (b) and number with their respective accumulated distribution (d).

and 5 exhibited diameters ($D_{0,9}$) smaller than 126 and 127 nm, respectively (Fig. 3c and d).

After 3 weeks of storage, no changes were observed in the mean diameter of the nanocapsules in formulations 4 and 5, and both formulations were considered physically stable. However, formulation 4 was chosen for further experiments because of the higher concentration of bixin measured, in addition to having satisfactory size and distribution characteristics.

The concentration of bixin in the nanocapsules affected the physical characteristics of the nanocapsules, such as their diameter, particle-size distribution and stability, hence, the results of our preliminary tests show that there is a limit of bixin solubilisation.

Determining the particle size distribution with respect to particle volume allowed us to verify the presence of particles with diameters greater than 1 μm. This verification is practically void when analysing the distributions in terms of number of particles because these particles (diameter >1 μm) are present in small amounts.

3.3. Characterisation and stability evaluation of bixin nanocapsules

The bixin nanocapsule suspension was prepared in triplicate with a mean bixin concentration of 16.92 ± 0.16 μg/mL. Venturini et al. (2011) produced lipid-core nanocapsules with higher concentration of indomethacin ethyl ester (1 mg/mL) using the same formulation components, which indicated that the type of compound which is encapsulated affected the amount incorporated into the formulation. However, the concentration of bixin was not considered low because food dyes are normally used in low concentrations.

The quantity of a compound that can be incorporated into nanoencapsulated systems is affected by the type of formulation and technique used (Ribeiro, Chua, Ichikawa, & Nakajima, 2008; Tan & Nakajima, 2005; Yuan, Gao, Zhao, & Mao, 2008). In the aqueous phase of the bixin nanocapsules formulation, the bixin concentration was below the limit of detection of 0.231 μg/mL (None bixin peak was found). The mean total concentration of bixin in the formulations was of 16.92 ± 0.16 μg/mL. The encapsulation efficiency

was determined by the equation: Encapsulation efficiency = [(16.92–0.00)/16.92] × 100. Thus, the value of encapsulation efficiency is around 100% or greater than 98.63% if the limit of detection is subtracted from the total concentration of bixin. The high encapsulation efficiency indicates that all bixin in the suspension was present in the nanocapsule structure (inner part and wall).

Such high encapsulation efficiency occurred probably due to the nanocapsule core which contains triglycerides (CCT), which facilitates solubilisation of bixin; this further indicates that nanoencapsulation is an effective technique for improving the solubilisation of bixin in aqueous media. The microencapsulation of bixin in different food polymers has been reported to achieve a maximum efficiency of 86.4% (Barbosa et al., 2005).

The optimal bixin nanocapsule suspension presented a yellow colour with the following CIELAB coordinates of L^* = 73.67 ± 0.34, a^* = 6.01 ± 0.24 and b^* = 48.60 ± 0.95. Compared to the pure bixin solution prepared in ethanol:water (20:80), with parameters L^* = 42.10 ± 0.35, a^* = 13.54 ± 0.98 and b^* = 25.50 ± 2.2, the bixin nanocapsule suspension presented an increase in luminosity and yellow colour, which was coupled with a decrease in red colour.

The viscosity of a suspension is important because the rheological properties affect all stages of manufacture such as mixing, pumping, filling and are valuable tools in quality control. The behaviour of the bixin nanocapsule suspension in this study is typical for a Newtonian fluid, since the increase of the shear stress was proportional to the increase of the shear rate. The optimal bixin nanocapsule formulation (16.92 ± 0.16 μg/mL) presented a viscosity of 11.4 ± 0.24 mPa.s.

Immediately after being produced, the bixin nanocapsule suspension showed a mean pH of 5.89 ± 0.70. Paese et al. (2009) used the same formulation to evaluate in vitro the effectiveness of nanoencapsulated benzophenone-3 and produced nanocapsule suspensions with pH values of 6.56 ± 0.09, while Pohlmann, Weiss, Mertins, Silveira, and Guterres (2002) produced indomethacin-loaded nanocapsule suspensions with pH values of 4.2 ± 0.1 in a study aiming to apply the spray-drying technique to produce dried nanocapsules and nanospheres prepared by the technique of interfacial deposition of preformed polymer, using a similar formulation to that used in this work.

One way to evaluate the chemical stability of a nanocapsule suspension is the measurement of the pH, since its decrease can be related to the degradation of the polymer or other ingredient (Kishore et al., 2011; Mallin, Vainio, Karjalainem, & Seppala, 1996). During the first 63 days of storage, no significant change was observed in the pH values ($p < 0.05$); however, on the 119th day, the pH levels decreased to 4.48 ± 0.32 (Fig. 4). One way to minimise the changes in pH is to use a buffering agent in the aqueous phase. In a previous study, indomethacin nanocapsule suspensions also showed reduced pH values during storage (3 months) that varied from 4.2 ± 0.1 to 3.4 ± 0.0 and 3.2 ± 0.0 at room temperature and at 50 °C, respectively (Pohlmann et al., 2002). The decrease in pH values is normal for this type of formulation because of the liberation of polyester monomer during poly-ε-caprolactone hydrolysis (Mallin et al., 1996).

Another explanation for the decrease in the pH is the probable formation of different compounds like acetic acid, formic acid, octanoic acid and nonanoic acid during the degradation of polysorbate 80 by auto-oxidation in aqueous media. The initiation of auto-oxidation in polysorbates could occur by the presence of residual peroxides, metal traces and incidence of light (Kishore et al., 2011).

The term "emulsion stability" refers to the ability of an emulsion to resist changes in its properties over time. An emulsion may become unstable due to a number of different types of physical and chemical processes. Physical instability results in an alteration in the spatial distribution or structural organisation of the molecules (creaming, flocculation, coalescence, partial coalescence, phase inversion, and Ostwald ripening), whereas chemical instability results in an alteration in the chemical structure of the molecules (oxidation and hydrolysis) (McClements, 1999).

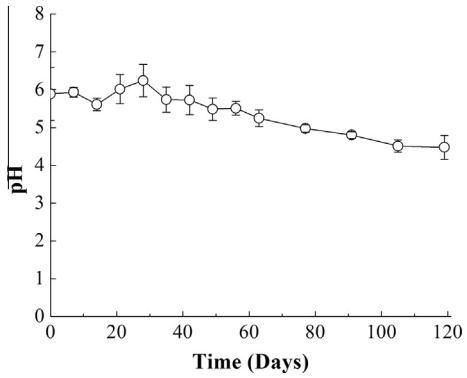

Fig. 4. pH variation of bixin nanocapsule suspension over 119 days of storage in ambient temperature. Standard deviations are represented by bars.

The zeta potential is the result of the components used in the production of particles, like the surfactants located at the interface between the continuous and disperse phases, and is commonly used to characterise the surface charge property of nanoparticles (Couvreur et al., 2002). Zeta potential reflects the electrical potential of particles and is influenced by the composition of the particle and the medium in which it is dispersed. Nanoparticles with a zeta potential above (±) 30 mV have been shown to be stable in suspension, as the surface charge prevents aggregation of the particles (Mohanraj & Chen, 2006). The magnitude and sign of the electrical charge on an emulsion droplet depend on the type of emulsifier, the concentration and the prevailing environmental conditions (e.g., pH, temperature, and ionic strength) (McClements, 1999).

The bixin nanocapsule suspension presented a mean zeta potential of −14.45 ± 0.92 mV immediately after preparation, and after 119 days of storage decreased to −25.85 ± 6.58 mV. Jäger et al. (2009) studied the influence of the concentration of sorbitan monostearate and polysorbate 80 on the indomethacin ethyl ester release kinetic and produced formulations using PCL with zeta potential values from −8.6 ± 0.1 to −12.7 ± 2.5 mV. Benzophenone-3-loaded lipid core nanocapsule suspension prepared with PCL and Tween 80 showed zeta potentials of −9.5 ± 1.0 mV. The change in the charge probably occurred due to the hydrolysis of polysorbate 80, since the negative zeta potential is a consequence of the negative charge density of the carboxylate groups in the PCL backbone (Paese et al., 2009).

The objective of producing particles exclusively on the nanometre scale was achieved, as indicated by the laser diffraction analyses, which showed a narrow monomodal distribution of bixin nanocapsules, with a volume-weighted mean diameter ($D_{4,3}$) of 195 ± 27 nm, a surface-weighted mean diameter ($D_{3,2}$) of 138 ± 13 nm and span value of 1.4 ± 0.1. In addition, 90% of the nanocapsules ($D_{0,9}$) presented diameters smaller than 124 ± 5 nm.

In evaluating certain process variables (homogenisation pressure, number of cycles and organic/aqueous phase relation), Tan and Nakajima (2005) produced β-carotene nanodispersions by solvent displacement method using Tween 20 as emulsifier, with mean diameters ($D_{4,3}$) varying from 60 to 135 nm and with span values varying from 0.4 to 0.7. Ribeiro et al. (2008) used food grade materials, gelatin and Tween 20 to produce polymeric nanodispersions of β-carotene with mean diameters ($D_{3,2}$) ranging from 74 to 77 nm.

The dynamic light scattering analyses also demonstrated that the bixin nanocapsules presented a monomodal distribution with

a mean diameter (*z*-diameter) of 190 ± 9 nm and a polydispersity index of 0.098 ± 0.03. The PDI values ranging from 0.1 to 0.25 indicates a narrow size distribution while a PDI greater than 0.5 is related to a broad distribution (Wu, Zhang, & Watanabe, 2011). Paese et al. (2009) produced nanocapsules with mean diameters (*z*-average) of 247 ± 4 nm and a polydispersity index lower than 0.2. Yuan et al. (2008), applied the technique of high pressure homogenisation and studied the influence of emulsifier type and concentration, homogenisation pressure, temperature and number of cycles, produced β-carotene nanoemulsions with diameters ranging from 132 to 184 nm (determined by DLS).

An unstable formulation of nanoparticles can form agglomerates and represent a risk to the health in the case of intravenous administration of a drug-loaded nanoparticle suspension, leading to blockage and embolism. The nanoparticle size control is a parameter that must be ensured during storage, since one form to verify if a nanoparticle formulation is physically stable is the periodic determination of the mean diameter (Wu et al., 2011). In the present work, no significant differences were observed ($p < 0.05$) between the volume-weighted diameters ($D_{4,3}$) determined by number and volume (via LD) and the mean diameters (*z*-diameter) measured by DLS. The bixin nanocapsules were considered stable because they did not exhibit any evidence of coalescence, creaming or flocculation in either analysis (LD or DLS) over 119 days of storage at 25 °C. These mechanisms of emulsion instability may be verified by an increase in mean particle diameter because the particles are in continual motion and collide with one another under normal conditions.

Silva et al. (2011) produced β-carotene nanoemulsions distributed in a monomodal profile with surface-weighted mean diameter ($D_{3,2}$) of 9.24 ± 0.16 nm and 228.63 ± 0.01 nm. The authors verified the increase in the size of nanoemulsions in two formulations, which varied from 9.24 ± 0.16 to 94.86 nm and from 10.27 ± 1.85 to 265.47 ± 120.86 nm after 21 days at 4 °C, respectively. They attributed the instability of the β-carotene nanoemulsions to the Brownian motion.

The bixin nanocapsule suspension was also considered physically stable regarding the mean diameter during the storage evaluated by laser diffraction (Fig. 5a) and dynamic light scattering (Fig. 5b), since no significant changes ($p < 0.05$) were observed in the mean diameter and the particle size distributions also remained constant, with no significant changes ($p < 0.05$), at 0, 28, 63, 91 and 119 days of storage. Other authors attributed to the steric effect provided by the surfactant polysorbate 80 the responsibility for the stability of this type of nanocapsule formulation (Jäger et al., 2009; Venturini et al., 2011).

Yuan et al. (2008) studying the effects of production parameters, developed β-carotene nanoemulsions with mean diameter (z-average) ranging from 132 to 184 nm that were stable for four weeks in amber bottle flushed with nitrogen and stored at 4 and 25 °C. Tan and Nakajima (2005) verified that β-carotene nanodispersions prepared using only Tween 20 as the emulsifier remained stable after 12 weeks of storage at 4 °C in amber bottles. Ribeiro et al. (2008) reported that the β-carotene nanoparticles prepared using poly-D,L lactic acid and poly-D,L-lactic-co-glycolic acid were stable over 5 months of storage at 4 °C in the dark.

The decrease in bixin content during the first days of storage most likely occurred due to the formation of free radicals in the oil (CCT) during the solubilisation of bixin in the organic phase (40 °C) (Tan & Nakajima, 2005) the presence of oxygen in the amber bottles and the bixin release from the nanocapsule structure during storage, which means free or unprotected bixin in the continuous phase (Jäger et al., 2009). From the 7th to the 28th day of storage, there was no significant variation in the bixin content ($p < 0.05$) (Fig. 6).

After 119 days of storage, a bixin content of 45.7 ± 1.1% was observed. This indicates that nanoencapsulation is highly effective in inhibiting carotenoid loss during storage, although, decrease in carotenoid content has also been demonstrated. Over 12 weeks of storage at 4 °C, the residual content of β-carotene in the nanodispersions varied from 25.2% to 56% (Tan & Nakajima, 2005).

Using different parameters of encapsulation, Yuan et al. (2008) produced and evaluated the stability of β-carotene nanoemulsions. After 4 weeks of storage at 4 and 25 °C, the residual β-carotene concentration ranged from 75% to 86% of β-carotene. Yin, Chu, Bobayashi, and Nakajima (2009) studied the effects of different emulsifiers on the stability of β-carotene nanodispersions. After 4 months, the content of β-carotene fell from 45.6 to 63.3%.

Tan and Nakajima (2005) reported that the surface area, which is higher in nanodispersions, was a contributing factor to β-carotene loss, which is larger than in a β-carotene crystalline solution, in addition to the formation of free radicals during the high-pressure homogenisation process. Silva et al. (2011) attributed the decrease in the β-carotene content to the high surface area of the nanoemulsions and the high degree of medium oxygenation occurring during the homogenisation step. Yuan et al. (2008) suggested that the degradation during storage may be a problem for the use of high-pressure homogenisation in commercial products.

An alternative method for reducing the bixin degradation rate in nanocapsules during storage might be to encapsulate bixin in the same manner as described by Ribeiro et al. (2008), who reported that the addition of α-tocopherol prevented β-carotene loss; the authors reported that the β-carotene content remained stable for at least 5 months. Qian et al. (2012) verified that the incorporation of the water-soluble antioxidants EDTA and ascorbic acid, and the oil-soluble vitamin E acetate, coenzyme Q10 reduced

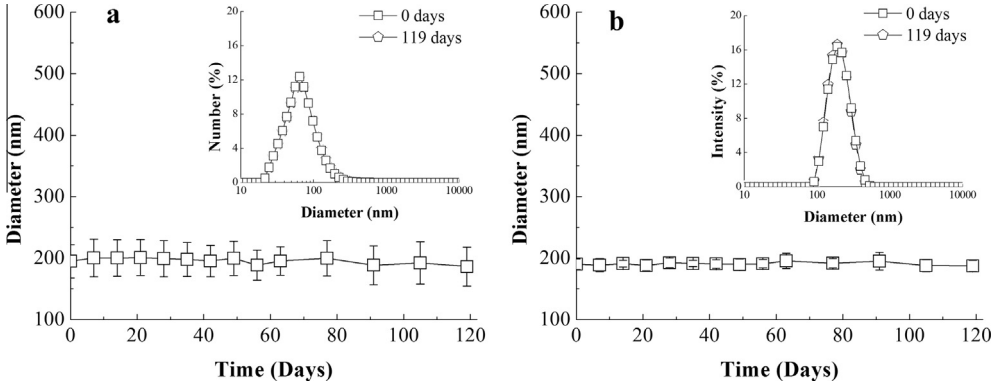

Fig. 5. Mean diameters of bixin nanocapsules evaluated by laser diffraction (a) and by dynamic light scattering (c) over 119 days of storage and respective size distribution of bixin nanocapsules at 0 (□) and 119 days (⬠) of storage in ambient temperature. Standard deviation is represented by bars.

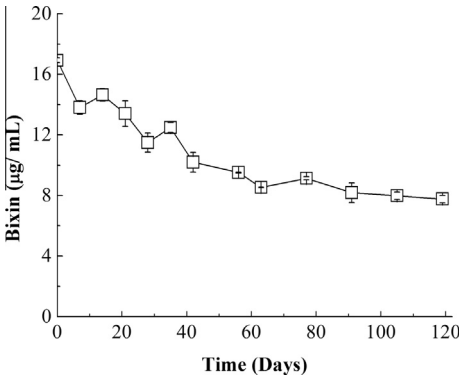

Fig. 6. Variation of bixin content in bixin nanocapsule suspension over 119 days of storage under ambient conditions. Standard deviations are represented by bars.

the degradation rate of β-carotene nanoemulsions under accelerated storage conditions (50 °C). These authors also reported that the addition of water- and oil-soluble antioxidants did not affect the particle size.

4. Conclusions

Bixin is an antioxidant and the predominant pigment found in fat-soluble preparations that are used to colourise butter, cheese, bakery products, oils, ice creams, sausages, cereals and extruded products. The technique of interfacial deposition of preformed polymer allowed for the production of bixin nanocapsules with high encapsulation efficiency (100%), satisfactory volume-weighted diameter ($D_{4,3}$) of 195 ± 27 nm and monomodal distributions. No significant changes ($p < 0.05$) were observed in the particle diameter over 119 days of storage when evaluated using both LD and DLS. The decrease in the pH levels most likely occurred due to poly-ε-caprolactone hydrolysis and the decrease in the bixin content most likely occurred due to poly-ε-caprolactone hydrolysis and the formation of free radicals, high surface area of the nanocapsules, and the presence of oxygen in the bottles. The solubilisation of bixin in aqueous media enhances the future possibility of using bixin in low-fat foods and in studies performed to evaluate its effects *in vivo*, which may expand the breadth of bixin's industrial application.

Acknowledgements

The authors are grateful to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the financial support.

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