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Effect of production parameters and stress conditions on beta-carotene-loaded lipid particles produced with palm stearin and whey protein isolate

Efeito dos parâmetros de produção e de condições de estresse em partículas lipídicas encapsulando beta-caroteno produzidas com estearina de palma e isolado proteico de soro

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Abstract

Microencapsulation is currently used by the food industry for different purposes, including the protection of ingredients against factors such as oxidation and volatilization, as well as to increase the bioavailability and bioaccessibility of nutrients. The current study aimed to encapsulate beta-carotene in solid lipid microparticles stabilized with whey protein isolate (WPI), and also investigate their integrity during storage and under stress conditions such as different ionic strengths, sucrose concentrations and thermal treatments. Solid lipid microparticles were produced using palm stearin, a food grade vegetable fat, using a single-step high shear process. Of the different formulations used for lipid microparticle production, characterization studies showed that the greatest stability was obtained with systems produced using 1.25% (w/v) whey protein isolate, 5% (w/v) palm stearin and 0.2% (w/v) xanthan gum. This formulation was applied for the production of beta-carotene-loaded solid lipid microparticles, with different concentrations of alpha-tocopherol, in order to verify its possible antioxidant activity. The results showed that the addition of alpha-tocopherol to the dispersions provided an increase in encapsulation efficiency after 40 days of storage that ranged from 29.4% to 30.8% when compared to the system without it. Furthermore, the solid lipid microparticles remained stable even when submitted to high ionic strength and to heating in the proposed temperature range (40 °C to 80 °C), highlighting their feasible application under typical food processing conditions.

Keywords: *Lipid particles; Beta-carotene; Stress conditions; Microencapsulation; Palm stearin; Whey protein isolate.*

Resumo

A microencapsulação é atualmente utilizada pela indústria de alimentos com diferentes propósitos, incluindo a proteção de ingredientes contra fatores, como a oxidação e a volatilização, assim como o aumento da biodisponibilidade e da bioacessibilidade de nutrientes. Este estudo teve por objetivo a encapsulação de betacaroteno em micropartículas lipídicas sólidas estabilizadas com isolado proteico de soro, além de verificar sua integridade sob armazenamento refrigerado e após submissão a diferentes condições de estresse, tais como força iônica, presença de açúcar e tratamentos térmicos. As partículas lipídicas sólidas foram produzidas utilizando estearina de palma, uma gordura vegetal de grau alimentício, a partir de homogeneização de alto cisalhamento. Dentre as diferentes formulações utilizadas durante a produção das partículas lipídicas sólidas, os estudos de caracterização mostraram que uma maior estabilidade foi obtida pelo sistema produzido com 1,25% (m/v) de isolado proteico de soro, 5% (m/v) de estearina de palma e 0,2% (m/v) de goma xantana. Essa formulação foi então escolhida para a posterior produção de partículas lipídicas sólidas encapsulando betacaroteno, etapa na qual foram testadas diferentes concentrações de alfa-tocoferol, a fim de verificar seu potencial antioxidante. Os resultados mostraram que uma adição de alfa-tocoferol nas dispersões de partículas lipídicas sólidas possibilitou um aumento na eficiência de

encapsulação dos sistemas, aumentando-a em cerca de 30%, quando comparada à eficiência de encapsulação do sistema no qual o alfa-tocoferol não foi adicionado. Ademais, as dispersões permaneceram estáveis mesmo depois de submetidas a altas concentrações de sal e à escala de temperatura proposta (40 °C a 80 °C), reforçando o potencial de aplicação desses sistemas em condições tipicamente encontradas durante o processamento de alimentos.

Palavras-chave: *Partículas lipídicas sólidas; Betacaroteno; Condições de estresse; Microencapsulação; Estearina de palma; Isolado proteico de soro.*

1 Introduction

Microencapsulation is currently used with different purposes in the food industry such as the prevention of nutritional losses and the oxidation of volatile compounds, as well as having a role in the preservation of flavors and pigments (LIU et al., 2015). Continuous research with microencapsulation techniques is also required to incorporate controlled release mechanisms into food formulations, which contribute to the increase in bioavailability and bioaccessibility of the bioactive compounds in human cells. Several lipid carriers have been studied to encapsulate hydrophobic compounds, such as emulsions, microemulsions, micelles, liposomes and lipid particles (RAO; McCLEMENTS, 2011; SARTORI; MENEGALLI, 2016). Solid lipid particles (SLP) are micro or nano carriers derived from oil-in-water emulsions in which the oil is replaced by a solid lipid or a mixture of solid lipids at room temperature (MÜLLER et al., 2000). Some advantages of using solid lipid particles are their higher loading capacity when compared to other lipid carriers and their extremely high biocompatibility (MEHNERT; MÄDER, 2001). In addition, if this type of carrier is designed in the micrometric range, it can present lower production costs compared to lipid nanoparticles, since there is no need for equipment such as high pressure homogenizers or microfluidizers (GOMES et al., 2013a; SILVA et al., 2014). The lipids used in the lipid particles are typically biodegradable and present low toxicity (HELGASON et al., 2009). In addition, the use of lipid mixtures in the production of lipid particles presents some advantages such as the formation of a less ordered microstructure of the lipid core, capable of accommodating larger amounts of bioactive compounds as well as preventing their expulsion to the more external parts of the lipid particles (TIKEKAR; NITIN, 2012). The prevention of expulsion to the more external parts of the lipid core leads to a lower probability of oxidation of the encapsulated bioactive compound (PAN et al., 2016).

Regarding the stability of the lipid particles, this depends mainly on their formulation, especially the type of surfactant used. Due to their amphipathic character and food-grade characteristics, proteins are often chosen to stabilize lipid microparticles; including beta-lactoglobulin, sodium caseinate, lactoferrin, soy protein isolate, whey protein concentrate and whey protein isolate (BENICHOU et al., 2007; CHAROEN et al., 2011; LIU; TANG, 2014; McCLEMENTS; GUMUS, 2016; BAI et al., 2016; TEO et al., 2016). The advantages of using whey protein isolate (WPI) as the surfactant include its flexibility and low cost, and the fact that it allows for the formation of a strong film between the oil/water interface during the emulsification process (McCLEMENTS; GUMUS, 2016). The stabilizer efficiency of a protein is strongly dependent on several factors, such as the concentration, pH and ionic strength, and the correct combination of these factors contributes to the formation of a film on the surface of the lipid particles which helps to inhibit the coalescence phenomenon (PALAZOLO et al., 2005).

Carotenes are isoprenoid molecules which contain a hydrocarbon with conjugated double bonds in the structure, and figure as one of the most important bioactive compounds used by the food industry as natural pigments (DONHOWE et al., 2014). These compounds are present in fruits and vegetables and are co-responsible for the yellow, orange and red colors of various vegetables (GONNET et al., 2010). The enhanced immune response, the regulation of the digestive system, and the control of the sugar concentration and cholesterol levels in the blood are some of the beneficial effects attributed to carotenoids (MILLAO; UQUICHE, 2016). Of around 600 different types of carotenoid, beta-carotene (BC) is the most active nutritionally, since it acts as the precursor of vitamin A (CAO-HOANG et al., 2010). These natural pigments are used to standardize the color of food products and also to recover color lost during processing and storage (ARIMBOOR et al., 2015). In this context, there is a continuous and widespread search by the food industry to replace artificial dyes by natural pigments, with a demand that will certainly grow in the future (PRIAMO et al., 2010). However, it should be mentioned that beta-carotene is highly hydrophobic and its physicochemical stability is relatively low, since its molecule can easily undergo oxidation and isomerization, especially in the presence of oxygen (FRASER; BRAMLEY, 2004). Lastly, the improvement of the bioavailability of beta-carotene is a challenge due to its high lipophilicity (YONEKURA; NAGAO, 2007), but this drawback can be overcome by incorporating beta-carotene into lipid-based matrices (PORTER et al., 2007).

In this context, the main objective of this study was to produce beta-carotene-loaded solid lipid microparticles (SLM) with palm stearin (PS) and stabilize them with whey protein isolate (WPI). The microparticles were stored for 40 days and their physicochemical stability evaluated

in the presence of different alpha-tocopherol (ATOC) concentrations. The samples were characterized with respect to size distribution, zeta potential and protective capacity of the encapsulated carotenoid. The most stable dispersions were then submitted to different stress conditions such as the addition of sugar and salt concentrations and changes in temperature. The results of this study will be useful in the production of solid lipid microparticles that are capable of stabilizing beta-carotene for application in food products, with the ultimate aim of replacing part of the artificial colorants commonly used by the food industry.

2 Material and methods

2.1 Materials

Lipid microparticles were produced with palm stearin (donated by Agropalma, Belém, PA, Brazil), whey protein isolate (WPI) (CL3987, donated by Alibra, Campinas, SP, Brazil) and xanthan gum (GRINDSTED Xanthan 80®, Du Pont, Cotia, SP, Brazil). Powdered crystalline beta-carotene was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water was obtained from a Direct Q3 system (Millipore, Billerica, MA, USA) and all other chemicals used in the experiments were of analytical grade. It is important to mention that palm stearin has melting points of approximately 41 °C and 52 °C, due to different lipid fractions (consisting of 50% palmitic acid, 30% oleic acid and a small fraction of 7% to 9% stearic acid). It was thus assured that the solid lipid microparticles presented a solid core at room temperature as well as at the storage temperature (BRITO-OLIVEIRA et al., 2017).

2.2 Production of solid lipid microparticles (SLM)

The microparticles were produced according to the method described by Cazado and Pinho (2016). A 5% (w/v) stock WPI dispersion was first prepared under the same conditions described by Kuhn and Cunha (2012) and at pH 7.0 (neutral and far from the isoelectric point of the WPI, which was determined as approximately 4.0). The WPI dispersion was stored under refrigeration for 12 h until used. The SLMs were prepared by dispersing the aqueous phase containing the surfactant (hydrolyzed WPI) in the lipid phase, composed of melted palm stearin (at 80 °C), by ultra-agitation (T25, IKA, Staufen Germany) at 12,000 rpm for 5 min. Beta-carotene (0.025% w/v) and alpha-tocopherol, when present, were added to the melted lipid phase and sodium benzoate (0.02% w/v) was also added to avoid microbiological contamination. The samples were produced in triplicate and stored in a refrigerator at 10 °C in vials protected from the light.

2.3 Effect of the SLM formulation

Three factors were evaluated to establish the proper conditions to produce stable solid lipid microparticles: WPI concentration, pH and concentration of xanthan gum (if needed) as thickener. Different WPI concentrations were tested (0.5% to 2% w/v) as well as a wide range of pH (8 to 12), aiming to reach the most stable sample for the subsequent tests. Xanthan gum was applied in concentrations that ranged from 0.1% to 0.5% w/v. The polysaccharide was added to the dispersions under magnetic stirring (at 3,000 rpm for 10 minutes) after the ultra-agitation step, while the temperature of the microparticles dispersion decreased to room temperature.

2.4 Effect of the alpha-tocopherol concentration

Different concentrations of alpha-tocopherol were added to the most stable SLM formulation as obtained in the previous step, aiming to verify its antioxidant effect on beta-carotene as follows, producing three formulations: Formulation (A), with no added tocopherol; Formulation (B), mass ratio 2:1 (BC:ATOC), and Formulation (C), mass ratio 1:1 (BC:ATOC). Some studies found in the literature stress the need to add an antioxidant to systems encapsulating beta-carotene (GOMES et al., 2013a,b).

2.5 Determination of the zeta potential and particle size distribution

The average particle size and size distribution curves of the SLM were obtained by laser diffraction (SALD-201V, Shimadzu, Kyoto, Japan), using the Mie theory of light scattering to calculate the particle size distribution, assuming a volume equivalent sphere model. The samples were previously diluted in deionized water and the measurements taken at 670 nm, ranging from 0 to 5 mW with a 0.01 mW resolution. The data were processed by the software included with the instrument (Wing-1) and the polydispersity index (PDI) calculated using Equation 1.

$$PDI = \frac{(d_{90} - d_{10})}{d_{50}} \quad (1)$$

where d_{10}, d_{50} and d_{90} are the diameters at 10%, 50% and 90% of cumulative volume, respectively.

The zeta potential was determined after diluting a drop of the beta-carotene-loaded dispersions in 3M KCl and adjusting the conductivity to 50 mS/cm. The calculation used the principle of electrophoretic mobility and the Helmholtz-Smoluchowksi equation. The data analyses were carried out using the software included with the system.

2.6 Quantification of the beta carotene encapsulated in the lipid microparticles

The beta-carotene in the lipid microparticles was quantified according to Cornacchia and Roos (2011). The samples were first diluted in deionized water (x 50). A 2 mL volume of the dispersion was then mixed with 1.5 mL of

ethanol and 1 mL of methanol saturated with KOH, vortexed, and heated to 45 °C for 30 min. The beta-carotene was extracted after washing the total volume of the vortexed mixture (4.5 mL) three times with 2 mL of n-hexane containing 0.1% w/v butylated hydroxytoluene. The organic solvent was added to the dispersion, the system stirred and left to stand for 10 min. The organic layer, which contained the beta-carotene, was carefully removed and its absorbance measured using a spectrophotometer (Libra 22S, Biochrom, Cambridge, UK) at 450 nm.

2.7 Optical microscopy

The morphology of all samples was characterized using a Bel Photonics BIO3 (Italy) optical microscope with a 1.3 MP camera. All images were captured by Bel View® software. The magnification of the lens corresponded to one hundred times (x100) and no coverslips were required. The samples were diluted x2 in deionized water before visualization.

2.8 Stability of the lipid microparticles under chemical and thermal stresses

The stability of the solid lipid microparticles was also assessed after exposition to different stress conditions commonly found in food-processing operations: different ionic strengths, sucrose concentrations and thermal treatments. These analyses were based on previous studies carried out by Thanasukarn et al. (2006) and Cazado and Pinho (2016).

Effect of the ionic strength: different amounts of sodium chloride (NaCl) were added to the SLM dispersions, resulting in salt concentrations ranging from 0.25 to 1.0 mol/L. The samples were vigorously shaken and stored under refrigerated conditions. Mixing was carried out at room temperature using different stirring methods: (i) mechanically (at 300 rpm, with a Cowles disk impeller) and (ii) magnetically.

Effect of the sugar concentration: different amounts of sucrose were added to the SLM dispersions, resulting in sugar concentrations ranging from 0.5% to 7.0% w/v. The samples were vigorously shaken and stored under refrigerated conditions and mixing was carried out under the same conditions used in the ionic strength tests.

Effect of thermal treatments: the SLM dispersions were heated to temperatures between 40 °C and 80 °C using a water bath. The size distribution of the particles was determined after 2, 5 and 10 min of each thermal treatment. The choice of the time and temperature binomials was based on the studies previously mentioned at the beginning of this section.

2.9 Statistical analyses

All experiments were carried out in triplicate and the data presented as the average ± standard deviation. Tukey tests were used to compare the mean values, adopting a 5% significance level, using SAS® (Cary, NC, USA) software version 9.2.

3 Results and discussion

3.1 Choice of the formula for the solid lipid microparticles

Six WPI concentrations (w/v) were initially tested: 0.5%, 0.75%, 1%, 1.25%, 1.5% and 2%, in the absence of xanthan gum. The dispersions produced with lower WPI concentrations (< 1% w/v) showed higher creaming heights but faster phase separation. This result was probably due to insufficient coverage of the lipid microparticles, since there was little incorporation of surfactant in these systems, allowing for the particles to aggregate by bridging. The remained samples can be seen in Figure 1. Samples prepared with higher concentrations of WPI (≥ 1.0 w/v) remained stable for longer periods of time, but also presented creaming. The lipid microparticles produced with 1.5% w/v and 2% w/v WPI presented intense coagulation due to considerable increases in their viscosities. In this case, this phenomenon arguably occurred due to the presence of an excess of surfactant in the continuous phase of the microparticle dispersions (HIEMENZ; RAJAGOPALAN, 1997).

The lipid microparticles produced with 1.25% w/v WPI were chosen since they presented a less visible and more fragile cream layer. The SLM dispersion produced was submitted to a wide range of pH values, as shown in Figure 2, aiming to find a condition that possibly contributed to a decrease in creaming. Therefore, pH 8 was chosen, since this was the one presenting the smallest cream layer and

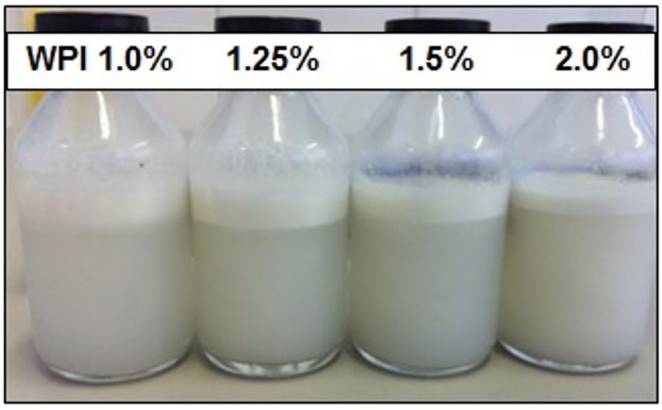

Figure 1. Visual aspect of the solid lipid microparticle dispersions, which were produced using palm stearin, and stabilized with different concentrations (in w/v) of whey protein isolate (WPI), after 24 hours of storage under refrigeration.

a satisfactory zeta potential (-35 mV). In addition, due to the use of WPI in its hydrolyzed form, the thick film formed by the globular proteins, that generally stabilizes the lipid interfaces, was probably not formed, hence contributing to the early destabilization of the systems.

In sequence, in order to eliminate the occurrence of creaming, different concentrations of xanthan gum (XG) were tested as thickener, from 0.1% to 0.5% w/v, and Table 1 shows the results in terms of average particle size of the lipid microparticles. This polysaccharide was added as a stabilizer (by way of its thickening mechanism) because it is commonly used for this purpose in oil-in-water food emulsions (CASAS et al., 2000).

The data shown in Table 1 indicate that only the sample produced with 0.5% w/v XG was significantly different in terms of the zeta potential. On the other hand, the sample produced with 0.3% w/v XG presented a higher average hydrodynamic diameter. After 24h, the samples prepared with more than 0.3% w/v XG exhibited extensive flocculation while the sample with 0.1% w/v XG exhibited slight phase separation. The SLM produced with 0.2% w/v XG appeared to be the most promising one since it was still stable at the end of 7 days of storage, with no signs of creaming or flocculation. The early destabilization of samples containing more than 0.3% w/v XG was probably related to flocculation by depletion, which is caused by the presence of an excess of non-adsorbing polymers in the aqueous phase, which resulted in the particles separating from each other by a distance short enough to exclude the polymer chains. This polymer exclusion led to an osmotic effect due to differences in the concentration of polymers both in the dispersed phase and in the narrow region between the particles, which resulted in attractive interactions and possible flocculation (HIEMENZ; RAJAGOPALAN, 1997; McCLEMENTS, 2005).

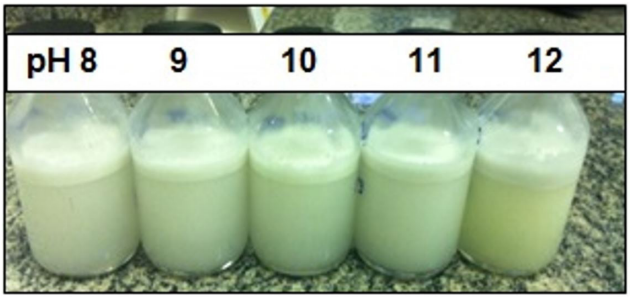

Figure 2. Visual aspect of the solid lipid microparticle dispersions produced using palm stearin and stabilized with 1.25% w/v of whey protein isolate (WPI) under a wide pH range, after 24 hours of refrigerated storage.

Thus, the polymer concentration is a highly important parameter that must be taken into account in systems thickened by non-adsorbing polymers, since the thickener must impose a viscosity high enough to reduce droplet-droplet encounters. On the other hand, the amount of polymer cannot reach saturation levels, since this leads to destabilization of the colloidal system by depletion. Such equilibrium of the effects (an increase in viscosity and a minimization of flocculation by depletion) seemed to occur in the case of lipid microparticle dispersions stabilized with 1.25% w/v WPI as the surfactant and 0.2% w/v XG as the thickener. The increase in viscosity caused by the

Table 1. Average diameter and zeta potential of freshly produced solid lipid microparticles made with 1.25% w/v WPI at pH 8 using different xanthan gum (XG) concentrations as thickener.

Xanthan gum (% w/v)	0.1	0.2	0.3	0.4	0.5
Average hydrodynamic diameter (μm)	$1.39^{B} \pm 0.16$	$1.61^{B} \pm 0.19$	$2.54^{A} \pm 0.42$	$1.67^{B} \pm 0.15$	$1.38^{B} \pm 0.14$
Zeta potential (mV)	$-42.1^{A} \pm 1.56$	$-46.9^{A} \pm 3.93$	$-41.4^{A} \pm 2.50$	$-42.3^{A} \pm 1.77$	$-34.2^{B} \pm 1.47$

Average values with identical letters in the same line were not significantly different ($p > 0.05$) according to Tukey's test.

Table 2. Summary of the characteristics of the beta-carotene-loaded lipid microparticles produced with palm stearin and stabilized with denatured whey protein isolate (WPI).

Parameter	Formulation A		Formulation B		Formulation C	
	1st Day	40th Day	1st Day	40th Day	1st Day	40th Day
Average hydrodynamic diameter (μm)	$0.91^{B} \pm 0.17$	$1.28^{B} \pm 0.19$	$1.16^{B} \pm 0.47$	$3.28^{A} \pm 0.18$	$1.11^{B} \pm 0.51$	$2.62^{A} \pm 0.18$
Polydispersity index	$1.07^{A} \pm 0.16$	$1.20^{A} \pm 0.11$	$0.98^{A} \pm 0.15$	$0.90^{A} \pm 0.03$	$1.00^{A} \pm 0.10$	$0.96^{A} \pm 0.17$
Zeta potential (mV)	$-48.2^{AB} \pm 4.61$	$-35.5^{C} \pm 1.69$	$-48.0^{AB} \pm 3.29$	$-47.2^{AB} \pm 3.38$	$-52.0^{A} \pm 2.82$	$-43.0^{BC} \pm 2.24$
Concentration of beta-carotene (mg/100 g SLM)	$6.11^{C} \pm 2.36$	$5.77^{C} \pm 1.11$	$13.9^{A} \pm 2.44$	$8.17^{BC} \pm 1.89$	$11.3^{AB} \pm 1.43$	$8.34^{BC} \pm 1.50$
% encapsulation efficiency	$24.4^{B} \pm 6.45$	-	$55.6^{A} \pm 9.75$	-	$45.2^{A} \pm 5.72$	-
% beta-carotene preserved	-	94.4	-	58.8	-	73.8

Average values with identical letters in the same line were not significantly different ($p > 0.05$) according to Tukey's test.

presence of 0.2% w/v XG was able to maintain the particles far apart from each other, with little Brownian movement, hence helping to avoid flocculation. Higher concentrations of XG (≥ 0.3% w/v) led to increases in the viscosities of the samples but were not sufficient to overcome destabilization by depletion.

3.2 Physicochemical characterization and stability of the lipid microparticles

The most stable formulation (1.25% w/v WPI, 5% w/v PS, and 0.2% w/v XG) was chosen to continue the beta-carotene encapsulating study. At the end of 40 days of refrigerated storage, the stability of the beta-carotene encapsulating solid lipid microparticles was evaluated according to their average hydrodynamic diameters, particle size distribution, zeta potential value, the amount of beta-carotene preserved and the encapsulation efficiency (% EE). The results were obtained on the first and last days of storage and are shown in Table 2.

The size distribution curves shown in Figure 3 indicate that the average diameters of the SLM were in the range from 1 µm to 2.5 µm. These sizes can be considered to be low, taking into account that the SLM were obtained using a single-step of high shear mixing. In addition, it can be seen that the diameter of the SLM with no alpha-tocopherol (formulation A) increased slightly with storage time since the particles started to agglomerate increasing their hydrodynamic diameter, showing a decay in the surfactant action promoted by the whey protein isolate. For the formulations containing tocopherol (formulations B and C), the appearance of a second particle population could only be seen on the fortieth day of storage, and there was a loss in the unimodal characteristic of the curves. This was also an indication of the particle aggregation phenomena, but was apparently not very extensive, since no phase separation occurred. Such behavior can be expected due to the loss of the stabilizer potential of the xanthan gum. In other words, the break-up of the polymer network allowed for approximation of the particles to each other, leading to the formation of other populations with different sized diameters. In this case, an increase in size led, as a consequence, to a significant increase in the PDI of the particle size distribution. In addition, the PDI of the formulations containing alpha-tocopherol slightly decreased after 40 days, but in general, the PDI did not change significantly with storage time. The zeta potential values, all below –35 mV, also indicated good stability of the systems stabilized with the proposed concentration of whey protein (1.25% w/v).

Figure 4 shows the beta-carotene concentration profile with storage time for all the formulations studied (A, B and C). According to the figure, it can be seen that on the first day of storage the amount of beta carotene retained in formulation A was practically half that encapsulated in the other formulations. Arguably, the alpha-tocopherol presented an antioxidant effect on the beta-carotene as from the production step, when high temperatures were used (80 °C), preserving it for a longer period. Although the exposure to a high production temperature was no longer than 10 minutes, the heat could be sufficient to start the heat-induced oxidation of beta-carotene, leading to the formation of pro-oxidant agents. These pro-oxidant agents may have been able to continue reacting and feed the auto-oxidation reactions of beta-carotene (GOMES et al., 2013a).

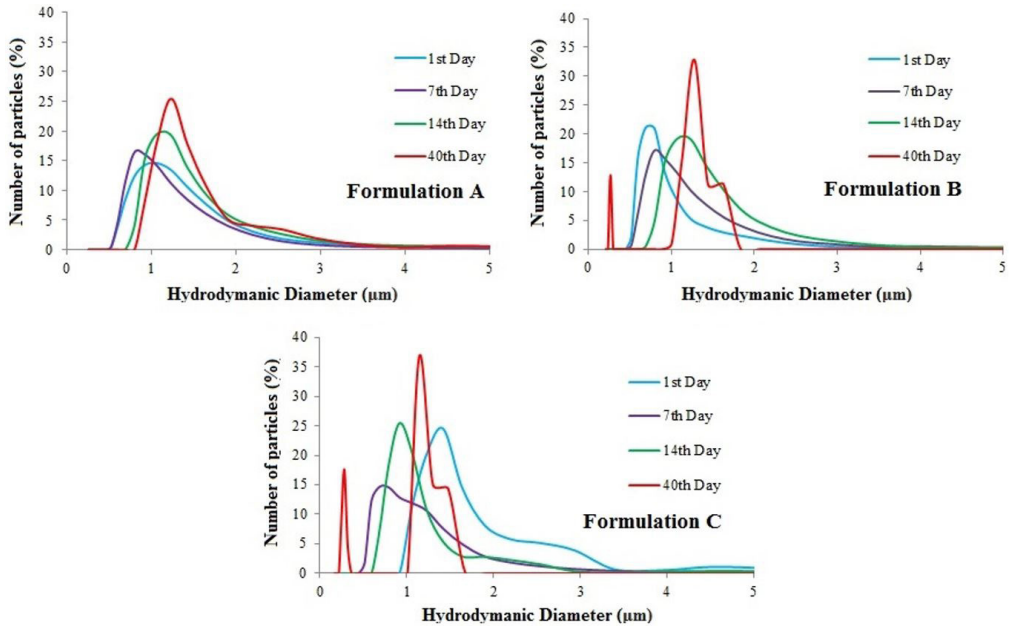

Figure 3. Size distributions of beta-carotene-loaded lipid microparticles obtained with formulations (A) without alpha-tocopherol; (B) mass ratio 2:1 beta-carotene:alpha-tocopherol; and (C) mass ratio 1:1 beta-carotene:alpha-tocopherol.

Comparing formulations B and C, the SLM dispersion with the greater capacity to protect the beta-carotene seemed to be formulation C, containing a larger amount of alpha-tocopherol. According to the data shown in Table 2, about 74% of the initial amount of beta-carotene was present in formulation C at the end of 40 days of storage, whereas only 59% remained in formulation B. In addition, the encapsulation efficiency (% EE) practically doubled in the formulations containing alpha-tocopherol. Other studies have already recognized the essential presence of an antioxidant such as alpha-tocopherol as a protector of beta-carotene in lipid carriers (HENTSCHEL et al., 2008; GOMES et al., 2013a, 2017; BRITO-OLIVEIRA et al., 2017).

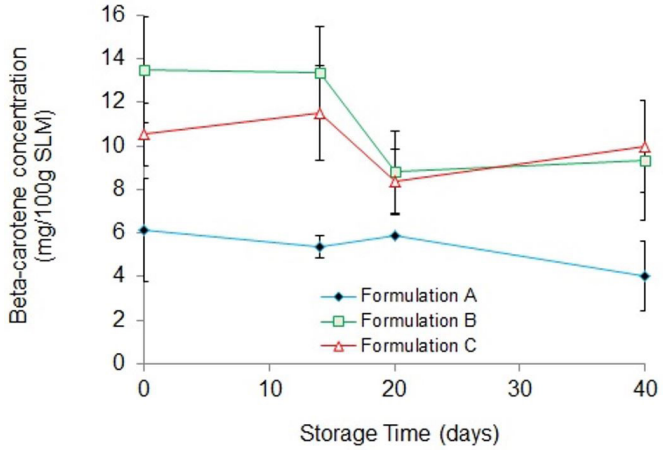

Figure 4. Beta-carotene concentration in solid lipid microparticles throughout the storage time for formulation (A) without alpha-tocopherol; formulation (B) mass ratio 2:1 beta-carotene:alpha-tocopherol; and formulation (C) mass ratio 1:1 beta-carotene:alpha-tocopherol.

3.3 Optical microscopy

Some optical micrographs are shown in Figure 5. The SLMs presented spherical shapes and a smooth appearance. As can be seen, even at the end of their shelf life, the surface morphology continued to present similar size distributions with no indication of flocculation or coagulation, characterizing a stable system from the point of view of particle size.

3.4 Stability of the lipid microparticles submitted to chemical and thermal stresses

Since higher ATOC concentrations did not result in significant improvements in encapsulation efficiency, only Formulation C (mass ratio 1:1 BC:ATOC) was submitted to the stress condition tests, as follows.

3.4.1 Effect of ionic strength

The samples were monitored in relation to BC concentration at the end of 14 days of storage, until the complete destabilization of all the systems. The results shown in Table 3 infer that even on the first day of storage, the samples containing sodium chloride showed less preservation of the encapsulated beta-carotene when compared to the control sample. It can be seen that the larger the amount of salt added to the microparticle dispersions, the smaller the amount of beta-carotene retained in the solid lipid microparticles. The SLM with an added NaCl concentration of 0.25 M preserved about 86% of the initial amount of the carotenoid, whereas the SLM containing 1 M of salt only retained 53% of the initial amount of beta-carotene. These results demonstrate the lower stability of the system in terms of preserving the beta-carotene, when submitted to higher ionic strengths. The results also indicated that the mechanical resistance

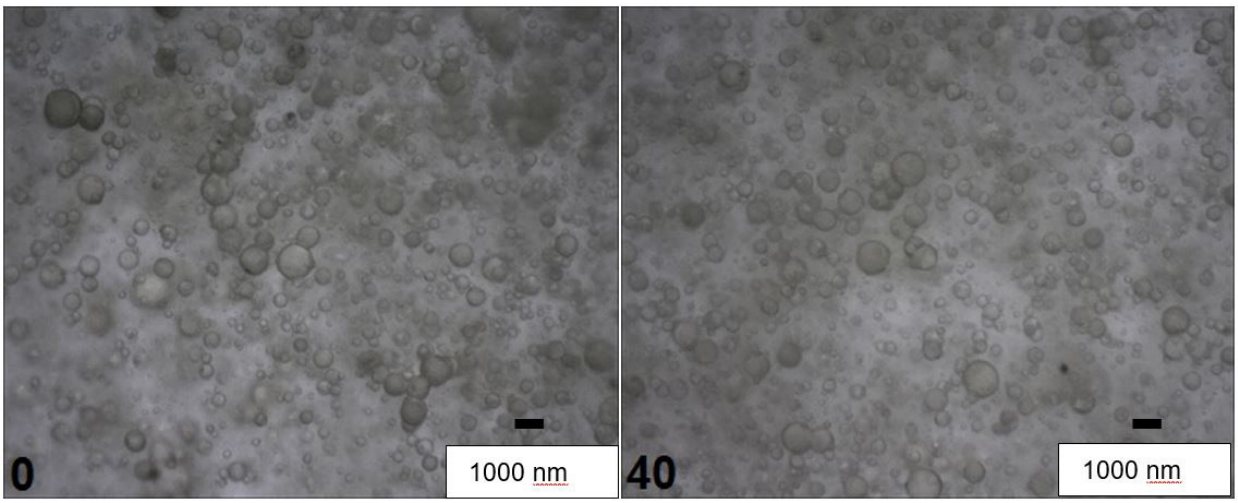

Figure 5. Micrographs of the solid lipid microparticles encapsulating beta-carotene obtained by optical microscopy (x100) for Formulation (C) mass ratio 1:1 beta-carotene:alpha-tocopherol; on the first (left) and last (right) days of storage. Scale: 1000 nm.

Table 3. Beta-carotene concentrations in the solid lipid microparticles produced with a 1:1 (w/v) beta-carotene:alpha-tocopherol ratio (Formulation C), submitted to various ionic strengths.

NaCl (M)	Beta-carotene concentration (mg/100g SLM)			
	Magnetic Stirring		Mechanical Stirring	
	Fresh Samples	After 14-day storage	Fresh Samples	After 14-day storage
Control	$13.5^{Aa} \pm 2.38$	$9.00^{Ba} \pm 0.96$	$9.33^{Ba} \pm 1.24$	$5.84^{Bab} \pm 0.04$
0.25	$8.91^{Ab} \pm 0.91$	$7.66^{ABab} \pm 0.49$	$7.14^{ABa} \pm 1.11$	$5.90^{Bab} \pm 0.41$
0.50	$9.40^{Aab} \pm 1.61$	$6.02^{Bb} \pm 1.34$	$6.94^{ABa} \pm 0.23$	$5.54^{Bb} \pm 0.22$
0.75	$9.09^{Ab} \pm 1.85$	$6.14^{Ab} \pm 1.44$	$7.94^{Aa} \pm 1.03$	$6.38^{Aa} \pm 0.35$
1.0	$11.7^{Aab} \pm 0.64$	$6.26^{Cab} \pm 0.68$	$8.37^{Ba} \pm 1.15$	$5.79^{Cab} \pm 0.25$

Average values with identical uppercase letters in the same line and lowercase letters in the same column were not significantly different ($p > 0.05$) according to Tukey's test.

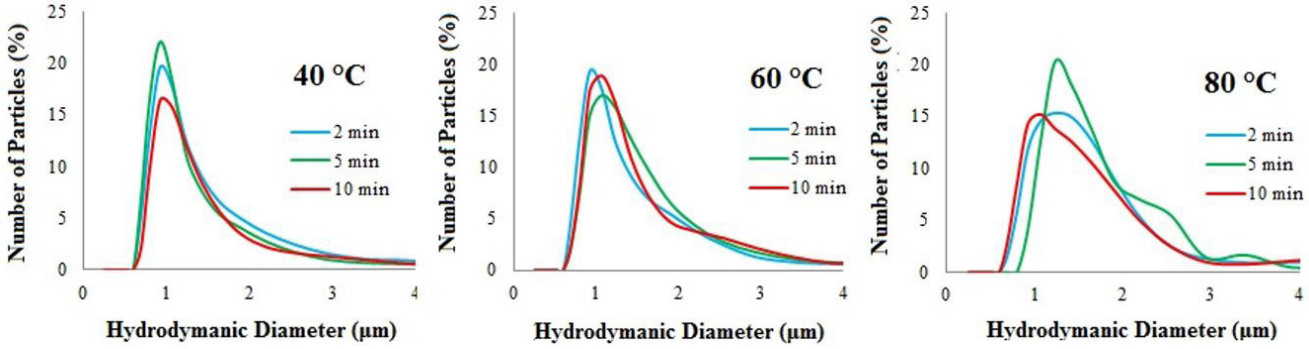

Figure 6. Particle size distribution curves of the beta-carotene-loaded lipid microparticles (Formulation C), when submitted to thermal stress at different temperatures.

produced by the WPI proteins at the interface was not seriously affected by higher salt concentrations, but the salt seemed to affect the permeability of the interface to oxidant species (GOMES et al., 2017).

Comparing the two stirring methods, it was found that of the samples produced by magnetic stirring only that containing 0.25 M of salt retained above 70% of the beta-carotene, whereas of the samples produced by mechanical stirring, all of them retained above 70% of the beta-carotene. These differences probably occurred because the mechanically stirred systems became homogeneous more rapidly and at higher speeds, reducing the probability of "spots" of high solute concentrations. These results showed that particles stabilized with WPI may be more stable in media containing high ionic strengths if the right stirring method is used. Similar results were previously shown by Cazado and Pinho (2016), also working with lipid microparticles produced with WPI, but encapsulating quercetin.

3.4.2 Effect of sucrose concentration

The results of the tests for the stability of beta-carotene-loaded lipid microparticles in the presence of sucrose showed that all the formulations were unstable under all of the conditions tested, since phase separation occurred in all cases after less than 24 h of storage. Disaccharides such as sucrose are known to be capable of destabilizing colloidal particles due to a mechanism similar to depletion flocculation, caused by changes in the balance of the intermolecular forces acting on the oil-water and fat crystal-water interfaces (THANASUKARN et al., 2006).

3.4.3 Effect of thermal stress

Figure 6 shows the size distributions of all the SLM formulations studied after the thermal treatments. As can be seen, no great differences were found in the size distributions even after the application of high temperatures and longer time periods. The microparticles showed no phase separation or signs of agglomeration or coalescence. This result could be of great interest since it indicates that SLMs produced with WPI as the surfactant may be able to resist the high temperatures typically used in important food processing operations, such as pasteurization.

4 Conclusions

The palm stearin lipid microparticles produced with hydrolyzed WPI as the surfactant were satisfactorily stable in protecting the beta-carotene against degradation over 40 days. The results obtained so far led to the conclusion that, with respect to carotenoid preservation, the addition of an antioxidant (such as alpha-tocopherol) to the lipid microparticles is essential. Regarding the stability of the lipid microparticles when submitted to chemical and thermal stresses, although they were relatively unstable

in media containing sucrose, they were highly stable when submitted to high ionic strengths and after thermal treatments. The dispersions showed good stability over a wide temperature range up to 80 °C for long periods such as 10 min, indicating the feasibility and suitability of their use in typical food processing treatments.

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