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Increasing solubility of red bell pepper carotenoids by complexation with 2-hydroxypropyl-β-cyclodextrin

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ABSTRACT

Red bell pepper carotenoids were complexed with 2-hydroxypropyl-β-cyclodextrin (2-HPβCD) in different mass ratios (1:4, 1:6, 1:8 and 1:10) through ultrasonic homogenization in order to increase carotenoid solubility and their use as natural pigment in food. Inclusion complexes, red bell pepper extract and physical mixtures were analyzed by DSC, FT-IR, ^{1}H NMR and DLS. Solubility assay was performed to identify the effect of complexation on the solubility of carotenoids. From characterization assays, results showed that inclusion process occurred for all tested ratios. Results for water solubility assays demonstrated clear differences between solubility index of inclusion complexes (8.06 ± 2.59–16.55 ± 4.40 mg/mL) and physical mixtures (3.53 ± 1.44–7.3 ± 1.88 mg/mL), while carotenoid extract was no water soluble, as expected. These results indicated that molecular inclusion of carotenoids in 2-HPβCD was efficient to enhance their solubility in water, enabling application of red bell pepper carotenoid as natural pigment and/or bioactive substances in food.

1. Introduction

Carotenoids are an important group of lipid-soluble pigments widely distributed in nature, responsible for the yellow, orange and red colors of flowers, fruits, vegetables and plants, where these compounds exert light harvesting function and preventing photo oxidative damage, participating in the photosynthetic system (Abdel Nasser & Omayma, 2013; Hannoufa & Hossain, 2012). In humans, consumption of a balanced diet rich in carotenoids has been associated with the prevention of diseases such as cancer in several tissues (Tanaka, Shnimizu, & Moriwaki, 2012) and macular degeneration (Bernstein, Delori, Richer, van Kuijk, & Wenzel, 2010). Due to the presence of conjugated double bonds, these substances have antioxidant action, deactivating free radicals and quenching reactive oxygen species. Furthermore, some carotenoids are known to exhibit pro-vitamin A activity, due to presence of β-ionone rings in their structures (Khoo, Prasad, Kong, Jiang, & Ismail, 2011). These beneficial effects become a powerful appeal for application of carotenoids in food.

However, their low water solubility and instability in front of light, thermal treatment and oxygen hinder their utilization in food or pharmaceutical formulations. An alternative to increase solubility and stability of organic compounds is the formation of complexes with cyclodextrins, substances able to protect the molecules of interest against external environment factors. Cyclodextrins are cyclic molecules formed by six, seven or eight (α, β, and γ-cyclodextrin) glucose units bound via α-1,4 glycosidic linkages, forming a truncated cone with a hydrophobic cavity and hydrophilic exterior (Kurkov & Loftsson, 2013). These properties allow the insertion of several hydrophobic substances inside the cyclodextrin cavity, turning possible the formation of complex for different purposes.

Sweet bell peppers (*Capsicum annuum* L.) are commonly cultivated and consumed all over the world. Besides being a rich array of carotenoids, bell pepper cultivation is one of the most widespread throughout Brazil and is considered one of the ten species most important in the vegetable market whose annual production reaches approximately 249,000 tons (de Azevedo-Meleiro & Rodriguez-Amaya, 2009). Capsanthin and capsorubin are the main carotenoids found in red bell pepper. These pigments are responsi-

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ble for red color and are synthesized during ripening (Hornero-Mendez, de Guevara, & Minguez-Mosquera, 2000). In our previous study, we investigated the molecular inclusion of red bell pepper pigments in β-cyclodextrin (βCD), with subsequent application in yogurt (Gomes, Petito, Costa, Falcao, & Araujo, 2014). In relation to the color stability of the inclusion complex evaluated in this food matrix, good results were obtained; however, difficulties for incorporating the inclusion complex in matrices of aqueous basis like beverages were observed due the low water solubility of the inclusion complex obtained using βCD. This finding led to the search for alternatives that besides protecting the guest molecules, make possible the application of complex in water-based foods. Therefore 2-hydroxypropyl-β-cyclodextrin (2-HPβCD), a water-soluble modified cyclodextrin, was chosen for this purpose.

Until now, the use of 2-HPβCD is authorized as excipient for drugs by Food and Drug Administration (U.S FDA), while native cyclodextrins (α, β, and γ) are classified like Generally Recognized as Safe (GRAS) for food application (Food & Drug Administration, 2015). In EU, only βCD is allowed as food additive with an ADI of 5 mg/kg/day (European Commission, 2016). Regarding toxicology of cyclodextrins, it is well-known that 2-HPβCD presents a better toxicity profile than the others. Furthermore, toxicological effects may vary according to the type of cyclodextrin and its route of administration. 2-HPβCD can be administered parenterally without generating nephrotoxicity, unlike βCD (Gould & Scott, 2005; Kurkov & Loftsson, 2013; Stella & He, 2008). Generally, oral bioavailability of cyclodextrins is very low, and due to its lack of absorption from the gastrointestinal tract, this route appears to be the safest (Stella & He, 2008).

With this consideration, the aim of this investigation was to prepare inclusion complexes between red bell pepper pigments and 2-HPβCD using four different mass ratios (1:4, 1:6, 1:8 and 1:10) through ultrasonic homogenization. Obtained complexes were characterized by differential scanning calorimetry (DSC), Fourier transform-infrared spectroscopy (FT-IR), proton nuclear magnetic resonance (^{1}H NMR) and particle size distribution. Furthermore, water solubility assays were performed for all inclusion complexes evaluated.

2. Materials and methods

2.1. Materials

The 2-hydroxypropyl-β-cyclodextrin, was purchased from Sigma-Aldrich. Ethyl alcohol (95%), hexane (mixed isomers), acetonitrile (P.A.) were purchased from Vetec (Duque de Caxias, Brazil). Deuterated water (D_2O) and deuterated dimethylsulfoxide (DMSO-D6) were purchased from Sigma-Aldrich.

2.2. Pigment extraction from red bell pepper

Ripe red bell peppers were purchased from a market in Rio de Janeiro. The material was ground in an industrial blender and extraction of the pigments was conducted by maceration in a solvent mixture of ethyl alcohol and distilled water (1:9, v/v) until exhaustion. The extract obtained was partitioned in hexane (1:1, v/v) and the hexane phase was recovered. Solvent was evaporated using a rotatory evaporator under 40 °C. All procedures were conducted in the dark.

2.3. HPLC analysis

Extract from red bell pepper was saponified with 10% KOH overnight in the dark at room temperature to hydrolyze carotenoid esters. After saponification, extract was washed, the residual water was removed with anhydrous sodium sulfate and dried under compressed air. Immediately before injection, the carotenoids pigments were dissolved in HPLC grade acetone and 15 μL aliquots were injected. The HPLC system consisted of a Waters® Alliance 2695 separation module, equipped with an analytical pump, automatic sample injector, a degasser and a DAD detector (PDA 2996). The column was a C30 YMC Waters®, 3 μm, 250 × 4.6 mm. The mobile phase consisted of a gradient elution with methanol and methyl-*tert*-butyl ether. The gradient consisting of solvent A (methanol) and solvent B (methyl-*tert*-butyl ether) was applied at a flow rate of 0.8 mL/min as follows: 80% A linear from 0 to 0.5 min; 75–15% A linear from 0.5 to 15 min; 15–10% A linear from 15 to 15.5 min; 10% A isocratic from 15.05 to 16.5 min; 10–80% A linear from 16.50 to 16.55 min; 80% A isocratic from 16.55 to 28 min. The identification of the carotenoids was based in the retention time and absorption spectrum in UV–Vis, compared with carotenoid standards.

2.4. Preparation of inclusion complexes

Inclusion complexes between red bell pepper extract and 2-HPβCD were prepared in four different mass ratios (1:4, 1:6, 1:8 and 1:10 w/w), aiming to identify higher performance and best efficiency inclusion. A method based on (Chen, Chen, Guo, Li, & Li, 2007) was applied. First, extract of red bell pepper was dispersed in ethanol (1:1, v/v) and 2-HPβCD was dissolved in 45 mL solution of ethanol:water (1:4, v/v) under magnetic stirring at 35 °C. Solutions were mixed using ultrasound probe (Omni Sonic Ruptor 250, Omni International, Kennesaw, GA) at 100 W for 5 min, followed by magnetic stirring until it reached room temperature. Mixtures were stored under refrigeration (10 °C) overnight. To discard extract not included in 2-HPβCD, the medium was centrifuged at 9000 rpm for 20 min. The red layer at the edge of the liquid was removed and discarded. Ethanol was evaporated using a rotatory evaporator. Samples were freeze-dried, weighed and stored protected from light at −18 °C. Yield (based on weight) obtained for each sample was calculated using the following equation:

$$\text{Powder recovery}\,(\%) = 100 \times [\text{recovered powder (complex)} / \text{initial material (red bell pepper extract} + \text{2-HP}\beta\text{CD})]$$

Physical mixtures were prepared by manual stirring of red bell pepper extract and 2-HPβCD in a mortar for 20 min. The mass ratios were the same used for inclusion complexes. Physical mixtures were stored under refrigeration and protected from light.

2.5. Determination of encapsulation efficiency

Inclusion complexes were submitted to repeated extraction steps in order to quantify the carotenoid moiety complexed with 2-HPβCD, according to a methodology previously published (Falcão, Santos, Ortiz-Silva, Seiceira, & Finotelli, 2011), with modifications. Extractions were performed using 10 mg of each complex with 1.0 mL acetonitrile and 0.2 mL ethanol, which were homogenized in an ultrasound bath for 10 min. After, samples were centrifuged (TG-1850 WS Bioridge) at 9000 rpm for 5 min to separate the supernatant of interest. These steps were carried on until the complete extraction of the pigments. Supernatants were adjusted to 25 mL with solvent ethanol:acetonitrile (1:5, v/v). In order to determine the concentration of each sample, spectrophotometric analyzes were conducted on a wavelength of 467.7 nm. Absorbances obtained were compared with a prepared standard curve. The experiment was performed in triplicate. To calculate

encapsulation efficiency, the use of the following equation was required:

$$IE(\%) = 100 \times (\text{measured extract content} / \text{theoretical extract content})$$

2.6. Characterization of inclusion complexes

2.6.1. FT-IR

Fourier transform infrared spectroscopic (FT-IR) spectra of the samples (extract from red bell pepper, 2-HPβCD, inclusion complexes and physical mixtures) were obtained in the range from 500 to 4000 cm^{-1} using Eco-ATR Alpha FT-IR spectrometer (Bruker Corporation), using attenuated total reflection (ATR) without sample preparation. The resolution was 0.01 cm^{-1} .

2.6.2. DSC

DSC measurements of the samples (red bell pepper extract, 2-HPβCD, inclusion complexes and physical mixtures) were performed using an 822 Mettler-Toledo (Mettler Toledo). Calibration was carried out using indium and zinc as reference materials. Samples weighting approximately 3 mg were analyzed in aluminum pan in which a pinhole was punched in the pan lid. During the study, the samples were heated from 25 to 400 °C at a rate of 10 °C/min under a nitrogen purge.

2.6.3. Dynamic light scattering (DLS)

Particle size of 2-HPβCD, inclusion complexes and physical mixtures was measured with ZetaSizer Nano ZS (Malvern Instruments Ltd, UK). 10 mg of each sample were diluted in 3 mL of ultrapure water and placed in acrylic cuvettes for measurement. Analyzes were performed in triplicate.

2.6.4. ^1H NMR

^1H NMR analyzes were performed using Varian VNMRS with frequency of 500 MHz. Solutions were prepared by dilution of 6 mg sample of complexes, physical mixtures, 2-HPβCD and red bell pepper extract in 600 μL of deuterated solvents. For solubilization of complexes, 2-HPβCD and physical mixtures D_2O was used, while DMSO-D6 was used for the extract. Spectra were analyzed using the software SpinWorks 4.

2.6.5. Solubility assay

Solubility assay was performed according to the methodology proposed by Tran, Guo, Song, Bruno, and Lu (2014) with modifications. A sample in excess of red bell pepper extract (0.05 g), inclusion complexes (0.1 g) and physical mixtures (0.1 g) were placed in 10 mL glass tubes jointly with 4 mL of distilled water. Tubes remained in a metabolic shaking bath for 48 h at 27 ± 2 °C to reach equilibrium. After 48 h, samples were centrifuged at 10,000 rpm for 10 min to separate the undissolved samples, followed by filtration through a 0.2 μm cellulose membrane attached to a syringe. An aliquot of 1 mL was selected and volume adjusted to 50 mL with water. After dilution, samples were analyzed by UV–visible absorption spectrophotometry at specific lengths for each sample. The experiment was performed in triplicate.

2.7. Statistical analyses

Yield results, inclusion efficiencies and particle size were statistically evaluated by analysis of variance (ANOVA one way), considering $p < 0.05$ to determine significant differences between means. Averages with significant differences were compared by Tukey test. For solubility assay, particle size and polydispersity index *t*-test was used for unpaired samples, comparing the physical mixtures groups versus complexes with the same proportions, considering $p < 0.05$. Statistical analyzes were performed using GraphPad Prism version 5.01.

3. Results and discussion

3.1. HPLC analysis of carotenoids from red bell pepper extract

Analyzes of HPLC were conducted after saponification of extract. Saponification is required to remove lipids and chlorophylls that may interfere in chromatographic analysis, improving chromatogram resolution. The main carotenoids identified were β-carotene, β-criptoxanthin, 9-*cis*-β-carotene, capsanthin and 13-*cis*-β-carotene (Fig. 1), which is in agreement with results obtained in our previous work (Gomes et al., 2014) and with other authors (Kim, Park, & Hwang, 2004) for most carotenoids found. However, we did not observe the occurrence of capsorubin in the extract used in the present work. Variation in amount and carotenoid profile reported by different authors may be related to maturation stage of the plant evaluated, even the soil conditions where they were cultivated. In immature stage, for example, lutein and neoxanthin are detected and their concentrations are reduced dramatically during ripening. In contrast, concentrations of β-carotene, anteraxanthin and violaxanthin increase and new pigments are synthesized as capsanthin, capsorubin, and β–cryptoxanthin (Hornero-Mendez et al., 2000).

Percent area for each peak was 36.94%, 11.89%, 10.42%, 6.31% e 5.68%, respectively, with predominance of β-carotene and β-criptoxanthin. Naturally, β-carotene and other carotenoids are found as all-*trans* isomers. However, when exposed to factors such as light and heat, they may isomerize to the *cis* form (Khoo et al., 2011). Occurrence of isomers 13- and 9-*cis*-β-carotene indicates that red pepper extract was exposed to these factors, probably during the process of extraction or during saponification.

3.2. Determination of encapsulation efficiency

After lyophilization, inclusion complex samples were weighed to calculate yield, i.e., the powder recoveries for each proportion tested. Percent yields of the complexes were obtained by comparing the mass of each complex obtained in relation to the sum of the masses of 2-HPβCD and extract used initially. Results showed no significant difference between the different complexes ($p > 0.05$), with yields of 91.15 ± 1.73 (1:4), 92.71 ± 1.24 (1:6), 90.75 ± 1.24 (1:8) and 93.03 ± 1.99 (1:10). These yields are higher than those obtained in our previous work with β-cyclodextrin (Gomes et al., 2014) which reached values as 40.79% ± 2.76 and 54.48% ± 3.60 for magnetic stirring and the ultrasonic homogenizing procedures, respectively. Regarding the inclusion efficiency, the percentage was obtained by comparing the mass of extract retained in the complexes relative to the extract mass used in the inclusion process. The obtained averages were 81.87% ± 2.44, 75.17% ± 7.53, 69.03% ± 3.27 and 69.67% ± 3.73 for the samples 1:4, 1:6, 1:8 and 1:10, respectively, with significant difference between only 1:4 and 1:8 proportions ($p < 0.05$), indicating that inclusion in molecular ratios used in general did not promote pronounced differences in relation to carotenoid inclusion performance. According to the literature, the inclusion efficiency and solubility increase as the amount of cyclodextrin is available to complex formation (Carvalho & Pinto, 2012; Piel et al., 2006). However, in this study this effect was not observed in relation to the inclusion efficiency.

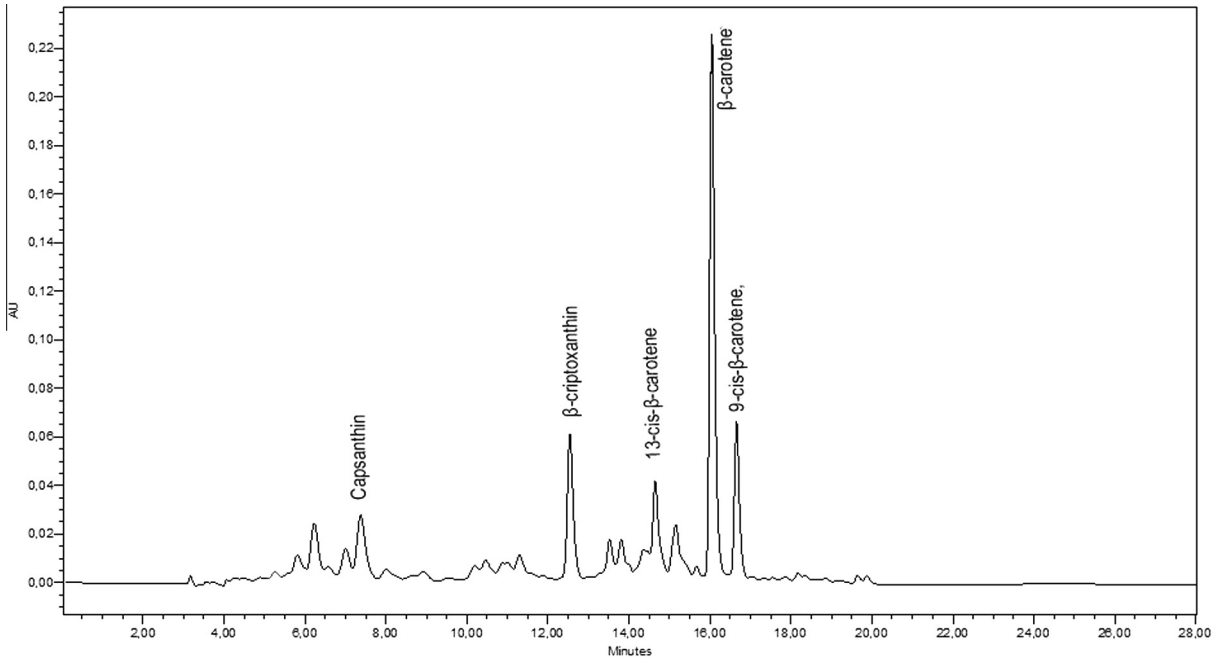

Fig. 1. Chromatographic profile of the saponified extract from red bell pepper.

3.3. Characterization of the complexes

3.3.1. FT-IR

FT-IR spectra of 2-HPβCD, red bell pepper extract, inclusion complexes, and physical mixtures can be seen in Fig. 2. In 3396 cm^{-1} region of red bell pepper extract spectrum (b), it can be observed a broad and weak band related to the presence of hydroxyl (OH), commonly found in the structures of xanthophylls. Two intense bands located at 2922 cm^{-1} and 2853 cm^{-1} can be identified as characteristics of the axial deformation of C—H bonds of carotenoids structural carbons. Another intense band at 1742 cm^{-1}, corresponding to the axial deformation of ketone carbonyl groups (C═O) characteristics of some pigments present in extract. The small band in the region of 1657 cm^{-1} refers to an axial deformation of C═C. Bands at 1460, 1376 and 724 cm^{-1} regions correspond to angular deformations of C—H bonds, representing the symmetrical deformation of methylene (CH_2), asymmetric deformation of methyl (CH_3) and asymmetric deformation of methylene, respectively. Referring to 2-HPβCD spectrum (a) it is observed an intense and broad band in the region of 3500–3000 cm^{-1}, characteristic of hydroxyl groups (—OH). The 2924 cm^{-1} region of cyclodextrin corresponds to axial strain C—H bonds and 1151, 1080 and 1024 cm^{-1} bands are stretching vibration characteristics of the C—H and C—O groups. Another important vibrational band is located in the region of 946 cm^{-1} related to the α-1,4 glucosidic bonds of cyclodextrin structure. Spectra for inclusion complexes (c, e, g, i) are very similar to that of 2-HPβCD. Characteristic extract bands such as those at 2922 and 2853 cm^{-1} and at 1742 cm^{-1} became weaker or almost disappeared in the spectra of complexes, indicating the occurrence of molecular inclusion. On the other hand, bands in the region of 2922 and 1742 cm^{-1}, identified as C—H and C═O of extract are most prominent in the spectra of the physical mixture (d, f, h, j) indicating the overlap of the extract signals on cyclodextrin, pointing deficiency in molecular inclusion. Usually, evidence of molecular inclusion is observed by comparing the spectra of cyclodextrin, the complex and the isolated sample of interest, in this case, red bell pepper extract. In complex spectra, attenuation of the characteristic extract peaks indicates an efficient molecular inclusion, while the prevalence of these on cyclodextrin peaks indicates deficiency in the process. Therefore, it is desirable similarity between complex and cyclodextrin spectrum, indicating the efficient allocation of extract molecules inside the cavity of cyclodextrin (Aleem, Kuchekar, Pore, & Late, 2008). A similar evaluation in relation to inclusion of sufetanil, a synthetic analgesic opioid, inside 2-HPβCD cavity was shown (Volobuef et al., 2012).

3.3.2. DSC

DSC curves of red bell pepper extract (a,b), 2-HPβCD (a,b), inclusion complexes (a) and physical mixtures (b) are presented in Fig. 3. 2-HPβCD thermogram exhibited a broad endothermic peak around 80 °C, which is related to water loss. Comparing cyclodextrin and complexes thermograms, it is observed the shift of peaks, which may indicate the molecular inclusion (Mohan, Sreelakshmi, Muraleedharan, & Joseph, 2012; Rajendiran, Mohandoss, & Saravanan, 2014). From approximately 300 °C, there are several endothermic peaks, characteristics degradation of the cyclodextrin. Moreover, the endothermic peaks observed in red pepper extract thermogram, located in the region between 230 and 390 °C, that appear to be degradation and melting point of the sample are not present in the complexes, fact that also shows the inclusion of pigments in cyclodextrin cavities of all complex samples (Liu, Zhu, Zeng, & Zhao, 2013; Mura, 2015). Regarding the physical mixtures thermograms (b), different effects were observed. Initially, there is absence of peaks on the endothermic release of water in 1:4 to 1:8 samples. In both samples, can be observed different peaks from those in cyclodextrin and overlap of the characteristic extract peak present in temperatures close to 230 °C for the physical mixture 1:8, and close to 250 °C for physical mixture 1:4. Furthermore, there was an atypical peak in the region of 190 °C for sample 1:8, characterizing degradation. These results indicate little or no interaction between extract and cyclodextrin in physical mixtures (Yao et al., 2014).

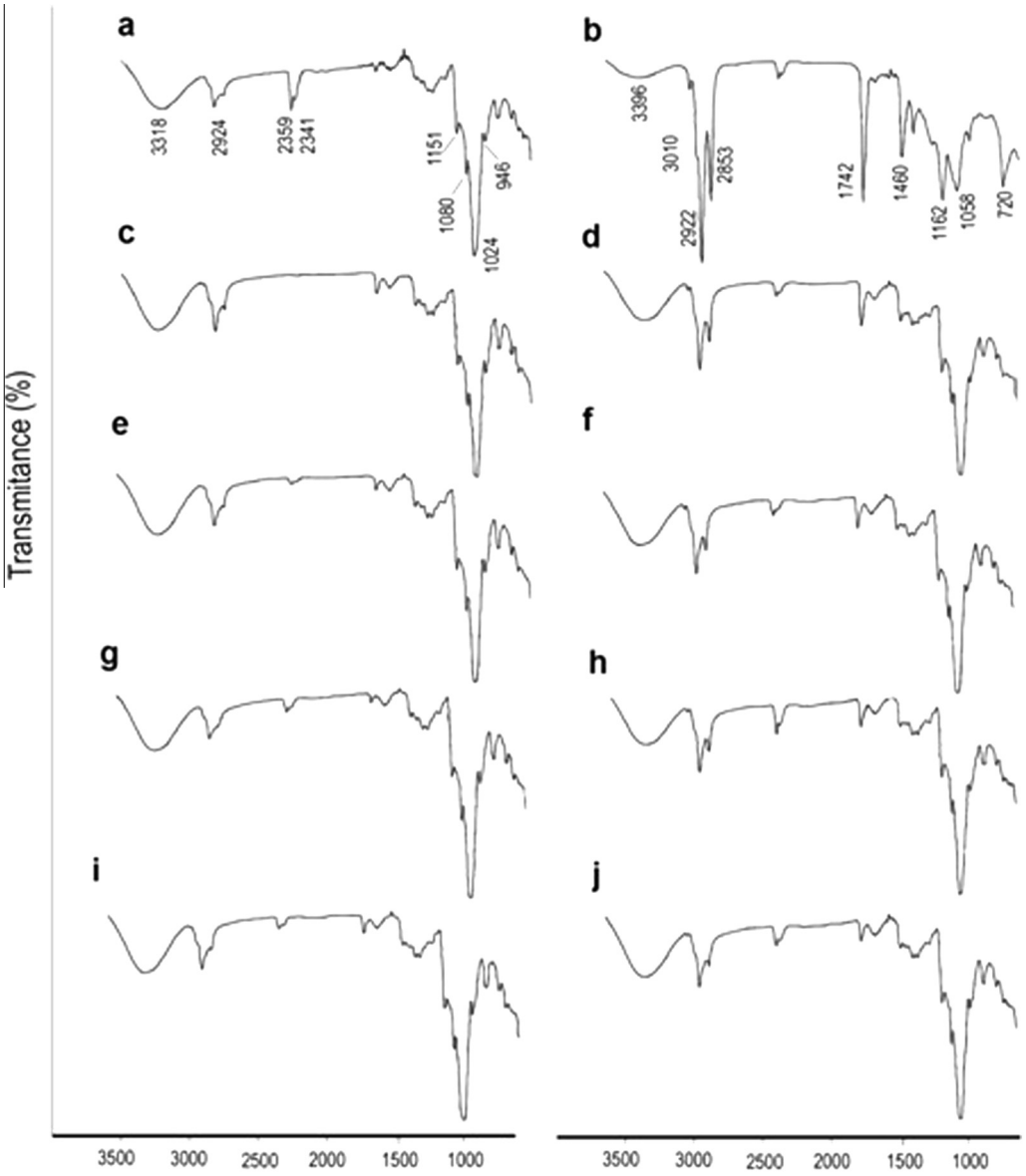

Fig. 2. FT-IR spectra of 2-HPβCD (a), red bell pepper extract (b), complex 1:4 (c), physical mixture 1:4 (d), complex 1:6 (e), physical mixture 1:6 (f), complex 1:8 (g), physical mixture 1:8 (h), complex 1:10 (i) and physical mixture 1:10 (j).

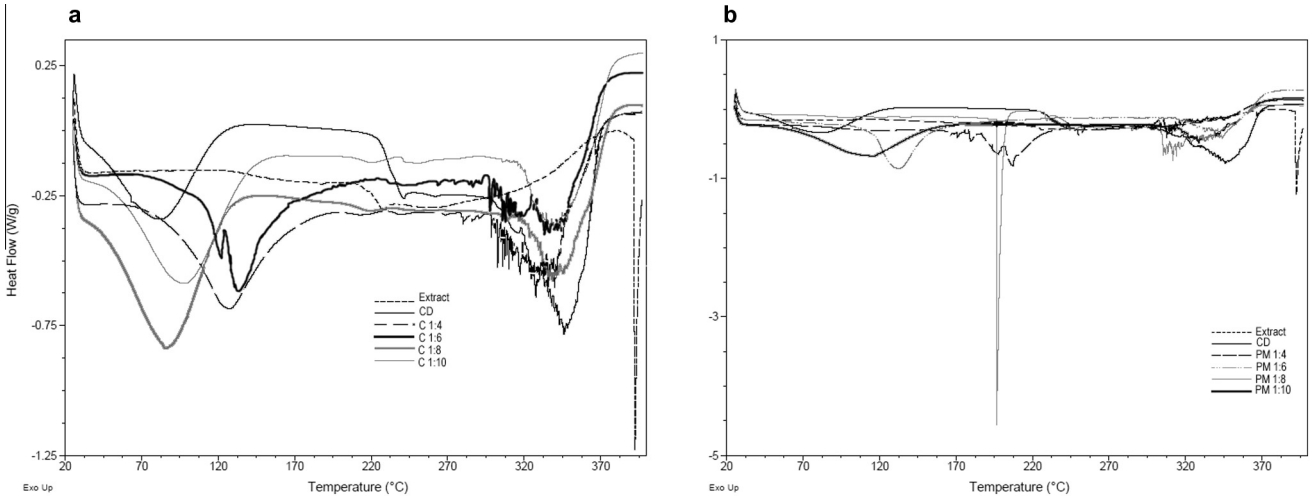

Fig. 3. DSC thermogram of red bell pepper extract (Extract), 2-HPβCD (CD), complex (C) 1:4, 1:6, 1:8, 1:10 and physical mixtures (PM) 1:4, 1:6, 1:8, 1:10.

3.3.3. DLS

DLS analysis is a widely used methodology in the research of nanoparticles, aiming to characterize the solution of interest, determining particle size and its distribution in suspension. Suspensions with small particle size and homogeneous distribution in the media are desired, which indicates greater stability. The analysis indicated that 2-HPβCD had a mean particle diameter of 255.6 ± 29.06 nm, while inclusion complexes had an average of 338.6 ± 40 nm (1:4), 363.6 ± 68.85 nm (1:6), 312.4 ± 12.78 nm (1:8), 287.8 ± 30.12 nm (1:10).

In our previous work, it was obtained for inclusion complexes an average size of 562 ± 36.7 nm (Gomes et al., 2014). According to Muñoz-Ruiz and Paronen (1997) 2-HPβCD has a very small particle size, while β-cyclodextrin has one of the biggest particle size reported. Furthermore, cyclodextrins are known to form aggregates, principally native, where outside hydroxyl groups participate in aggregation process. Meanwhile, modified cyclodextrins, are less susceptible to form aggregates, explaining their smaller particle size (Messner, Kurkov, Jansook, & Loftsson, 2010).

Physical mixtures showed an irregular profile with different particle sizes, then for the most part of the population, an average size of 1232 ± 219 nm (1:4), 1623 ± 316 nm (1:6), 1273 ± 385 nm (1:8) and 1130 ± 256 nm (1:10) was obtained, also indicating aggregation. Comparing particle sizes of complexes with physical mixtures in the same ratio groups there were significant differences for all samples. Among the samples of inclusion complexes and 2-HPβCD there was no significant difference in particle size ($p > 0.05$).

Results indicated that simple mixing of the components is not able to form stable complexes that can be applied in solution. Moreover, if there was no molecular inclusion, free carotenoids which have remained on the surfaces of the 2-HPβCD may agglomerated due to their hydrophobic character, being an aggregation promoter or forming a micellar-type aggregates, which contributes to the increase of particle size (Garnero, Zoppi, Longhi, & Genovese, 2010).

Polydispersity index (PDI), which is the measure of distribution particle size, was determined. It is desirable that the values for this index are lower than 0.5, which indicates the homogeneity of the sample. Complexes and physical mixtures distribution size are shown in Fig. 4. The values obtained for the samples of inclusion complexes in the proportions 1:4 (0.390 ± 0.056), 1:6 (0.308 ± 0.091), 1:8 (0.354 ± 0.032) and 1:10 (0.319 ± 0.054) had polydispersity indexes around 0.3, indicating that these are monodisperse suspensions. However, physical mixture samples showed values above 0.7 (0.796 ± 0.177, 0.989 ± 0.018, 0.403 ± 0.119, 0.971 ± 0.050, respectively for 1:4, 1:6, 1:8 and 1:10 proportions) with the exception of sample 1:8. Results indicated there was no uniformity among the particle size of physical mixtures suspensions, featuring a heterogeneous profile. Among inclusion complexes samples and physical mixtures of same proportion groups, there was significant difference comparing complexes and physical mixtures, except for the samples 1: 8.

3.3.4. ^{1}H NMR

Proton Nuclear Magnetic Resonance (^{1}H NMR) is a methodology widely used in the characterization of molecular inclusion. This becomes possible by observation of the chemical shifts caused by inclusion complex formation. Protons located inside the structure of cyclodextrins (H3, H5 and H6) are sensitive to environmental change, so there is chemical shift, indicating the presence of the substance of interest in the cavity. In contrast, protons present on the surface of cyclodextrins (H2 and H4) must undergo subtle deviations, because they do not participate in the process of inclusion (Kfoury, Auezova, Greige-Gerges, Ruellan, & Fourmentin, 2014; Yuan, Du, Jin, & Xu, 2013).

In agreement with the results obtained by various authors (Ma et al., 2012; Qiu et al., 2014; Yang, Lin, Chen, & Liu, 2009) it can be observed the presence of the characteristic protons in cyclodextrins structure (H1, H2, H3, H4, H5, H6) in the present work, which served as reference for analysis of the chemical shifts obtained, as shown in Table 1.

However, NMR spectra of complexes showed no significant chemical shifts, resulting in displacements from 0.000 to 0.012 ppm ($\Delta\sigma$, in Table 1), both for external as for internal protons. Regarding physical mixtures, large chemical shift was expected in external protons due to the possibility of carotenoids allocation on cyclodextrins surface. However, similar to the displacements obtained for inclusion complexes, small variations were observed (from 0.001 to 0.016 ppm) for these samples.

Although several authors have reported the use of this methodology for assessing the effect of inclusion on molecules (Chin et al., 2015; Siva, Kothai Nayaki, & Rajendiran, 2014), it was not possible to extract relevant information from analysis by ^{1}H NMR. Observed chemical shifts were minimum and similar between inner and outer protons of 2-HPβCD, which precludes any statement about the occurrence of molecular inclusion. These results are in agreement with those ones obtained in our previous work, in which we evaluate the inclusion of red bell pepper carotenoids inside βCD by ultrasonic stirring (Gomes et al., 2014) and we also could not observe any change between βCD and inclusion complexes in relation the chemical shifts. However, it is described that inclusion complexes with low association constants often display insignificant chemical shifts (Wimmer, Aachmann, Larsen, & Petersen, 2002), and this statement could explain the small chemical shifts differences obtained for the inclusion complexes found in the present work. In despite of the results obtained by use of ^{1}H NMR, direct evidence of inclusion was obtained through the use of other methodologies described above.

3.3.5. Solubility assay

Results of solubility assay are shown in Fig. 5. It was found that there was no significant difference ($p > 0.05$) between physical mixtures and complexes of 1:4 and 1:6 samples although all values obtained for complexes were of higher magnitude in relation to the respective physical mixture. Regarding samples 1:8 and 1:10 there was significant difference ($p < 0.05$) between complexes and physical mixtures. All complexes samples were significantly different of extract sample. Results showed an average solubility about 8.00 ± 2.6 mg/mL for the sample complex 1:4, 8.24 ± 4.36 mg/mL for the complex 1:6, 14.54 ± 3.21 mg/mL for the complex 1:8 16.55 ± 4.42 mg/mL for 1:10 complex.

In relation to physical mixtures, there was no significant difference ($p > 0.05$) between samples or with respect to the extract. The average solubility values found for physical mixtures samples were 3.53 mg/mL for the sample 1:4, 2.63 mg/mL for the 1:6, 6.31 mg/mL for the 1:8 and 7.32 mg/mL for the 1:10. For red pepper extract sample solubility was 0.025 mg/mL. After the molecular inclusion of red bell pepper pigments, it was observed for complexes samples a minimum aqueous solubility of 8.00 mg/mL and the maximum 16.55 mg/mL, which indicates an increase of 320–660 times the solubility of extract, demonstrating the effectiveness in the increasing of carotenoids solubility by molecular inclusion.

In previous work of our research group, the difficulty of incorporating the red bell pepper:β-cyclodextrin complex in water-based media was observed. Samples easily precipitated, limiting the possibility of incorporation in various foods, especially beverages. Solubility data obtained in this study expands the applicability of carotenoids in a wide variety of foods such as desserts, juices, teas, soft drinks, jellies, candy, popsicles, soluble powders, among others.

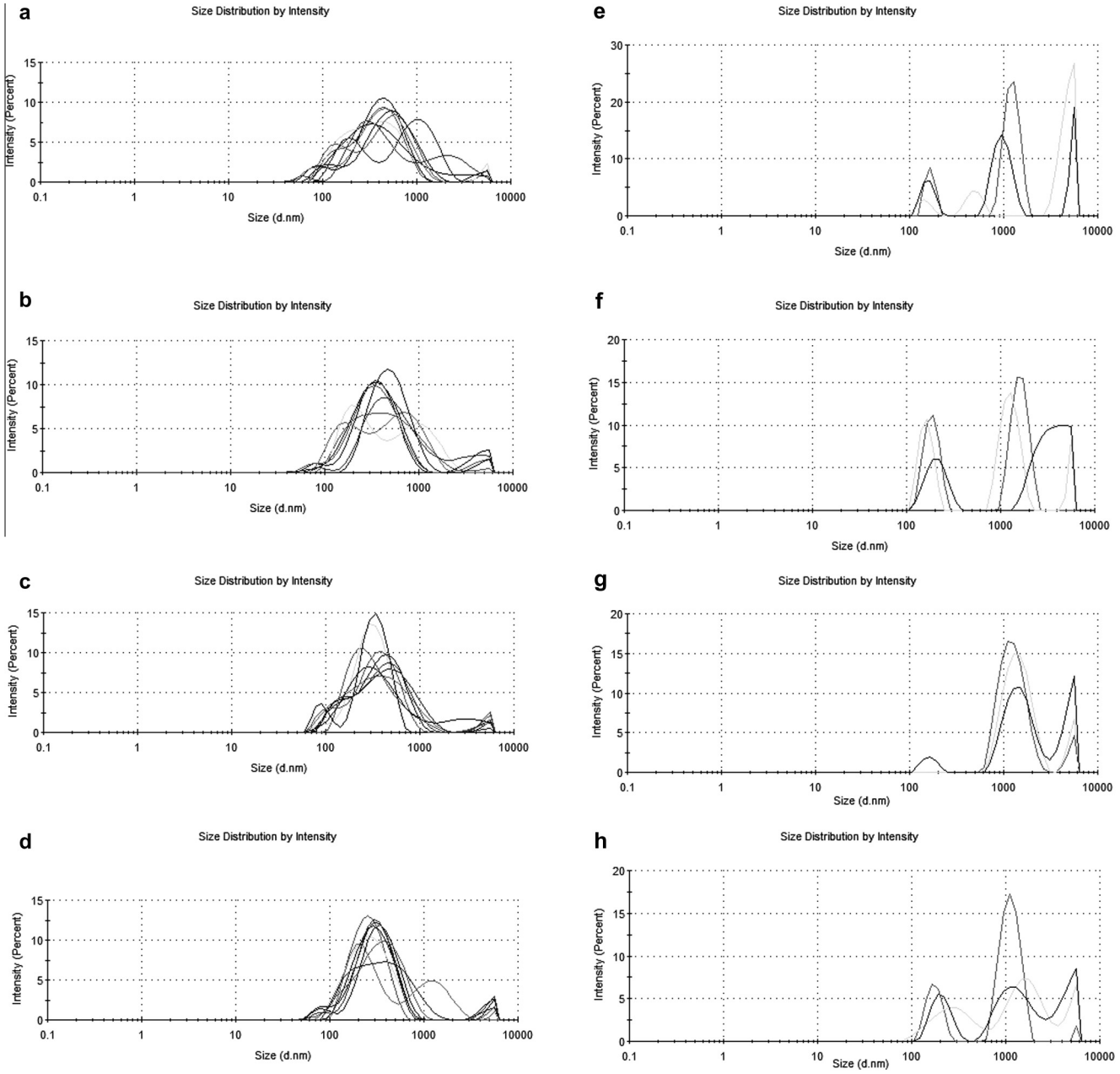

Fig. 4. Particle size distribution of complex 1:4 (a), complex 1:6 (b), complex 1:8 (c), complex 1:10 (d), physical mixture 1:4 (e), physical mixture 1:6 (f), physical mixture 1:8 (g), physical mixture 1:10 (h).

Table 1
Chemical shifts (ppm) of specific protons obtained from the ^{1}H NMR spectra of 2-hydroxypropyl-β-cyclodextrin (2-HPβCD), inclusion complexes and physical mixtures and differences ($\Delta\sigma$) compared to 2-HPβCD spectrum.

		2-HPβCD	1:4	$\Delta\sigma$	1:6	$\Delta\sigma$	1:8	$\Delta\sigma$	1:10	$\Delta\sigma$
Complexes	H_1	5.162	5.160	**0.002**	5.162	**0.000**	5.157	**−0.005**	5.160	**0.002**
	H_2	3.811	3.803	**0.004**	3.810	**0.001**	3.802	**−0.009**	3.799	**0.012**
	H_3	4.109	4.101	**0.008**	4.107	**0.002**	4.100	**−0.009**	4.100	**0.009**
	H_4	3.722	3.729	**−0.007**	3.721	**0.001**	3.729	**−0.007**	3.726	**−0.004**
	H_5	3.957	3.951	**0.006**	3.956	**0.001**	3.948	**−0.009**	3.947	**0.010**
	H_6	3.957	3.951	**0.006**	3.956	**0.001**	3.948	**−0.009**	3.947	**0.010**
Physical mixtures	H_1	5.162	5.176	**−0.014**	5.161	**0.001**	5.164	**0.002**	5.159	**0.003**
	H_2	3.811	3.819	**−0.008**	3.804	**0.007**	3.806	**0.005**	3.798	**0.012**
	H_3	4.109	4.118	**−0.009**	4.103	**0.006**	4.106	**0.003**	4.098	**0.011**
	H_4	3.722	3.731	**−0.009**	3.714	**0.008**	3.717	**0.005**	3.706	**0.016**
	H_5	3.957	3.966	**−0.009**	3.950	**0.007**	3.954	**0.003**	3.945	**0.012**
	H_6	3.957	3.966	**−0.009**	3.950	**0.007**	3.954	**0.003**	3.945	**0.012**

The bold values are the differences in chemical shifts of specific protons between 2-HPβCD and inclusion complexes or physical mixtures.

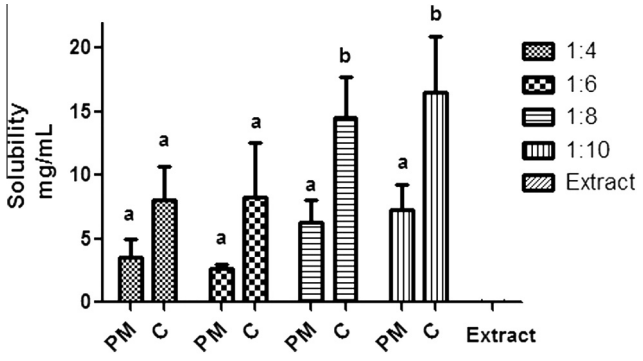

Fig. 5. Solubility indexes of complexes (CD), red bell pepper extract (Extract) and physical mixtures (PM). Values given are averages of 3 replicates ± standard deviations. Average values with differing superscript letters (between columns of same proportion group) indicate significantly different values ($p < 0.05$).

The analysis used for inclusion complexes characterization revealed the occurrence of molecular inclusion for all the mass ratios studied, compared to physical mixtures. DSC, FT-IR, DLS and solubility assay were able to identify chemical and spatial changes after the complex formation. Solubility assay demonstrated that complexation with 2-HPβCD increased aqueous solubility of red bell pepper extract up to 660 times, proving to be promising the application of complexes in water-based food.

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