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Inclusion complexes of red bell pepper pigments with β-cyclodextrin: Preparation, characterisation and application as natural colorant in yogurt

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ABSTRACT

This work aimed to prepare inclusion complexes between red bell pepper pigments and β-cyclodextrin using two different procedures (i.e., magnetic stirring and ultrasonic homogenisation), to characterise the prepared inclusion complexes and to evaluate the colour stability of a selected complex added to yogurt. The mass ratio of extract to β-cyclodextrin was 1:4. The formed extract: β-cyclodextrin complexes and a physical mixture of extract and β-cyclodextrin were evaluated by differential scanning calorimetry, Fourier transform-infrared spectroscopy, proton nuclear magnetic resonance, particle size distribution and Zeta potential. The obtained data showed that ultrasonic homogenisation resulted in better yield and inclusion efficiency compared to magnetic stirring. The yogurt with the added complex produced by ultrasonic homogenisation showed slower variations for the a^* (redness) and b^* (yellowness) indices compared to yogurt with added extract, indicating a higher protection of the colour during storage.

1. Introduction

Among the Solanaceae family, the sweet bell pepper (*Capsicum annuum*) is used prominently in the culinary world due to its strong flavour and intense colour. In Brazil and in some other countries, the sweet bell pepper is considered one of the most important vegetables in terms of both volume production and commercial value (Azevedo-Meleiro & Rodriguez-Amaya, 2009). Aside from its sensory properties, sweet bell pepper is also a good source of bioactive compounds, such as the carotenoids (Kim, Park, & Hwang, 2004). These substances can reduce the risk of developing degenerative disease, such as macular degeneration in women (Moeller et al., 2006), inhibit carcinogenic activity (Nishino et al., 2002) and reduce (50% reduced rate) posterior subcapsular cataracts in subjects with higher plasma lutein concentration (Gale, Hall, Phillips, & Martyn, 2003). These effects have sparked interest in the food industry to add such compounds to functional foods, allowing them to be more bioavailable than their natural sources (Shi & Le Maguer, 2000).

Capsanthin (3,3′dihydroxy-β,κ-carotene-6′-one) and capsorubin (3,3′dihydroxy-κ,κ-carotene-6,6′-dione) are the pigments responsible for the red coloration of the capsicum species (Kim et al., 2004); they are synthesised during maturation (Azevedo-Meleiro, Rodriguez-Amaya, 2009; Hornero-Méndez, Gomez-Ladron, & Mínguez-Mosquera, 2000). Capsanthin may represent as much as 50% of the total carotenoids in red bell pepper (Locey & Guzinski, 2000) and has been associated with prevention of colon carcinogenesis (Narisawa et al., 2000).

Synthetic food additives have been used for the purposes of flavouring, colouring and extending the useful shelf-life of food. However, using colorants is one of the most controversial activities of the food industry because of the toxicological potential of synthetic colorants (Reyes, Valim, & Vercesi, 1996; Mizutani, 2009). Studies report the development of allergies in children (Inomata, Osuna, Fujita, Ogawa, & Ikezawa, 2006) and the increased risk of cancer associated with the consumption of artificial colorants (Sasaki et al., 2002). Therefore, the need to use safe colorants has prompted researchers to extract colorants from natural pigment sources that would be suitable for food applications (Moreira et al., 2012; Rutkowska & Stolyhwo, 2009). However, some natural pigments, such as carotenoids, exhibit extremely high lipophilicity, low aqueous solubility and sensitivity to air and light that may impair their employment as food additives (Vertzoni, Tkartezini, Reppas, Archontaki, & Valsami, 2006).

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A proven alternative for increasing the solubility, stability, and bioavailability of lipophilic organic molecules is their inclusion in the cavity of cyclodextrins (Garnero, Zoppi, Genovese, & Longhi, 2010). Cyclodextrins are cyclic molecules containing six, seven or eight D-glucose units, namely α-cyclodextrin, β-cyclodextrin, and γ-cyclodextrin, respectively (Chen et al., 2011); they may also be chemically modified to change their properties (Bricout, Hapiot, Ponchel, Tilloy, & Monflier, 2009). Whilst the external surfaces are hydrophilic, the internal cavities of cyclodextrins are relatively hydrophobic; these properties allow them to form stable inclusion complexes with several interesting compounds, for health products ranging from drugs (Garnero et al., 2010) to food compounds (Navarro et al., 2011). Despite their poor solubility in aqueous media and low stability during food processing and storage, natural pigments may be optimised by complexation with cyclodextrins, which may allow for the possible development of new colorants for food applications.

With this consideration, the aim of this investigation was to prepare inclusion complexes between red bell pepper pigments and β-cyclodextrin using two different procedures, namely magnetic stirring and ultrasonic homogenisation. The obtained complexes were characterised by differential scanning calorimetry (DSC), Fourier transform-infrared spectroscopy (FT-IR), proton nuclear magnetic resonance (^{1}H NMR), particle size distribution and Zeta potential. It is well known that changes in physicochemical properties can be observed when a guest molecule is incorporated inside the cyclodextrin cavity (Singh, Bharti, Madan, & Hiremath, 2010). Furthermore, an inclusion complex was added to yogurt and its stability as a natural colorant was evaluated.

2. Materials and methods

2.1. Materials

β-Cyclodextrin was purchased from Sigma–Aldrich (St. Louis, MO). Reagent grade ethyl alcohol (95%) and hexane (mixed isomers) were purchased from Vetec (Duque de Caxias, Brazil). Deuterated water (D_2O) was purchased from Sigma–Aldrich. Cellulose acetate membranes (0.2 μm and 47 mm) were purchased from Sartorius Stedim (Göttingen, Germany.

2.2. Pigment extraction from red bell peppers

Ripe red bell peppers (*C. annuum* cv. annuum) were purchased from a market in Rio de Janeiro and dried in a ventilated oven at 55 °C for 24 h. The dried material was powdered using an industrial blender then used for extraction. The pigments were extracted by maceration of 1.63 kg of red bell pepper powder using a mixture of ethyl alcohol and water (90:10, v/v) until exhaustion. The extract obtained was partitioned in hexane PA (1:1, v/v). The organic solvent was evaporated from the hexane extract under reduced pressure at 40 °C using a rotatory evaporator. The extract obtained was analysed by HPLC for carotenoids then complexed with β-cyclodextrin. Its application as a natural colorant in yogurt was evaluated for colour stability.

2.3. HPLC analysis

The extract from the red bell peppers was saponified with 10% KOH overnight in the dark at ambient temperature to hydrolyse the carotenol esters. After saponification, the extract was washed, and the residual water was removed with anhydrous sodium sulfate and dried under compressed air. Immediately before injection, the carotenoid pigments were dissolved in HPLC-grade acetone. Aliquots of 15 μL were injected into an HPLC system consisting of a Waters Alliance 2695 separation module equipped with an analytical pump (Waters Corporation, Milford, MA), an automatic sample injector, a degasser, and a diode array detector (PDA 2996). The column used was a 3 μm C30 YMC Waters® (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 15.5 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 chromatogram was monitored at 450 nm. The identification of the carotenoids was based on the retention time and absorption spectrum in UV–Vis, compared with carotenoid standards previously isolated in the laboratory according to Pacheco et al. (in press).

2.4. Preparation of inclusion complexes of red pepper pigments with β-cyclodextrin

A precipitation method was used to prepare the inclusion complexes according to Chen, Chen, Guo, Li, and Li (2007) with modifications, using a ratio of 1:4 (w/w) of bell pepper extract to β-cyclodextrin. The red bell pepper extract (0.5 g) was dispersed in ethyl alcohol (0.5 mL), and β-cyclodextrin (2 g) was dissolved in 45 mL of an ethyl alcohol-distiled water mixture (1:2 (v/v)), under magnetic stirring with heating until a temperature of 35 ± 0.1 °C was achieved.

Two procedures were used to prepare the inclusion complexes, magnetic stirring and ultrasonic homogenisation. The first procedure consisted of mixing the two solutions using magnetic stirring until they reached room temperature (22 ± 1 °C). The procedure in the ultrasonic environment was conducted by mixing the solutions of extract and β-cyclodextrin using an ultrasonic homogeniser (Omni Sonic Ruptor 250; Omni International, Kennesaw, GA) at 100 W for 5 min, followed by magnetic stirring until the mixture reached room temperature. Both mixtures were stored overnight in a refrigerator at 10 °C. Afterwards, they were filtered under vacuum using a cellulose acetate membrane with a porosity of 0.2 μm. The precipitate was washed three times with ethanol and distiled water then freeze-dried. The obtained powder was weighed and stored at −18 °C in an airtight bottle. The amount of powder recovered (dry weight basis) was calculated using the following equation:

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

The molecular inclusion procedure was carried out in quadruplicate. A physical mixture was prepared by manual agitation, adding red bell pepper extract to a mortar containing powdered β-cyclodextrin. The mass ratio of red bell pepper extract to β-cyclodextrin was maintained as described for the inclusion complexes, and the mixture was stored under refrigeration in the dark.

2.5. Determination of inclusion efficiency

2.5.1. Extract content in the inclusion complexes

The total extract content of red bell pepper in the obtained complexes was determined by the solvent extraction method according to Falcão et al. (2011) with modifications. An extraction was performed on 5 mg of the lyophilised complex with ethyl alcohol (1 mL), followed by an ultrasonic bath at 100 W for 10 min, and finally centrifugation at 6200 rpm for 15 min. Six successive extraction were carried out. The supernatant obtained was immediately analysed by spectrophotometry at 451 nm. The extract content

was calculated by comparing A_{451} with a prepared standard curve. The extraction procedure was carried out in triplicate. The inclusion efficiency (IE) was calculated using the following equation according to Falcão et al. (2011):

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

The carotenoids present in the extracts from the two obtained complexes were analysed by HPLC according to the procedure in Section 2.3.

2.6. Characterisation of the inclusion complexes

2.6.1. FT-IR

FT-IR spectra of the samples (i.e., the red bell pepper extract, β-cyclodextrin, the physical mixture of the extract and β-cyclodextrin and the inclusion complexes) were obtained in the range of 500–4500 cm^{-1} using a Shimadzu IRPrestige-21 FT-IR spectrophotometer (Shimadzu, Kyoto, Japan). The resolution was 1.0 cm^{-1}, and each spectrum was the result of averaging 32 scans. The samples were diluted in the ratio of 1:100 with potassium bromide (KBr), an IR transparent salt.

2.6.2. DSC

The DSC measurements of the samples (i.e., the red bell pepper extract, β-cyclodextrin, the mixture and the complexes) were performed using a Mettler-Toledo 822 differential scanning calorimeter (Mettler-Toledo Inc., Columbus, OH). Calibration was carried out using indium and zinc as reference materials. Samples weighing approximately 3 mg were analysed in an aluminium pan in which a pinhole was punched into the pan lid. The samples were heated from 25 to 400 °C at a rate of 10 °C/min under a nitrogen purge.

2.6.3. Particle size, size distribution and Zeta potential

The diameters of the particles of the samples (i.e., β-cyclodextrin, the physical mixture and the complexes) were analysed by dynamic light scattering (DLS) (ZetaPlus™, Zeta Potential Analyzer; Brookhaven Inst. Corp., Holtsville, NY). Dilute suspensions were prepared in ultra-pure Milli-Q water and sonicated for 30 s. Each sample was placed in a cuvette and subjected to particle size measurement in the ZetaPlus™ for 30 s. The analyses were performed in quintuplicate. The average particle size was expressed as the mean diameter. Following the particle size analyses of the samples, the electrode was placed inside the cuvette, and five measurements of the Zeta potential were recorded.

2.6.4. ^1H NMR

The complexes, β-cyclodextrin and the physical mixture were analysed by ^1H NMR with a Varian VNMRS operating at a frequency of 500 MHz for hydrogen (Varian, Palo Alto, CA). D_2O was used as a solvent. Aliquots of 600 μL were placed in NMR tubes of 5 mm diameter. The chemical shifts (δ) were expressed in parts per million (ppm). The spectra were analysed using the software SpinWorks 3.1.5.0.

2.7. Colour stability of yogurt with inclusion complexes of red pepper pigments with β-cyclodextrin

Yogurt was prepared by the lactic fermentation of 7 L of sterilised milk with 700 g of fat-free powdered milk at 45 °C until a pH value of 4.1 was achieved. The starter culture was inoculated from a commercial yogurt sample containing *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. Either extract, the complex or artificial yellow colorant (FD & C n. 6) was added to a batch of yogurt until a similar colour to commercial papaya yogurt was obtained (L^* = 77.91; a^* = 22.99; and b^* = 29.63). The yogurts were stored for 60 days at 4 °C. Instrumental colour measurements were performed every 10 days from the start of the experiment.

There were four yogurts: yogurt without colorants (YWC), yogurt coloured by the extract from red bell peppers (YE), yogurt coloured by the inclusion complex obtained by sonication (YC) and yogurt coloured by the artificial colorant Sunset Yellow FD&C n. 6 (YA). Instrumental analysis was performed by reflectance using a spectrophotometer (Colorview 9000 Byk-Gardner, Columbia, MD,) with QC Manager Software. A CIE Illuminant D65 was used in all the colour measurements. The colour measured was expressed by the CIE L^*, a^*, b^* (CIELAB) colour space values. The colour indices determined for each analysis time were compared by ANOVA, using $p < 0.05$ to determine significant differences between the storage days for each group. If there was a significant difference for the mean values of each sample studied, it was compared to time zero using the Dunnet test. The experiments and measurements were performed in triplicate.

3. Results and discussion

3.1. Identification of the carotenoids in the pigment extracts

The HPLC analyses of the red bell pepper extract were performed after sample saponification, as shown in Fig. 1. The saponification procedure was performed to improve the chromatographic resolution and remove lipids and/or chlorophylls that may interfere with the chromatographic analysis. The main pigments of the red bell pepper extract were found to be capsanthin, capsorubin, β-cryptoxanthin, β-carotene and the isomers 13- and 9-*cis*-β-carotene, and the percent area for each peak was 35.16%, 3.73%, 6.87%, 13.61%, 1,67% and 3.69%, respectively, with capsanthin and β-carotene predominant. Kim et al. (2004) identified capsorubin, capsanthin, β-carotene, zeaxanthin and β-cryptoxanthin as the major pigments in red bell pepper fruits during ripening. Likewise, the major carotenoids responsible for the coloration of red bell pepper were identified as capsanthin and capsorubin (Hornero-Méndez et al., 2000). The pigments identified in this work are in agreement with the results of the other authors cited. Furthermore, 13- and 9-*cis*-β-carotene were most likely formed by *trans–cis* isomerisation during extraction or saponification and analysis because the occurrence of *trans* isomers of carotenoids in foods and others naturals products have been previously reported (Mercadante & Egeland, 2004).

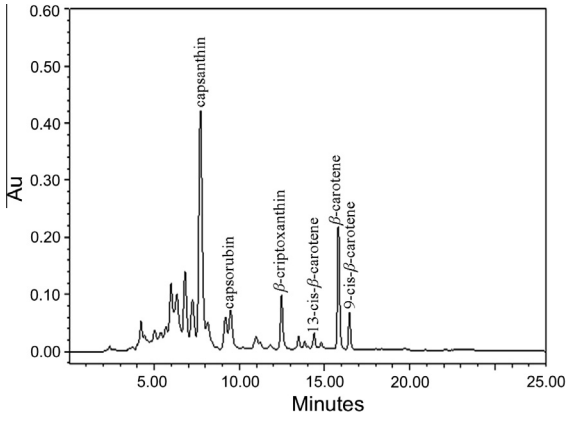

Fig. 1. Chromatographic profile of the saponified extract from red bell pepper showing the presence of the main carotenoids described in this vegetable.

3.2. Determination of inclusion efficiency

The aim in this step was to evaluate the best molecular inclusion procedure (i.e., magnetic stirring or ultrasonic homogeniser) with respect to the recovery of the powder complex obtained and inclusion efficiency. The choice of the complex to be used in the subsequent yogurt colouring application was based on these parameters and on the results of the characterisation analysis. The powder recoveries for magnetic stirring and the ultrasonic homogenising procedures were 40.79% ± 2.76 and 54.48% ± 3.60, respectively. There was a significant difference ($p < 0.05$) between the recovery of the freeze-dried complexes prepared by magnetic stirring and the ultrasonic procedure. The homogenising ultrasonic procedure provided a higher recovery rate compared to the magnetic stirring process. The inclusion efficiency means the proportion of the used extract recovered in the complexes, and the evaluation of the inclusion efficiencies showed no significant difference ($p > 0.05$) between the two procedures, 52.95% ± 9.39 and 62.43% ± 12.89 for magnetic stirring and ultrasonic, respectively. The lack of significant difference between the inclusion efficiencies of the two procedures may be due to the high standard deviation (SD) found for the media. Previous work on the molecular inclusion of carotenoids in β-cyclodextrins by magnetic stirring resulted in inclusion efficiencies as low as 48.96% (Chen et al., 2007) and 50% (Nunes & Mercadante, 2007). Ultrasonic homogenisation may be a better way to form inclusion complexes with acceptable recoveries of the carotenoid extract and cyclodextrin used in the process; it would be of great interest for the placement of natural pigments in foods. Due to cost and production limitations, small amounts of cyclodextrin are used in health products such as medicines and foods. Thus, it is important to develop methods with high inclusion efficacies. The inclusion efficiency of the ultrasonic procedure obtained in this study could be due to a longer contact time between the carotenoids and the carrier; mixing may have be achieved by cavitation, which causes bursts of bubbles at the interface that facilitate contact between the cyclodextrin and the guest molecules.

HPLC analysis of the extracts obtained from the two inclusion complexes produced revealed that the carotenoids present in the magnetic stirring complex were capsantin (58.06% peak area (p.a.)) and β-carotene (3.06% p.a.), with the 13-*cis* and 9-*cis*-β-carotene present at up to 1%. For the ultrasonic procedure complex, the carotenoids found were capsanthin (52.69% p.a.), β-carotene (16.88% p.a.) and 9-*cis*-β-carotene (2.02% p.a.). Capsorubin and β-cryptoxanthin were not detected in the complex extracts, but capsanthin and β-carotene were the main carotenoids present, likely to be present in higher proportions in the extract of red bell pepper, which should have favoured their inclusion. We hypothesise that in the inclusion complex, the carotenoid molecules are contained inside the cyclodextrin cavity and stabilised by van der Waals forces and hydrophobic interactions, which is supported by Oliveira et al. (2011). Furthermore, capsanthin in the red bell pepper extract is a molecule with two electron donator groups, and is able to form hydrogen bonds within the cyclodextrin cavity because it is an oxygenated carotenoid. Marcolino, Zanin, Durrant, Benassi, and Matioli (2011) used a similar explanation for the formation of inclusion complexes between curcumin, bixin and β-cyclodextrin. For long carotenes such as β-carotene, the β-cyclodextrin migrates to the extremities through the secondary hydroxyl side. In the case of carotenoids which does not present the bulk terminal group, the β-cyclodextrin migrates through the primary hydroxyl side, suggesting that more than one host molecule would be likely to interact with such long carotenes. Based on Raman spectroscopy analysis and molecular modelling, Oliveira et al. (2011) could demonstrate the overall distortion in the carotenes' backbone upon inclusion in β-cyclodextrin. Carotenes such as β-carotene, which has bulky groups at the ends and consequently lower backbone flexibility, it is suggested a structure for the inclusion complex found close to the ends of the guest molecule (Oliveira et al., 2011).

3.3. Characterisation of the complexes

3.3.1. Particle size and distribution and Zeta potential

The particle size distribution results for the physical mixture, the complexes and β-cyclodextrin are shown in Fig. 2. The analysis indicated that the inclusion complex obtained through the homogenising ultrasonic procedure had a mean particle diameter of 562 ± 36 nm, while the result for the physical mixture was 603 ± 43 nm and for the complex formed by magnetic stirring was 596 ± 132 nm. Although there was no significant difference among the results ($p > 0.05$) they suggest a reduced particle size for the complex produced by ultrasonic homogenisation, maybe due to the interaction between the apolar carotenoids and the cyclodextrin hydrophobic cavity (Falcão et al., 2011). The β-cyclodextrin exhibited a mean diameter of 644 ± 44.1 nm. The β-cyclodextrin and the physical mixture both showed a bimodal profile indicating two different particle populations. The magnetic stirring procedure produced more than two populations while the homogenising ultrasonic procedure had the most homogeneous population.

The complex prepared by ultrasonic homogenisation had the lowest polydispersity index (0.005) in relation to all the other samples (physical mixture: 0.079; β-cyclodextrin: 0.345; stirring magnetic: 0.383), indicating a more uniform particle size distribution due to the ultrasonic procedure.

The values of the Zeta potential obtained for β-cyclodextrin, the complex prepared by ultrasonic homogenisation, the complex prepared by magnetic stirring and the physical mixture were −24.0 ± 3.03 mV, −34.1 ± 2.64 mV, −42.3 ± 3.11 and −36.1 ± 5.98, respectively. The results vary considerably when compared to the Zeta potential value of the β-cyclodextrin, suggesting that the presence of substances adsorbed on the surface of the cyclodextrin may alter its charge.

3.3.2. ^1H NMR

^{1}H NMR has become an important method for the characterisation of inclusion complexes because it is direct and it can distinguish the differences between surface interactions or the inclusion of the guest molecule inside the cyclodextrin. If a guest molecule is incorporated into the cavity, the screening constants of the cyclodextrin protons inside the cavity (H_3 and H_5) should be sensitive to the changed environment, but those of the outside protons (H_1, H_2 and H_4) should not, resulting in chemical shift changes of the inside protons (Marcolino et al., 2011). Fig. 3 shows the NMR spectra of the protons of interest for the identification of complex formation. Unfortunately, it was not possible to obtain significant NMR results for the inclusion complex prepared by ultrasonic homogenisation. Moreover, the chemical shifts between β-cyclodextrin and the inclusion complex prepared by ultrasonic homogenisation were less than 0.01 ppm. Because the changes in the peaks were minimal, it was not possible to affirm the formation of complex by this method. According to Wimmer, Aachman, Larsen, and Peterson (2002), complexes with low association constants often display insignificant chemical shifts; this result could explain the small chemical shifts in the peaks for this complex. The NMR spectrum for the inclusion prepared by magnetic stirring showed changes in the peaks of the regions corresponding to the inner cavities (H_3, H_6 and H_5) and the external part (H_2 and H_4) of the cyclodextrin, indicating interactions of the extract with both regions. The chemical shifts between β-cyclodextrin and the inclusion complex prepared by magnetic stirring were less than 0.12 ppm. The NMR spectrum for the physical mixture showed

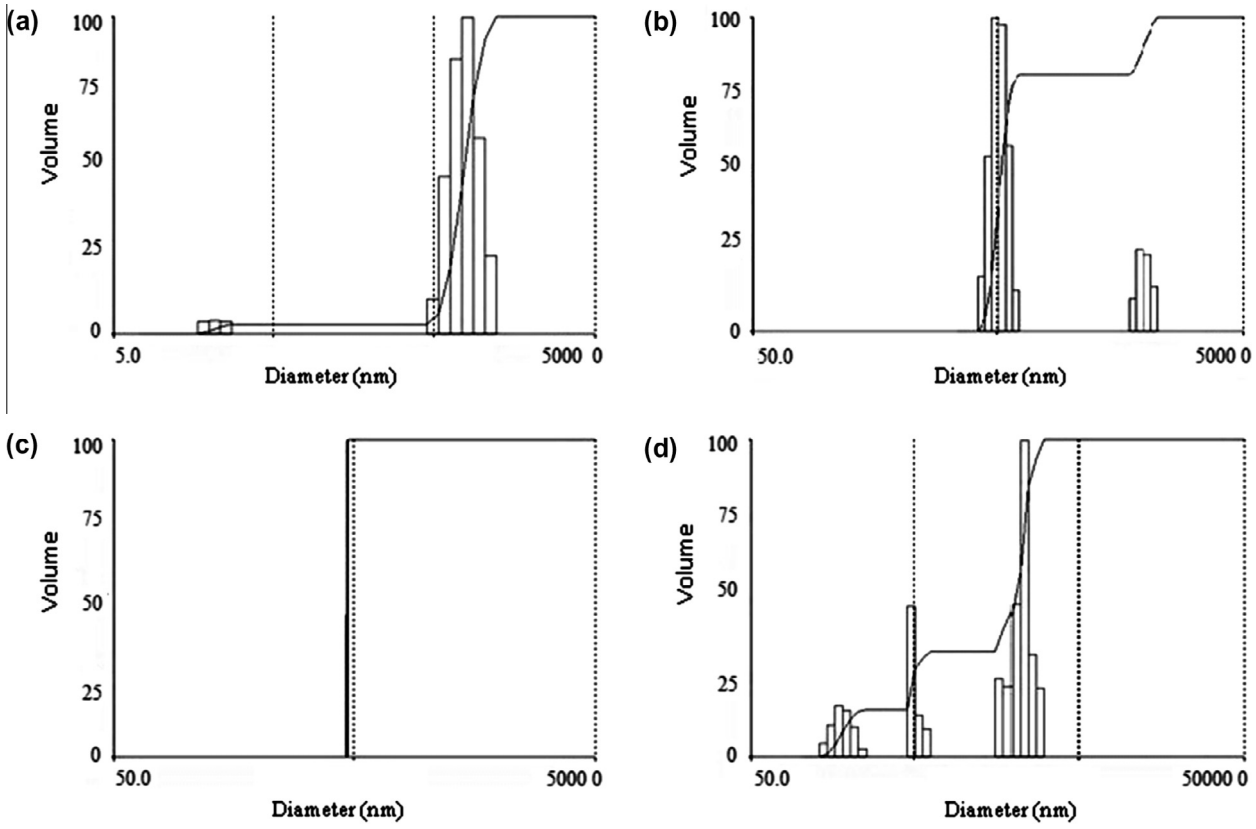

Fig. 2. Particle size distribution: (a) β-cyclodextrin, (b) physical mixture, (c) complex formed by ultrasonic homogenisation and (d) complex formed by magnetic stirring.

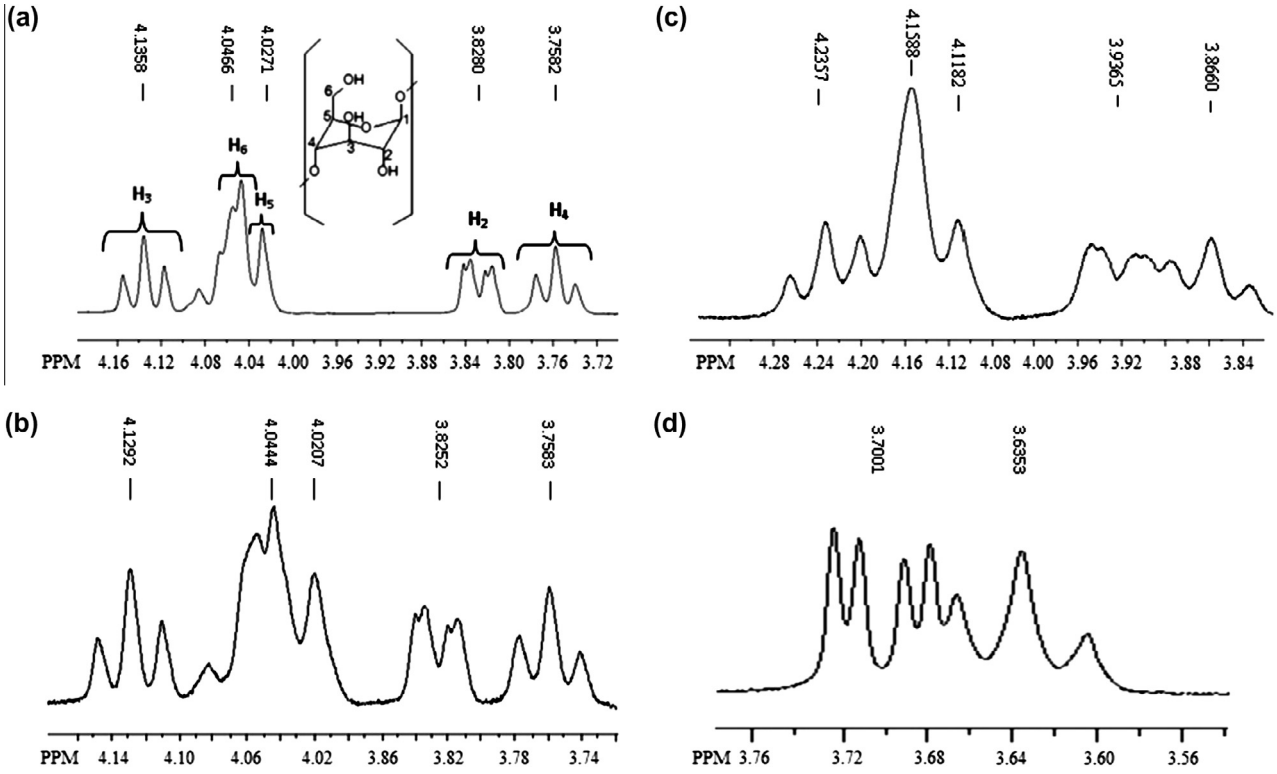

Fig. 3. ^{1}H NMR spectra: (a) β-cyclodextrin (top and left: the drawing explains the numbering of the hydrogens positioned in the molecule), (b) complex formed by ultrasonic homogeniser, (c) complex formed by stirring magnetic and (d) physical mixture.

changes in the peaks of the external region (H_2 and H_4) of the cyclodextrin, indicating a strong interaction of the extract with this region only. The chemical shifts between β-cyclodextrin and the physical mixture were less than 0.13 ppm. The ^{1}H NMR analysis of an inclusion complex formation of β-carotene and cyclodextrins was previously described in the literature. It was demonstrated that the β-carotene/cyclodextrins associations can have a neat amphiphilic character due to the hydrophilic hosts and the hydrophobic guest. In this case it is postulated that the observed NMR signals are only due to the free cyclodextrins molecules which are in fast exchange, on the NMR timescale, with the micelle-bound host molecule. In the presence of β-carotene, chemical shifts of the glucose protons of native β-cyclodextrin do not show any appreciable variation and it can be related to the high conformational rigidity of the seven-membered ring structure of β-cyclodextrin. These results could not be considered conclusive proof of a host–guest interaction, they are consistent with an inclusion complex which can be expected to have a low value of equilibrium constant for its formation; a low degree of penetration of the guest into the host's cavity for the sterical hindrance of the guest and a small anisotropic effect on the part of β-carotene, which is likely to interact with the cavity (Mele, Mendichi, & Selva, 1998). Although the ^{1}H NMR results did not show any inclusion evidence between the extract and the β-cyclodextrin for the ultrasonic homogenisation procedure, it is noteworthy that changes were observed in protons on the external regions of the cyclodextrin, H_2 and H_4, in relation to the physical mixture that was not complexed.

3.3.3. FT-IR

Fig. 4 shows the comparison between the FT-IR spectra obtained for β-cyclodextrin (a), the extract from red bell pepper (b), the physical mixture (c), the inclusion complex prepared by ultrasonic homogenisation (d), and the inclusion complex prepared by magnetic stirring. Important changes in the polymeric hydroxyl stretching region (3000–3500 cm^{-1}) were more pronounced in the inclusion complex that was prepared by ultrasonic homogenisation compared to the physical mixture and the complex prepared by magnetic stirring. These changes seemed to be related to the complex formation between the OH groups of the host molecule and the red bell pepper extract. C–H aliphatic bands were apparent in the 2926 cm^{-1} region, as seen in the five spectra. The band of the inclusion complex prepared by ultrasonic homogenisation (d) exhibits a differential profile compared to the physical mixture (c), β-cyclodextrin (a) and the complex formed by magnetic stirring (e), suggesting that a different behaviour is related to this kind of link when there is inclusion complex formation. In the 1.738 cm^{-1} region, the red bell pepper extract (b), the physical mixture (c), the inclusion complex produced by ultrasonic homogenisation (d) and the inclusion complex produced by magnetic stirring (e) can be observed to have a vibrational band in the region of the C=O bond in the carbonyl group of the compounds in the red bell pepper extract. The vibrational bands corresponding to the 942 cm^{-1} region in (a), (c), (d) and (e) are related to the skeletal vibration involving the α-1, 4 linkage of the β-cyclodextrin molecule. The vibrational band of the complex (d) shows a similar intensity to the band of the β-cyclodextrin. In the physical mixture (c) and the inclusion complex formed by magnetic stirring (e), this band exhibited a lower intensity. In the region between 1.033 and 1.162 cm^{-1}, a couple of bands related to C–O and C–C can be observed in the (a), (c), (d) and (e) spectra. The profile of the bands of the inclusion complex prepared by ultrasonic homogenisation (d) was similar to β-cyclodextrin (a) but different from the bands profile of the inclusion complex prepared by magnetic stirring (e), showing a slightly lower intensity; the physical mixture (c) showed the lowest intensity, suggesting a different role of this linking in the physical mixture and in the complexes.

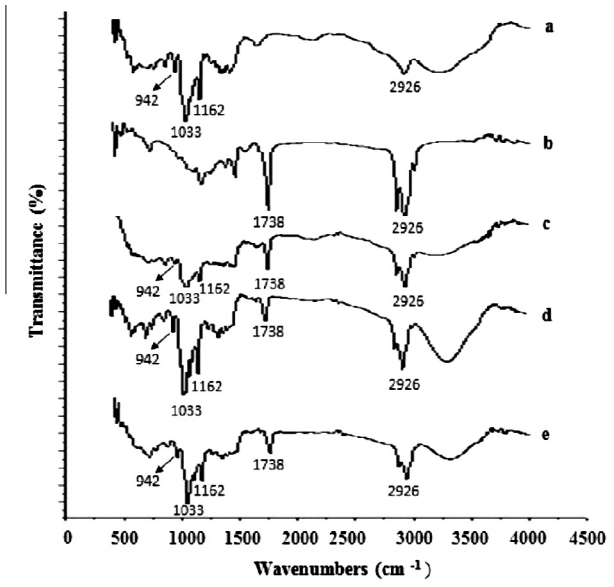

Fig. 4. FTIR spectra of (a) β-cyclodextrin, (b) red bell pepper extract, (c) physical mixture, (d) complex by ultrasonic homogeniser and (e) complex by magnetic stirring.

3.3.4. DSC

The calorimetric analysis of β-cyclodextrin, the red bell pepper extract, the physical mixture, the inclusion complex prepared by ultrasonic homogenisation and the inclusion complex prepared by magnetic stirring is presented in Fig. 5. The β-cyclodextrin thermogram exhibited a broad thermal peak between 115 and 160 °C, with a maximum at around 140 °C. The presence of a thermal rise near 300 °C is generally attributed to the initiation of β-cyclodextrin decomposition. The extract exhibited an exothermic peak between 115 and 160 °C that may be related to degradation. This peak is not present in the DSC scan of the other samples, which means that the extract interacting with β-cyclodextrin is protected from degradation. The thermogram of the inclusion complex produced by ultrasonic homogenisation showed that the change in the water evaporation enthalpy, represented by the endothermic peak, was lower compared to the inclusion complex formed by magnetic stirring and the physical mixture; this finding indicates a better interaction between the carotenoids and β-cyclodextrin in the ultrasonic homogenisation procedure than

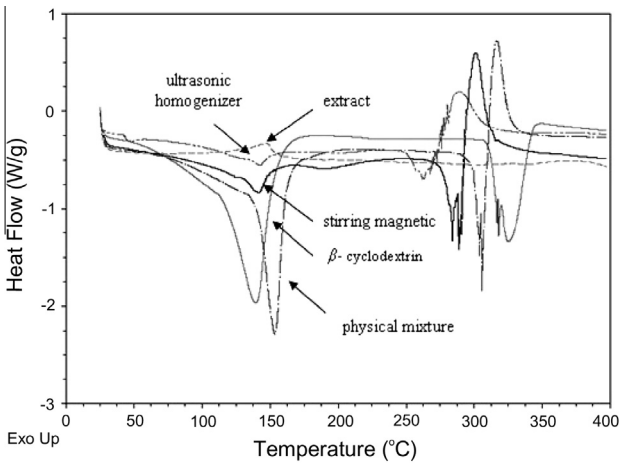

Fig. 5. DSC thermograms of β-cyclodextrin, red bell pepper extract, the physical mixture, the complex formed by ultrasonic homogenisation and the complex formed by magnetic stirring.

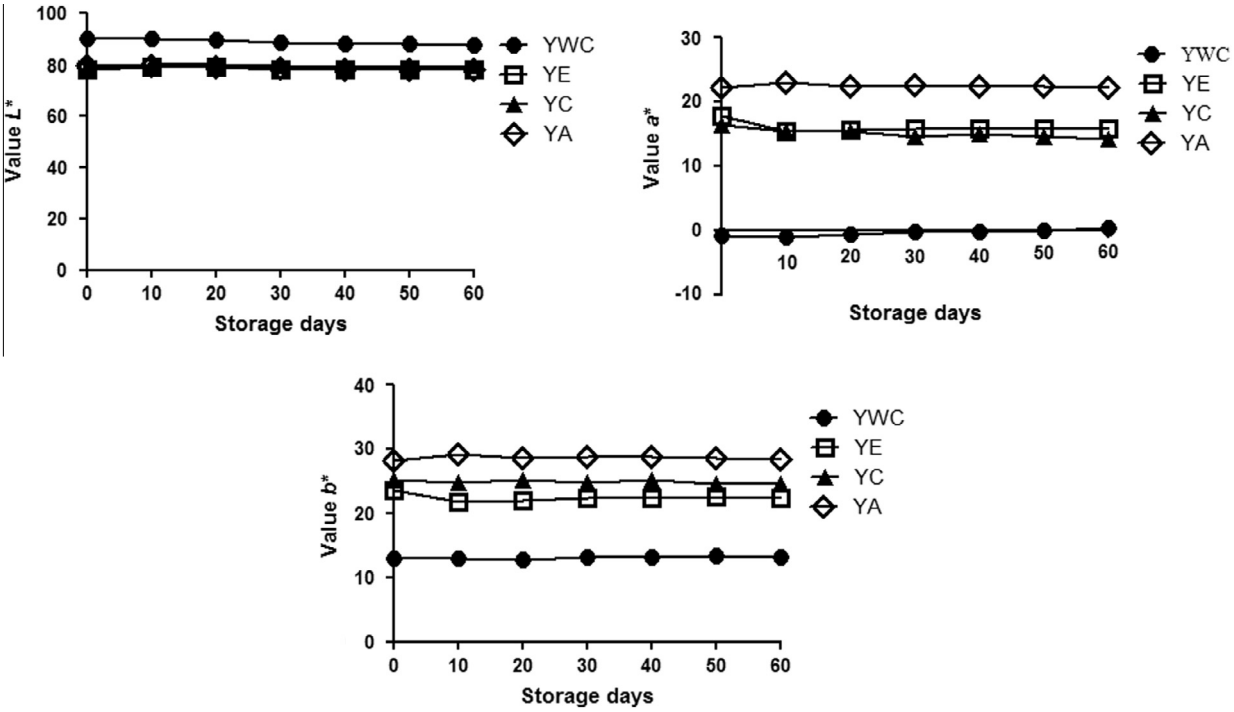

Fig. 6. Variation of (a) L^* index (lightness), (b) a^* index (redness) and (c) b^* index (yellowness) in yogurt without colorants (YWC), yogurt coloured by extract from red bell pepper (YE), yogurt with complex (YC) and yogurt coloured by artificial colorant (YA) stored for 60 days (mean ± standard deviation).

in the other procedures. The DSC curve of the physical mixture was similar to that of pure β-cyclodextrin, indicating that β-cyclodextrin is present in free form in this sample.

Although the ^{1}H NMR analyses did not show evidence of inclusion between the carotenoids and β-cyclodextrin in the complex formed by the ultrasonic procedure, the information from this characterisation analysis show evidence of complex formation by both inclusion procedures (i.e., magnetic stirring and ultrasonic homogenisation). Thus, from this characterisation and the powder recovery rate and inclusion efficiency results, the complex formed by ultrasonic homogenisation was chosen to be applied in yogurt; the evaluation of its colour stability during shelf life will determine its possible use as a natural colorant for yogurt.

3.4. Colour stability of yogurt with inclusion complexes of red pepper pigments with β-cyclodextrin

Yogurt is a food with high nutritional quality due to the variety and amounts of nutrients present. It is consumed by individuals of all ages. Therefore, the artificial colours that are added to yogurt aim to improve sensory characteristics, making the product more appealing. The extract of red pepper when added to yogurt, can simulate the colour of commercial yogurt of papaya flavour and others, if used in mixture, but due to its low water solubility, there is difficulty in dispersing the extract in the product. Therefore, in an attempt to improve stability and solubility of the carotenoids from red bell pepper, we conducted the inclusion of the extract in β-cyclodextrin and subsequent application in yogurt. It was observed that the homogenisation of the extract in yogurt was more difficult than the mixing of β-cyclodextrin complex since, using the extract, complete distribution was slower. Additionally, during the 60 days of storage there was no homogeneity in appearance in yogurt added of the extract, since orange spots were noted, which were not observed in yogurt added with the complex. According to the ingredients described on the labels of papaya, strawberry and red fruit yogurts sold in Brazil, the most commonly used synthetic dyes are Ponceau 4R (FD&C Red No. 1), Bordeaux (FD&C Red No. 9), Brilliant Blue FCF (FD&C Blue No. 2) and Sunset Yellow (FD&C Yellow No. 6). The pH value and acidity of yogurt are important parameters because high acidity may cause the instability of natural colorants (Obón, Castellar, Alacid, & Fernáde, 2009). The pH of the yogurt prepared in this study was 4.1. The pH values of the food matrix may affect the variety of pigments present in plants and may cause instability and loss of colour.

The colour stability of yogurt coloured with red bell pepper extract, the inclusion complex obtained by ultrasonic homogenisation, and synthetic food dye FD&C Yellow No. 6, as well as uncoloured yogurt, was assessed by the colour parameters (L^*, a^* and b^*) determined by reflectance spectrophotometry. Fig. 6 shows the variations of the L^* index (lightness) with time for all the yogurt groups. The statistical analysis was performed by one-way ANOVA, and significant differences ($p < 0.05$) were found between 0 and 60 days for all groups. For YWC, the differences between 0 and 60 days were greater than for the other groups. For the other groups, the differences related to the L^* index were so low they could not be visually perceived on the graphs in Fig. 6, indicating that all the colorants evaluated in this work had no tendency to lighten the colour of the samples during 60 days of storage. In addition, the values of SD were too low to be perceived on the graphs.

The a^* index represents the redness of the samples and is directly influenced by the carotenoids of the red bell pepper. The statistical analysis indicated that there was a significant difference ($p < 0.05$) between 0 and 60 days for all of the groups.

The results show that there was a variation of the a^* index of 0.7% in YA, 11% in YE, 13% in YC and 24% in YWC between 0 and 60 days. Although the variation of the a^* index between 0 and 60 days of YC was 2% higher than YE, the plotted data show that the variation of the a^* index was 12.6% compared to YE and 6.6% compared to YC between the 0 and 10 days. Furthermore, the variation in the a^* index of the YC group was slower than in the YE group, indicating a higher protection of the redness compared to the YE group because a greater difference in the redness can be observed in this group for the 10 days of storage.

The variation for the b^* index (yellowness) for all the yogurt groups is shown in Fig. 6. Statistical analysis indicated that there was a significant difference ($p < 0.05$) between 0 and 60 days for the YA and YE groups. With regards to the YC group, there was a significant difference ($p < 0.05$) between 0 and 10, 20, 30, 50, 60 days, but not between 0 and 40 days. The percentage changes observed between zero and the times cited above were 1.8%, 0.4%, 2.3%, 2.1% and 2.3%, respectively, indicating a low loss of the yellowness for this sample. However, it is noteworthy that the b^* index variation between 0 and 10 days for the YC group was lower (1.8%) than the YE group (8.3%); between 0 and 60 days, the variations were 2.3% for YC and 5.0% for YE, indicating that the complex has provided a greater stability of the yellowness in the yogurt compared to the extract, which showed a more pronounced reduction of the yellowness throughout the experiment. In conclusion, we feel that based on the evaluation of the obtained data for the colour parameters studied, the red pepper pigments included in β-cyclodextrin by the ultrasonic procedure may be useful as natural colorants in an acidic food such as yogurt.

Children are reported to be the main consumer group exposed to synthetic dyes that are added to foods. The index of recommended daily intake for each artificial dye must not be exceeded; these indexes are calculated in milligrams per kilogram weight. Because children have a lower weight compared to adults, the daily amount allowed may be quickly reached (Polônio & Peres, 2009). The red pepper pigments included in β-cyclodextrin by the ultrasonic procedure can simulate the colour of papaya flavours in yogurt, providing a higher stability for the colour compared to the crude bell pepper extract. However, the inclusion complex process needs to be optimised to attain higher protection of the carotenoids. Moreover, the inclusion procedure should be evaluated for other kinds of foods, such as those in which thermal treatment is involved in microbiological stabilisation, to verify the extension of use in several food systems.

4. Conclusion

The ultrasonic homogenisation procedure for producing the inclusion complex between red bell pepper pigments and β-cyclodextrin had a higher yield and inclusion efficiency compared to magnetic stirring. The characterisation analyses revealed the occurrence of inclusion by both procedures; infrared spectroscopy and differential scanning calorimetry confirmed a better interaction between the extract and the β-cyclodextrin in the ultrasonic homogenisation procedure. The evaluation of the instrumental colour parameters L^*, a^* and b^* demonstrated higher stability of the colour indices in the yogurt coloured with the inclusion complex compared to yogurt coloured with crude red bell pepper extract, showing that the inclusion procedure that was evaluated in this study may be useful for the application of natural additives in foods.

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