Food Chemistry 202 (2016) 373–382

Contents lists available at ScienceDirect

Food Chemistry

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

Encapsulating betalains from *Opuntia ficus-indica* fruits by ionic gelation: Pigment chemical stability during storage of beads

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ARTICLE INFO

Article history:
Received 30 September 2015
Received in revised form 26 January 2016
Accepted 27 January 2016
Available online 2 February 2016

Chemical compounds studied in this article:
Indicaxanthin (PubChem CID: 6096870)
Betanin (PubChem SID: 10733)
Isobetanin (PubChem CID: 6325438)
Betalamic acid (PubChem CID: 5281176)

Keywords:
Capsules
Cactus pear
Betacyanins
Encapsulation
Antioxidants
Antiradical activity

ABSTRACT

Betalain encapsulation was performed by ionic gelation as a stabilization strategy for these natural pigments. Betalains were extracted from purple cactus fruits and encapsulated in calcium-alginate and in combination of calcium alginate and bovine serum albumin. Beads were characterised by scanning electron microscopy and thermal analysis using differential scanning calorimetry and thermogravimetry. Moisture sorption isotherms were determined. Bead morphology was affected by matrix composition. Pigments storage stability was evaluated at different equilibrium relative humidity and temperatures. Pigment composition of beads was determined by HPLC–MS–MS and degradation products were also analysed after storage; betalamic acid being the major one. Both types of matrices protected the encapsulated pigments, being their storage stability better at low relative humidity than that of the non-encapsulated control material. Antiradical activities of beads were proportional to remaining betalain contents. At high relative humidity, there was no protection and low storage stability was observed in the samples.

1. Introduction

Opuntia ficus-indica belongs to the Cactaceae family. The fruits of this plant species are oval-shaped berries which present varied colors such as lime green, yellow, orange, red, or purple. Betalains are the main pigments occurring in cactus fruits. According to their chemical structure, they can be classified as betacyanins that are red-violet pigments, and betaxanthins that exhibit a yellow–orange color. Stability is an important aspect to consider before using these natural compounds as colorants and antioxidants in foods. Betalain stability is strongly affected by pH, water activity, temperature, oxygen, light exposure, among others. In red beet preparations, temperature has been reported as a crucial factor affecting betalain stability (Herbach, Stintzing, & Carle, 2004).

The encapsulation can be used for stabilization, protection, and extending the shelf life of sensitive compounds as well as for many applications in the food industry, including controlled release, delivery and masking of flavors, colors, and odours (Anal & Singh, 2007).

One of the most common methods of encapsulation is the ionic gelation with formation of alginate gels by ionic cross-linking with multivalent cations. Alginate is an anionic biopolymer with carboxyl end groups produced by brown algae and bacteria and consists of α-L-guluronic acid (G) and β-D-mannuronic acid (M) residues linearly linked by 1,4-glycosidic linkages (Yang, Xie, & He, 2011). The gelation of alginate occurs by the exchange of sodium ions from the G blocks with multivalent cations, like

Abbreviations: BE, betalain-rich extract; BSA, bovine serum albumin; CA, calcium alginate beads; CAB, calcium alginate-bovine serum albumin beads; CP, non-encapsulated lyophilized pulp of cactus pear; DSC, differential scanning calorimetry; GAB, Guggenheim–Anderson–de Boer; HPLC, high performance liquid chromatography; LC–MS–IT–TOF, liquid chromatography hybrid ion trap and time of flight mass spectrometry; RH, relative humidity; SEM, scanning electron microscopy; TGA, thermogravimetric analysis.

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http://dx.doi.org/10.1016/j.foodchem.2016.01.115
0308-8146/

calcium ions, and subsequent stacking of these G blocks to form a characteristic "egg-box" structure (Lee & Mooney, 2012; Poojari & Srivastava, 2013). To the best of our knowledge, encapsulation of cactus betalains by ionic gelation has not been explored to date. By contrast, microencapsulation by spray-drying of betalains from *Opuntia* fruits has been reported as strategy to improve their stability (Sáenz, Tapia, Chávez, & Robert, 2009; Castro-Muñoz, Barragán-Huerta, & Yáñez-Fernández, 2015; Obón, Castellar, Alacid, & Fernández-López, 2009; Otálora, Carriazo, Iturriaga, Nazareno, & Osorio, 2015). The literature includes scarce scientific studies using combinations of proteins and polysaccharides as spray drying microencapsulation agents for cactus pear pulp (Robert, Torres, García, Vergara, & Sáenz, 2015). Among the main factors that affect the stability of the microencapsulated pigments during storage, water activity has been found as one of the most relevant for betalain microcapsules (Pitalua, Jiménez, Vernon-Carter, & Beristain, 2010).

Thus, the main objectives of the present work were to obtain cactus betalain encapsulates by ionic gelation, and to evaluate the storage stability and antiradical activity (ARA) retention of pigment beads at different storage temperatures and equilibrium relative humidity (RH) values. Two different bead matrices were prepared using calcium alginate (CA) as well as calcium alginate combined with a model protein (CAB), in order to evaluate the effect of possibly interactions between proteins and carbohydrates on the stability of betalains.

2. Materials and methods

2.1. Materials

Opuntia ficus-indica fruits (purple pulp) corresponding to clone number 1279 were collected from the cactus collection belonging to the Faculty of Agronomy and Agroindustries (National University of Santiago del Estero) of the province of Santiago del Estero located in the Argentinean region of the dry Chaco (27° 45′S, 64° 180′W, 170 m over sea level). Sodium alginate (Emulgel CP 3792) was provided by Saporiti (Buenos Aires, Argentina) and used as received without further purification. Calcium chloride dihydrated ($CaCl_2{\cdot}2H_2O$) was purchased from Cicarelli Laboratories (Buenos Aires, Argentina). Bovine serum albumin (BSA) (Fraction V. pH: 7) was provided by Sigma–Aldrich (Buenos Aires, Argentina).

2.2. Methods

2.2.1. Preparation of cactus betalain extracts

Cactus pear fruits were peeled; the fruit pulp was crushed using a Pro200 homogenizer (Proscientific Inc., CT USA) operated at the maximum speed for 5 min at room temperature and the seeds were separated. Cactus pulp was macerated for 5 min in a 1:2 w/w ratio with 0.1 M phosphate and 0.05 M citric acid pH 5 buffer to obtain betalain-rich extract (BE). The choice of the pH 5 buffer is based on a compromising situation between the best conditions for betalain stability and the ionization of the G-groups of alginate to optimize their interaction with calcium ions. BE also contains sugars, phenolic compounds and other antioxidants as ascorbic acid, as described in Coria-Cayupán, Ochoa, and Nazareno (2011). Control sample (CP) was the lyophilized extract of cactus pear pulp.

2.2.2. Preparation of beads by external ionic gelation

2.2.2.1. Calcium alginate beads. The hydrogel beads were prepared by external ionic gelation through a calcium alginate matrix. For this, sodium alginate (15 g/L) was dissolved in the BE extracts in pH 5 buffer using a food processor (Philips, Buenos Aires, Argentina) for 2 min at low speed. Once homogenized the solution, it was passed using a 5 mL macro pipette through a 1.43 ± 0.03 mm internal diameter tip, letting it drop on a 0.15 M calcium chloride solution, at 15 cm height between the tip and the calcium chloride solution. The hydrogel beads were maintained in the calcium chloride solution for 1 min (hardening time) and washed with distilled water, as recommended by Deladino, Anbinder, Navarro, and Martino (2008). Subsequently, they were filtered and washed with distilled water. This BE hydrogel beads will be referred as CA beads. Hydrogel beads dehydration was performed by air drying at 30 ± 1 °C for 24 h in a forced-air circulating oven (Termo Dalvo SRL, Model TDC 30, Buenos Aires, Argentina).

2.2.2.2. Calcium alginate-bovine serum albumin beads. Sodium alginate was added to 100 mL of the BE extract prepared in the pH 5 phosphate-citrate buffer to obtain a final concentration of 15 g/L; subsequently, an aliquot of 10 mL of a BSA suspension (10 g/L) at pH 4.6 was added under mild stirring for 10 min. A pH value of 4.6 allows the electrostatic interaction between the ammonium groups of the protein and the carboxylic groups of the polysaccharide to enhance the stability of the matrix structure. This mixture containing BE and sodium alginate-BSA in a 15:1 w/w ratio was passed using a 5 mL macro pipette through a 1.43 ± 0.03 mm internal diameter tip letting the solution drop into a 0.15 M calcium chloride solution. Then, beads were kept in the calcium chloride solution for 1 min, filtered, washed with distilled water, and dried as it was previously described. The BE beads containing BSA will be referred as CAB.

2.3. Characterisation of beads

2.3.1. Bead sizes and moisture content

Mean sizes of fresh and dried pigment beads were determined by measuring the diameter of 20 beads with a digital micrometer and taking the average value. Moisture content of the betalain beads was determined gravimetrically in triplicate by drying 30 mg of beads at 70 °C until constant weight (AOAC, 1997).

2.3.2. Moisture sorption isotherms

The sorption isotherms were determined by the gravimetric static method using 1 g of CA or CAB beads. These were transferred to desiccators containing the saturated solutions of $MgCl_2$, NaBr, NaCl, and KCl which provide RH values of 32.6%, 57.6%, 74.8%, and 84.3%, respectively, at 25 °C. The desiccators were stored in a forced-air oven (Termo Dalvo SRL, Model TDC 30, Buenos Aires, Argentina) at 25 ± 1 °C. Once equilibrium was reached after 3 or 4 weeks, moisture contents of the beads were gravimetrically measured by drying at 105 °C until constant weight. All data were acquired in triplicate. The sorption isotherms were predicted by using the Guggenheim–Anderson–De Boer (GAB) mathematical model according to the following Eq. (1) (Van den Berg, 1985).

$$X_{eq} = \frac{CkXma_w}{(1 - ka_w)(1 - Ka_w + Cka_w)} \quad (1)$$

where X_{eq} is the equilibrium moisture content (g water/g dry beads), X_m is the monolayer moisture content (g water/g dry beads), a_w is the water activity; while C and k are the model constants related to adsorbed monolayer and multilayer properties, respectively.

2.3.3. Betalain contents

Beads (120 mg) were dispersed in a 7 mL of a (1:1) methanol: water mixture for 1 h at room temperature with mild stirring, and then, centrifuged at 10,000 rpm for 20 min. In this media, pigment release takes place by diffusion and not erosion and the

bleached capsule remains intact. When pigment release was tested in pure ethanol as solvent, the erosion of the capsule was observed and the capsule disintegrated forming a powder. CP (60 mg) sample was also analysed as control. The released betalains were quantified by using a UNICAM UV2 UV/Vis spectrophotometer (Cambridge, UK) at 536 nm according to the method used in Coria-Cayupán et al. (2011). The values obtained were expressed using betanin as reference compound (ε = 65,000 M^{-1} cm^{-1} at λ_{max} = 536 nm).

2.3.4. Betalain analysis by HPLC-MS-MS

BE used as starting material and the hydrogel beads were analysed according to the procedure described by Otálora et al. (2015) to identify the main pigments using a liquid chromatograph coupled to a mass spectrometer (Shimadzu, Kyoto, Japan) equipped with an electrospray ionization (ESI) probe, which was operated in positive ion mode (LC MS-IT-TOF). UV and MS data were acquired and processed using Shimadzu LC–MS Solution software. UV–Vis detection was carried out at 484 and 535 nm as recommended by Fernández-López, Castellar, Obón, and Almela (2002).

2.4. Storage stability evaluation of the beads

The protective effect of the calcium-alginate matrix on the encapsulated cactus betalains against degradation reaction was evaluated under non-favorable storage conditions.

2.4.1. Pigment stability as function of relative humidity

The CA and CAB beads as well as CP, as the control sample, were stored in desiccators in darkness at 25 ± 1 °C containing saturated solutions of NaBr, NaCl, and KCl to obtain RH values of 57.6%, 74.8% and 84.3%, respectively. Taking the initial concentration as reference of the zero point, the samples were withdrawn every 2, 5, 10, 15, 20, and 25 days for the measurement of betalain contents (as it was described in Section 2.3.3). The pigment retention was calculated according to Eq. (2).

$$\text{Betalain retention}(\%) = 100 \times (M_t/M_0) \quad (2)$$

where M_t and M_0 are the absolute amounts of betalains dispersed in distilled water–methanol solution at every storage time and when starting the experiment (initial time), respectively.

The observed first-order rate constant (k) and half-life time ($t_{1/2}$) of the samples were determined by a semi-log plot of betalain retention (%) versus time. The value of k was obtained as the slope of the graphs and $t_{1/2}$ as 0.693/k.

2.4.2. Pigment stability as function of storage temperature

The CA and CAB beads as well as the CP as control sample were stored in the absence of light, in a forced-air oven (Termo Dalvo SRL, Model TDC 30, Buenos Aires, Argentina) for 25 days with controlled temperature at 25 and 50 °C. Dried samples (0.1 g each) were transferred to dark glass vials and this moment was chosen as the zero time point to determine the betalain relative retention as it was described in Section 2.4.1. In the case of samples kept at 50 °C, they were removed every hour for the first five hours; then, every 2 h for the following ten hours and, finally, every 12 h until the study was completed (39 h) for the determination of betalain content. The same kinetic model of Section 2.4.1 was used.

2.4.3. Determination of antiradical activity by DPPH· method

A 500 μL aliquot of the supernatant obtained after pigment release from beads (prepared in Section 2.3.3) was mixed with 2.5 mL of DPPH· methanol solution (31.6 mg DPPH·/L; Abs 515 nm c.a. 1.00). The decrease in absorbance at 515 nm was monitored in 20 cycles every 30 s for 10 min using a UNICAM UV2 UV/Vis spectrophotometer (Cambridge, UK).

ARA was calculated according to the Eq. (3), as follows:

$$\%\text{ARA} = 100 \times (1 - A_{SS}/A_0) \quad (3)$$

where A_{SS} is the absorbance of the solution at the steady state, and A_0 is the absorbance of DPPH· solution before adding the antioxidant. A_{SS} was estimated by the mathematical fitting of kinetic curves performed with Origin 7.0 software as indicated in Coria-Cayupán et al. (2011). The samples were analysed in triplicate.

2.5. Analyses of pigment composition and degradation products after bead storage

2.5.1. Analyses of pigment stability after bead storage by HPLC-PDA

Samples of betalain beads and CP were removed at zero time and after storage for 25 days at different temperatures and %RH to determine the pigment retention percentage by liquid chromatography coupled with a photo diode array detector. Lab Alliance Series III-5 mL chromatographic system (Syracuse, USA), equipped with binary solvent system, a Rheodyne injector with a 20 μL loop was coupled to a Shimadzu photo diode array detector (Shimadzu, Kyoto, Japan). A reverse phase C18RS column (SS WAKOSIL 5 μm, 250 × 4.6 mm, SGE, Australia) was used for the analysis of the betalains of every sample. The mobile phase was a mixture of acetonitrile/formic acid/water (7:1:92 v/v, solvent A) and acetonitrile/water (1:99 v/v, solvent B) at a flow rate of 1 mL/min. The initial 3 min, elution was performed with 100% A, followed by a linear gradient from 0% to 5% B in 10 min, then, from 5% to 10% B in 7 min, followed by an isocratic elution for 10 min. Prior to injection, all samples were filtered through a 0.45 μm pore size Millipore membrane filter. Detection wavelengths selected for PDA were 536, 480 and 410 nm. Betanin and isobetanin were monitored at 536 nm, indicaxanthin and their degradation product, betalamic acid, were monitored at 480 nm and 410 nm, respectively.

2.5.2. Identification of degradation products after bead storage by HPLC-MS analysis

HPLC-MS analysis of betalain degradation products was performed evaluating the pigment composition of beads and CP at initial time and after 25 days of storage using a Shimadzu LC–MS-2010 System equipped with a UV/Vis detector (SPD-10A) and two pumps (LC-10AD) coupled on-line with an MS-2010 mass spectrometer with an electrospray ionisation (ESI) probe. UV and MS data were acquired and processed using Shimadzu LC–MS Solution software. UV–Vis detection was performed at λ 280, 410, 484 and 535 nm, according to that reported by Fernández-López et al. (2002). The equipment also included an on-line DGU-14A degasser and a Rheodyne injection valve with a 20-μl loop. A LUNA RP-18 5-μm column (150 × 2.0 mm i.d.; Phenomenex, Torrance, CA) was used for the analysis. The solvent system was a mixture of water/formic acid (1% v/v, solvent A) and acetonitrile (solvent B) and the flow rate was 0.4 mL/min. The linear gradient used was set up as follows: from 3% to 20% B (0–35 min), 20–100% B (35–40 min), 100–3% B (40–45 min). The electrospray ionisation (ESI) probe was operated in positive mode: CDL, 300 °C; block at 240 °C; flow gas (N_2) at 4.5 L/min; CDL voltage, 150.0 kV; Q array voltage RF 150 V; detector voltage, 1.5 kV; and scan range m/z 100–800. Prior to injection (volume of 20 μL), all samples were filtered through a 0.45-μm Millipore membrane filter.

2.5.3. Morphology of beads

The morphology of the betalain beads was evaluated by using a scanning electron microscope (SEM) FEI QUANTA 200 (operating at 30 kV), coating the samples with gold–palladium sputtering before their examination.

2.5.4. Thermal analysis

Thermal analyses and glass transition temperature measurements of CA and CAB beads were carried out by differential scanning calorimetry (DSC) and thermo gravimetric analysis (TGA) at initial time and after 25 days storage. A TA Instruments, Model 2910 and 2050 (New Castle, USA) previously calibrated with indium of high purity (Tm = 429.8 K, ΔHm = 28.4 J g^{-1}) was used for that purpose. The experiments were performed under nitrogen flow (50 cm^3/min). The samples (5 mg) were heated from 0 to 300 °C for DSC and from 20 to 600 °C for TGA, in aluminium crucibles with a linear heating rate of 10 °C/min. An empty aluminium crucible was used as the reference material. Sodium alginate and BSA as commercial reagents were used for comparative purposes.

2.6. Statistical analysis

Results were reported as the mean ± standard deviation (n = 3). Analysis of variance (ANOVA) was performed to identify differences among the means with the statistical package InfoStat/P version1.1 computing program (Di Rienzo et al., 2012). Differences at probability level $p < 0.05$ were considered significant.

3. Results and discussion

In this report, the effect of ionic gelation encapsulation on the stability of betalains was studied in calcium alginate beads with and without BSA presence.

3.1. Characterisation of beads

3.1.1. Bead sizes

The average diameter of fresh beads was 4.27 ± 0.23 mm for CA and 4.09 ± 0.20 mm for CAB. After drying, the average diameter was 2.18 ± 0.26 mm and 2.04 ± 0.22 mm for CA and CAB dried beads, respectively. The BSA addition in the matrix CAB did not affect the average diameter in respect to CA.

3.1.2. Moisture

The moisture contents of CA and CAB beads were 2.862 ± 0.004% and 2.466 ± 0.002%, respectively. Differences were not found ($p > 0.05$) by the addition of BSA in the matrix.

In order to apply the GAB model, a second-degree polynomial equation resulting from the Eq. (1) was used. The estimated GAB parameters were the monolayer moisture content X_m, and the constants C and k. The experimental and predicted data under GAB model were in good agreement, showing correlation coefficients (R) of 0.996 for CA and 0.995 for CAB beads.

Small values for the monolayer moisture content X_m were found; being 0.017 ± 0.001 and 0.015 ± 0.001 g H_2O/g d. b. for CA and CAB beads, respectively. This implies that the alginate content in the formulations does not induce open bead morphology what would increase the number of active sites (Xiao, Limb, & Tonga, 2012). X_m value did not significantly increase ($p > 0.05$) with the presence of BSA in the matrix indicating that the addition of protein did not reduce the number of active sites.

C values calculated for both beads were 1.64 ± 0.06 for CA and 1.77 ± 0.02 for CAB, without significant differences ($p > 0.05$) between those beads; indicating that water molecules were adsorbed with similar energy on the active site. C values were low suggesting that the monolayer moisture interaction was weak. The parameter k is a correction factor for multilayer molecules relative to the bulk liquid; when $k = 1$, the molecules beyond the monolayer have the same characteristics as pure water (Quirijns, van Boxtel, van Loon, & van Straten, 2005); k values for both beads were 0.96 ± 0.02 for CA and 0.96 ± 0.01 for CAB without significant differences ($p > 0.05$) between those beads.

3.2. Initial pigment composition of beads

The HPLC-MS-IT-TOF analysis of beads and BE extract showed a similar betalain profile constituted by three peaks, corresponding to two betacyanins, betanin and isobetanin ($[M+H]^+$ at m/z 550.8038 and 550.8240, respectively; λ_{max} = 535 nm), and indicaxanthin ($[M+H]^+$ = 309.0540; λ_{max} = 484 nm); being betanin the major one. This is in good agreement with those results reported before (Otálora et al., 2015).

3.3. Storage stability of beads

Stability of pigments in CA and CAB beads were compared with CP as control, after 25 days of storage at different RH conditions (34.6%, 57.6%, 74.8%, and 84.3%) at 25 °C. Results are shown in Table 1; they clearly evidence that the betalain retention of stored

Table 1
Betalain content and antiradical activity retentions after 25 days of bead storage under different conditions and their correlation along 25 days.

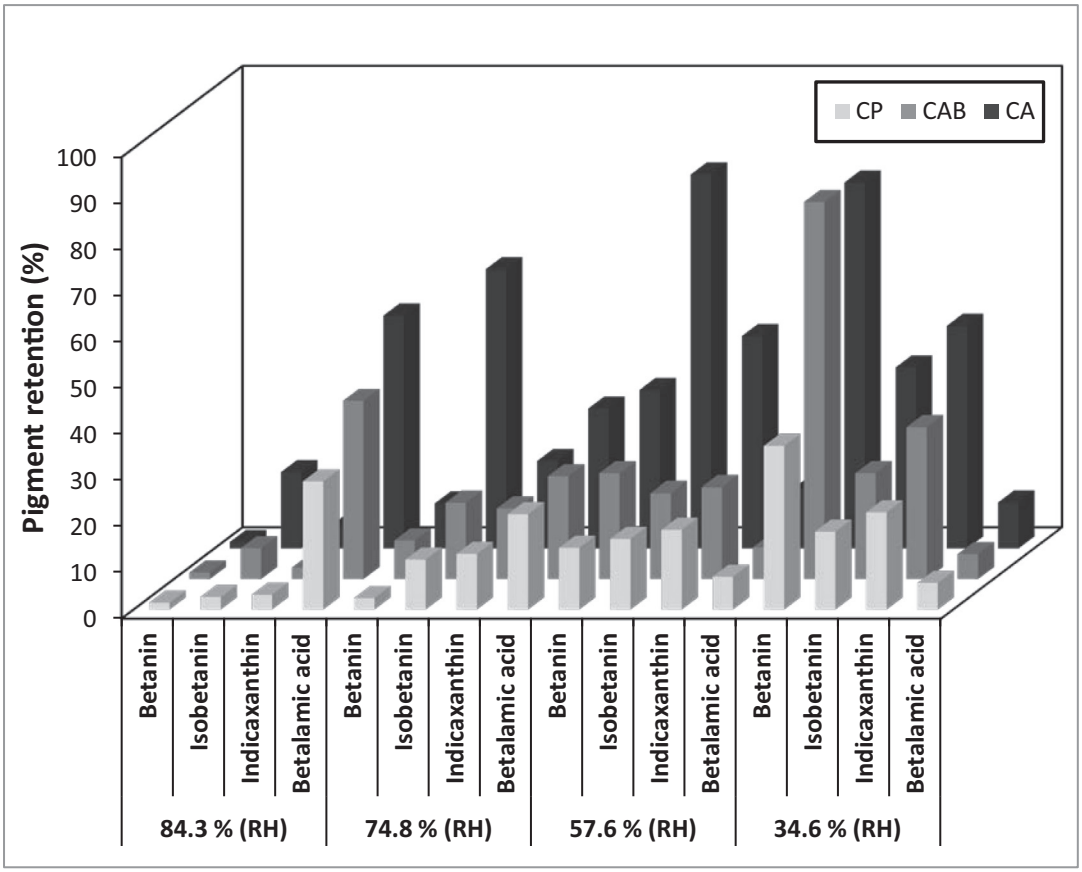
Storage Conditions	Samples	Betalain retention (%)	ARA retention (%)	ARA (μg vit. C/mg)	Correlation ARA – betalain retentions (R)
25 °C	CP	3.2 ± 1.6^{ab}	36.7 ± 0.7^{b}	9.7 ± 0.6^{b}	0.98
84.3%RH	CAB	2.8 ± 0.1^{b}	45.4 ± 0.3^{a}	13.3 ± 0.7^{a}	0.97
	CA	7.2 ± 1.6^{a}	47.1 ± 0.2^{a}	12.9 ± 0.5^{a}	0.96
25 °C	CP	12.7 ± 0.6^{b}	65.4 ± 2.9^{b}	17.2 ± 0.4^{b}	0.99
74.8%RH	CAB	19.0 ± 2.9^{a}	71.2 ± 3.5^{a}	20.9 ± 1.1^{a}	0.98
	CA	15.2 ± 1.7^{a}	71.9 ± 3.7^{a}	19.7 ± 1.2^{a}	0.99
25 °C	CP	34.6 ± 1.1^{b}	73.4 ± 1.7^{b}	19.3 ± 0.3^{b}	0.98
57.6%RH	CAB	39.9 ± 1.7^{a}	76.9 ± 2.5^{a}	22.6 ± 1.5^{a}	0.99
	CA	40.3 ± 1.1^{a}	81.7 ± 3.5^{a}	22.2 ± 1.6^{a}	0.99
25 °C	CP	27.6 ± 3.8^{c}	75.1 ± 2.9^{b}	20.0 ± 0.7^{b}	0.98
34.6%RH	CAB	37.2 ± 1.2^{b}	80.6 ± 3.9^{a}	23.6 ± 1.1^{a}	0.99
	CA	48.8 ± 0.4^{a}	88.5 ± 5.9^{a}	24.2 ± 1.6^{a}	0.98
50 °C	CP	14.7 ± 0.1^{b}	33.1 ± 1.0^{c}	8.7 ± 0.2^{c}	0.98
34.6%RH	CAB	19.6 ± 0.1^{a}	36.3 ± 0.6^{b}	10.7 ± 0.1^{b}	0.98
	CA	20.6 ± 0.1^{a}	49.7 ± 0.5^{a}	13.6 ± 0.2^{a}	0.99

CA: Calcium alginate beads; CAB: Sodium alginate-bovine serum albumin beads; CP: lyophilized cactus pulp as control sample. Different letters in the same column indicate a significant difference among CA, CAB beads and CP after 25 days at each storage condition ($p < 0.05$).

Table 2
Kinetic parameters of the betalain degradation reaction in CA and CAB beads in contrast to CP after storage at different conditions for 25 days.

Storage conditions	Samples	$10^2 k_{obsd}$ (days^{-1})		$t_{1/2}$ (days)		Correlation coefficients (R)	
		Superficial	Internal	Superficial	Internal	Superficial	Internal
25 °C	CP	42.23 ± 4.75^a	9.13 ± 1.90^a	1.6 ± 0.2^b	7.4 ± 2.3^b	0.91	0.97
84.3%RH	CAB	31.58 ± 0.19^b	7.94 ± 1.05^a	2.2 ± 0.1^a	8.7 ± 1.0^b	0.96	0.98
	CA	30.57 ± 0.17^b	3.92 ± 1.10^b	2.3 ± 0.1^a	17.7 ± 2.8^a	0.92	0.95
25 °C	CP	31.96 ± 2.80^a	4.79 ± 1.22^a	2.2 ± 0.2^c	14.5 ± 3.0^b	0.92	0.95
74.8%RH	CAB	16.92 ± 0.89^b	4.45 ± 1.47^a	4.0 ± 0.1^a	15.6 ± 1.8^b	0.99	0.97
	CA	24.85 ± 1.25^b	3.72 ± 0.94^b	2.8 ± 0.1^b	18.6 ± 1.4^a	0.99	0.98
25 °C	CP	21.45 ± 1.41^a	4.35 ± 0.43^b	3.2 ± 0.2^c	15.9 ± 1.6^b	0.87	0.99
57.6%RH	CAB	14.56 ± 1.16^b	3.98 ± 0.78^a	4.8 ± 4.0^a	17.4 ± 0.2^b	0.98	0.96
	CA	17.85 ± 5.43^b	3.21 ± 0.37^a	3.9 ± 1.0^b	21.5 ± 1.6^a	0.94	0.95
25 °C	CP	11.81 ± 1.38^b	1.37 ± 0.34^b	5.9 ± 1.0^b	50.6 ± 4.2^b	0.98	0.88
34.6%RH	CAB	13.55 ± 0.46^a	1.31 ± 0.09^a	5.1 ± 0.1^c	52.9 ± 4.1^b	0.99	0.92
	CA	11.14 ± 0.34^a	1.27 ± 0.09^a	6.3 ± 0.3^a	54.6 ± 1.0^a	0.99	0.99
50 °C	CP	16.73 ± 1.91^a	5.38 ± 0.21^b	4.1 ± 3.1^a	12.9 ± 0.5^b	0.93	0.97
34.6%RH	CAB	15.69 ± 1.40^a	3.02 ± 0.07^a	4.4 ± 0.5^b	22.9 ± 1.0^a	0.89	0.99
	CA	18.19 ± 0.44^b	2.80 ± 0.09^a	3.8 ± 0.1^c	24.7 ± 1.0^a	0.90	0.98

CA: Calcium alginate beads CAB: Calcium alginate-bovine serum albumin beads and lyophilized cactus pulp as control sample (CP). Values were obtained from plots of the slopes of ln (%retention) vs. time. Different letters in the same column indicate significant differences among CA, CAB and CP at each storage condition ($p < 0.05$).



CA: Calcium alginate hydrogel beads; CAB: Calcium alginate-bovine serum albumin hydrogel beads and CP: Control sample.

Fig. 1. Composition of pigments and the degradation product in CA and CAB beads and CP after 25 days storage at 57.6%, 74.8% and 84.3%RH at 25 °C.

beads depended on both, the RH and the type of matrix used in the beads. Betalain stability decreased with the increase of moisture content in the environment. At a given RH, the betalain contents of both types of beads, as well as of CP, decreased after storage. The betalain retention percentages after 25 days storage in the non-encapsulated material decreased when the RH was raised.

These results confirmed the negative effect of moisture on the stability of betalains. Betalain retention (%) in CA and CAB beads at RH of 57.6% and 74.8% were significantly different ($p < 0.05$); whereas in CP, it was significantly lower ($p < 0.05$). In contrast, at 84.3%RH no significant differences were found in the betalain retention between CA or CAB with CP ($p > 0.05$). The best storage conditions resulted to be encapsulated in CA beads at 34.6%RH at 25 °C with retentions of betalain content of 48.8% and ARA of 88.5%. CA and CAB encapsulated betalains showed higher storage stability at low %RH. The rise of storage temperature to 50 °C reduced the pigment protection. This behavior is ascribed to the betalain degradation due to a hydrolytic reaction when moisture increased. At high RH values, sorption phenomenon produces conformational swelling and changes in the structure of the dry beads (Serris & Biliaderis, 2001) due to the formation of water hydration spheres around the hydrophilic groups of alginate (Fringant, Desbrieres, Milas, Rinaudo, & Esgoubes, 1996).

A good correlation between the betanin retention and the antiradical capacity was observed in the beads (CA and CAB) and control CP when stored a different %RH and temperature conditions. These results show that the ARA of beads had a quasi-lineal relationship with the amount of remaining betanin; although, after complete pigment loss, residual ARA could be found, probably due to the reactivity of degradation products. The ARA at 57.6 and 74.8%RH for CA, CAB, were significantly different compared to CP samples ($p < 0.05$). At 84.3%RH no difference was observed for the ARA between CA and CAB beads ($p < 0.05$); whereas that for CP was significantly different ($p < 0.05$).

Table 1 shows that the ARA diminished when the bioactive compound content decreased. The change observed in the slope of the correlation curves suggests the degradation product formation with different ARA (data not shown). The formation of polyphenols from betalains could be attributed to the storage conditions (Nicoli, Anese, & Parpinel, 1999) and possibly due to the hydrolysis of glycosylated or condensed polyphenols into aglycones, increasing the number of free hydroxyl groups during the drying process (Sáenz et al., 2009; Turkmen, Sari, & Velioglu, 2005). This hydrolysis reaction yields the betanin degradation products, being betalamic acid (yellowish) and the *cyclo*-Dopa-D-glucoside (colorless) the major reaction products (Herbach, Rohe, Stintzing, & Carle, 2006).

As Table 1 indicates, ARA after bead storage at 50 °C can be ascribed not only to the presence of betalains but also to other phenolic compounds which could have appeared. Betalain degradation in beads and CP stored at different conditions (%RH and temperature) followed pseudo-first order kinetics. The same order was reported for the degradation of betacyanin in cactus pear microparticles (Sáenz et al., 2009). Table 2 shows the kinetic parameters, the pigment degradation rate constant (k) and the half-life time ($t_{1/2}$), for the CAB and CA beads as well as for CP under the different storage conditions. Kinetic data show that $t_{1/2}$ of the betanin in CP was much shorter than those for the beads; and the k value was higher for CP, indicating a higher degradation rate in these samples under different conditions of storage. In conclusion, beads protected the bioactive compounds during storage at 25 °C at low %RH as 34.6%. At 57.6% and 74.8%RH, encapsulation

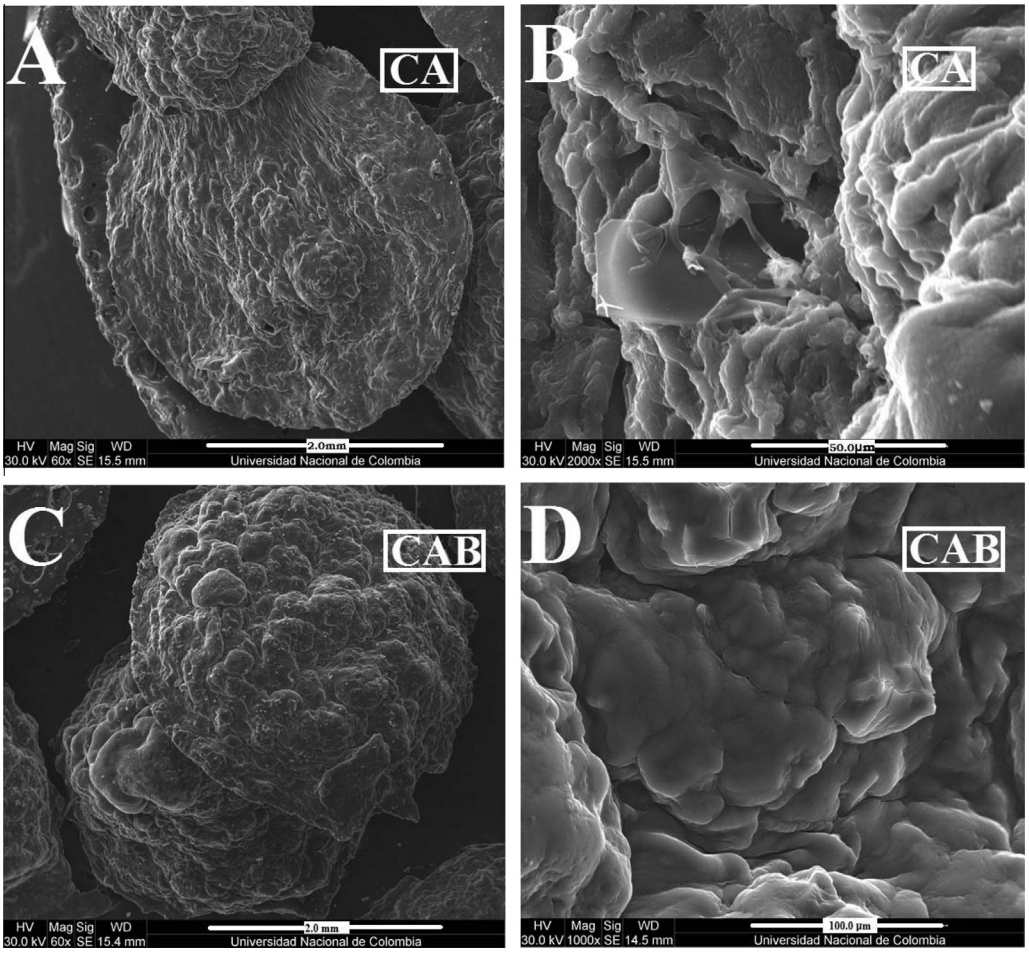


Fig. 2. SEM images of CA and CAB beads at initial time. (A) CA at 60×, (B) CA at 2000×, (C) CAB at 60× and (D) CAB at 1000×.

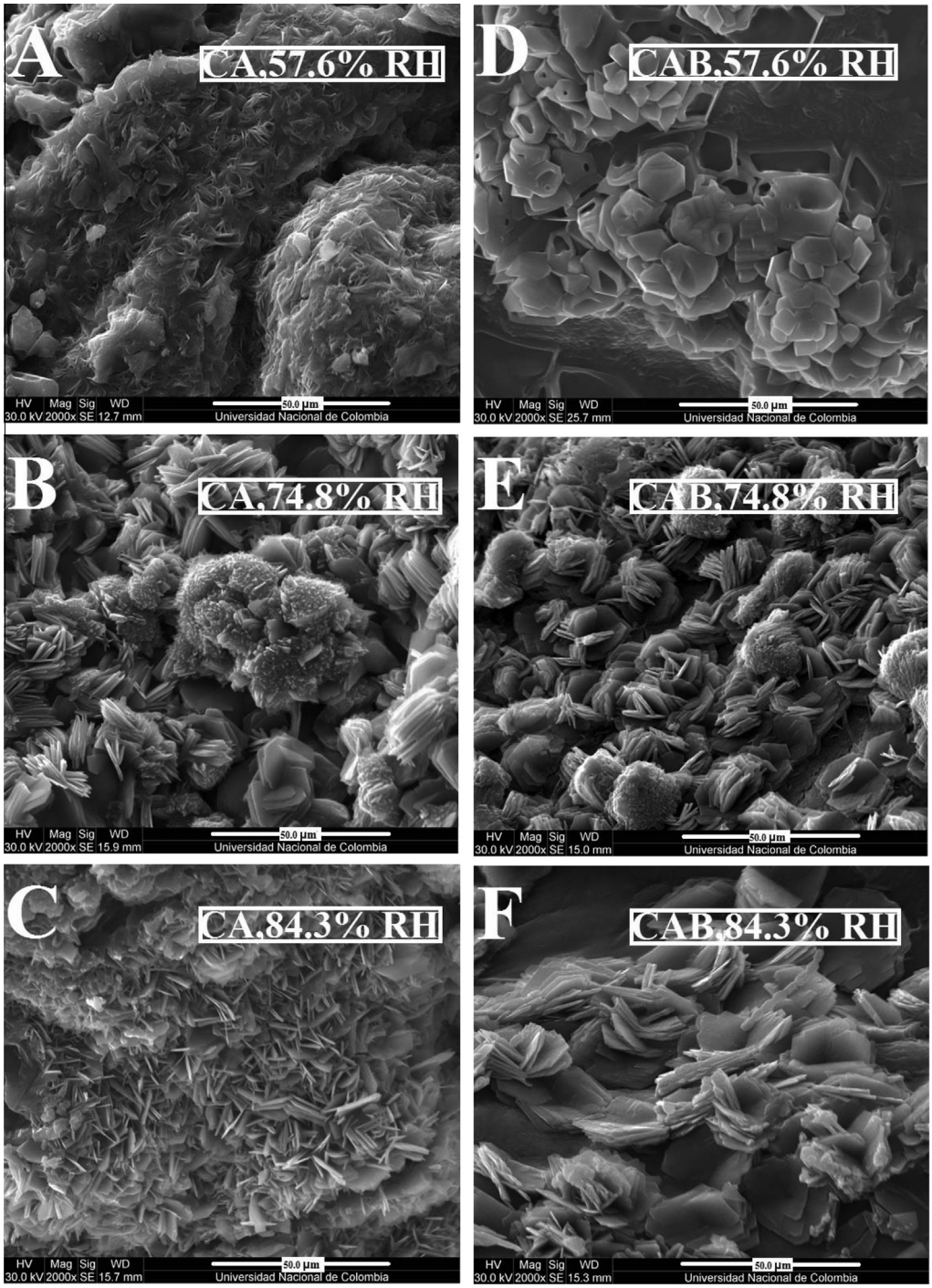


Fig. 3. SEM images at 2000× of CA (A, B, C) and CAB (D, E, F) beads after 25 days of storage at 25 °C and 57.6%, 74.8%, and 84.3%RH, respectively.

could provide good protection under storage conditions, although for a shorter period of time.

3.4. Pigment composition after bead storage

The betalain composition at initial time of CA and CAB beads showed three compounds being betanin the major one, in agreement with those results recently published by Otálora et al. (2015) for the original extract of cactus pear betalains. After 25 days of storage of CA and CAB beads, the presence of a betalain degradation product which could be determined at 410 nm. Betalamic acid formation was confirmed by a peak at higher retention time than the original pigments showing a pseudomolecular ion $[M+H]^+$ at m/z 212.

Based on the chromatographic analysis, the relative retention percentages of betanin, isobetanin and indicaxanthin as well as the formation of betalamic acid were determined in CA and CAB beads and CP after different storage conditions. Fig. 1 shows good retention of betanin and indicaxanthin at lower %RH values being the highest in CA sample. The ratio of betanin/isobetanin diminished when RH increases, because the betanin isomerization takes place usually during long-term storage of betalainic material (Strack, Vogt, & Schliemann, 2003). Fig. 1 also shows a steadily increase in the amount of betalamic acid after 25 days of storage

for all the samples when %RH was raised. The betanin and isobetanin degradation is induced by hydrolytic cleavage of the aldimine bond of betalains leading to the generation of bright yellow betalamic acid (Herbach et al., 2004; Schwartz & Von Elbe, 1983). At higher storage temperatures the retention of betanin, isobetanin, and indicaxanthin markedly diminished and the increase of the degradation product was observed. The formation of betalamic acid at 50 °C is higher than at 25 °C, being more noticeable in CP.

3.5. Microscopy of beads before and after storage

SEM micrographs revealed the surface morphologies of beads (Fig. 2). CA beads after drying showed collapsed irregular capsules with abundant roughness and cracks on the surface (Fig. 2A). This morphology was similar to those reported by Fundueanu, Nastruzzi, Carpov, Desbrieres, and Rinauto (1999), and López-Córdoba, Deladino, and Martino (2013). It can be observed that drying process influenced the morphology of beads concerning the surface characteristics. This suggests the cracking of the polymer network probably due to tensions promoted by shrinking during drying.

The CAB beads showed abundant protuberances and furrows as shown in Fig. 2C. Before the drying process, the bead shapes were spheroidal regardless of the presence of BSA; after drying, the beads almost kept the initial shape. This suggests that the filler was able to act as a structural support to control the shrinkage and to maintain the bead shape after drying. López-Córdoba, Deladino, and Martino (2014) have observed similar results in starch–alginate capsules.

Alginate matrix is very hygroscopic, a critical factor affecting the stability during bead storage. SEM images of all stored samples showed either eroded or exfoliated surfaces with smooth platelets formed in higher or lesser quantity according to the encapsulating agent. In general, this event is consequence of degradation of the beads under storage conditions. The SEM images of CA beads after 25 days of storage at 57.6%RH (Fig. 3A) showed eroded surfaces with small exfoliated grains, in contrast to 74.8, and 84.6%RH (Fig. 3B and C) these beads showed a flake-like structure as result of higher exfoliation of the surface. Exfoliated particles with smaller size were observed at higher RH (84.6%RH), indicating a higher damage of these beads (Fig. 3C).

The SEM micrographs of CAB beads after 25 days storage at 57.6%RH (Fig. 3D) showed the formation of smooth particles as larger platelets compared to the particles of CA beads. At 84.6%RH (Fig. 3F), CAB beads showed the formation of particles with larger size than those observed for CA beads (Fig. 3C); indicating a lesser damage on the surface of CAB beads.

The influence of storage temperature (25 and 50 °C) on the surface morphology for CA beads was also evaluated (%RH 34.6, see Supplementary Material). Clearly, small changes on the roughness and erosion of surfaces were observed, revealing that, at this low RH value, the effect of these temperatures (25 and 50 °C) is minor and, thus, small bead deterioration was found.

3.6. Thermal properties of beads before and after storage

Complex and similar TGA and DSC curves were observed in all cases (Fig. 4) and compared with those of the commercial samples of sodium alginate and BSA used as reagents (shown as Supplementary Material). Initially, TGA curves clearly indicate that sodium alginate is more thermally stable than BSA. About 20% of alginate degrades in the temperature range of 25–258 °C. This may be due to the volatilization of adsorbed water, water of hydration and residual molecules from the breaking of M and G segments (Sand, Yadav, Mishra, & Behari, 2010). These results are in line with findings reported by Devi and Kakati (2013), Mimmo, Marzadori, Montecchio, and Gessa (2005). The BSA showed a first endothermic peak around 47 °C, which has been previously

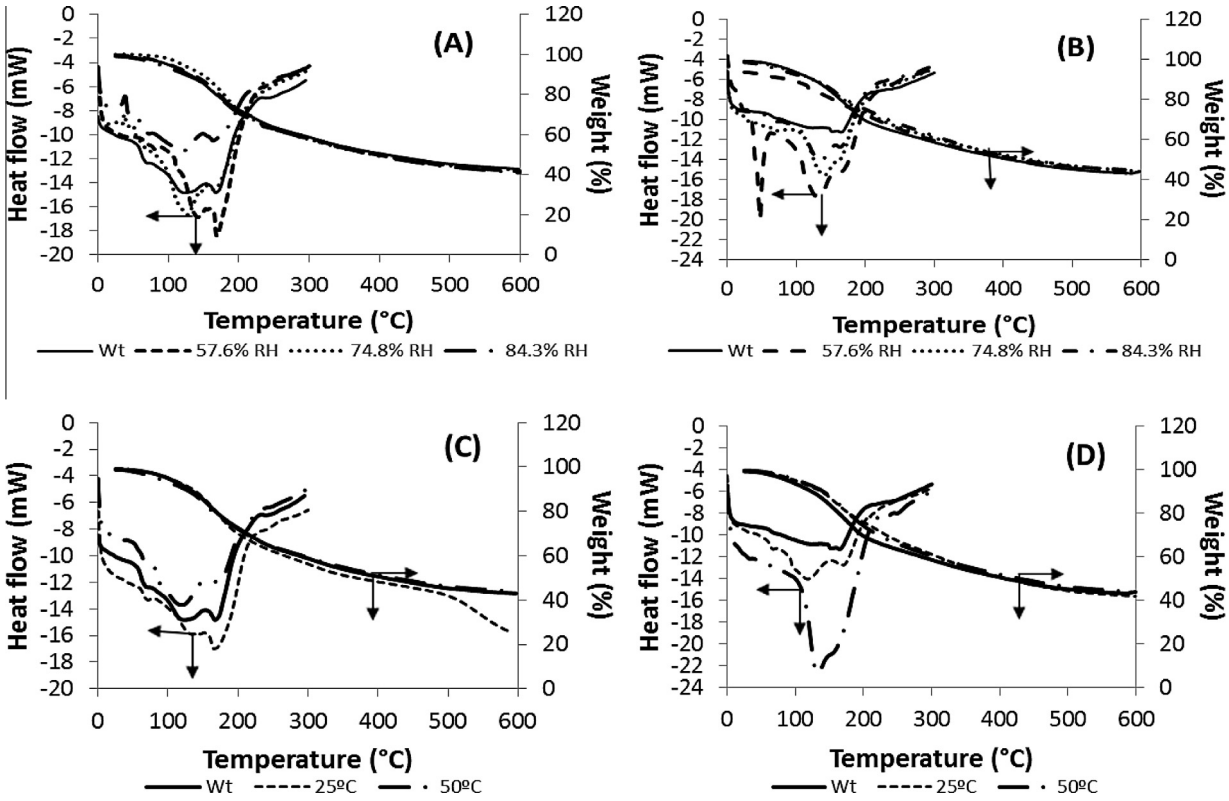


Fig. 4. DSC and TGA of CA and CAB beads stored 25 days at 25 °C at different %RH (A and B, respectively) and stored 25 days at 34.6%RH and 25 and 50 °C (C and D, respectively). Wt: bead without treatment.

reported as glass transition (T_g) event, whose value is situated approximately at 50 °C. At this temperature, it is considered that the protein internal dynamics is activated. A second (weak) endothermic peak at 200 °C, corresponding to combination of denaturalization and decomposition of BSA was also been observed. A similar result was found by Mizuno and Pikal (2013) at 40–60 °C in freeze dried pure BSA equilibrated at 11%RH.

TGA profiles were similar for CA beads after storage at different RH (Fig. 4A), revealing a loss of mass around 40% at 200 °C. This indicates that their thermal stability is similar. This loss of mass may involve dehydration of coordinated water molecules and the destruction of glycosidic bonds. The second decomposition process, above 200 °C, may be associated with de-polymerization, leading to the formation of levoglucosan ($C_6H_{10}O_5$), which is further thermo degraded to low molecular weight intermediate substances (Teixeira et al., 2014) that can be volatilised. In CA beads, DSC curves show complex endothermic peaks (Fig. 4A) which can be attributed to several successive processes such as, desorption, dehydration, gelatinization and perhaps melting of bead components. For CAB beads subjected at different RH values, similar TGA profiles was obtained revealing comparable thermal stability among the samples (Fig. 4B). The addition of BSA in the matrix did not interfered in the weight loss steps of beads. Thermograms of CAB beads at different %RH show similarly degradation to CAB beads without treatment (zero time). On the other hand, DSC curves show similar behavior of endothermic phenomena with respect to those of CA beads. Processes such as water evaporation, dehydration, gelatinization and melting of bead components lead to complex shape of curves. However, an endothermic peak at 57 °C was observed for the CAB sample after storage at 57.6%RH, which probably represents a glass transition of this material after partial degradation of the bead components and remaining BSA molecules.

Fig. 4A and D show the thermograms obtained after 25 days of storage (34.6%RH) at 25 and 50 °C for CA and CAB beads, respectively. Similar behavior of TGA and DSC profiles were observed regarding phenomena discussed above. Loss of mass is comparable in all cases; although, DSC curves show small differences concerning the storage temperatures used. In Fig. 4C, it is observed that the CA beads stored at 50 °C shows small area under curve (small change of enthalpy) than CA beads stored at 25 °C, perhaps as consequence of a higher deterioration of beads. However, an opposite behavior was observed for CAB beads, perhaps as result of some unknown species emerging from interactions between BSA and CA at 50 °C.

4. Conclusions

The present study evidenced the protective effect of encapsulation by ionic gelation on cactus betalains in respect to the lyophilized betalain extract. Incorporation of BSA to alginate in the formulation of CAB matrix did not affect the pigment stability, keeping its antioxidant activity, beads morphology and their physicochemical characteristics. Hydrolysis was the main mechanism of betalain degradation leading to the betalamic acid formation during storage under non-favorable conditions, such as either high temperature or high %RH. The encapsulated cactus betalains by ionic gelation can be considered a potential natural red-violet colorant to be used as functional colorant additive for the food industry.

Acknowledgments

M.C.O acknowledges CONICET for her fellowship. Authors greatly acknowledge financial support from COLCIENCIAS-MINCYT.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.01.115.

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