Food Chemistry 131 (2012) 231–238

Contents lists available at SciVerse ScienceDirect

Food Chemistry

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

A comparative study of the purification of betanin

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ARTICLE INFO

Article history:
Received 18 April 2011
Received in revised form 23 August 2011
Accepted 23 August 2011
Available online 30 August 2011

Keywords:
Betalains
Betacyanins
Betanin
E162
Natural Red 33

ABSTRACT

Betanin is a natural pigment with antioxidant properties used as a food colourant. This work describes the spectrophotometric and chromatographic quantification of betanin (2*S*/15*S*) and its epimer isobetanin (2*S*/15*R*) in fresh beetroot juice, food-grade beetroot powder and betanin standard diluted in dextrin. Absorption spectra of all three samples were deconvoluted using a mixed three-function model. Food-grade beetroot powder has the largest amount of violet-red impurities, probably formed during processing. The purification of betanin from these complex matrices was carried out by seven different methods. Ion exchange chromatography was the most efficient method for the purification of betanin from all samples; however, fractions contain high amounts of salt. Reversed-phase HPLC as well as reversed-phase column chromatography also produced good results at a much faster rate. The longer retention time of isobetanin when compared to betanin in reversed-phase conditions has been investigated by means of quantum-mechanical methods.

1. Introduction

Betalains are water-soluble vacuolar chromoalkaloids found in plants of the order Caryophyllales as well as in some Basidiomycota (Azeredo, 2009; Herbach, Stintzing, & Carle, 2006; Moreno, Garcia-Viguera, Gil, & Gil-Izquierdo, 2008; Stintzing & Carle, 2004; Strack, Vogt, & Schliemann, 2003; Zryd & Christinet, 2004). According to their chemical structure, these pigments can be subdivided into red–violet betacyanins or yellow betaxanthins (Scheme 1). Betacyanins are derivatives of betanidin, an iminium adduct of betalamic acid and cyclo-DOPA (Delgado-Vargas, Jimenez, & Paredes-Lopez, 2000), whereas betaxanthins result from the condensation of α-amino acids or amines with betalamic acid. *In natura*, betalains occur predominantly in fruits and flowers, including some fluorescent varieties of the latter (Gandia-Herrero, Escribano, & Garcia-Carmona, 2005; Gandia-Herrero, Garcia-Carmona, & Escribano, 2005a), and around seventy natural derivatives have been described so far (Stintzing & Carle, 2008b; Strack et al., 2003; Tsai, Sheu, Wu, & Sun, 2010).

Betanin (CI Natural Red 33, E-number E162, betanidin 5-*O*-β-glucoside, Scheme 1) is the only betalain approved for use in food and it is almost entirely obtained from red beet crops (Delgado-Vargas et al., 2000; Gaertner & Goldman, 2005; Goldman, Eagen, Breitbach, & Gabelman, 1996). The isolation of large amounts of any betalain is difficult due to its instability; yet around 40–200 mg of betanin are usually obtained per 100 g of beetroot (Herbach et al., 2006; Stintzing & Carle, 2008c). Although betalains are natural antioxidants, these pigments are used in the food industry exclusively as colourants (Georgiev et al., 2010; Kanner, Harel, & Granit, 2001; Kujala, Loponen, Klika, & Pihlaja, 2000).

Sources of betanin used for food-colouring purposes contain, amongst other substances, a mixture of betanin (Bn, 2*S*/15*S*) and its epimer isobetanin (iBn, 2*S*/15*R*). Nemzer and collaborators have compared the betalainic composition of pigment-enriched red beetroot dried extracts and analysed their nutritional profile (Nemzer et al., 2011). However, there is no systematic comparison of the methods for the purification of betanin from commercial betalain sources. Therefore, in this work we quantify the amount of betanin and isobetanin in fresh beetroot juice, in lyophilised beetroot (commercial food-grade beet powder), and betanin diluted with dextrin (commercial betanin), as well as compare the performance of seven different methods for the purification of betanin from these matrices.

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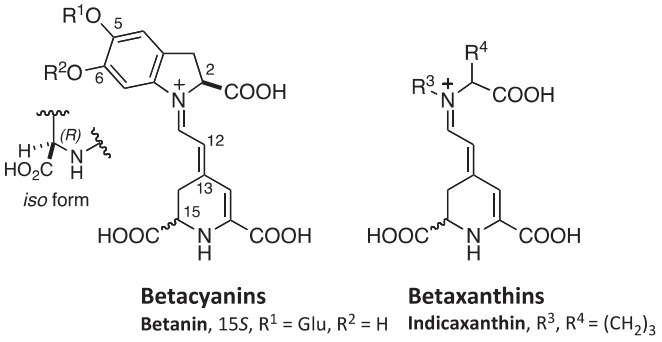
0308-8146
doi:10.1016/j.foodchem.2011.08.067



Scheme 1. Chemical structure of betalains in the fully protonated form.

2. Experimental

2.1. Chemicals

Polyethylene glycol (PEG, $\bar{M}$ = 5000 g mol^{-1}), $(NH_4)_2SO_4$, trifluoroacetic acid (TFA), acetic acid (HOAc), Sephadex G-25, Sephadex LH-20, silica gel 60 (70–230 mesh), silica gel 90 C_{18}-RP (230–400 mesh) and Q-Sepharose HP were obtained from Sigma–Aldrich (St. Louis, MO). Methanol (MeOH) and acetonitrile (MeCN) were HPLC-grade and were obtained from Merck (Darmstadt, Germany). All solutions were prepared using deionised water (18.2 MΩ cm, Milli-Q, Millipore, Billerica, MA).

2.2. Beetroot samples

Fresh beetroots (*Beta vulgaris* subsp. *vulgaris* var. *vulgaris*), also known as red beet, were obtained from a local market in Santo André, SP, Brazil (sample A), commercial lyophilised beetroot (food-grade, sample B), and commercial betanin in dextrin (sample C) were purchased in Jena, Germany.

2.2.1. Sample preparation

Sample A: beetroots (0.5 kg) were peeled, sliced and homogenised in a centrifugal juice extractor (Phillips–Walita, RI1858) at maximum speed. The homogenate was centrifuged (3500 rpm, 30 min, 25 °C) and filtered (Whatman qualitative filter paper, grade 4). The supernatant was stored at −20 °C and used within 5 days. Samples B and C: lyophilised beetroot and betanin in dextrin were resuspended in water (40–200 mg/mL) and filtered through a PTFE filter membrane (25 mm, pore size 0.45 μm) before purification.

2.3. Betanin purification

Samples A, B and C were submitted to purification by the following methods: gel permeation chromatography (GPC), normal phase column chromatography (NPC), reversed-phase column chromatography (RPC), reversed-phase high-performance liquid chromatography (RP-HPLC), ion-exchange chromatography (IEX) and aqueous two-phase extraction (ATPE). All experiments were performed in independent triplicates and purification yields are reported as mean ± standard deviation (mg/100 g of fresh (A) or dry (B and C) weight, namely raw weight) across all replicates. After purification, magenta fractions containing betanin were collected, pooled and the solution was concentrated (final volume of 1 mL) under reduced pressure (18 mbar, 25 °C). Afterwards, samples were submitted to UV–Vis spectroscopy and analytical HPLC analysis.

2.3.1. Purification by gel permeation column chromatography (GPC)

Sephadex G-25 (6 g) and Sephadex LH-20 (5 g) were used as the stationary phases in a glass column and packed under deionised water. The elution was performed with deionised water as the mobile phase, flow rates of 2.2 mL/min (GPC-G25) and 0.25 mL/min (GPC-LH20). After complete elution, the column was regenerated by washing with 5 column volumes of deionised water. Cleaning and re-equilibration steps were performed between each elution.

2.3.2. Purification by normal phase column chromatography (NPC)

Silica gel 60 (15 g) was used as the stationary phase in a glass column and packed with the binary solvent mixture of methanol/water 8:2 v/v with 1% v/v glacial acetic acid. The elution was performed with the same binary solvent mixture at a flow rate of 0.7 mL/min. The silica gel 60 column was not regenerated.

2.3.3. Purification by reversed-phase column chromatography (RPC)

Silica gel 90 C18 (20 g) was used as stationary phase in a glass column and conditioned with methanol followed by deionised water. The elution was performed with deionised water at a flow rate of 0.3 mL/min. After complete elution, the column was regenerated by washing with 6 column volumes of methanol and re-equilibrated with water. Cleaning (MeOH) and re-equilibration (water) steps were performed between each elution.

2.3.4. Purification by ion-exchange chromatography (IEX)

Q-Sepharose High Performance (cross-linked agarose with quaternary ammonium as exchanger group) was used as stationary phase (25 mL of a suspension in 20% ethanol) in a glass column and conditioned with water. The samples were washed with water and eluted with a gradient of NaCl (0.1–0.5 M) at a flow rate of 0.1 mL/min. Cleaning (1.0 M NaCl) and re-equilibration (water) steps were performed between each elution.

2.3.5. Purification by aqueous two-phase extraction (ATPE)

PEG 5000 (2 g) and $(NH_4)_2SO_4$ (2 g) were weighed and added to sample A (2 mL), sample B (0.4 g) or sample C (0.4 g). The samples were dissolved in water (10 mL) and filtered. The contents were mixed thoroughly using a magnetic stirrer for 1 h for equilibration and were allowed for phase separation for 3 h. After the separation of the two phases, the dull red bottom aqueous phase was discarded and the bright magenta top phase containing PEG and betanin was submitted to extraction with chloroform. The brown polyphenolic components present in the juice accumulate at the interface and were discarded. The PEG was regenerated after extraction with chloroform and the betanin was submitted to UV–Vis and analytical HPLC analysis (Chethana, Nayak, & Raghavarao, 2007).

2.3.6. Purification by reversed-phase high performance liquid chromatography (RP-HPLC)

Reversed-phase chromatography was performed in a Waters (Milford, MA) 600 system equipped with a UV–Vis detector (dual-wavelength, Waters 2489) and a Jupiter-15 (300 Å, 15 μm, 250 × 21.2 mm, Phenomenex, Torrance, CA) C_{18} column. Gradients were formed between two helium-degassed solvents: solvent A: water with 1% v/v HOAc, solvent B: 60% v/v MeCN/water with 1% v/v HOAc; linear gradient from 5% to 20% B in 60 min at 25 °C, flow rate: 10 mL/min. Samples were monitored by UV–Vis absorption at 254 nm.

2.4. Analysis of purified betanin

2.4.1. Spectrophotometric analysis and spectra deconvolution

Absorption spectra were recorded in the UV–Vis region (200–800 nm) at 25 ± 1 °C on a Varian Cary 50 Bio spectrophotometer equipped with a Peltier-thermostatted cell holder (Varian, Palo

Alto, CA). The betanin concentration was determined by assuming a molar absorption coefficient (ε) of 6.5×10^4 L mol^{-1} cm^{-1} at 536 nm (Schwartz & Von Elbe, 1980). Spectra were deconvoluted using the Fytik analysis software (Wojdyr, 2010).

2.4.2. RP-HPLC analysis

Analytical RP-HPLC separation and analysis were performed on a Waters 2695 Alliance system equipped with a UV–Vis detector (dual-wavelength, Waters 2489) and a Supelcosil LC-18 (300 Å, 5 μm, 150 × 46 mm; Supelco, Bellefonte, PA) C_{18} column. Solvent A was water with 0.1% v/v TFA, solvent B was 60% v/v MeCN/water with 0.1% v/v TFA; a linear gradient was performed from 5% to 95% B in 20 min at 25 °C, at a flow rate of 1 mL/min, injection volume was 10 μL, with spectrophotometric detection set at 254 and 536 nm. Due to the high polarity of most betalainic pigments (Escribano, Cabanes, & Garcia-Carmona, 1997; Gandia-Herrero, Garcia-Carmona, & Escribano, 2004; Stintzing, Conrad, Klaiber, Beifuss, & Carle, 2004; Wybraniec, Jerz, Gebers, & Winterhalter, 2010), the retention times of Bn and iBn are short under these experimental conditions.

2.4.3. LC-MS analysis

A Bruker Daltonics Esquire 3000 Plus was used for the ESI-MS analyses. Elution conditions were the same as those used in the RP-HPLC analysis. The vaporiser temperature was 325 °C and the voltage was maintained at 4.0 kV. The sheath gas was nitrogen, operated at a pressure of 26 psi (6.0 L/min). Compounds were ionised in the positive mode and ions were monitored in the full scan mode (range of *m/z* 200–600). HPLC-DAD-ESI(+)-MS/MS analysis was carried out on a Bruker Daltonics (Billerica, MA) Esquire HCT ion trap mass spectrometer equipped with an electrospray source and coupled to a Shimadzu Prominence liquid chromatograph (Shimadzu, Kyoto, Japan). The chromatograph was equipped with a Luna C18 column (150 mm × 2.0 mm, 3 mm, Phenomenex) maintained at 30 °C and a PDA SPD-M20A detector. Nitrogen was used as nebulising (45 psi) and drying gas (6 L/min, 300 °C) and helium as buffer gas (4×10^{-6} mbar). The capillary high voltage was set to 3500 V. To avoid space–charge effects, smart ion charge control (ICC) was set to an arbitrary value of 50,000.

2.5. Statistical analysis

All values are expressed as mean ± standard deviation (SD) of three completely independent replicates. Statistical data analysis was performed by one-way analysis of variance (ANOVA). The level of statistical significance was taken to be $p < 0.05$.

3. Computational methods

The geometries of all species were fully optimised at the semi-empirical PM6 level of theory without any constraints (Stewart, 2007). The optimised structures were confirmed as real minima by frequency calculations (no imaginary frequency). Single-point energies were estimated at the M06-2X/6-311++G(d,p) level (Zhao & Truhlar, 2008a, 2008b), corrected for the zero point energies and thermal corrections. Electronic transition energies and oscillator strengths of the molecules were determined using the ZIndo/S method (Zerner, 1991). Solvation energy was calculated as the difference between the calculated Gibbs free energy in the gas-phase and that estimated using the IEFPCM (integral equation formalism PCM) parameterised for water according to the SMD protocol (Marenich, Cramer, & Truhlar, 2009). All calculations were performed using Gaussian 09.

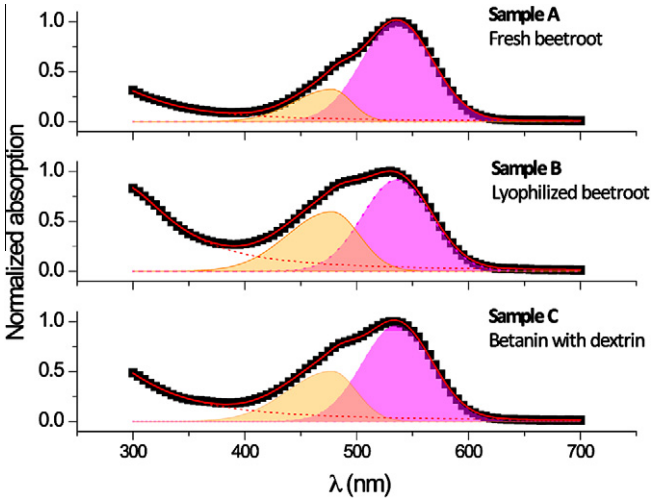


Fig. 1. Normalised absorption spectra of samples A, B and C. Deconvoluted bands correspond to Bns (magenta) and Bx (yellow); the continuous red line shows the fitted spectra, $r^2 = 0.9991$ (sample A), 0.9994 (sample B) and 0.9992 (sample C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Results and discussion

4.1. Analysis of betanin sources

Betanin sources are as follows: fresh beetroot juice (sample A), commercial lyophilised (freeze-dried) food-grade beetroot (i.e., beet powder, sample B), and commercial betanin diluted with dextrin (sample C). These samples represent the main betalainic sources used commercially for colouring purposes in Europe and North America (Otterstätter, 1999; Stintzing & Carle, 2008c). The UV–Vis spectra of raw samples are depicted in Fig. 1. Each spectrum was deconvoluted into a Gaussian line centred at 536 nm, a split-Gaussian line with maximum height fixed at 478 nm and a non-constrained split-Voigt line, corresponding, respectively, to the betanin/isobetanin mixture (Bns, $\lambda_{max} = 536$ nm, $\varepsilon_{535} = 6.5 \times 10^4$ L mol^{-1} cm^{-1}) (Schwartz & Von Elbe, 1980), betaxanthins (Bx, $\lambda_{max} = 478$ nm, $\varepsilon_{480} = 4.8 \times 10^4$ L mol^{-1} cm^{-1}) (Schliemann, Kobayashi, & Strack, 1999; Trezzini & Zryd, 1991), and other components ($\lambda_{max} < 300$ nm, including browning substances absorbing at around 600 nm). The non-linear curve fitting of the experimental absorption spectra using this approach resulted in very good coefficients of determination for all samples ($r^2 > 0.999$). In particular, the fitting of the 350–420 nm and >600 nm regions of the spectra is improved when compared to previous results (Nilsson, 1970; Saguy, Kopelman, & Mizrahi, 1978). Analysis of the deconvoluted bands indicates that sample A has the highest relative amount of Bns; i.e., Bns/Bx molar ratios: 2.3 (sample A), 1.1 (sample B) and 1.4 (sample C).

Raw samples were submitted to RP-HPLC analysis coupled with UV–Vis (254 and 536 nm, Fig. S1) and MS (ESI+, *m/z* 200–600) detec-

Table 1
Comparison of the concentration of betanins (in mg/100 g of raw material) determined by UV–Vis spectrophotometry and RP-HPLC/UV–Vis. Mean values of triplicate experiments ± SD (in parenthesis).

Sample	^{V}Bns+	^{LC}Bns+	^{LC}Bn	^{LC}iBn	$^{LC/V}$ Bns+(%)[a]
A	66.3 (17.6)	60.7 (15.7)	52.9 (13.4)	6.2 (1.8)	91.6 (0.7)
B	77.4 (3.8)	59.1 (1.3)	16.9 (0.2)	18.3 (0.2)	76.4 (5.8)
C	179.8 (2.2)	172.6 (3.4)	80.5 (2.0)	74.4 (1.7)	96.0 (2.3)

[a] Considering error propagation, $\sigma_{A/B} = A/B \times ((\sigma_A/A)^2 + (\sigma_B/B)^2)^{0.5}$, errors are 33.9%, 4.1% and 2.3% for samples A, B and C, respectively.

tion. Quantitative analysis of the spectrophotometric and chromatographic data is given in Table 1. The concentration of species absorbing at 536 nm in RP-HPLC elution (^{LC}Bns+) was determined by assuming $\varepsilon = 6.5 \times 10^4$ L mol^{-1} cm^{-1}, to allow direct comparison with data obtained by UV–Vis spectrophotometry (^{V}Bns+). The concentrations of betanin (^{LC}Bn, $t_R = 6.1 \pm 0.2$ min) and isobetanin (^{LC}iBn, 6.4 ± 0.2 min) were determined using a calibration curve (Fig. S2). The Bn/iBn ratios are 8.6 ± 3.3, 0.9 ± 0.1 and 1.1 ± 0.1 for samples A, B and C, respectively. The amount of iBn is higher than Bn in sample B and almost equivalent in sample C. Also, the relative amount of Bns is higher in sample C (0.15 ± 0.01%) than in samples A and B (0.06 ± 0.01% and 0.04 ± 0.01%, respectively).

The discrepancies in the quantification of betalains by spectrophotometric and chromatographic methods can reach 15% (Schwartz, Hildenbrand, & Von Elbe, 1981). We have found that the determination of the Bns+ concentrations by UV–Vis spectroscopy produced a much less dramatic error for samples A and C than for sample B. The determination of the betanin concentration by direct absorption measurement at 536 nm resulted in overestimates of 8% for sample A, 25% for sample B (lyophilised beetroot) and 4% for sample C. The use of a correction factor based on the absorption of impurities at 600 or 605 nm improves the agreement of the spectrophotometric and chromatographic results for samples A and C (von Elbe, 2005); however, even using corrected absorption, the discrepancy for sample B is still around 9% (Table S1). This result could be due to the decomposition of Bn into decarboxylated (at C2, C15, and C17) and oxidised (i.e., neobetalains) derivatives absorbing at 536 nm during the lyophilisation process (see Table S2), as well as to the large amount of betaxanthins absorbing at 480 nm in sample B (for an example of the effect of impurities, i.e., decarboxylated betacyanins and neobetalamic derivatives, on the spectra of Bns, see Fig. S3). Although sample B is a commercial product, lyophilisation of sample A immediately after juice extraction (initial pH 6) also resulted in sample browning, probably due to the increase in the concentration of polyphenol oxidase enzymes (PPOs) during freeze-drying (Mayer, 2006). It is known that PPOs can catalyse the oxidation of *o*-hydroquinones to *o*-quinones, which polymerise, producing black, brown and red pigments related to fruit browning (Mayer, 2006).

Moreover, the presence of light, metals, oxygen or lack of pH control during concentration almost always leads to betanin decomposition (Mosshammer, Stintzing, & Carle, 2005; Stintzing, Kugler, Carle, & Conrad, 2006; Stintzing et al., 2004; Strack et al., 1987). The analysis of the raw samples A, B and C was carried out by RP-HPLC-DAD-ESI(+)-MS/MS and the chromatographic peak assignment is given in Table S2 and Figs. S4, S5 and S6. As reported previously (Herbach, Stintzing, & Carle, 2004; Liu, Gao, Xu, Wang, & Yang, 2008; Nemzer et al., 2011), processing of fresh beetroot juice (sample A) results in the decarboxylation of Bns, decreases the amount of vulgaxanthin I (Gln-betaxanthin) as well as other betaxanthins and increases the amount of neobetalains.

The spectrophotometric quantification of betalain content according to the method proposed by Nilsson was shown to be inappropriate in the study of cactus fruit juices, due to the large amount of betaxanthins compared to beetroot extracts (Nilsson, 1970; Stintzing, Schieber, & Carle, 2003). However, this method is still recommended for red beet samples today (Stintzing & Carle, 2008a). Our results indicate that the betanin/isobetanin mixture can only be unequivocally quantified by spectrophotometric methods when the amount of other substances absorbing at 400–480 nm and at 536 nm is reduced.

4.2. Purification of betanin

Betanin purification was carried out by seven different methods, which have been previously described in the purification of betalains, namely normal and reversed (C_{18}) phase adsorption column chromatography (NPC and RPC, respectively) (Delgado-Vargas et al., 2000; Herbach et al., 2006; Kobayashi, Schmidt, Wray, & Schliemann, 2001; Kugler, Stintzing, & Carle, 2004; Rudrappa, Neelwarne, & Aswathanarayana, 2004), reversed-phase high performance liquid chromatography (RP-HPLC) (Alcalde-Eon, Saavedra, de Pascual-Teresa, & Rivas-Gonzalo, 2004; Gandia-Herrero, Garcia-Carmona, & Escribano, 2005b; Wybraniec et al., 2009), gel permeation chromatography with Sephadex G-25 and Sephadex LH-20 (GPC-G25 and GPC-LH20, respectively) (Adams & Elbe, 1977; Schliemann et al., 1996), ion-exchange chromatography with Q-Sepharose (IEX) (Stintzing, Schieber, & Carle, 2002) and two-phase aqueous extraction with PEG/$(NH_4)_2SO_4$ (ATPE) (Chethana et al., 2007; Neelwarne & Thimmaraju, 2009).

To allow direct comparison of results after purification, the following points were considered: (i) all column chromatographic experiments were carried out on identical columns and fractions (1 mL) of the magenta portion were collected and combined after preliminary HPLC analysis; (ii) samples were manipulated in a very similar manner and no additive for betalain preservation was used. The addition of chain-breaking antioxidants (e.g., ascorbic acid) and chelants (e.g., EDTA, citric acid) to avoid the decomposition of betanin can compromise subsequent studies of antioxidant capacity (Bilyk & Howard, 1982; Kugler et al., 2004; Schliemann et al., 1999); (iii) analytical HPLC analysis of the purified samples was carried out using solutions of betanins with an absorption at 536 nm between 0.4 and 0.5. This reduces discrepancies in the determination of the betanin concentration by spectrophotometric and quantitative HPLC analysis (von Elbe, 2005); (iv) no additional precautions (e.g., protection from light, low temperature) was taken to avoid the decomposition of betanin; therefore, the yields reported here can be considered as "minimum" yields.

Fig. 2 shows the RP-HPLC elution profile of samples A, B and C after purification. Monitoring of the purified samples by absorption at 254 nm indicated that the analysis of purification profiles of betalains exclusively at 536 nm can be deceptive. RPC and IEX are the only methods able to purify betanin in sample A. In samples B and C, a significant amount of betanidin and its epimer are present after purification by almost all methods, with the exception of IEX and RPC. Detection at 536 nm shows that the Bn/iBn ratio is higher in sample A than in samples B and C, probably because of thermal treatment during sample processing and storage. Analysis of peaks detected by fluorescence and ESI(+)-MS/MS indicates the presence of vulgaxanthin I ($t_R = 4.2$ min, m/z $[M+H]^+ = 340$, $MS^2[340]$: 323 (100%), 277 (32%)), and small amounts of other betaxanthins, after purification of sample A by most methods, except IEX. Interestingly, the amount of betaxanthins in samples B and C is very small, probably due to their lower thermal stability when compared to betacyanins (Cai, Sun, Schliemann, & Corke, 2001; Herbach et al., 2004). Also, neobetalains and decarboxylated degradation products are formed at the expense of red–purple genuine beetroot pigments during processing, as reported previously by Herbach et al. (2004).

The efficiency of betanin purification from samples A, B and C is given in Table 2. Betanin was successfully purified from samples A and C using at least one of the methods studied. Unfortunately, although a mixture of betanin and isobetanin was efficiently purified from sample B, we were not able to obtain high purity betanin (>97%) from this sample by any means.

Sephadex LH-20 is reported to remove colourless phenolics efficiently from betalain samples, as well as fractionate betaxanthins, betacyanins and betacyanin aglycones (Kujala, Loponen, & Pihlaja, 2001; Kujala et al., 2000; Stintzing & Carle, 2008a). In samples B and C, the largest amount of species absorbing at 536 nm (Bns+) was purified by GPC-LH20, indicating that this method is the most adequate for the pre-purification of betacyanins in processed

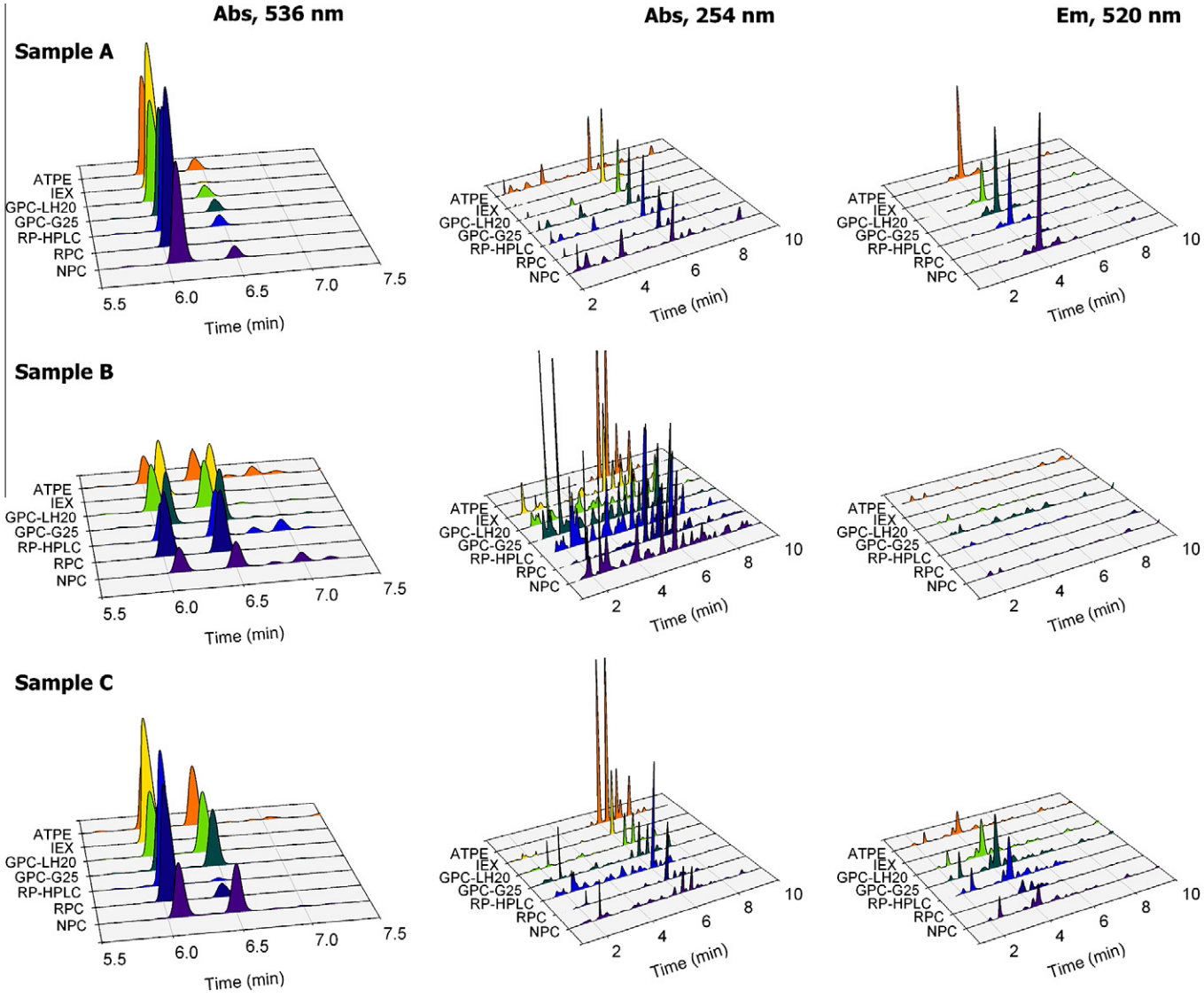


Fig. 2. HPLC chromatograms of purified samples monitored by both UV–Vis absorption at 254 and 536 nm, and fluorescence at 520 nm. Plots within each column are on the same scale, $\lambda_{max}^{536} = 0.6$, $\lambda_{max}^{254} = 0.1$ and $\lambda_{max}^{520} = 1000$.

samples. However, this method is apparently not adequate for the resolution of betanin and isobetanin. Interestingly, RPC, RP-HPLC and IEX methods were the most efficient in the purification of betanin in samples A and C, i.e., with the highest ^{LC}Bn. Ion-exchange chromatography (IEX), both anion and cation exchange methods, requires the use of large concentrations of acid or salt, which could result in pigment degradation or require a desalting step, depending on the desired application (Piattelli, Minale, & Prota, 1964). As expected, the samples purified by IEX show the highest specific conductance (up to 9.4 mS/cm). Besides, IEX shows poor scalability and is the slowest method investigated (see Section 2). The use of NPC and ATPS in the purification of betanin produced the worst results. The PEG/$(NH_4)_2SO_4$ system is based on the differential partitioning of betalains and sugars between the aqueous phases (Chethana et al., 2007; Neelwarne & Thimmaraju, 2009). Consequently, the presence of large amounts of dextrin in sample C compromises the partitioning, making this approach inefficient. Results reported here are yet to be compared to those obtained using countercurrent chromatography, which has been applied effectively for the purification of betanin and its epimer on the preparative scale (Degenhardt & Winterhalter, 2001; Schliemann & Strack, 1998; Wybraniec et al., 2010).

4.3. Physicochemical properties of betanin and isobetanin

Betalain optical isomers can be resolved by several chromatographic methods in the absence of chiral stationary phases (Schliemann et al., 1999; Wybraniec et al., 2009). For example, betanin has a shorter retention time than isobetanin ($\Delta t_R = 0.3$ min) in the RP-HPLC analysis reported in this work. This difference has been rationalised in terms of a stronger interaction of iBn with the non-polar stationary phase, but no quantitative data is given whatsoever (Schwartz & Von Elbe, 1980; Wybraniec, 2005). Therefore, we examined this effect quantitatively through theoretical calculations.

Due to the size of betanin, geometry optimisation and frequency calculations were conducted using the semi-empirical method PM6 (Stewart, 2007); however, the energies and the physicochemical properties in water were determined at the SMD/M06-2X/6-311++G(d,p) level (Marenich et al., 2009; Zhao & Truhlar, 2008a, 2008b). Furthermore, we limited ourselves to two conformational isomers of both Bn and iBn, namely the axial carboxyl and equatorial carboxyl conformers (Scheme S1, coordinates of optimised geometries are given in the Supplementary data).

Due to the lack of structural parameters for betanin and isobetanin, the structure optimisation was verified by comparison of the

Table 2
Betanin/isobetanin contents of samples A, B and C (in mg/100 g of raw material) using different purification methods.[a] Mean values of triplicate experiments ± SD (in parenthesis).

	^{V}Bns+	^{LC}Bns+	^{LC}Bn	^{LC}iBn	Bns (%)	Bn (%)
Sample A						
NPC	19.2 (2.1)	17.5 (2.5)	15.1 (2.2)	1.8 (0.4)	97	86
RPC	30.6 (2.2)	30.2 (2.8)	29.8 (2.8)	0.3 (0.1)	100	99
RP-HPLC	13.3 (0.7)	13.2 (0.9)	12.2 (0.6)	0.9 (0.3)	99	92
GPC-G25	27.7 (7.0)	27.4 (6.5)	24.0 (5.9)	3.1 (0.5)	99	88
GPC-LH20	13.6 (4.1)	13.5 (4.1)	12.0 (3.7)	1.4 (0.4)	99	89
IEX	12.8 (1.6)	12.7 (1.4)	12.5 (1.4)	0.1 (0.1)	99	99
ATPS	8.9 (1.7)	8.4 (1.2)	7.3 (1.3)	0.9 (0.1)	98	87
Sample B						
NPC	15.1 (3.0)	10.3 (1.7)	3.3 (0.8)	3.2 (0.3)	64	32
RPC	15.2 (2.0)	14.7 (2.7)	7.7 (1.4)	7.0 (1.3)	100	52
RP-HPLC	16.3 (4.2)	15.6 (3.4)	5.3 (1.2)	6.5 (1.4)	76	34
GPC-G25	7.2 (2.7)	6.5 (3.0)	3.1 (1.4)	2.9 (1.3)	92	47
GPC-LH20	23.3 (3.2)	20.5 (2.4)	10.4 (1.7)	9.5 (0.7)	97	51
IEX	17.1 (3.4)	16.2(2.9)	8.7 (2.0)	7.2 (1.0)	98	54
ATPS	6.4 (1.4)	4.9 (1.4)	1.5 (0.5)	1.7 (0.5)	66	31
Sample C						
NPC	67.3 (10.6)	59.7 (8.5)	31.4 (2.6)	24.3 (5.6)	93	52
RPC	89.6 (17.2)	93.7 (17.7)	81.3 (15.0)	11.6 (2.2)	99	87
RP-HPLC	85.3 (19.2)	83.9 (18.1)	81.3 (17.8)	2.1 (0.5)	99	97
GPC-G25	57.5 (9.5)	62.0 (14.6)	31.9 (7.0)	28.4 (7.3)	97	51
GPC-LH20	116.5 (1.9)	128.4 (6.4)	67.9 (6.4)	55.4 (2.1)	96	53
IEX	90.2 (22.7)	89.5 (20.9)	89.1 (20.9)	0.4 (0.4)	100	100
ATPS	66.4 (6.9)	67.9 (1.9)	30.9 (2.3)	30.7 (1.0)	91	45

[a] GPC, gel permeation chromatography; NPC, column chromatography; RPC, reversed-phase column chromatography; RP-HPLC, reversed-phase high performance liquid chromatography; IEX, ion-exchange chromatography; ATPE, aqueous two-phase extraction.

Table 3
Calculated spectroscopic and physicochemical properties of the main conformational isomers of Bn and iBn.

Entry	ξ (Å^3)[a]	ΔG_S^0 (kJ mol^{-1})[a]	μ_V (D)[b]	μ_W (D)[a]	Z (Bohr^3)[b]	λ^{Th} (nm)[c]	f^c
Bn eq	482.1	59.4	11.9	14.8	392.5	535	1.13
Bn ax	482.5	60.6	11.7	14.5	389.3	526	1.15
iBn eq	482.1	59.2	11.7	14.4	392.6	536	1.13
iBn ax	482.6	60.6	11.5	14.1	388.9	525	1.15

[a] SMD/M06-2X/6-311++*G*(d,p)/PM6.
[b] PM6.
[c] SMD/ZIndo-S/PM6; experimental λ = 536 nm in water.

theoretical electronic transition wavelengths (λ^{Th}), determined using the SMD/ZIndo/S approach (Marenich et al., 2009; Zerner, 1991), with experimental absorption maxima. The equatorial carboxyl conformers of both Bn and iBn are more planar than the corresponding axial isomers.

Results obtained for the equatorial carboxyl conformers of both Bn and iBn are in excellent agreement with experimental data (Table 3). This correspondence is obtained exclusively when fully protonated forms of Bn and iBn are used (pK_a of the carboxylic groups of betanin is 3.4) (Nilsson, 1970). The absorption maximum is not pH-dependent in the 3–7 pH range (Azeredo, 2009); however, the results can be rationalised based on the occurrence of hydrogen bonds with strong ion-dipole character between the carboxylate groups and water. The variation in the solvent cavity volume (ξ), the solvation free-energy (ΔG_S^0) and the isotropic polarisability (Z) is very small between Bn and iBn and, therefore, these are poor descriptors for the retention time of these isomers. However, the calculated dipole moments in the gas phase and in water (μ_V and μ_W, respectively) indicate that Bn is slightly more polar than iBn, a fact that would explain the higher retention times under reversed-phase conditions of the latter.

5. Conclusions

The deconvolution of absorption spectra using a mixed-function approach results in good fitting of experimental spectra for samples containing betanin and different amounts of other substances. Determination of the concentration of the mixture of betanin/isobetanin by corrected spectrophotometry is in agreement with quantitative chromatographic data when the amount of impurities absorbing at 400–480 nm and at around 530 nm is small. Processed samples containing betanin have higher amounts of its epimer isobetanin than fresh extract.

Comparison of methods for the purification of betanin indicates that ion-exchange chromatography is very efficient and is able to resolve the betanin/isobetanin mixture. However, this method is time-consuming and the amount of salt in the purified fractions results in high specific conductance. Consequently, both RP-HPLC and RP column chromatography methods provide the best balance between speed and efficiency. The longer retention time of isobetanin when compared to betanin, under reversed-phase conditions, results from higher interaction of the former with the non-polar stationary phase, as implied by the dipole moment calculated for these substances in the gas-phase and in water.

Acknowledgements

We thank Prof. Dr. Antonio de Miranda (UNIFESP), Prof. Dr. Etelvino J.H. Bechara (IQ-USP/UNIFESP-Diadema) and Prof. Dr. Nilson Antonio de Assunção (UNIFESP-Diadema) for their help in the early stages of this work, Prof. Ernani Pinto Junior (FCF-USP) and the Instituto Nacional de Ciência e Tecnologia de Análise Integrada

do Risco Ambiental (INAIRA) for allowing us to use the HPLC-MS/MS equipment in his laboratory, and Prof. Frank H. Quina for a critical reading of the manuscript. Betanin in dextrin and lyophilised beetroot samples were kindly provided by Prof. Dr. Rainer Beckert and PD. Dr. Dieter Weiss (FSU-Jena, Germany). This work was partially supported by FAPESP (JP #07/00684-6, DD #07/59407-1), CAPES (PE/2007) and UFABC; ELB thanks the CNPq for a research productivity fellowship (#304887/2010-2).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodchem.2011.08.067.

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