Original article
Red Beetroot Thematic Issue

Thermal Stability of Betalains in By-Products of the Blanching and Cutting of *Beta vulgaris* L. var *conditiva*

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The objective of this study was to evaluate the thermal stability (5°C, 25°C, and 45°C) of betalains present in by-products of the blanching and cutting of *Beta vulgaris* L. var *conditiva*, to evaluate the possibility of taking advantage of them as a source of natural colorants to be incorporated in food products. The identification of the betalain compounds present in these by-products was also performed. Blanching waters showed pigment degradation at all the temperatures evaluated. The remnant tissues were freeze dried rendering beetroot powders whose pigments only presented thermal degradation at 45°C. Sixteen betalain compounds were identified in powders by chromatography and it was concluded that a thermal treatment at 45ºC during six days affected the chemical stability of some of these compounds, producing a diversity of betalain degradation products. Results obtained allowed concluding that the red beetroot powder would have a better performance as a natural coloring additive than the blanching water at temperatures below 45°C. Probably, the low water activity of the powder and its lignin content ensured an effective protection of the pigments up to this temperature.

INTRODUCTION

The concern for healthy eating has driven the search for more natural ingredients and additives. As part of this reality, the use of natural pigments in food industrialization has been extended.

Natural pigments are extracted from plant tissues rich in such compounds as betalains, anthocyanins, carotenoids, and chlorophylls. Red beet (*Beta vulgaris* L. var *conditiva*) contains betacyanins and betaxanthins that are characterized by the presence of betalamic acid in their chemical structure [Polturak & Aharoni, 2018; Sakuta, 2014; Stintzing *et al.*, 2002]. The betacyanins (Bc) are a source of red-violet color with λ_{max} of absorption spectrum at 530 nm [Saenz *et al.*, 2012] whereas betaxanthins (Bx) provide food with yellow-orange color having a λ_{max} of absorption spectrum at 470 nm [Khan & Giridhar, 2015]. More than 80% of the red pigments in beet are betacyanins, mainly betanin (betanidin 5-*O*-β-glucoside) and its isomer isobetanin [Nemzer *et al.*, 2011; Sawicki *et al.*, 2016]. In addition, there are approximately 15 natural betaxanthins in red beet, with vulgaxanthin I and indicaxanthin being the main ones [Khan & Giridhar, 2015].

The stability of betalains is affected by different factors, such as temperature, pH, water activity, light, presence or absence of oxygen, and enzymatic action [Celli & Brooks, 2017; Herbach *et al.*, 2006; Wybraniec & Mizrahi, 2005]. Betacyanins in beet extracts have been noted as having pH stability in the range of 3–7 [Mikołajczyk-Bator & Czapski, 2017] and to be readily susceptible to thermal degradation [Gengatharan *et al.*, 2016]. Temperatures above 50°C are reported to produce the loss of color and antioxidant capacity. In the heat treatment, the betacyanins can be degraded by isomerization and/or decarboxylation [Kumorkiewicz & Wybraniec, 2017]. A slight hypsochromic and hypochromic change can occur displacing the maximum absorption in the spectrum, therefore imparting an orange-red color [Azeredo, 2009]. Also, betanin and isobetanin can be dehydrogenated and hydrolyzed causing the formation of neobetanin (4, 15-dehydrobetanin), which is bright yellow [Herbach *et al.*, 2006]. On the other hand, betaxanthins are also thermally sensitive and have a lower stability than betacyanins do [Pires Goncalves *et al.*, 2013].

In the food area, betalains application is accepted by the European Community [2008] and these pigments (named E162) are used in the production of jellies, jams, strawberry yogurt, ice cream, fruit cocktails, candies, and cookies [Esatbeyoglu *et al.*, 2015].

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Plant by-products are promising sources of high-value compounds with antioxidant and/or antimicrobial properties, such as fibers and polyphenols [O'Shea *et al.*, 2012]. Red beet processing generates large quantities of underutilized biomass [Fernandez *et al.*, 2017]. Bengardino *et al.* [2019] studied the extraction of bioactive compounds from beet leaves for the valorization of this by-product.

Red beet roots are consumed fresh, fermented, dried or after thermal processing. Polyphenol oxidase (PPO) and peroxidase (POX) are present in plant tissues and during preservation and storage of food products based on red beets, these enzymes are responsible for changes in color and nutritive value. For the reduction of enzymes activity, in general, a blanching treatment is applied in the frame of raw material industrialization [Latorre *et al.*, 2012]. It is also usual to subject these tissues to cutting and trimming to give them the desired geometrical characteristics [Jideani *et al.*, 2017]. Blanching and cutting give the origin to remnant water and solids that can give rise to additives and ingredients to be used in the food industry itself.

The objective of this study was to evaluate the thermal stability of betalains present in by-products of the blanching and cutting of *Beta vulgaris* roots, to evaluate the possibility of taking advantage of them as a source of natural colorants to be applied in food products. The identification of the betalain compounds present in these by-products was performed as well.

MATERIALS AND METHODS

Chemicals

Chemicals used were of analytical quality and provided by Sigma-Aldrich (Saint Louis, USA) or Merck Química (Buenos Aires, Argentina). The solvents for chromatography were of HPLC quality. Deionized water was used (Milli-Q™, Billerica, MA, USA).

Plant material

Samples of beet (*Beta vulgaris* L. var *conditiva*) roots were obtained from local markets in Buenos Aires city (Argentina).

Obtaining beet root by-products

Red beets were washed, peeled, and cut into slices 1 cm thick, 4.9 cm to 6.0 cm in diameter. Slices were subjected to a blanching treatment by immersion in water at 90°C for 7 min with a tissue/water ratio of 0.5 kg/L according to Latorre *et al.* [2010]. The remnant water was frozen (-18°C). The tissues were considered equivalent to those from the cutting operation in the industrialization and were also frozen at -18°C. Their water was sublimated in a Pennsalt freeze dryer (Pennsalt, Philadelphia, USA) at a chamber pressure of 100 mm and shelf temperature of 25°C. They were then milled in a domestic blade mill (DeLonghi, Buenos Aires, Argentina) and sieved to obtain powders with a particle size smaller than 105 μm.

The peroxidase and polyphenol oxidase activities in the powders were evaluated according to Latorre *et al.* [2010] and expressed as absorbance unit/(min × mg protein).

Chemical analysis of cell wall components in beetroot powders

Uronic acids, total (non-cellulosic) carbohydrates, cellulose, lignin, and protein contents were evaluated in red beet powders, according to Ng *et al.* [1998] by means of sulfuric acid hydrolysis. From the final residues, cellulose and lignin were determined gravimetrically, whilst the non-cellulosic carbohydrates, uronic acid as well as protein contents were determined in supernatants with the methods reported by Dubois *et al.* [1956], Filisetti-Cozzi *et al.* [1991], and Lowry *et al.* [1951], respectively.

Moisture content and water activity of beetroot powders

Moisture content of the powders was determined, in duplicate, by means of and infrared scale (Moisture Analyzer MB45 Ohaus Corporation, New Jersey, USA), using a ≈0.500 g sample.

Water activity was measured two times at 25°C in a Decagon AquaLab (Series 3 Water Activity Meter, Pullman, WA, USA), as explained by Basanta *et al.* [2016].

Evaluation of thermal stability of betalains in beetroot by-products

The powder was fractionated in amounts of ≈3.000 g in caramel glass flasks (volume 30 mL) and stored, for 6 days, at 5°C, 25°C, and 45ºC. In the case of blanching water (pH=6.3), ≈ 2.00 mL were stored for 4 days in the same type of flasks, at the same temperatures. Storage was performed in duplicate.

After each storage day, the samples were characterized for their UV/Vis spectrum, total betalains content, and color parameters.

Betalain extraction and quantification

For the powder characterization, a quantity of ≈0.5000 g was extracted with 15 mL of Milli-Q™ water, stirred for 2 h, and centrifuged at 7700× *g* and 4°C for 15 min (Eppendorf 5804R, Hamburg, Germany). The supernatant was separated and used for measurement. In the case of blanching water, the measurement was performed directly on the sample.

According to Moßhammer *et al.* [2006], powder extracts obtained or blanching water were diluted in McIlvaine buffer (pH=6.5) to adjust the maximum absorption at 1.00 ± 0.05 at wavelengths of 536 nm (betacyanins) or 476 nm (betaxanthins). The measurement was carried out in a UV-mini 1240UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan). The content of betacyanins (Bc) and betaxanthins (Bx) was calculated as:

$$\text{Bc or Bx} = [(A \times DF \times Mw \times 100/\varepsilon \times l)].$$

where: A is the absorption value of the betanin at its λ_{max} of 536 nm or vulgaraxanthin at its λ_{max} of 476 nm, corrected by the absorption at 600 nm; DF is the dilution factor and l is the pathlength (1 cm) of the cuvette; Mw is the molecular weight of betanin (550 g/mol) or vulgaraxanthin I (339 g/mol); and ε is the molar extinction coefficient of betanin (60,000 L/(mol × cm)) or vulgaraxanthin (48,000 L/(mol × cm)).

The contents of betacyanins and betaxanthins were expressed as mg/100 g for powder and as mg/L for blanching water.

Determinations were performed three times, and average and standard deviation (SD) are reported.

Absorption spectra

The spectra were determined for the extracts obtained from powders as described in the *Betalain extraction and quantification* section or directly on blanching water. Both were previously diluted with Milli-Q™ water to adjust the absorption maximum to 1.00 ± 0.05. The whole visible spectrum (300–700 nm) was recorded at constant intervals ($\Delta\lambda=2$ nm) using a UV-mini 1240UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan) and 2 mm pathlength glass cells.

Color

Measurement of powders and water color was performed with a Minolta colorimeter (Minolta CM-600 Co. Ltd., Osaka, Japan) with natural daylight illuminant D65 and standard observer angle α: 10°. Each sample was placed on a white tile, registering the color through the chromatic coordinates of the CIELab space, L^* (ranging from 0, black, to 100, white), a^* (positive values for reddish colors and negative values for greenish ones), and b^* (positive for yellowish colors and negative for the bluish ones).

The average and SD for triplicate measurements are reported.

Chromatographic analysis of betalains in beetroot powders

To clarify the mechanism of betalains degradation, a chromatographic analysis was performed for beetroot powders at 45°C.

Extraction of betalains

A quantity of ≈1.0 g of powder was extracted twice with 10 mL of methanol/water (80:20, *v/v*) under continuous agitation, then centrifuged at $7700\times g$ and 4°C for 15 min (Eppendorf 5804R, Hamburg, Germany). The supernatants were mixed and concentrated to a final volume of 2 mL, under reduced pressure at room temperature (25°C), then filtered through a 0.45 μm nylon filter and directly analyzed by HPLC [Swarna *et al.*, 2013].

HPLC-DAD analysis

HPLC-DAD analyses of the extracts were performed with a Waters HPLC 1525 system (Waters, Milford, USA), equipped with a binary pump system (model M0925P), a degasser (model M09DG2 455M), and with a photodiode array detector (model A10998). An Eclipse XDB-C18 column (150 mm x 4.6 mm, 5 μm particle size, Agilent, USA), with a mobile phase of formic acid (1 mL/100 mL water) (A) and acetonitrile (B) [Swarna *et al.*, 2013], at 0.3 mL/min flow rate and 20 μL injection volume was used in the study. A linear gradient was used, starting with 1% B and up till reaching 33% after 40 minutes. Chromatograms were recorded at 470 and 530 nm which correspond to the betaxanthins and betacyanins maximum absorption wavelengths.

HPLC-ESI-MS-MS analysis

HPLC-ESI-MS/MS analyses were performed with an Agilent 1200 HPLC system (Agilent Technologies, Wilmington, USA) provided with a binary pump (model G1312B), an automatic injector (model G1367D), a degasser (model G1379B), and a photodiode array detector (model G1315C) registering the chromatograms at 470 nm and 530 nm. The HPLC system was coupled with a high-resolution mass spectrometer Bruker micrOTOF-QII (Bruker Daltonics, Billerica, MA, USA) with an electrospray ionization source (ESI). The ionization conditions were 200°C and 4.5 kV for the capillary temperature and voltage, respectively. The nitrogen pressure as the nebulizer gas and its flow rate as the drying gas, was 3.0 bar and 6.0 L/min, respectively. The mass scan was performed between 50 and 950 m/z in positive mode. The acquisition and processing of data were done using the software Bruker Compass Data Analysis ver. 4.0 (Bruker Daltonics, Billerica, MA, USA). Peak identification was carried out by means of the UV spectra and mass spectra with identification of the $[M+H]^+$ ions of the individual compounds, as well as their fragmentation.

Statistical analysis

Results are reported as average and SD. The number of replicates is stated for each analysis. The comparison of the results was carried out by means of an analysis of variance, ANOVA, with the level of significance at α=0.05. The Tukey test was used "a posteriori" [Sokal & Rohlf, 2000].

Statistical analysis was performed using the Prism 5 utility (Statistical Software for Windows GraphPad, La Jolla, USA).

RESULTS AND DISCUSSION

Efficiency of blanching treatment

It is important to note that the results obtained in relation to the activity of POX and PPO (Figure 1) showed that the blanching method used was effective in inhibiting the activity of both enzymes. Therefore, the results reported below are not affected by them.

Chemical composition and physicochemical characteristics of the by-products

The powder was obtained with an average size <105 μm and a yield of 12.3 g/100 g tissue. The low moisture content (4.9 g/100 g powder) and a water activity of 0.28 guarantees the stability against deterioration during storage [Muggeridge & Clay, 2001].

Lightness (L^*) of the powder was low (23.1 ± 0.1) and a^* (33.3 ± 0.1) and b^* (3.9 ± 0.3) color coordinates were both above zero, with a^* being higher than b^*, which is coincident with the red color visually observed.

Non-cellulosic carbohydrates (hemicelluloses and pectins) were the main powder components (30.9 g/100 g) and, of them, 9.8 g/100 g were constituted by uronic acids. The rest of the components included proteins (8.5 g/100 g), lignin (1.7 g/100 g), and cellulose (7.0 g/100 g) (Table 1). It is worth noticing that, due to the technique used to obtain beetroot powders, water-soluble pectin and other residual water-soluble components belonging to the cytoplasmic medium (*i.e.*
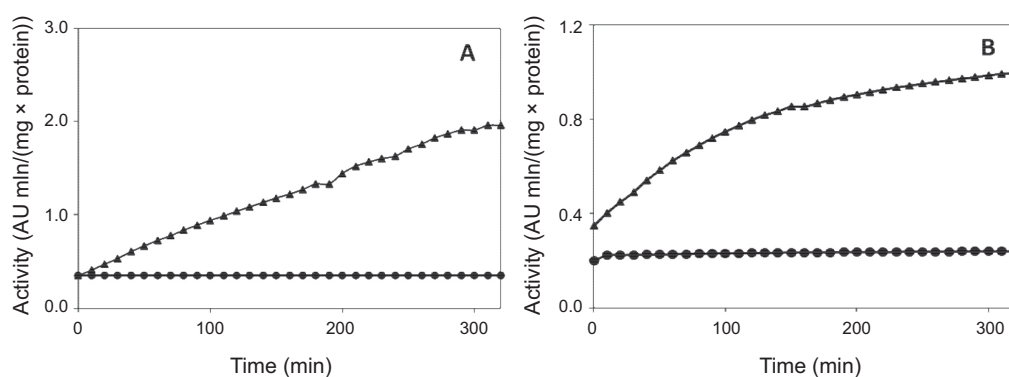
FIGURE 1. Efficiency of blanching procedure for enzymes inactivation.
(A) Peroxidase activity. (B) Polyphenol oxidase activity. AU: Absorbance unit. ▲ Non-blanched tissues. ● Blanched tissues.

TABLE 1. Chemical composition of the red beetroot powder prior to storage at different temperatures.

Component	Content
Moisture (g/100 g)	4.9±0.1
Cellulose (g/100 g)	7.0±0.1
Non-cellulosic carbohydrates (g/100 g)	30.9±0.8
Uronic acids (g/100 g)	9.8±0.8
Proteins (g/100 g)	8.5±0.4
Lignin (g/100 g)	1.7±0.1
Betacyanins (mg betanin/100 g)	0.47±0.01
Betaxanthins (mg vulgaxanthin/100 g)	0.26±0.01

Results are presented as mean ± SD (n=2). Values are reported per 100 g of powder.

globular proteins, amino acids, mono and di-saccharides) might have been lost during the procedure applied [Vincken *et al.*, 2003].

It can be observed in Table 1 that the powders had a higher content of betacyanins than betaxanthins (0.47 mg betanin/100 g *vs.* 0.26 mg vulgaxanthin/100 g), which is coincident with the trend observed for color parameters *a** and *b**. Betalains are found in the vacuoles of plant cells belonging to the order of Caryophyllales [Polturak & Aharoni, 2018]. According to Wiczkowski *et al.* [2018] and Sepúlveda-Jiménez *et al.* [2004], red beetroot accumulates mainly betacyanins in the form of betanin which is found at high concentration in the root (0.5 g/kg of betanin).

In the case of blanching water (pH 6.3), the *L** showed values of 25±1 and the *a** (50±1) and *b** (37±3) parameters were positive, with *a** being higher than *b**. The concentration was 5.57±0.56 mg betanin/L for betacyanins and 5.36±0.38 mg vulgaxanthin /L for betaxanthins.

Thermal stability of betalains in beetroot by-products

UV/Visible spectra
Figure 2 (A, B and C) shows the UV/Visible spectra, in a wavelength range between 300 nm and 700 nm, for beet-

root powder extracts stored at 5°C, 25°C, and 45°C. The wavelengths where the peaks of maximum absorption occurred were 470 nm and 530 nm, corresponding to the absorption of betaxanthins and betacyanins, respectively. Similar spectrophotometric behavior was reported by other authors [Cejudo *et al.*, 2014; Cejudo-Bastante *et al.*, 2014; Stintzing & Carle, 2004]. All the extracts showed that the peak at 530 nm had a higher absorbance than the peak at 470 nm. The temperature exerted a null effect when the systems were stored at temperatures of 5°C and 25°C. Nevertheless, the absorbance values for the maximum wavelengths decreased throughout storage at 45°C, revealing the effect of this temperature on betalain stability.

In the case of blanching waters stored at the three temperatures, it can be observed (Figure 2D, E and F) that the peak at 470 nm showed greater absorbance than the peak at 530 nm. During storage at 5°C, it was observed that the peak at 470 nm decreased with storage time. At storage temperatures of 25°C and 45°C, it was observed a strong decay of both peaks with time and the decay increased with temperature. Khan & Giridhar [2014] stated that the degradation of betalains accelerated with increasing temperature and heating period.

Content of betalains
Figure 3 (A and B) shows the content of betacyanins and betaxanthins in red beetroot powders. For each storage time at different temperatures, the content of betacyanins was greater than the one of betaxanthins. The betalains were found to be much more stable at 5°C and 25°C with losses of less than 1% during storage time. Cai & Corke [2000] also studied the stability of betalains in amaranth powders, establishing that the stability of betalains increases as the dry matter increases and with a moisture content below 5%. Serris & Biliaderis [2001] reported that the stability of these pigments increases with lower water activity. In the present work, the red beetroot powders presented a moisture content lower than 4.9 g/100 g and a water activity of 0.28, facts that can justify the high stability of betacyanins and betaxanthins at 5°C and 25°C. It must be also considered that lignin was detected in the beetroot powder (Table 1), which can act as an antioxidant, protecting pigments from degradation [You *et al.*, 2019]. However, the content of betalains, varied during storage time at 45°C. A loss of 60% was observed for the be-
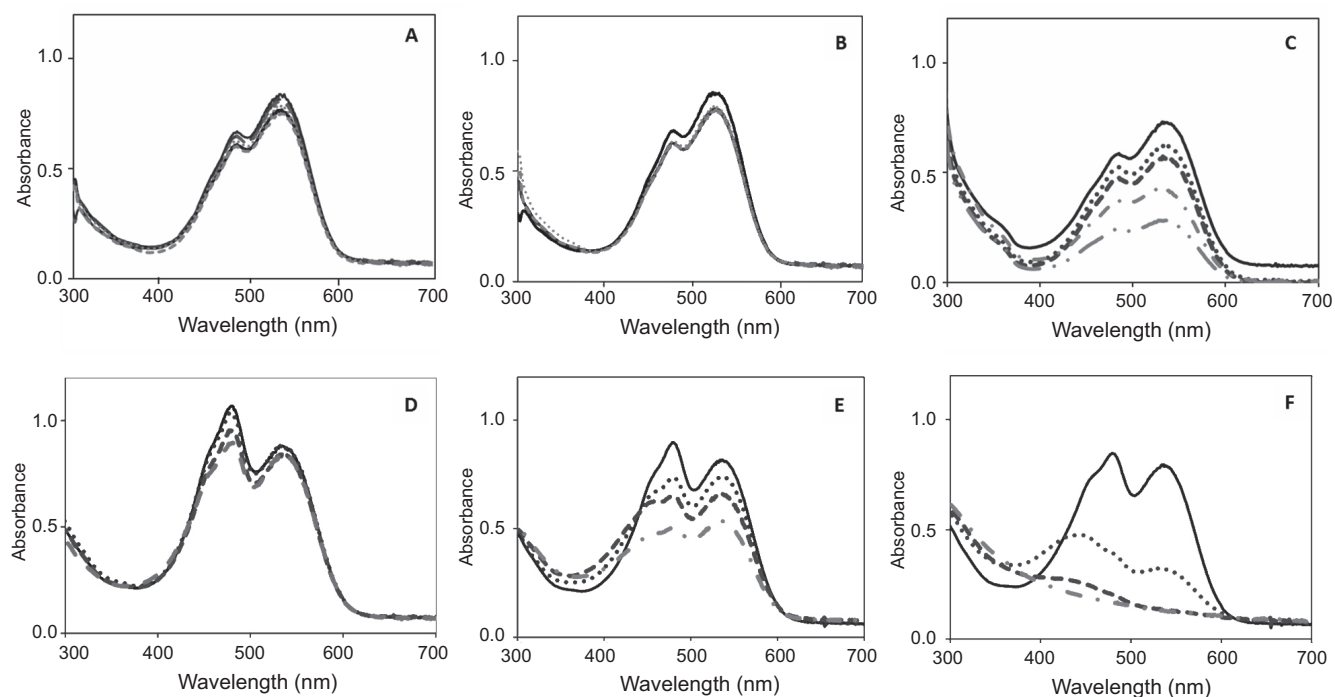
FIGURE 2. UV/Visible spectra at different storage temperatures.

Powder stored at 5°C (A), 25°C (B), 45°C (C). Blanching water stored at 5°C (D), 25°C (E), 45°C (F). Time: ———— day 0, ·········· day 1, — — — day 2, ·—·—· day 4, ·—··—·· day 6.
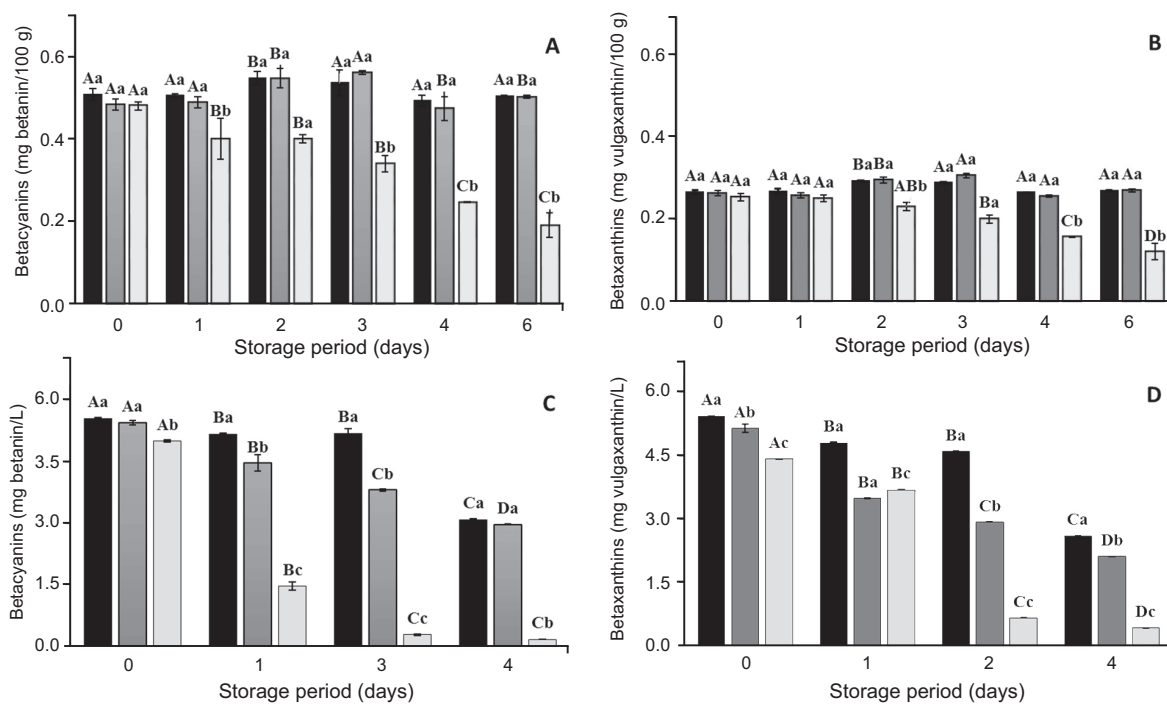

FIGURE 3. Betalain content at different storage temperatures.

Betacyanins in powder (A). Betaxanthins in powder (B). Betacyanins in blanching water (C). Betaxanthins in blanching water (D). Storage temperatures: ■ 5°C; ■ 25°C; ■ 45°C. Results are presented as mean ± SD (n=3). Different lower case letters indicate significant differences (p<0.05) between storage temperatures. Different capital letters indicate significant differences (p<0.05) between storage days.
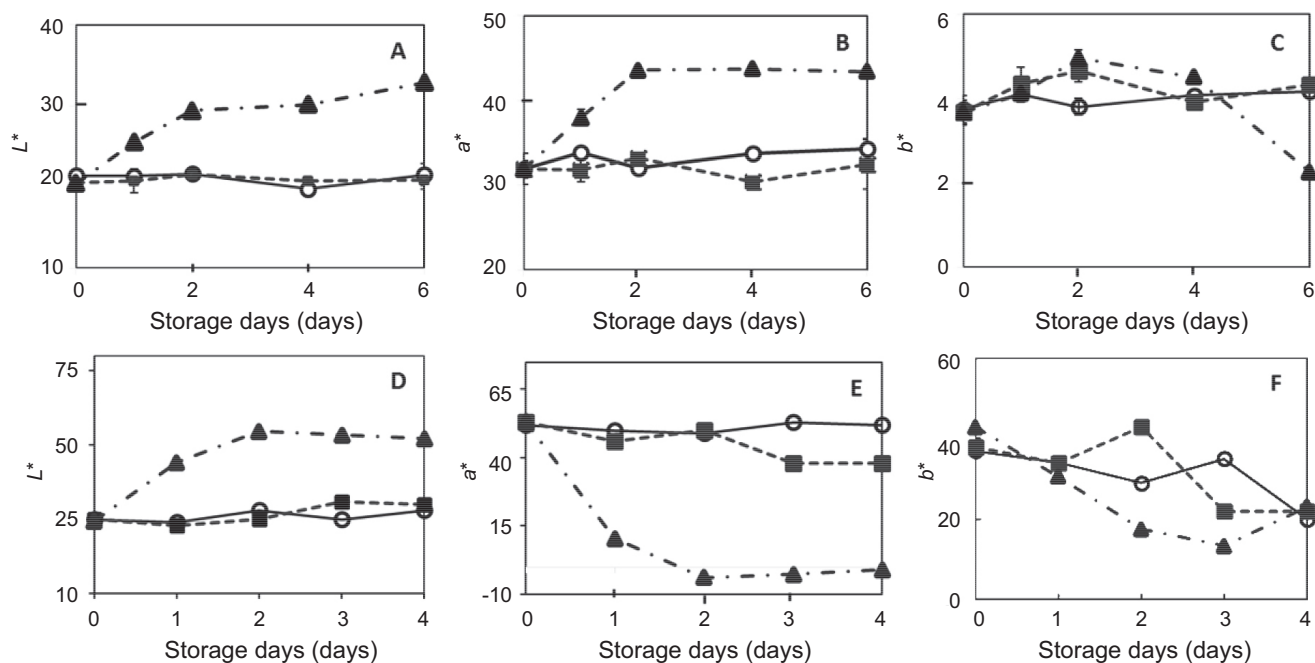
FIGURE 4. Color parameters at different storage temperatures.

Powder: L^* (A), a^* (B), b^* (C). Blanching water: L^* (D), a^* (E), b^* (F). Storage temperatures: ○ 5°C; ■ 25°C; ▲ 45°C.

tacyanins and 53% for betaxanthins, showing that these compounds were unstable at this higher temperature.

In the case of blanching water, the betalains concentration was affected by all the three temperatures studied, as can be observed in Figure 3 (C and D). The losses observed were 44% (betacyanins) and 52% (betaxanthins) at 5°C, 45% (betacyanins) and 59% (betaxanthins) at 25°C as well as 97% (betacyanins) and 90% (betaxanthins) at 45°C. Cai *et al.* [1998] reported a higher stability of pigments in amaranth powders compared to aqueous solutions, attributing this trend to the lower water activity of the powders.

Color parameters

The CIELab color parameters (L^*, a^*, b^*) are reported in Figure 4. The powders stored at 5°C, 25°C, and 45°C (Figure 4 A, B and C) showed high values for the a^* parameter, which is expressed as a strongly red color of the powders. At 5°C and 25°C there was practically no variation in the color parameters with storage time. During storage at 45°C, an increase for L^* and a^* was observed, in contrast with a decrease for b^* parameter. It can be concluded that the color stability of the powders was not affected during storage at 5°C and 25°C but was in fact affected when the powders were stored at 45°C. This trend matches the ones observed for spectra and betacyanins and betaxanthins content which varied significantly during storage time at 45°C.

In the case of blanching water (Figure 4 D, E and F), only the parameter b^* showed a clear trend to a decrease at 5°C. At 25°C, both a^* and b^* decreased and, at 45°C, L^* increased while both a^* and b^* decreased showing negative values for parameter a^* at the higher storage times which means a change from red to green color.

It can be concluded from the studies of spectra, pigment concentration and color parameters, that the changes suffered by blanching waters with storage time at 5°C, 25°C and 45°C are greater than those occurring in beetroot powders. While blanching waters showed changes at all temperatures tested, the powders only suffered changes at 45°C. This can be ascribed to their low water activity and to the presence of lignin of the cell wall material which can diminish, by means of its antioxidant activity, the pigments oxidation at 5°C and 25°C. As a consequence, among the products evaluated, the powders would have a better performance as a natural coloring additive than the blanching waters at temperatures below 45°C.

Therefore, it is interesting to clarify the changes suffered by betalains present in powders during storage at 45°C to have a more complete picture of the mechanisms involved in their decay as well as of their potential as coloring additives.

HPLC-DAD-ESI-MS/MS analysis of the betalains in beetroot powders

The betalain compounds of beetroot powders non stored (I) and stored at 45°C for 6 days (II), were evaluated by HPLC-DAD and HPLC-MS/MS, to analyze the effect of temperature on these pigments contained in a solid matrix. The results are shown in Figure 5. The compounds were identified by comparing their retention times (tr) and MS/MS spectra with bibliographic information [Herbach *et al.*, 2004; Nemzer *et al.*, 2011; Sawicki *et al.*, 2016].

From the analysis of the HPLC-DAD chromatograms registered at 470 and 530 nm wavelength, it was confirmed that these powders contained betacyanins, with a maximum absorbance in the visible spectrum at 530 nm being responsible for the intense red color, and compounds of the family of betaxanthins, with a maximum absorbance at 470 nm.

Within the family of betaxanthins (Figure 5 and Table 2, I-470 nm), vulgaxanthin I (glutamine bx) corresponding to
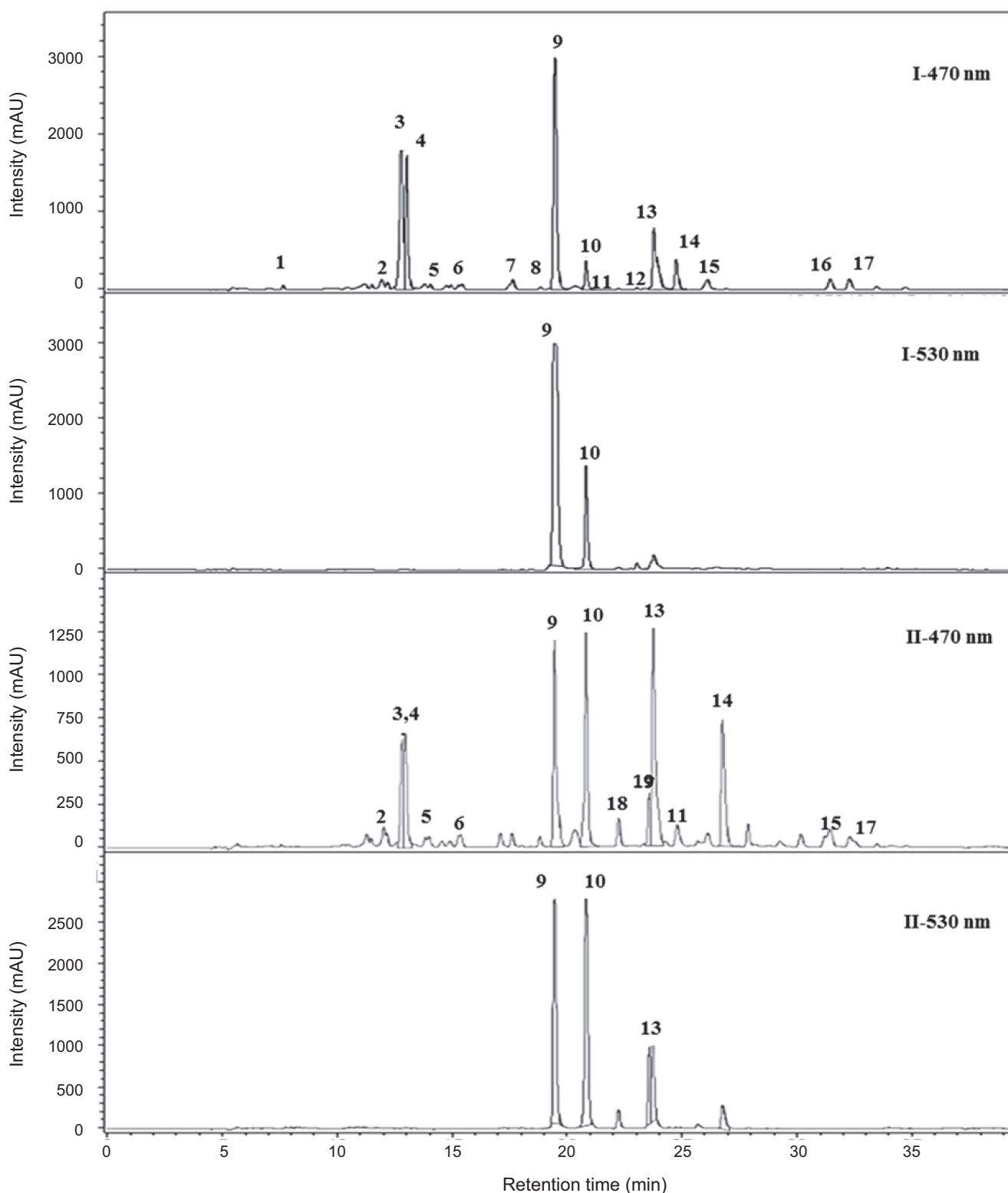
FIGURE 5. HPLC chromatograms of the betalain compounds present in powders.

Non-heat-treated (I) and 45°C heat-treated powders (II). The detection was performed at 470 nm and 530 nm. mAU: one-thousandth of an absorbance unit.

peak 3 and 4 was found through the pseudomolecular ions [M+H]⁺ at retention times of 12.8 and 13 min both with m/z 340.11 ($C_{14}H_{18}N_3O_7$). The MS/MS spectra showed two characteristic fragments, one at m/z 323.09 [M+H – 17]⁺ that could be formed by the elimination of hydroxyl group, and another at m/z 277.08 [323 – 46]⁺ originated by decar-

boxylation and di-deprotonation [Sawicki *et al.*, 2016]. It can be inferred that these two compounds (peaks 3 and 4) that present the same λ$_{max}$, and MS/MS spectra, are the two possible diasteroisomers of vulgaxanthin I due to the different configurations that C11 can adopt. According to the literature [Kujala *et al.*, 2002; Mégard, 1993; Singer & von Elbe,

TABLE 2. Analysis of the betalamic compounds by HPLC-DAD and HPLC-ESI- MS/MS of the non-heat-treated (I) and 45°C heat-treated powders (II).

Peak no.		Betalain name	Chemical formula	Retention time (min)		λ_{max} (nm)	m/z MS[M+H]$^+$	m/z MS/MS of [M+H]$^+$
I	**II**			**I**	**II**			
1	**-**	Asparagine bx (Vulgaxantin III)	$C_{13}H_{16}N_3O_7$	7.8	-	471	326.10	-
2	**2**	Serine bx	$C_{12}H_{15}N_2O_7$	11.9	11.8	470	299.09	-
3,4	**3**	Glutamine bx (Vulgaxanthin I)	$C_{14}H_{18}N_3O_7$	12.8/13	12.8	470	340.11	323.09 277.08
5	**5**	2,17-Bidecarboxy-2,3--dehydro-neobetanin	$C_{22}H_{23}N_2O_9$	14	14	463	459.16	330.11
6	**6**	Glutamic acid bx (Vulgaxanthin II)	$C_{14}H_{17}N_2O_8$	14.9	15	471	341.10	194.04 150.06
7	**-**	γ-Aminobutyric acid bx	$C_{13}H_{17}N_2O_6$	17.5	-	463	297.11	-
8	**-**	Proline bx (Indicaxanthin)	$C_{14}H_{17}N_2O_6$	18.7	-	477	309.11	-
9,10	**9,10**	Betanin /Isobetanin	$C_{24}H_{27}N_2O_{13}$	19.5/20.8	19.5/20.8	536	551.15	389.09
11	**11**	Dopamine bx (Miraxanthin V)	$C_{17}H_{19}N_2O_6$	21.8	24.8	456	347.12	303.13 211.07 137.06
12	**-**	Tyrosine bx (Portulacaxanthin II)	$C_{18}H_{19}N_2O_7$	22.8	-	472	375.12	211.08 178.07
13	**13**	4,15-Dehydro- betanin (Neobetanin)	$C_{24}H_{25}N_2O_{13}$	23.8	23.9	464	549.13	387.07
14	**14**	Valine bx	$C_{14}H_{18}N_2O_6$	24.6	26.8	466	311.12	267.13 221.13 193.13 150.05
15	**15**	Leucine bx (Vulgaxanthin IV)	$C_{15}H_{21}N_2O_6$	26.2	31.9	470	325.14	281.15
16	**-**	Phenylalanine bx	$C_{18}H_{19}N_2O_6$	31.2	-	470	359.12	-
17	**17**	Tryptophan bx	$C_{20}H_{20}N_3O_6$	32.1	32.2	472	398.13	-
-	**18**	17-Decarboxy-betanin	$C_{23}H_{27}N_2O_{11}$	-	20.8	515	507.16	345.11
-	**19**	2-Decarboxy-neobetanin	$C_{22}H_{25}N_2O_{11}$	-	21.9	a	505.15	343.09

a: λ_{max} was not determined due to co-elution with another compound or to low concentration. PI: Peak number corresponding to the chromatogram I-470/530 nm (non-heat-treated). PII: Peak number corresponding to the chromatogram II-470/530 nm (45°C heat-treated powders).

1980], the predominant yellow pigment in red beetroot juice is vulgaxanthin I. There could also be identified, serina bx, vulgaxanthin II, III and IV, isoindicaxanthin and miraxanthin V, but in a lower proportion. This family of compounds presented fragmentations that correspond to the loss of amino acids, desamination, and decarboxylation.

Betacyanins (Figure 5 and Table 2, I-530 nm) were detected at retention times of 19.5 and 20.8 min (peaks 9 and 10) and were identified as betanin and isobetanin [Sawicki *et al.*, 2016; Wybraniec, 2007], showing the pseudomolecular ion [M+H]$^+$ at m/z 551.15 ($C_{24}H_{27}N_2O_{13}$) and in the MS/MS spectrum, a characteristic fragment at m/z 389.09 [M+H – 162]$^+$ which was formed by the loss of the glucose molecule resulting in the presence of aglycones [betanidin+H]$^+$ or [isobetanidin+H]$^+$. Neobetanin was also found (I-470 nm, peak 13), with a λ_{max} of 464 nm because it has an extra double bond, which changes the resonance, generating a λ_{max} shift. In the mass spectrum, it gave an origin to the [M+H]$^+$ ion at m/z 549.13 and in the MS/MS spectrum to a fragment at m/z 387.07, also formed by the loss of the glucose molecule,

characteristic rupture of these compounds [Sawicki *et al.*, 2016].

Comparing the information for the non-heat-treated (I) and the heat-treated (II) powders (Figure 5 and Table 2), it can be observed that peaks 1, 7, 8, 12, and 16 corresponding to asparagine bx, γ-aminobutyric acid bx, proline/Iso bx, tirosine bx, and phenylalanine bx, respectively, did not appear in the heat-treated samples, showing that some betaxanthins are highly sensitive to temperature. In addition, two new compounds derived from betacyanins appeared (peaks 18 and 19). Peak 18 corresponded to 17-decarboxy-betanin giving the pseudomolecular ion [M+H]$^+$ at m/z 507.16 and an MS/MS fragment at m/z 345.11 (507–162) and λ_{max} of 515 nm. Peak 19 corresponded to 2-decarboxy-neobetanin, with [M+H]$^+$ at m/z 505.15 and an MS/MS fragment at m/z 343.09 (505–162) and resulted from decarboxylation of neobetanin (549–44). 17-Decarboxy-betanin was the product of the decarboxylation of betanin (551–44). Decarboxylation is induced by the thermal effect as it was reported by Herbach *et al.* [2004]. The displacement of the maximum absorbance

of 17-decarboxy-betanin (λ_{max} of 515 nm) with respect to the one of betanin/isobetanin (λ_{max} of 536 nm) was produced by the delocalization of electrons π due to the decarboxylation of the C17 [Minale *et al.*, 1965; Stintzing *et al.*, 2004]. Previous studies on red beetroot showed that decarboxylations can occur in the C2, C15, and C17 although these positions differ in their susceptibility [Herbach *et al.*, 2004; Wybraniec, 2007; Wybraniec & Mizrahi, 2005].

Analyzing the ratio of the isobetanin/betanin and neo-betanin/betanin areas for the non-heat-treated and the heat-treated powders (Figure 5), it could be observed an increase in the isobetanin/betanin ratio after the heat treatment (from 0.25 to 1) due to isomerization of betanin to isobetanin by the thermal effect [Herbach *et al.*, 2004]. On the other hand, for the neobetanin/betanin ratio, its increase was found to be non-significant (from 0.21 to 0.25).

CONCLUSIONS

The thermal stability at 5, 25, and 45° C of betalains present in by-products of the blanching and cutting of *Beta vulgaris* tissues has been studied.

Blanching waters showed pigment degradation at all the temperatures evaluated. The red beetroot powders suffered pigments thermal degradation only at 45°C. This can be ascribed to their low water activity and to the presence of lignin which can protect pigments from degradation, through its antioxidant activity, allowing to use these powders as a coloring additive up to 45°C.

Chromatographic studies showed that storage at 45°C for six days, affected the chemical stability of betalains. Degradation reactions might impair the use of these powders as natural pigments in foods that are heat treated at temperatures higher than 45°C after pigment inclusion in the formulation.

It is expected that the results of this research will contribute to the addition of value to the *Beta vulgaris* tissues, thus contributing to its integral use as well as to the development of both sustainable processing technologies and healthy foods.

RESEARCH FUNDING

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