Monitoring by Liquid Chromatography Coupled to Mass Spectrometry the Impact of pH and Temperature on the Pigment Pattern of Cactus Pear Fruit Extracts

José A. Fernández-López[1,*], Rosario Castellar[1], José M. Obón[1], and **Luis Almela[2]**

[1]Department of Chemical Engineering, Polytechnic University of Cartagena, Paseo Alfonso XIII 52, E-30203 Cartagena (Murcia), Spain and
[2]Department of Agricultural Chemistry, University of Murcia, E-30071 Murcia, Spain

Abstract

The influence of pH and moderate heating (50°C) on the color and individual betacyanin content of pigment extracts from cactus pear fruits (*Opuntia stricta*) is studied in the course of this paper. The study is carried out by using a high-performance liquid chromatograph equipped with a photodiode array detector and coupled to a mass spectrometer. The results point to a pH-dependent degradation mechanism, which is reflected in the chromatographic patterns obtained at different exposure times (0–28 h). At pH 3, 15-descarboxy-betanin is the most resistant betacyanin derivative. At pH 5, seven peaks are detected after 8 h, the most prominent being betanin, cyclo-dopa-5-*O*-β-glucoside, and betalamic. In the assay conducted at pH 7, rapid color loss affects all the pigments, except for betanin.

Introduction

The legal restrictions introduced into the use of artificial food colorants lead to a growing interest in the studies of natural pigments in recent years. The main areas investigated are: analytical characterization (1,2), prospects for new natural pigments (3), and their applications in food systems (4). Public opinion is also becoming more sensitive to the use of additives in general, colorants in particular. This has led to an increasing tendency to use natural colorants, rather than synthetic ones. Natural colorants provide an image of health and quality, whereas the safety of synthetic colorants is questioned (5,6).

The use of colorants in foods is regulated by the legislation of every country. The existing legislation on this matter is becoming progressively more restrictive, and the number of colorants allowed is being limited and strictly controlled. The pigment extract from red beet root (*Beta vulgaris* L.) is approved as an additive colorant in foods, both in the United States (Title 21 of the Code of Federal Regulations, 21 CFR 73.40) and in the European Union (7). It is mainly used in the manufacture of ice creams, frozen desserts, liquors, soft drinks, yogurts, biscuit filling, and confectionery. Eighty to ninety percent of the total pigment content of red beet root extracts is attributed to betacyanins, the red-purple, water-soluble immonium derivatives of betalamic acid with cyclo-dopa, with betanin (betanidin 5-*O*-β-glucoside) and its C_{15}-isomer isobetanin being the most abundant. Yellow betaxanthins are also present and joined to betacyanins, which are known collectively as betalains. These pigments behave as redox acid–base indicators and are structurally unstable at extreme pH values. Therefore, they lose their color in alkaline conditions and undergo irreversible decomposition (red to yellow) in acid solutions (8–10).

Betalains are widely distributed in the Cactaceae family, among which the *Opuntia* genus is the best known. Studies carried out with several species of *Opuntia* fruits revealed a high betacyanin content, especially in cactus pears from *Opuntia stricta* (11,12). The betanin concentration in these fruits is similar to that found in some cultivars of *Beta vulgaris* cultivated for pigment production. However, *Opuntia stricta* fruit extracts have several technological and sensory advantages with respect to red beet. The high nitrate and microbiological levels of red beets, as well as, the earth-like flavor of geosmin and 3-sec-butyl-2-methoxypyrazine are not present in cactus pears (13).

Opuntia stricta fruits mainly contain betanin and its C_{15} diastereoisomer, isobetanin (Figure 1), but not indicaxanthin, a typical betaxanthin present in other widespread *Opuntia* species, such as *O. ficus-indica* and *O. undulata* (12,14). Currently, about 30 structures of betacyanins are known and well documented (15), from the simplest 5-*O*-glucosylated betacyanins (the major red-purple pigments both in red beet root and cactus pears), to those esterified with ferulic, *p*-coumaric, or malonic acids (15,16). Several papers have been developed on this matter in recent years, revealing an increasing interest in the chromatographic analysis, as well as structural elucidation of betacyanin derivatives. This includes new compounds, such as hylocerenin (17), hydrolysated, and decarboxylated degradation products (18–22).

*Author to whom correspondence should be addressed: email josea.fernandez@upct.es.

To complete the investigations performed by our research group on pigments from *Opuntia stricta* cactus pears, the possibility of using them as a novel source of natural food colorants has been considered. Changes in the betacyanin content of *O. stricta* extracts at different pH values have been determined during moderate heating. Degradation mechanisms were characterized by using high-performance liquid chromatography (HPLC) with photodiode array detection and mass spectrometric (MS) analysis to monitor the betacyanin derivatives generated.

Experimental

Plant material

Mature cactus pear (*Opuntia stricta* Haw.) fruits growing in the wild in Murcia (Spain), with a deep red-purple color, were chosen for this study. They were collected, washed, and drained. The thorns and the glochides on the peel were removed under running water. By homogenizing whole fruits, a red-purple cactus pear homogenate was obtained, which was kept at –21°C in an air tight container until processing. Pigments were extracted from the homogenate with water, and 20 g were stirred magnetically with 100 mL of water for 5 min. The extract was then extracted by centrifugation at 15,000 × *g* for 10 min in a refrigerated Z383K Hermle centrifuge (Wehingen, Germany), and finally, the supernatant was passed through a 0.45-µm nylon filter.

Chemicals

Acetonitrile (ACN) was of HPLC grade from Lab Scan (Dublin, Ireland). Acetic acid, citric acid, and di-sodium-hydrogen-phosphate, all of analytical grade, were obtained from Merck (Darmstad, Germany). HPLC-grade water was obtained from a Milli-Q system from Millipore (Bedford, MA).

Pigment degradation assays

The studies on pigment degradation were conducted in a temperature-controlled water bath at 50°C and at pH values of 3, 5, and 7, obtained by using citric acid and di-sodium-hydrogen-phosphate buffers. After 2, 4, 6, 8, 10, 16, 22, and 28 h, aliquots (6 mL) were taken with a syringe, cooled in an ice bath for 1 min, filtered (0.45 µm), and immediately analyzed, both by HPLC and spectrophotometrically. The UV–vis spectra (200–800 nm) of the samples were recorded using an Agilent 8453 spectrophotometer (Waldbronn, Germany).

Total pigment quantitation

Total betacyanin concentration was estimated as betanin using an extinction coefficient ($E^{1\%}_{1\,cm}$) of 1120 (23).

HPLC system and operating conditions

A Waters liquid chromatographic system, model Alliance (Milford, MA) equipped with a M996 photodiode array detector and coupled to a ZQ quadrupole MS was used. The analysis was performed at 25°C. An analytical-scale Atlantis dC_{18} (15 cm × 4.6 mm, 5 µm) column was used (Waters, Wexford, Ireland). The injection volume was 20 µL. Mobile phase A consisted of 0.5% (v/v) acetic acid in water, whereas mobile phase B consisted of 0.5% acetic acid in ACN. At a flow-rate of 0.8 mL/min, a linear gradient from 100% A to 88% A in 12 min was followed by isocratic elution with 88% A. Simultaneous monitoring of the betacyanins and their degradation compounds was carried out at 538 nm and 476 nm, respectively. Betalamic acid was detected at 415 nm and cyclo-dopa 5-*O*-β-glucoside at 276 nm.

The quadrupole MS fitted with an electrospray ionization (ESI) source was operated in the positive ionization mode using nitrogen as the drying gas. The ESI parameters were calibrated to optimize the response and to obtain high sensitivity for the molecular ion.

Identification of compounds

Identification of the compounds was based on their molecular mass determined by HPLC–ESI-MS and UV–vis spectral characteristics. Both the mass spectral and UV–vis spectral data were compared with values reported in the literature (18–20).

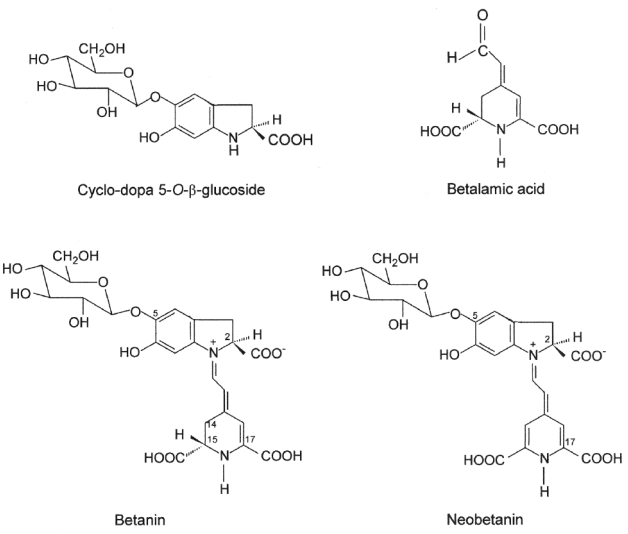

Figure 1. Chemical structures of betacyanins and degradative products.

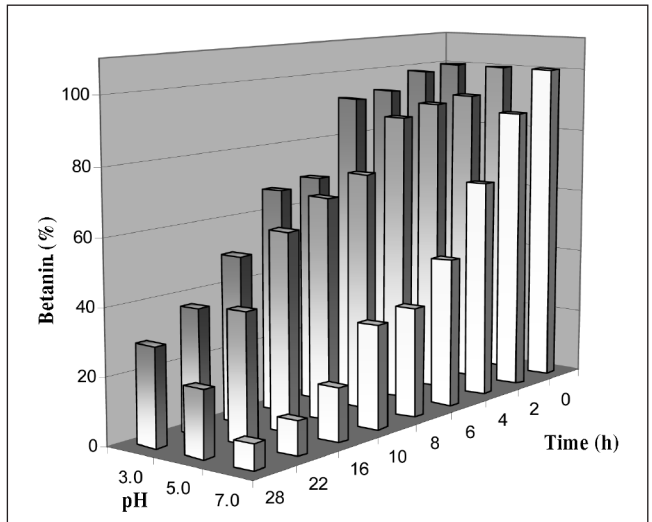

Figure 2. Influence of pH on the thermodegradation of betacyanins from *Opuntia stricta* fruit extracts. In all cases, the initial pigment content, expressed as betanin, was adjusted to 20 mg/L.

Results and Discussion

Color degradation

Any thermal degradation of the pigment extract obtained from cactus pear fruit (*Opuntia stricta*) was investigated to assess its suitability as a natural food colorant. The use of citric acid–phosphate buffer slightly improves general pigment stability (24), an effect which was similar in the three assays carried out. Changes in betacyanin content of cactus pear fruit extracts during heating at 50°C for 28 h at different pH values are shown in Figure 2. It is established that betacyanin degradation depends, not only on temperature and pH, but also on the initial pigment content in the extracts (25). As can be seen in Figure 2, pH clearly had an influence on the retention of pigments during this treatment. The betacyanin content was gradually degraded as a function of exposure time. After 28 h of heat exposure, approximately 30% of initial betacyanins were retained at pH 3, 20% at pH 5 and only 7.5% at pH 7. Note that after 6 h approximately 92% and 88% of the initial pigment levels were retained at pH 3 and 5, respectively. This decrease in pigment stability, while increasing pH, was in accordance with previous studies focused on *Opuntia ficus-indica* fruits (14).

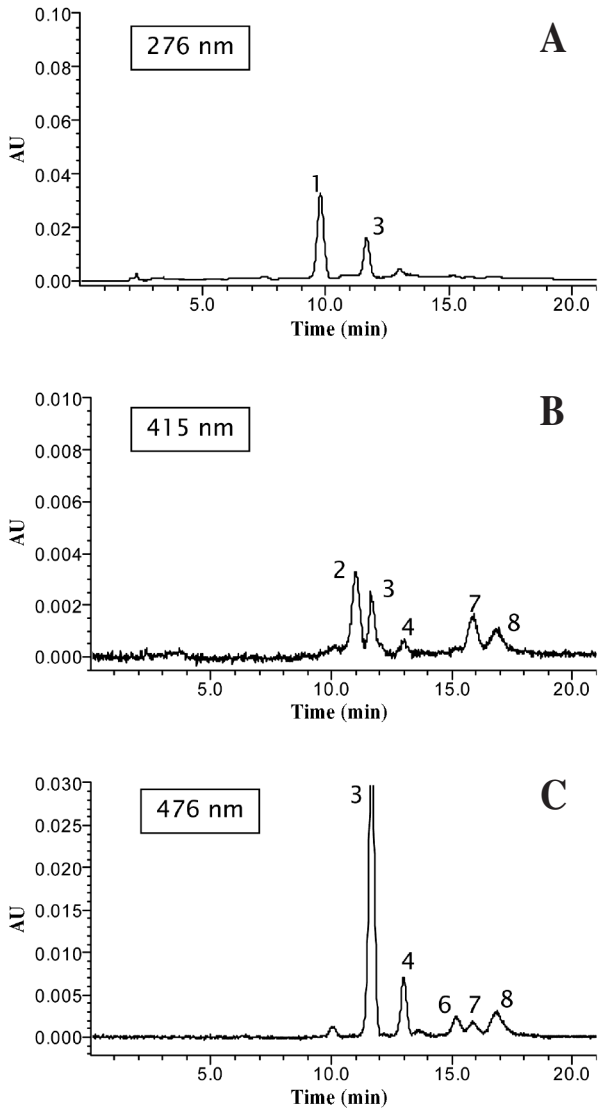

Figure 3. Chromatographic patterns of a *Opuntia stricta* fruit extract thermally treated for 8 h at pH 5.0: diode-array detection at 276 nm for cyclo-dopa-glucoside (A); diode-array detection at 415 nm for betalamic acid (B); and diode-array detection at 476 nm for betacyanins and other degradative compounds (C). Peak numbers are the same as Table I.

Pigment qualitative analysis

The chromatographic program detected eight peaks in the thermodegraded cactus pear fruit extracts. Figure 3 shows the different chromatograms corresponding to the extract heated at pH 5 for 8 h and monitored at 276 nm (for the detection of cyclo-dopa 5-*O*-β-glucoside), at 415 nm (for betalamic acid), and at 476 nm (for betacyanins and their degradation pigments). Tentative identification of the peaks could be made by observing their chromatographic behavior, and corroborative data were provided by analyzing their absorption spectra. Moreover, the use of MS coupled with HPLC provided molecular mass and structural information of the chromatographic bands, ensuring highly sensitive and selective detection.

Peaks 1 and 2 were assigned to the betanin cleavage compounds, cyclo-dopa 5-*O*-β-glucoside and betalamic acid (Figure 1), with absorbance maxima at 276 and 415 nm, respectively. Their protonated parent ions ($[M+H]^+$) were found at *m/z* 358 for peak 1 and at *m/z* 212 for peak 2 (Table I), which confirmed the identity of both compounds.

The presence of betanin (peak 3) and isobetanin, its 15R-isoform (peak 4), were confirmed firstly by their identical spectral properties (maximum absorbance at 538 nm), secondly by the presence of their protonated molecular ions $[M+H]^+$ with *m/z* 551, and lastly by the prominent secondary ion at *m/z* 389 due to the presence of the protonated aglycones [betanidin+H]$^+$ or [isobetanidin+H]$^+$.

The maximum absorbance (539 nm) and molecular ion ($[M+H]^+$ at *m/z* 551) of peak 5 (Figures 4) suggested that this peak should correspond to a betacyanin structure very close to betanin and isobetanin. Taking into consideration that a

Table I. Chromatographic and Spectral Data of Betacyanins and Degradative Compounds From *Opuntia stricta* Fruit Extracts

Peak number	Retention time (min)	Name	HPLC–DAD* λmax (nm)	*m/z* $[M+H]^+$	*m/z* Other ions
1	9.9	Cyclo-dopa 5-*O*-β-glucoside	276	358	196
2	11.0	Betalamic acid	415	212	166
3	11.8	Betanin	538	551	389
4	12.9	Isobetanin	538	551	389
5	13.5	Gomphrenin I†	539	551	389
6	15.1	15-Descarboxy-betanin†	538	507	345
7	15.9	17-Descarboxy-neobetanin†	447	505	343
8	16.7	Neobetanin	480	549	387

* DAD = diode array detection.
† Tentatively identified.

reversed-phase column was used, its longer retention time (due to a higher interaction with the stationary phase) would account for the lower polarity of this pigment. According to these data, this peak might correspond to some other isomer of betanin, isomerization which may take place in the C_2 position. However, up to now, no betanin diastereoisomer at C_2 has been found in any plant; therefore, it seems very improbable to be present in the *Opuntia* species. Another possibility would be that this peak corresponded to gomphrenin I (betanidin 6-*O*-β-glucoside), a pigment frequently found in plants (22,26–28), which has also been recently detected in Cactaceae (20). The co-occurrence of betanidin 5- and 6-*O*-glycosides in the same plant tissue is not usual, as it has previously been reported in *Gomphrena globosa* (26, 27) and in *Phytolacca americana* (28). The bathochromic shift of 1 nm relative to betanin (Table I), as well as these last findings (20), added weight to the opinion that this peak would correspond to gomphrenin I.

Peak 6 corresponds to a compound with an absorption maximum at 538 nm (identical to that of betanin and isobetanin), which suggests that the chromophore is not disrupted. This characteristic, joined to the presence of a protonated parent ion ($[M+H]^+$) at *m/z* 507 (Table I), points to a descarboxylated betanin structure (betanin-CO_2; 551 – 44 = 507). Its longer retention time compared with betanin suggests a less polar structure. The maximum absorption wavelength, identical to that of betanin, implies that decarboxylation does not occur at C_{17} position, because this would imply a hypsochromic shift as a result of a reduction in the π-electron delocalization system and a change in the betanin chromophore (18,29). Furthermore, only a single peak with these characteristics was detected from the epimers of betanin, which suggests a loss of chirality due to descarboxylation, implying that the C_{15} center (Figure 1) was altered (29). These findings indicate that a CO_2 molecule is lost at C_{15} for peak 6, corroborating the observations made by Schwartz and von Elbe (29) and Herbach et al. (18), who proposed that the heating of betacyanin extracts would lead to the formation of 15-descarboxy-betanin.

Peak 8 was identified as neobetanin (14,15-dehydrobetanin) because of its spectral maximum (480 nm), molecular ion ($[M+H]^+$=549=551-2=betanin-2H), and longer retention time (lower polarity) in comparison with betanin (Table I) (18,30).

In the light of previous findings (18), the thermally treated betacyanin extract from cactus pear fruits yielded a compound (peak 7) that was tentatively identified as a descarboxylated neobetanin derivative. Its maximum absorbance was determined at 447 nm, representing a hypsochromic shift of 33 nm compared with neobetanin. As previously mentioned, this would be indicative of descarboxylation at C_{17} position, which would agree with its molecular mass ($[M+H]^+$=505=549-44=neobetanin-CO_2). This suggests that it was 17-descarboxy-neobetanin (Table I).

Pigment pattern of thermodegraded extracts

The HPLC method developed was applied to examining the influence of pH on the pattern of the major betacyanin degradative compounds generated during heating. Figure 4 shows the HPLC profiles of cactus pear fruit extracts before and after thermodegradation at the three pH values assayed. The greatest number of degradative compounds was detected at pH 5. Cyclodopa 5-*O*-β-glucoside was a very stable metabolite and could be monitored throughout the time assayed (28 h). Its stability was higher at pH 5, slightly lower at pH 3, and noticeably lower at pH 7. Betalamic acid could not be detected at pH 3 because of its instability and rapid decomposition at acidic pH (29); however, it was recorded at pH 5 between 8 and 28 h of thermal treatment (Table II). Despite the fact that betalamic acid is known to increase in terms of its stability at basic pH (31,32), it could not

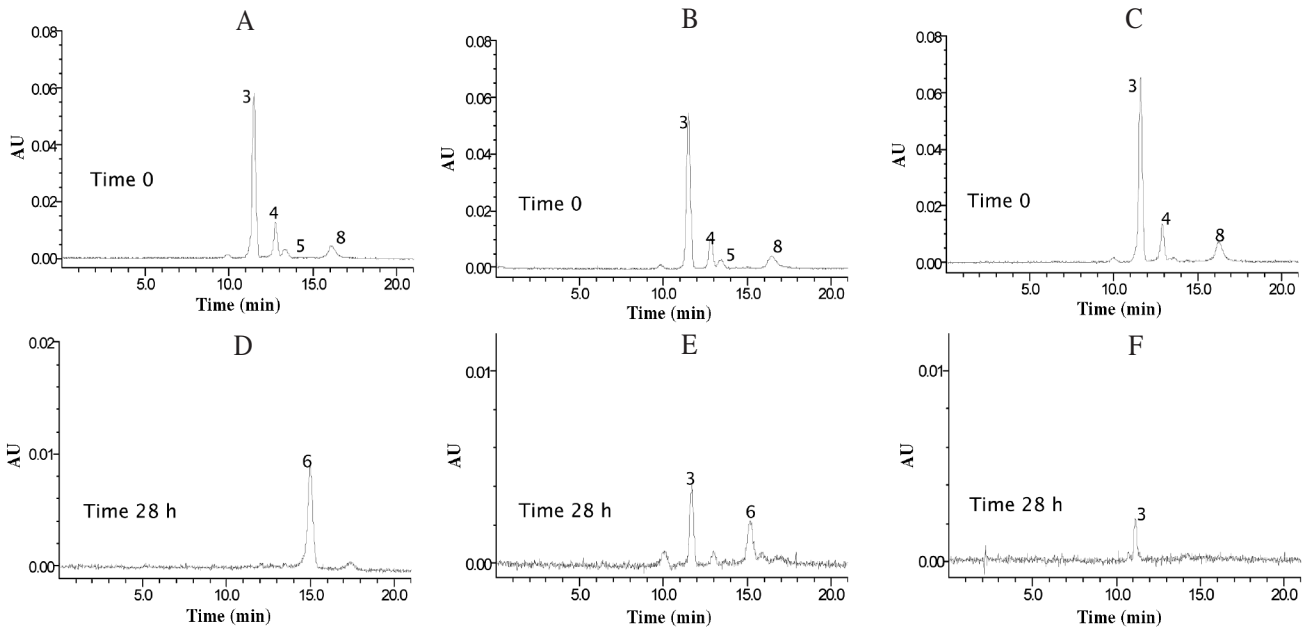

Figure 4. HPLC chromatograms (476 nm) of *Opuntia stricta* fruit extracts at pH 3.0 (A and D), pH 5.0 (B and E), and pH 7.0 (C and F) thermodegraded at 50°C. Peak numbers are the same as Table I.

Table II. Presence of Betacyanins and Degradative Compounds in *Opuntia stricta* Fruit Extracts Thermodegraded at 50°C

	pH 3.0			pH 5.0			pH 7.0		
Compound	**0 h**	**8 h**	**28 h**	**0 h**	**8 h**	**28 h**	**0 h**	**8 h**	**28 h**
Cyclo-dopa-5-*O*-β-glucoside	+*	–†	–	+	+	–	+	+	–
Betalamic acid	–	–	–	+	+	–	–	–	–
Betanin	+	+	–	+	+	+	+	+	+
Isobetanin	+	+	–	+	+	–	+	+	–
Gomphrenin I	ND‡	–	–	ND	–	–	–	–	–
15-Descarboxy-betanin	–	ND	ND	–	ND	ND	–	–	–
17-Descarboxy-neobetanin	–	–	–	–	ND	–	–	–	–
Neobetanin	+	–	–	+	ND	–	+	+	–

* + = present.
† – = not detectable.
‡ ND = not unambiguously detected.

be detected in the experiment conducted at pH 7.

In regards to the betanin and isobetanin content, it is assumed that betanin readily isomerizes to isobetanin during moderate heating without affecting visual color (29). This isomerization was more noticeable at pH 3, whereas at pH 5 the betanin–isobetanin ratio was almost constant throughout the assay (28 h) at around 80:20. At pH 7, this ratio remained constant (80:20) for the first 10 h of thermal treatment, after which, betanin was the only pigment that could be detected, but at very low levels (6% of the initial content).

Consequently, pH had a large influence on pigment and color stability during heating, which was deduced from the chromatographic patterns obtained for the extracts (Figure 4). Note that at the end of the assay the only peak recorded at pH 3 corresponded to the 15-descarboxy-betanin (Figure 4A), with similar light-absorption characteristics to betanin. This means that the level of residual color in these extracts was relatively high. Two peaks (betanin and 15-descarboxy-betanin) were detected in the extract thermodegraded at pH 5 after 28 h (Figure 4B). The assay conducted at pH 7 revealed a rapid color loss, which affected all the pigments except for betanin, which could still be detected although only slightly (Figure 4C).

These results revealed that pH clearly had an effect on both color loss and the number of degradative pigments formed, confirming that the degradation mechanism of betacyanins in cactus pears was subordinated to pH.

Conclusion

Opuntia stricta fruits appear to be a promising novel source of natural food colorant. Investigations into changes in color stability and individual betacyanin content under moderate heating (50°C) at controlled pH (3, 5, and 7) revealed a pH-dependent degradation mechanism. This was reflected in the chromatographic patterns obtained at different exposure times (0–28 h) in the assayed conditions. The reported HPLC method devoted to the assay of betacyanins permitted the identification and analysis of eight peaks (cyclo-dopa 5-*O*-β-glucoside, betalamic acid, betanin, isobetanin, gomphrenin I, 15-descarboxy-betanin, neobetanin, and 17-descarboxy-neobetanin) within 18 min. The presence of gomphrenin I in these extracts would confirm recent investigations reported in the bibliography. Further studies to confirm the presence of this pigment are in progress.

Acknowledgments

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