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Extraction, Stability, and Separation of Betalains from *Opuntia joconostle* cv. Using Response Surface Methodology

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ABSTRACT: Betalains were extracted and analyzed from *Opuntia joconostle* (the prickly pear known as xoconostle in Mexico). For the extraction, two solvent systems were used, methanol/water and ethanol/water. A three-variable Box−Behnken statistical design was used for extraction: solvent concentration (0−80%, v/v), temperature (5−30 °C), and treatment time (10−30 min). The extraction and stability of betalains from xoconostle were studied using response surface methodology (RSM). Techniques such as UV−vis, column chromatography, and HPLC were employed for the separation and analysis of the main pigments present in the extracts. Maximum pigment concentration (92 mg/100 g of fruit) was obtained at a temperature of 15 °C and a time of 10 min for methanol/water (20:80), whereas maximum stability of the pigment was observed at pH 5 and a temperature of 25 °C. HPLC chromatograms showed the main betalains of the xoconostle characterized were betalain, betanidin, and isobetalain.

KEYWORDS: *Opuntia, xoconostle, solvent extraction, stability, separation, HPLC, natural colorants*

■ INTRODUCTION

Betalains are natural, water-soluble pigments derived from the condensation of a primary or secondary amine with betalamic acid.[1,2] Due to their powerful antioxidant ability and their capacity to absorb free radicals, betalains can be used in the treatment of inflammatory and cardiovascular diseases, cancer, asthma, arthritis, oxidative stress, intestinal inflammation, diabetes, and other diseases associated with aging.[3−11]

The main source of betalains, particularly betanins, is the beet root (*Beta vulgaris*); nonetheless, the preparations obtained from this root have undesirable flavors,[9] and the presence of high concentrations of labile betaxanthins restricts their use as a food coloring.[12−14] An alternative source, which has been less studied for the extraction of this type of pigments, is the xoconostle prickly pear.

The xoconostle (*Opuntia joconostle* cv.) is a drought-resistant cactus that is grown in a large portion of the central area of the state of Jalisco and in the Mezquital Valley region of the state of Hidalgo, Mexico. Among the physiochemical properties of the xoconostle's fruit, its acidity stands out; its pH is between 3.7 and 4.5, resulting in its name (xococ means sour in Nahuatl) and limiting its use. In contrast to the sweet prickly pear, which has pH values between 5.2 and 6.0, the extremely low pH value of the fruit of the xoconostle means that it can be stored for longer periods without decomposing.[15] This factor also permits the fruit to remain on the plant longer, even several weeks after the fruit has ripened. Nonetheless, the use of this fruit is limited to the consumption of the fresh fruit due to the difficulties of postharvest handling and lack of knowledge of its nutritional potential.[16] Some species are barely exploited and are not marketed, as it is not very profitable for the farmer. In this scenario, research must be done to study the potential use of xoconostle as source of antioxidants that could be employed in therapeutic treatments or as natural food coloring, adding value to the fresh fruit. To elucidate the functional mechanisms and validate and improve their biological activity, it is necessary, first, to assess the optimal conditions for extraction, stability, and purification of the main pigments of xoconostle. Furthermore, studies should be done to fractionate the extracts into the substances they are composed of to evaluate their activity individually and in combination.

The objective of this study was to assess the optimal conditions in terms of pH, temperature, time, and appropriate solvents for the extraction and stability of the pigments present in the fruit of the xoconostle by using response surface methodology (RSM). Moreover, UV−vis spectroscopy and high-precision liquid chromatography (HPLC) were also carried out to characterize the pigments present in the pulp of the xoconostle to study the potential use of these pigments as food coloring and their further use in chronic diseases.

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MATERIALS AND METHODS

As raw material, the fruit of the xoconostle prickly pear *O. joconostle* Weber in Diguet, of the Cactaceae botanical family, grown in November 2004 in Zempoala, Hidalgo, Mexico, was used in this study. To compare the stability and content of the pigments extracted from xoconostle, beet root (*B. vulgaris*) and three species of *Opuntia ficus-indica* (red, yellow, and white) were also used. These plant materials were purchased in a local market in November 2005 in Mexico City.

Morphology of the Fruit (Physical Characterization). For this determination, 20 intact pieces of xoconostle fruit were selected; each was weighed, and the height and width of each were measured using a digital caliper (Truper, Mexico).

pH of the Pulp. The pH of the pulp was determined in a Corning/ion meter 450, previously calibrated with standard regulating solutions with pH 4.0 and 10.0 (Corning).

Activity of Water (a_w). The a_w was obtained with an AquaLab equipment (Decagon, USA); the determinations were done in triplicate using only the pulp of the xoconostle as the sample.

Proximate Analysis. The proximate analysis of the xoconostle (*O. joconostle* cv.) was carried out in accordance with Official Standards Methods of the AOAC.[17]

Moisture. Moisture was evaluated in accordance with Official Standard AOAC Method 925.09.

Protein. Total nitrogen was determined in accordance with Official Standard AOAC Method 950.48.

Ash. Ash was calculated in accordance with the method reported in 930.05 AOAC.

Fat. Fat was deetermined by the method reported in AOAC 920.39.

Carbohydrates. Carbohydrates were calculated by difference.

Plant Material Preparation. Prior to extraction, the fruit of the xoconostle was placed in chlorine solution (5 ppm) to disinfect it. Afterward, the water was drained and the fruit was treated for extraction or stability studies.

For extraction studies, the whole fruit of xoconostle was sliced longitudinally into small sections to facilitate its drying. The sample was placed on stainless steel trays in a drying chamber (F. J. Stokes, USA) under vacuum conditions and a maximum temperature of 40 °C for 24 h. Finally, the dried xoconostle was ground in a disk mill (Bauer, USA), and it was stored in polyethylene bags in a cool place protected from light.

For stability and separation studies, the extraction was carried out only from the pulp of the xoconostle to obtain better yields and a higher percentage of betalains. After washing, the fruit of the xoconostle was sliced longitudinally to facilitate the removal of peel and uncolored sides. After peel removal, the fruit of the xoconostle was placed in a laboratory pulping station (Langsenkamp, Indiana Laboratory Pulper, USA) to eliminate the seeds of the fruit. Then, the pulp of xoconostle was passed through a sieve to remove coarse particles, obtaining a thick liquid sample, which was used for a methanol/water/acetic acid (50:49:1) extraction. Pigment extraction from the pulp of xoconostle was carried out at 12 °C during 10 min. The methanolic extracts were centrifuged (3000 rpm/20 min), filtered and concentrated to dryness under reduced pressure (25 psi). Samples were stored at 4 °C in amber vials under nitrogen atmospheres to avoid degradation of the pigment due to oxygen. The extraction process described before was also used for pigment extraction from the whole beet root (*B. vulgaris*) and the pulp of the fruit of red, yellow, and white prickly pears (*O. ficus-indica*).

Extraction of the Pigments from Xoconostle. To determine the effect of the solvents on the efficiency of the extraction, two solvent systems were used: methanol/water and ethanol/water, in different proportions (0–80%, v/v). For the extraction, 1.0 g of the powder of the whole fruit was diluted in 20 mL of the solution, and the pH was adjusted to 5 with a 1% citric acid solution to maintain an acidic pH and avoid decomposition of the pigment. The sample was mixed for 1 min at maximum velocity in a Vortex (Thermolyne, USA), and it was placed in a water bath at the temperature and for the time specified in the design of the experiments (Tables 1 and 4). The solution underwent centrifugation at 3500 rpm for 15 min at room temperature. Afterward, the supernatant liquid was decanted and filtered through a membrane with 45 μm pores (Millipore). The content of the pigment (mg of pigment/100 g of fresh fruit) in the extract was determined using an extinction coefficient for betanin, the major component of the extracts, at 535 nm ($E^{1\%}_{1cm}$ = 1120) in accordance with the method proposed by Kujala et al.[18]

Table 1. Experimental Factors for Extraction of Betalains

variable	levels −1	0	1
x_1 = percentage of solvent in the water (%, v/v)	0	40	80
x_2 = extraction temperature (°C)	5	17.5	30
x_3 = extraction time (min)	10	20	30

Studies of Stability. To statistically evaluate the stability of the pigment at certain pH levels, temperatures, and heating times, 0.5 g of the dehydrated pigment extracted from the pulp of the xoconostle was dissolved in 10 mL of double-distilled water, under the conditions established in the experimental design (Tables 2 and 5). The stability of the pigments from xoconostle was compared with the stability of those obtained from beet root. For this assay, the dehydrated pigment extracted from beet root (0.5 g) was dissolved in 10 mL of double-distilled water and evaluated under the same conditions as the experimental design established in Table 2. The effect of each of the parameters on the stability of the pigment was qualitatively evaluated by measuring the absorbance in the visible spectrum, using a UV–vis spectrophotometer (Varian cary 50, USA), of each of the assays, and it was compared with the beet extract (without prior heating at pH 5).

Table 2. Variables and Variation Levels for Stability Studies of Betalains

factor	levels −1.682	−1	0	1	1.682
x_1 = time (min)	1	16	38	60	75
x_2 = temperature (°C)	6.25	25	52.5	80	98.75
x_3 = pH	1.64	3	5	7	8.36

Separation of the Pigments from Xoconostle. Samples were fractioned by using column chromatography and analyzed by HPLC. For the column chromatography, 25 cm high glass flash columns with 2 cm interior diameter were used; they were packed with three different stationary phases: (a) Amberlite XAD-7, (b) Sephadex G-25, and (c) silica gel 60, as described below.

Amberlite XAD-7. Amberlite XAD-7 is recommended to remove proteins, mucilage, and pectins from solutes of low molecular weight present in the pulp of the xoconostle. The extract previously dissolved in the water/methanol (50:50) solution was made to pass through a column packed with Amberlite XAD-7, previously activated with double-distilled water (pH 7). A 50:50 water/methanol solution was used as the eluent. The flow, resulting from the force of gravity, was continuous. Ten milliliter fractions were obtained and placed in 35 mL amber-colored jars. The fractions obtained from the separation by column chromatography were evaluated by thin layer chromatography (TLC), and then those fractions that showed similar retention coefficients (R_f) in the TLC were concentrated in a rotary evaporator (Yamato, Japan).

Sephadex G-25–80. The extract obtained from the xoconostle was passed through the column packed with Sephadex G-25–80 with a particle diameter of 20–80 μm (gel filtration chromatography), which, due to its high level of cross-linking, allows for the fractionation of mixtures of polysaccharides, polypeptides, globular proteins (1000–5000 MW), and nucleic acids.[19] The packing of the column was done after hydrating the gel for 24 h in double-distilled, degassed water for 60 min with a vacuum pump. Once the column had been prepared, the pigment dissolved in 5 mL of methanol was added. Water (500 mL) was used as the eluent. The flow, obtained due to the force of gravity, was continuous. Fractions of 5 and 10 mL were obtained and placed

into 50 mL amber-colored jars. These fractions were evaluated using thin layer chromatography, and those that showed similar R_f values were concentrated.

Silica Gel 60. In addition to the columns already discussed, a silica gel 60 column (0.040−0.063 mm) packed with 100 mL of ethanol was used. The extract was placed in a 50 mL beaker and dissolved in methanol. Seven grams of silica gel was added to the solution. Then, the methanol was evaporated to concentrate the pigment in the silica, making sure that no traces of moisture remained that would affect the elution. At once, the pigment was added to the column, forming a thin, uniform layer; the elution was done initially with ethanol at 95%, then with methanol at 95%, and finally with acidified (pH 3.2) methanol/water (80:20) to collect 6−10 mL fractions in 50 mL amber-colored jars, which were then concentrated in a Rotavap (Yamato, Japan) until dry, for subsequent evaluation.

The fractions obtained from the separation of pigments by column chromatography were analyzed with HPLC, in accordance with the method reported by Fernández-López et al. for Cactaceae.[20] The experimental conditions are described below.

Setup 1. HPLC with a UV detector (Dual Waters) monitored a wavelength of 535 nm. An isocratic method with a flow of 2.0 mL/min and a trial time of 20 min was used. The column was C_{18} μBondapak, particle size = 10 μm. The elution solvent for the separation method was the following: CH_3OH/0.05 M KH_2PO_4 (18:82, v/v) adjusted to a pH of 2.75 with H_3PO_4.

UV−Vis Spectroscopy Characterization. The characterization of betalains from xoconostle was carried out with UV−vis spectroscopy. The absorption spectrum of the betalains from xoconostle was compared to the absorption spectrum of betalains from beets, red prickly pear, yellow prickly pear, and white prickly pear. The previously purified, dehydrated samples of xoconostle and beet (0.01 g) were dissolved in a 10 mL volumetric flask to be characterized by UV−vis spectroscopy (250−600 nm). In the case of red, yellow, and white prickly pears, the pigments were extracted from the pulp using the methodology previously described. The prickly pear extract was concentrated to 10 and 20 mL, followed by a filtering through chromatographic paper of the liquid extract obtained. Half a gram was weighed, gauging to 25 mL. Once the gauged solutions had been obtained, 3 mL was taken and filtered through a 0.45 μm Millipore membrane to obtain its UV−vis absorption spectrum and compare it to the previous two.

HPLC Characterization. The separation and identification of the pigments were done with HPLC in accordance with the method reported by Schwartz and von Elbe.[21] Briefly, 0.03 g of the dried pigment was dissolved in 25 mL of HPLC grade water, for liquid samples, and 0.5 g was placed in 25 mL of HPLC water. Prior to injection into the column, the samples were filtered through a 0.45 μm Millipore membrane. The HPLC was carried out with the aid of the equipment described below. As no commercial standards of betacyanins or betaxanthins were found, the HPLC elution profiles of the pigments were compared with the results (retention time) reported by Schwartz and von Elbe.[21]

Setup 2. HPLC with a diode array detector (model 168), a gradient method was used, with monitoring at two wavelengths, 484 and 535 nm, with an RT 250-4 LiChrosorb column. The following solvent system was used for this method: CH_3OH/0.05 M KH_2PO_4 (18:82, v/v), adjusted to a pH of 2.75 with H_3PO_4 (solvent A). The solvents in the separation by gradient elution were initially 100% solvent A (at 9 min), 80%/20% methanol (solvent B) (10 min), 100% solvent A (5 min), and 100% solvent A (5 min).

Statistical Analysis. RSM was used for the analysis of the results through Design Expert version 5 statistics software, applying a three-variable Box−Behnken statistical design (Table 1) for extraction and a central composite experimental design (Table 2) for studies of stability. The significance of the models was tested using variance analysis (F test) and the determination coefficient (R^2).

Figure 1. Picture of *Opuntia joconostle* cv. fruit longitudinally cut: epicarp, mesocarp, endocarp, and seeds.

Table 3. Proximate Analysis of the Whole Fruit of Xoconostle

composition	percentage
moisture	82.56 ± 0.55
lipids	1.21 ± 0.01
protein	2.22 ± 0.06
ash	0.78 ± 0.05

Table 4. Experimental Design and Results for Xoconostle's Pigment Extraction

	independent variables[a]			response variables	
				methanol	ethanol
trial	x_1	x_2	x_3	betacyanin (mg/100 g)	betacyanin (mg/100 g)
1	0	5	20	89.7	74.6
2	0	30	20	84.3	75.2
3	80	5	20	55.3	31.8
4	80	30	20	59.5	46.2
5	0	17.5	10	89.3	85.5
6	0	17.5	30	89.7	89.5
7	80	17.5	10	57.5	31.7
8	80	17.5	30	57.5	34.9
9	40	5	10	88.2	72.6
10	40	5	30	80.5	66.7
11	40	30	10	77.9	66.7
12	40	30	30	77.9	74.9
13	40	17	20	80.6	76.4
14	40	17	20	77.9	81.4
15	40	17	20	80.5	82.4

[a] x_1 = percentage of methanol or ethanol in the water (%); x_2 = extraction temperature (°C); x_3 = extraction time (min).

■ RESULTS AND DISCUSSION

Morphology of the Fruit of Xoconostle. The shape of the fruit of the *O. joconostle* was between ellipsoid and pear-shaped; the skin of the rind is pinkish-red (Figure 1). The average weight of the fruit was 58.7 ± 13.9 g, with equatorial and polar diameters of 4.4 ± 0.45 and 5.1 ± 0.95 cm, respectively. The pieces of fruit in the study had 43.4 ± 5.1% pericarp (1.5 ± 0.2 cm thickness), 6.1 ± 2% pulp, and 4.3 ± 2.1% seeds. The pH of the pulp was 3.37 ± 0.05, and water activity was 0.982 ± 0.002.

Proximate Analysis of the Xoconostle. The results of the proximate analysis of the whole fruit are presented in Table

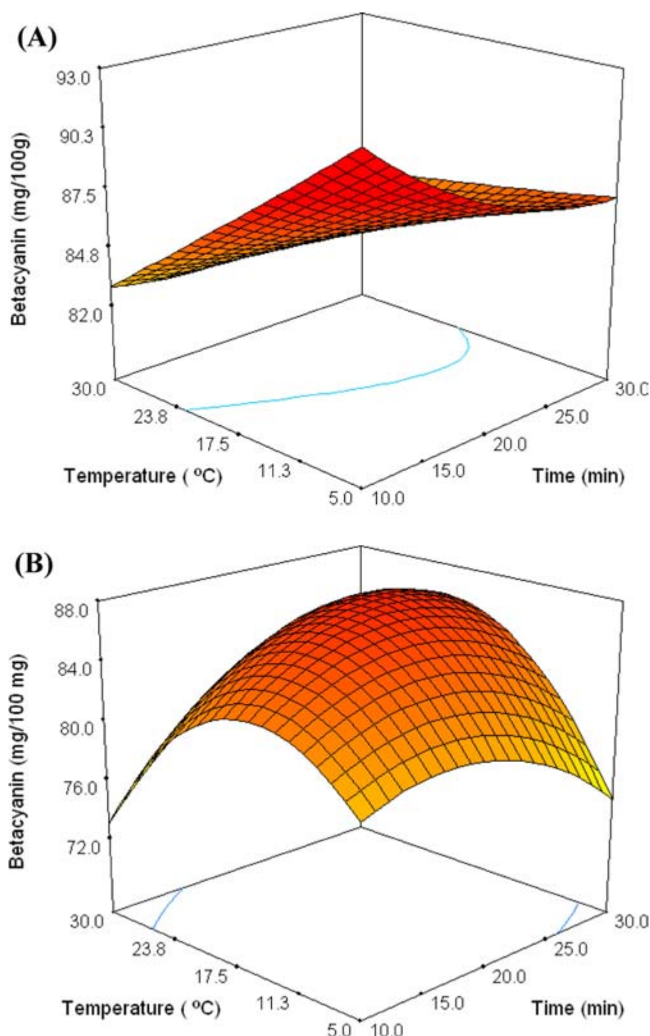

Figure 2. Extraction of betalains from xoconostle, effect of time and temperature of extraction: (A) water/methanol (80:20); (B) water/ethanol (20:80).

3. The fruit of xoconostle (*O. joconostle*) presents high amounts of moisture (82.5%) and carbohydrate (13.2%, obtained by difference). The protein and fat contents were 2 and 1%, respectively, which are in agreement with the results reported by García-Pedraza et al. for different species of *Opuntia*.[22] The amounts of these components depend on the cultivation conditions and the degree of ripeness of the fruit.[23]

Extraction from the Whole Xoconostle. Of the solvents evaluated in the extraction of betalains from the xoconostle, aqueous extraction was the most efficient, achieving a greater concentration of the pigments (Table 4). The extractions done with solutions with low concentrations (20% v/v) of methanol and ethanol in water also resulted in a high concentration of pigments, but still lower than that obtained in the aqueous extractions.

Extraction System I: Methanol Solvent. The total pigment content of the extracts represented as betacyanins (mg of betanin/100 g of fresh fruit) obtained with methanol is described by eq 1

Table 5. Experimental Design for Stability Studies of Betalains Extracted from Beet Root and Xoconostle

	independent variables[a]			response variables	
				beet root	xoconostle
trial	x_1	x_2	x_3	Abs	Abs
1	16.0	25.0	3.0	0.4661	0.4833
2	16.0	25.0	7.0	1.5079	0.4748
3	16.0	80.0	3.0	0.2788	0.1436
4	16.0	80.0	7.0	0.8297	0.2387
5	60.0	25.0	3.0	0.5165	0.4119
6	60.0	25.0	7.0	1.1889	0.4793
7	60.0	80.0	3.0	0.1615	0.0858
8	60.0	80.0	7.0	0.4168	0.1420
9	01.0	52.5	5.0	1.1900	0.4930
10	75.0	52.5	5.0	0.9785	0.4367
11	38.0	6.25	5.0	1.2040	0.4794
12	38.0	98.7	5.0	0.3207	0.1265
13	38.0	52.5	1.6	0.1636	0.1173
14	38.0	52.5	8.4	0.9930	0.4121
15	38.0	52.5	5.0	1.0672	0.4411
16	38.0	52.5	5.0	1.1077	0.4519
17	38.0	52.5	5.0	1.1165	0.4384
18	38.0	52.5	5.0	1.0712	0.4628
19	38.0	52.5	5.0	1.1345	0.4845
20	38.0	52.5	5.0	1.1406	0.4411

[a] x_1 = heating time (min); x_2 = heating temperature (°C); x_3 = pH.

$$\text{betacyanins (mg/100 g)} = 106.04 - 0.66x_1 - 0.90x_2 - 0.087x_3 - 0.0005x_1^2 + 0.014x_2^2 - 0.004716x_3^2 + 0.015x_1x_2 + 0.0048x_1x_3 + 0.00025x_2x_3 \quad (1)$$

where x_1 is the percentage of methanol in the water (%), x_2 is the extraction temperature (°C), and x_3 is the extraction time (min).

The regression model for the extraction of the pigments using methanol had a determination coefficient (R^2) of 0.9852, a variation coefficient of 3.36, and a probability ($P > F$) of 0.0005, indicating that the factors were an appropriate fit for this model. The statistical analysis indicated that the concentration of methanol, linear term ($p < 0.0001$) and quadratic term ($p < 0.0024$), was the factor that significantly influenced the extraction process. As can be seen in the response surface diagrams (Figure 2A), the greatest pigment extraction (92.7 mg/100 g) was obtained when a water/methanol solution (80:20, v/v) was used at 5 °C for 10 min. The extraction yield diminished with an increase in temperature and extraction time for concentrations of 20−40% methanol. When the temperature was raised from 5 to 30 °C for these concentrations of methanol, the concentration of the pigment decreased by 10 mg. Nonetheless, for a methanol concentration of 80% (data not show), there was a decrease in the total pigment concentration. In this case, when the temperature and extraction time were increased, the efficiency of extraction improved, probably due to the fact that when an extraction system has a low concentration of water, the process requires more energy and time for the hydration of the plant matter and the separation of the sugars associated with the aglycone, allowing for better extraction.

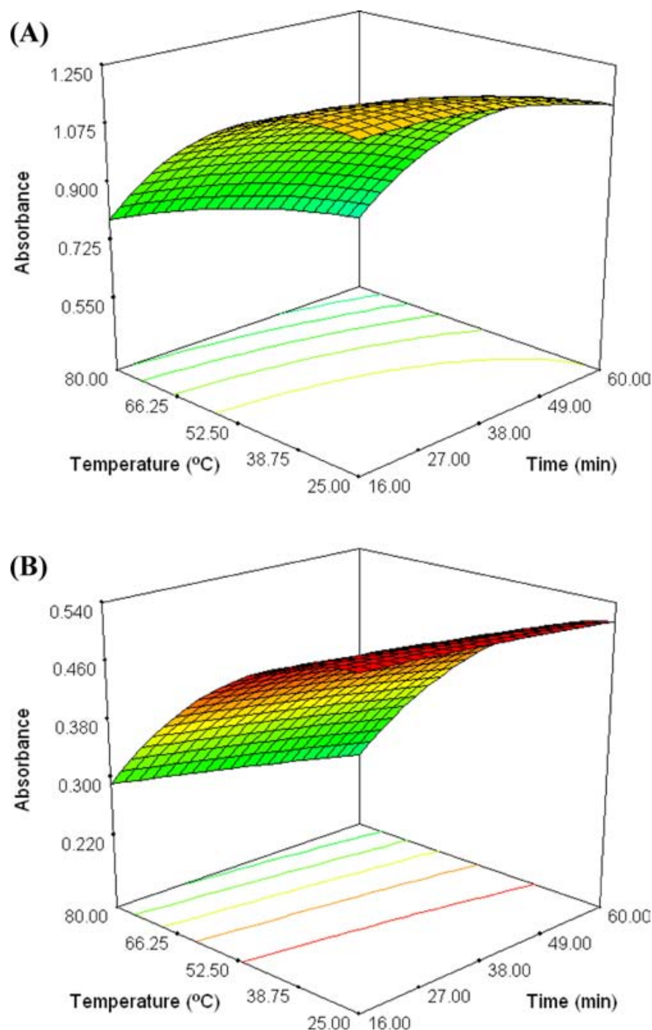

Figure 3. Stability of the pigments of (A) beet root and (B) xoconostle: effect of temperature and heating time at pH 5.0.

Extraction System II: Ethanol Solvent. The total pigment content (mg of betanin/100 g of fresh fruit) of the extracts obtained with ethanol is described by eq 2

$$\begin{aligned}\text{betacyanins (mg/100 g)} \\ = 69.17 + 1.02x_1 - 0.68x_2 - 0.20x_3 - 0.035x_1^2 \\ + 0.021x_2^2 - 0.011x_3^2 + 0.011x_1x_2 + 0.0069x_1x_3 \\ - 0.00005x_2x_3 \qquad (2)\end{aligned}$$

where x_1 is the percentage of ethanol in the water (%), x_2 is the extraction temperature (°C), and x_3 is the extraction time (min).

The value of the determination coefficient was 0.9620, the variation coefficient was 9.81, and the significance level of the model was 0.0048, indicating that the model was an appropriate fit for the experimental data. As in the previous case, the ANOVA indicated that the ethanol concentration and the linear and quadratic parameters ($p < 0.0002$ and $p < 0.00369$, respectively) significantly affected the extraction yield. Compared with methanol, the concentration of the pigments in the aqueous ethanolic extracts went down from 92.7 mg with methanol to 86 mg with ethanol (Figure 2B). This tendency was observed for all concentrations of ethanol. For this solvent, neither the extraction time nor the temperature significantly affected the efficacy of the extraction. The maximum value of pigment concentration (86 mg) was obtained when the extraction was carried out for 25 min at 17.5 °C using a 20% (v/v) ethanol solution. With higher percentages of ethanol the efficiency of the extraction decreased, changing the trend. For 40% ethanol (data not shown), the maximum concentration was obtained at higher temperature (23.75 °C) for a maximum time of 25 min. Meanwhile, for 80% ethanol solutions, the maximum was attained at a temperature of 30 °C and 30 min. This behavior can be explained, as in the case of methanol, due to the fact that in solutions with greater alcohol percentages, greater temperature and extraction time are required to break the hydrogen bonds that the quaternary structure of the betalains form with the other components of the xoconostle.

On the other hand, with the use of greater percentages of water in both solvents, the extraction efficiency increased. This is in agreement with the results reported by Castellar et al.,[9] who found that water extracted the highest concentrations of pigments from three different species of *Opuntia* fruits (80.1, 19.6, and 15.2 mg/100 g of fresh fruit from *O. stricta*, *O. ondulata*, and *O. ficus-indica*, respectively). Nonetheless, the solvents prepared with greater percentages of water are less selective in dissolving other water-soluble compounds such as proteins and phenolic compounds. Overall, the concentration of pigments extracted from *O. joconostle* was higher than those reported from other *Opuntia* fruits[9] and in some cases from some commercial beet root (40−77 mg/100 g).

Stability of Betalains Extracted from Beet Root. Equation 3 describes the variation in coloration (absorbance) of the beet extracts.

$$\begin{aligned}\text{absorbance} = -1.94 + 0.014x_1 + 3.023x_2 + 0.85x_3 \\ - 6.817x_1^2 + 0.0001941x_2^2 - 0.053x_3^2 \\ - 0.00005405x_1x_2 + 0.001889x_1x_3 \\ - 0.002064x_2x_3 \qquad (3)\end{aligned}$$

where x_1 is heating time (min), x_2 is heating temperature (°C), and x_3 is the pH of the solution.

The probability of the model was highly significant ($P > F = 0.0001$), indicating that the experimental values showed a high correlation with the theoretical model. The determination coefficient was 0.9777, and the variation coefficient was 10.05. From the ANOVA, it was seen that the heating temperature and the pH on the linear term ($P > F = 0.0001$ and 0.0001, respectively) and the quadratic term ($P > F = 0.0001$ and 0.0001, respectively) were the factors that had the greatest influence on stability of the beet pigments (Table 5). The graph in Figure 3A shows the stability of the beet extracts. The stability of the pigment was affected when the temperature and time of heating were increased with a pigment loss of up to 40% at the pH studied (3, 5, and 7). This could be due to the presence of a high concentration of betaxanthins in red beet, which have showed a higher susceptibility to temperature than betacyanins.[24]

Stability of Betalains Extracted from Xoconostle. Equation 4 describes the stability of xoconostle determined by the absorbance for different evaluation factors.

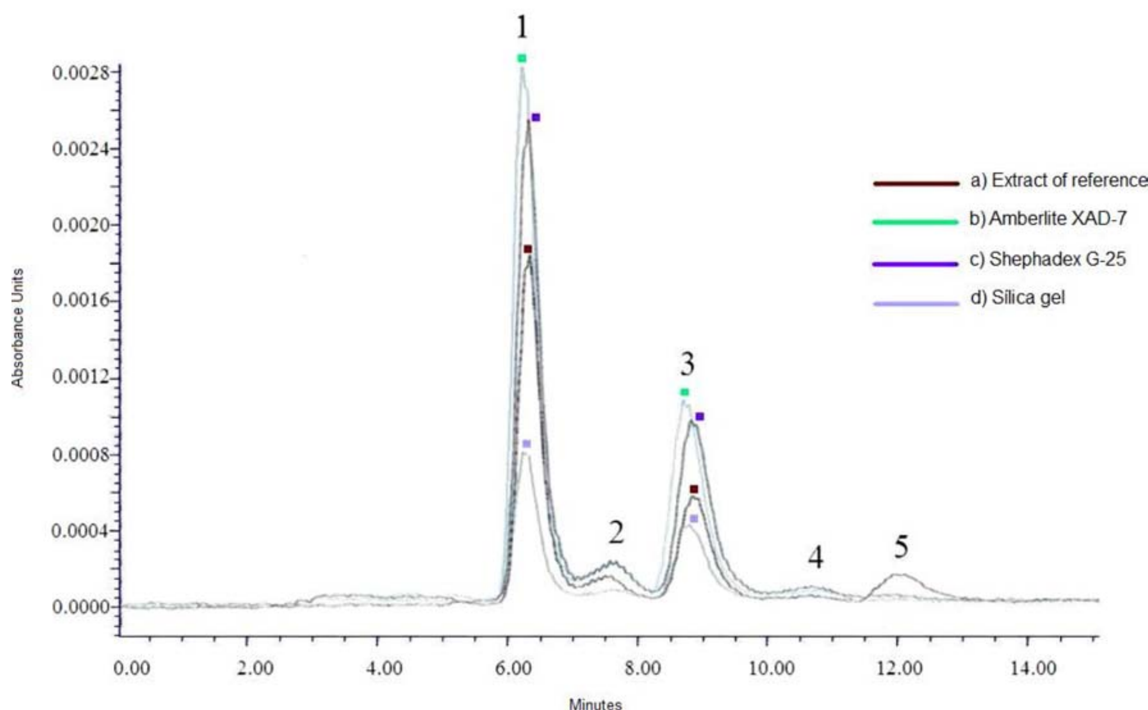

Figure 4. HPLC chromatograms of the fractions obtained in the separation of pigments by column chromatography (Amberlite, Sephadex, and silica gel).

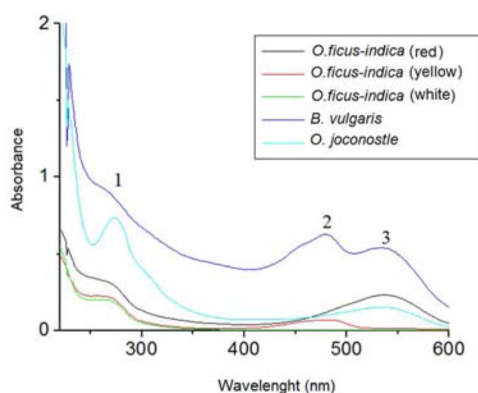

Figure 5. UV−vis spectra of (1) cyclo Dopa, (2) betaxanthin, and (3) betacyanin for three prickly pears, beet root, and xoconostle.

$$\text{absorbance} = -0.20 - 0.0002705x_1 + 0.003021x_2 + 0.19x_3 - 0.000004696x_1^2 + 0.0000787x_2^2 - 0.018x_3^2 - 0.0000181x_1x_2 + 0.0001051x_1x_3 - 0.00021x_2x_3 \quad (4)$$

where x_1 is heating time (min), x_2 is heating temperature (°C), and x_3 is the pH of the solution.

The probability of the model was $P > F = 0.0001$, this being highly significant and indicating that the experimental values showed a high correlation with the theoretical model, with a determination coefficient of 0.9376 and a variation coefficient of 14.42. As before, from the ANOVA, it was observed that the heating temperature and the pH on the linear term ($P > F$ = 0.0001 and 0.0044, respectively) and the quadratic term ($P > F$ = 0.0015 and 0.0003, respectively) were the factors that had the greatest influence on the stability of the pigments from xoconostle. Values of stability (absorbance) are summarized in Table 5.

Figure 3B shows the response surface graphs for the stability of xoconostle at pH 5. When the samples at pH 3 (data not shown) were heated at high temperatures (80 °C) for 1 h, the absorbance of the extracts fell drastically (from 0.43 to 0.08). In the graphs obtained at pH 5 and 7 a similar tendency was observed corroborating that at pH 5 the pigment was more stable, with a maximum absorbance value (0.54, 25 °C and 60 min) slightly greater compared with the value of 0.501 at pH 7. The tonality of the pigment under drastic treatment conditions changed; nonetheless, it was clear that the heating temperature influenced the degradation of the pigment. At room temperature, the pigment was stable for up to 60 min. Under these conditions a minimum change in the absorbance values of the extracts was observed at all pH values. This is in agreement with the results obtained by other authors.[24−26] Pigments from xoconostle showed a better stability compare to those extracted from beet root, particularly at low pH (3.0), at which a pigment loss of only 20% was observed.

The effect of temperature, time, and pH on the stability of the main pigments of the xoconostle, evaluated through the UV−vis spectra, indicates that when the pigment solution is at an alkaline pH (9−11) there is a decrease in absorbance of these pigments. This is probably due to the fact that with betanins as with isobetanins there can be a rupture in the aldimine bonds creating two compounds, one being betalamic acid (bright yellow color) and the other being cyclo Dopa 5-*O*-*β*-glucoside (colorless); the combined colors of these compounds reduced the absorbance value of the absorption spectrum.[25,27] Furthermore, at greater temperature, the betalains of the xoconostle generally lose color due to a probable dehydrogenation and decarboxylation, resulting in the neobetanin (yellow) and 17-decarboxy-betanin (orange red) compounds.[25]

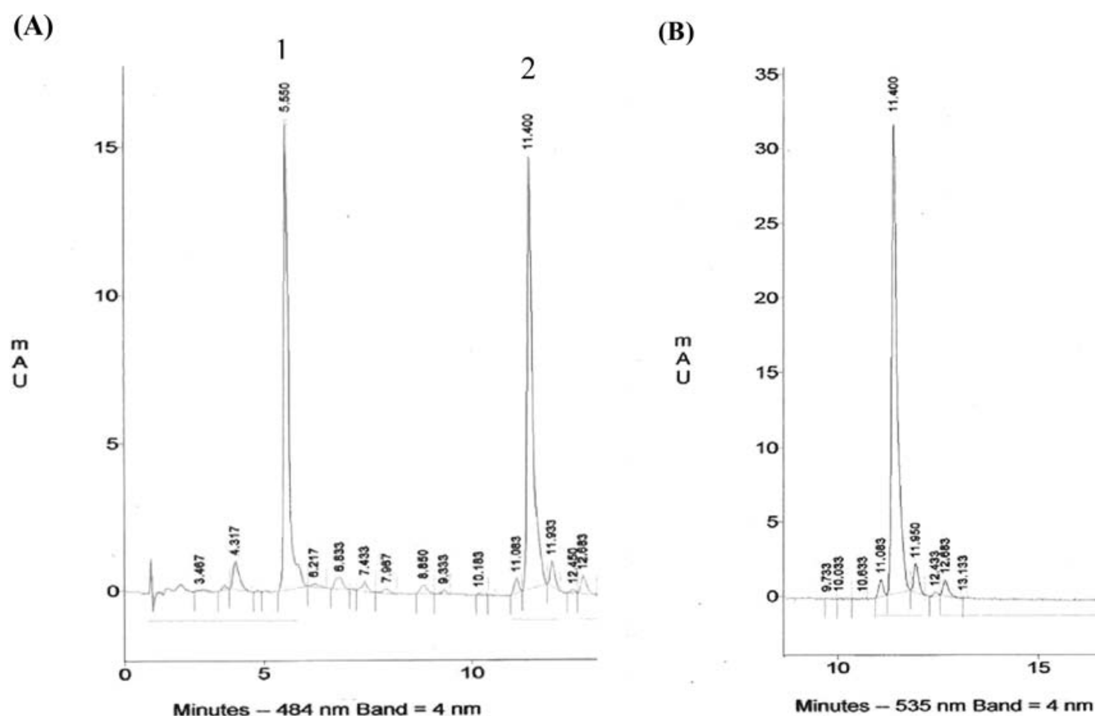

Figure 6. HPLC chromatograms for gradient elution for the beet root pigments: (A) λ = 484 nm; (B) λ = 535 nm.

As other researchers suggest[25−28], the combined effect of the pH and the temperature, greater dehydrogenation and decarboxylation of the xoconostle betalains are observed, evidenced by greater loss of color and, thus, lower absorbance values. When there is an alkaline pH and high temperature, the color loss is even greater due to increased rupture of aldimine bonds, resulting in colorless compounds; a similar effect is observed at very acid pH levels and high temperatures.

Betalains in solution at alkaline pH (9−11) have a decrease in absorbance of these pigments; this is probably due to the fact that in betanins as in isobetanins there can be a rupture of the aldimine bond, resulting in two compounds, one being betalamic acid (bright orange color) and the other being cyclo Dopa 5-*O*-β-glucoside (colorless). Similar results were observed at acid pH levels (<3).

Thermal degradation of betanin has been evaluated by several authors.[14,28,29] They observed that upon heating of betanin solutions there is a gradual reduction in the characteristic red color of this pigment and a slightly brown color begins to appear. If the betanin is heated to high temperatures (>60 °C) and for prolonged periods (longer than an hour), hydrolysis of this compound in solution is accelerated, producing betalamic acid and cyclo Dopa-5-*O*-glucoside as intermediary products. Nonetheless, this reaction is partially reversible depending on the pH. Altamirano et al.[28] reported that in water/ethanol model solutions the stability of betanin was very low due to the nucleophilic attack of the N═CH group that is present in the structure of the betanin. Ethanol is a strong nucleophilic agent that, given its high electron density in the oxygen atom, lowers the stability of the betanin.

Overall, the pigments from the xoconostle were stable at acid−neutral pH (3−7), showing greatest stability at pH 5. Due to this stability, these natural coloring agents could have a potential use in low-acid and neutral foods such as meat and dairy products as a replacement for artificial colors.

Column Chromatography Evaluated with HPLC. HPLC chromatograms of the fractions obtained in the separation of pigments by column chromatography (Amberlite, Sephadex, and silica gel) are shown in Figure 4. The curves were compared with a xoconostle extract, without prior separation in chromatographic column. Five characteristic peaks (1−5) were seen in the chromatogram of the reference extract, whereas in the fractions obtained by column chromatography only four of the peaks initially observed were evident.

When the extract was separated with Amberlite, the area of the peaks increased, probably due to the Amberlite retaining pectins and mucilage present in the extract, thereby increasing the betalain content. As seen in Figure 4, in the separation with Sephadex can be also seen an increase in the area of the peaks of the extract; peaks 1 and 3 have areas slightly smaller than those obtained with Amberlite. Nonetheless, with this column, peak 4, which can barely be differentiated in the reference extract, became evident. In the column packed with silica gel, a greater separation of the pigments was obtained; a clear differentiation of the fractions previously separated through TLC (data not shown) can be seen. However, in the HPLC chromatogram the peaks had lower intensity, probably because of the acidity of the column initially. Thus, ionic exchange resins, particularly Amberlite, were shown to be useful in the fractionation and separation of this type of pigment.[30] This simple and fast procedure consists of placing the plant extract in contact with the ionic exchange resin, which allows for the betalains to be fixed by adsorption (nonionic interaction).

Characterization of the Pigments by UV−Vis Spectroscopy. In the UV−vis spectra seen in Figure 5 for three prickly pears, beet, and xoconostle, a local maximum can be seen between 270 and 280 nm, corresponding to the structure of cyclo Dopa. For the xoconostle at this wavelength a peak of greater intensity (1) can be observed. Yellow prickly pear and beet showed more defined peaks at approximately 475 nm,

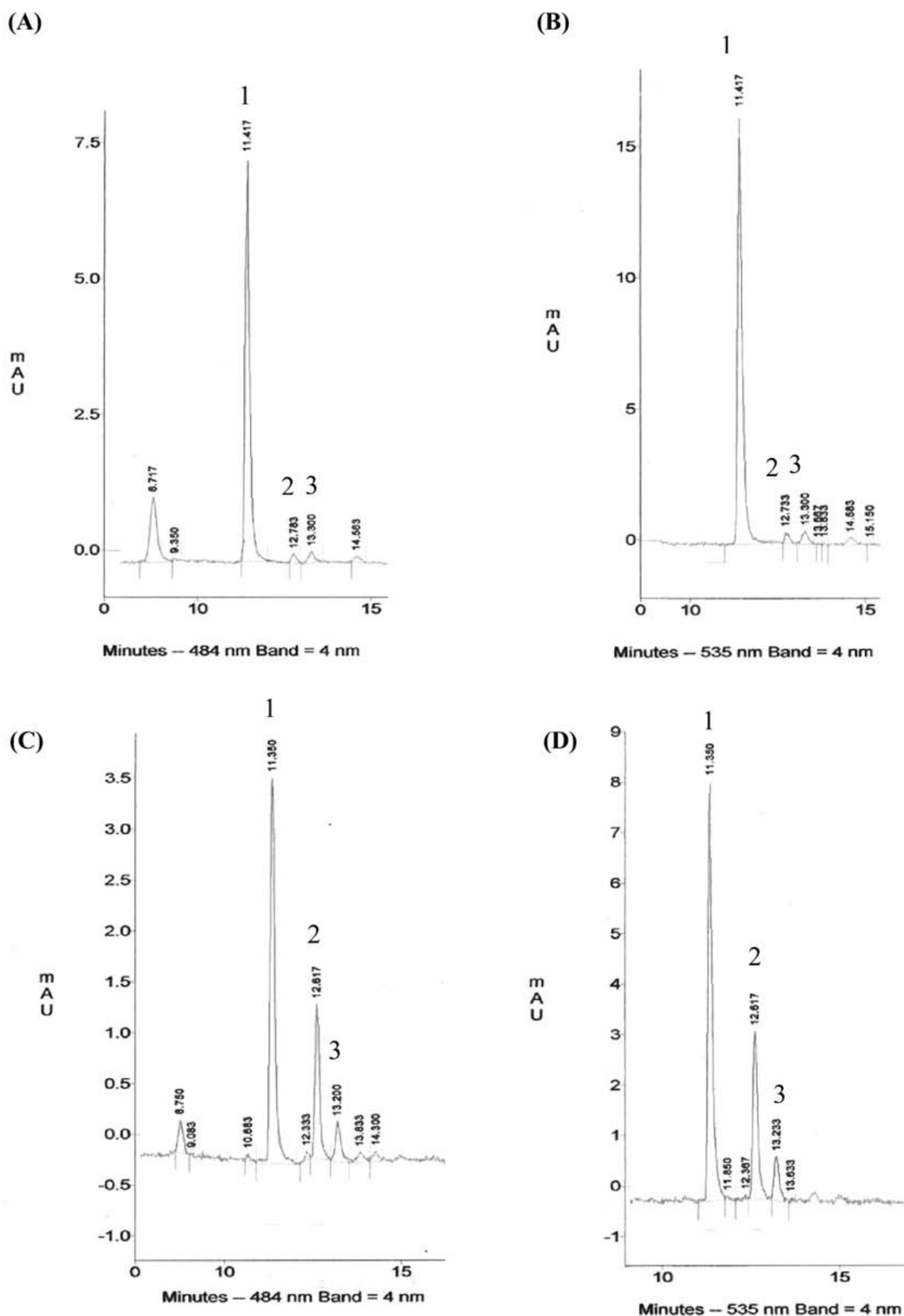

Figure 7. HPLC chromatograms for gradient elution with a retention time between 8 and 15 min for *Opuntia ficus-indica* ((A) λ = 484 nm; (B) λ = 535 nm) and for *Opuntia joconostle* cv. ((C) λ = 484 nm; (D) λ = 535 nm).

corresponding to betaxanthins (2), and a third maximum absorption only for beet in the visible range, around 535−538 nm (3), corresponding to betacyanins, mainly betanin.[26] The color of betalains is stable at pH between 3.5 and 7.0. Betalain solutions in this pH interval showed a visible spectrum similar to those of betacyanins and betaxanthins. The maximum wavelength (λ) for betacyanins was between 537 and 538 nm, whereas the maximum for betaxanthins was approximately 475 nm. At acid pH (3.5), λ is displaced to a lower value; above a pH of 7, λ changes to a higher value; outside this interval, the intensity of the visible spectrum decreases. Huang and Von Elbe[14] showed that the optimal pH for maximum stability of

betalain in the presence of oxygen was between 5.5 and 5.8. The beet solutions showed maximum stability at pH 5.5, corresponding to the normal pH for fresh beets. Additionally, vulgaxanthin I was more stable in the pH interval between 5 and 6 and showed greater stability in extracts that were not purified, whereas the optimal stability of the pigment in reconstituted powder was reached at a pH of 5.7.

Characterization of the Pigments by HPLC. For the characterization of xoconostle pigments, first HPLC chromatograms for beet root were done using as a reference the retention times of these pigments at two wavelengths, $\lambda = 484$ nm and $\lambda = 535$ nm, using the gradient method proposed by Schwartz and Von Elbe.[21] By this method, two well-defined peaks were obtained at 484 nm (Figure 6A). Peak 1 could probably be identified as vulgaxanthin I (5.55 min), and peak 2 could be betanin (11.40 min), as has been indicated by Schwartz and Von Elbe and Reynoso et al.[21,31] When the chromatogram was done at 535 nm (Figure 6B), a single peak is seen at 11.4 min, which could correspond to betanin.

Once the method was adapted using the beet pigments as a reference, the HPLC chromatograms for xoconostle and red prickly pear were obtained as can be seen in Figure 7. In the red prickly pear chromatograms (Figure 7A) at 480 nm, three main peaks are seen and they could correspond to betanin (1), betanidin (2), and isobetanin (3), these being responsible for red coloration. When the chromatogram was done at 535 nm (Figure 7B), the first peak (8.71 min) was not observed, but the other peaks, corresponding to the pigments indicated above, were maintained. In the case of xoconostle (Figure 7C,D), at 480 nm three peaks were also observed with the same retention times as red prickly pear, which indicates the presence of these three betalains, but in lower concentrations. Also in the chromatograms at 535 nm, only three peaks were observed, corresponding to betanin (1), betanidin (2), and isobetanin (3). In these samples, betanin (1) was the majority, but in xoconostle there is a greater amount of betanidin and isobetain (peaks 2 and 3) than in red prickly pear, with lower resolution.[31]

Because betacyanins and betaxanthins have similar properties, HPLC is an invaluable method for their separation and analysis. Tentative identification of betalains can be deduced from its chromatographic behavior and proven through analysis of its absorption spectrum and retention time (t_R). The first use of this technique with betalains was carried out by Vicent and Scholz.[32] The most useful support columns were C_8 and C_{18}, in reverse phase with particle sizes between 3 and 10 μm, whereas the solvents that have been used the most are water/methanol or mixtures of water/acetonitrile, acidified with acetic, formic, or phosphoric acid.[2] The order of elution in HPLC of the pure, crystalline pigments was as follows: betanin, betanidin, isobetanin, and isobetanidin.[33] This evidence was based on prior acid hydrolysis of the glucosides to break the glucoside bonds and obtain aglycones, with the isomerization of betanin into isobetanin.[33]

In summary, the best solvent for the extraction of pigments from xoconostle was water/methanol (80:20), compared with the different solvents evaluated. The greatest extraction yield and the least degradation of the pigments were obtained at 15 °C and a time of 10 min. The number of extractions to exhaust the pigments from xoconostle depends on the solvent system used. However, when methanol was employed, the extraction was more selective, requiring more steps to exhaust the pigments present in the whole fruit of xoconostle.

For stability studies, it has been seen that the combined effect of pH and temperature causes likely greater dehydrogenation and decarboxylation of the xoconostle betalains. When there is alkaline pH and high temperature, color loss is greater due to increased rupturing of the aldimine bond, resulting in two colorless compounds.

The main betalains of the xoconostle characterized by the techniques used in column chromatography, UV−vis, and HPLC were betalain, isobetalain, betanidin or filocactin, and isobetanidin, with the additional possibility of obtaining betaxanthins in natural form. All of them have been reported to have a great antioxidant capacity that could be used in further epidemiological studies to assess their application as functional products in both food and pharmaceutical industries.

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Notes

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