Optimization of the pitaya (Stenocereus spp.) betalain extraction process using Response Surface Methodology: Evaluation of their stability versus beetroot (Beta vulgaris)

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Abstract

A study was conducted to optimize the extraction of betalains from pitaya (Stenocereus spp.) and evaluate their stability compared to those from beetroot (Beta vulgaris). Optimal extraction conditions for betalains from pitaya were determined: pH 6, a solvent ratio of 30% ethanol, and a solvent ratio of 1 g/9.5 ml. These conditions yielded estimated concentrations of 76.44 and 54.01 mg/kg, as well as experimental concentrations of 76.45 mg/kg of BX and 52.64 mg/kg of BC in fresh fruit. pH analysis revealed that the hue stability range in aqueous media for pitaya was 3 to 9, maintaining an orange hue, while for beetroot, it was 2 to 8, maintaining a bright pink hue. Therefore, the degradation of BX and BC from both botanical sources was observed at pHs lower and higher than those mentioned above. A higher degradation rate was associated with ethanolic solutions. Thermal stability analysis showed that, at 40 °C, the hue of pitaya betalains remained unchanged. At 50 °C and 60 °C, a hue change from orange to yellow-orange was observed, with the greatest hue loss at 60 °C after 2.5 hours. Meanwhile, beetroot betalains showed hue changes at 60 °C after 1.5 hours, turning brown. Furthermore, the activation energy of pitaya and beetroot BCs was higher than that of BX, indicating that BC degradation reactions are more sensitive to temperature. These results highlight the importance of expanding our knowledge of pigments derived from diverse plant sources for their optimal utilization in industrial applications.

Introduction

Food products on the market have a wide range of colors that attract consumers. Color is a physical-physiological phenomenon that, as a sensory characteristic, is involved in the aesthetics, acceptability, and perception of food safety. Most processed foods incorporate synthetic colorants in their formulation; however, it has been observed that some of these are associated with neurobehavioral impacts [1] and other effects such as hypersensitivity, atopic dermatitis, and allergic rhinitis [2]. This has resulted in the retirement of certain colorings from the market, including FD&C Red #1, 2, 3, and 4; FD&C Green #1 and 2, and FD&C Violet #1 [3]. This situation necessitates the use of colorants that do not pose risks to consumers' health.

Plant-derived pigments are an alternative that, in addition to providing color, are attributed with pharmacological properties such as anticancer, antitumor, antimicrobial, and antihyperglycemic effects [4, 5]. Among these pigments are carotenoids, anthocyanins, chlorophylls, and betalains, with the latter being the least studied. The European Union identifies betalains as E-162, and in the United States as 21 CFR-73.40 (Beet powder). They are used in meat products (such as sausage), frozen foods, and dairy products [6]. The main extraction source of this pigment is the *Beta vulgaris* species known as beetroot. However, it has some disadvantages, since being a bulb that grows under the soil, it presents a high microbial load and imparts an earthy taste (caused by geosmin and derivatives) [7], in addition to the nitrate content, which is still a topic under discussion [8, 9].

Recently, cacti fruits have emerged as an alternative for extracting these pigments [10]. The genus *Stenocereus* is a columnar cactus that grows from the north to the south of Mexico [11]. Several studies have shown that the fruits of these cacti are a promising source for obtaining betalains [5, 10, 12, 13]. However, this area has been relatively understudied.

Betalains are nitrogenous and water-soluble chemical compounds, which are divided into two sub-groups: betaxanthins (yellow-orange hue) and betacyanins (red-purple hue), both sharing a common structure: betalamic acid (4-(2-oxo-ethylidene) -1,2,3,4- tetrahydropyridine -2,6 -dicarboxylic acid). When betalamic acid binds to the cyclo-DOPA (cyclo-3-(3,4-dihydroxyphenylalanine) and its glycosylated derivatives, it forms betacyanins. Conversely, when betalamic acid binds to amines or amino acids, it forms betaxanthins [9, 14].

These pigments can undergo various structural changes, such as isomerization, deglycosylation, dehydrogenation, decarboxylation, and hydrolysis, when exposed to specific conditions of pH, temperature, oxygen, water activity (Aw), and the presence of certain metals [15]. Alterations of these conditions could potentially change the color and functionality of these pigments [16, 17].

The color properties of betalains are widely documented to be affected by pH and temperature [10, 15, 16, 18]. Temperature promotes the decarboxylation of betacyanins at the C17 position, resulting in a hypsochromic effect at their maximum absorption point, reflecting color changes towards orange hues [19]. A dehydrogenation process is also reported that, as a consequence, forms neobetanin, responsible for yellow tones [20]. On the other hand, pH conditions have been reported to maintain the stability of betalains in situations of high temperatures [17, 18, 21, 22]. Therefore, understanding the behavior of betalains under these factors allows us to establish stability conditions and narrow down their extraction conditions to optimize the process and obtain higher yields.

Most studies have focused on analyzing the factors that affect the stability of betalains extracted from beetroot [17 – 19, 23, 24]. However, it is inappropriate to generalize their behavior towards these factors, as there is variability in betalains depending on the matrix in which they are found. This inherent diversity makes it relevant to investigate the stability of betalains in cacti fruits, specifically those belonging to the genus *Stenocereus* spp.

The objectives of this research were to: a) optimize the extraction conditions of pitaya betalains considering the factors pH, solvent ratio, and mass ratio, and b) evaluate the effect of pH, type of solvent, and extraction temperature, as factors that affect the stability of pitaya betalains (*Stenocereus* spp.) compared to those of beetroot (*Beta vulgaris*), through kinetic behavior and variables associated with their stability (half-life, activation energy, degradation constant).

Materials and Methods

Chemicals and Reagents

Sodium hydroxide, hydrochloric acid, and ethanol were analytical grade and purchased from J.T. Baker (CDMX, Mexico). Deionized water provided by Reasol (CDMX, Mexico) was used in all the experiments.

Biological Sample Collection

Red fruits of pitaya (*Stenocereus* spp.) with commercial maturity (which occurred when spines were easily released, and the fruits had a shiny surface) were obtained from Tula in the state of Tamaulipas, Mexico, with GPS coordinates of 22° 59' 0" N and 99° 43' 00" W, and an elevation above sea level of 1,173 m. Lithosol soil is used mainly in agriculture, livestock, and grassland activities. The annual mean rainfall and temperature were 400 mm and 25.7 °C, respectively. The climate was characterized as warm semiarid, with rainfall occurring half a year, classified as type BSh in the Köppen classification.

The fruits were selected of homogeneous size and without physical damage and were disinfected with 200 ppm sodium hypochlorite solution.

The peel was removed from 5 kg of red pitaya fruits to obtain the pulp, which was then macerated. Briefly, 250 g of samples were frozen for later analysis. Beetroot juice was used as a Control, extracted from tubers purchased from the local market of Ciudad Mante, Tamaulipas, Mexico.

Preparation of Aqueous Extract

The aqueous extracts of pitaya and beetroot were obtained after thawing the samples and subjecting them to centrifugation at 8500 rpm (Corning LSE, 6766-HS, USA) for 10 minutes.

Extraction Process Optimization

Briefly, 1 g of pulp was weighed and placed in a 50 mL polypropylene tube (Falcon®). The volume and solvent ratio were added, and the pH was adjusted as indicated in Table 1, using NaOH solutions (0.5%, 2%, and 5%) and HCl (0.5%, 1%, and 2%). It was shaken in a vortex (Science MED, MD-MX-S, México) for 5 minutes at medium speed. The mixture was centrifuged at 10,000 rpm (Corning LSE, 6766-HS, USA) for 10 minutes. The supernatant was collected in amber vials to quantify betalains immediately. The concentration of BX and BC (i.e. betalains) was calculated using Eq. 1 of Castellanos-Santiago and Yahia [25] as cited by García-Cruz [12].

$$B = \frac{(1000 * A * Df * W * Vm)}{(\varepsilon * ms * \lambda)} \qquad (Eq.\ 1)$$

where B is the concentration of BC or BX in mg per kg, A is the absorbance at 538 and 483 nm for BC and BX, respectively. Df is the dilution factor, W is the molecular weight (550 for betanin and 308 g/mol for indicaxanthin), Vm is the volume of the extract (L), ε is the molar extinction coefficient (6000 and 4800 L/mol × cm for BC and BX, respectively), ms is the mass of the sample (Kg), and λ is the length of the cell (0.01 m).

RSM Analysis

Response Surface Methodology (RSM) was conducted to obtain the optimal extraction conditions, using the randomized Box-Behnken design with 5 central points, and 3 factors with three levels, for the optimization of BC and BX concentration. The factors considered were pH (ranging from 3 to 8), ethanol: water solvent ratio (RS; from 30% to 100%), and the mass-solvent ratio (RM; 1g of pulp per 3 to 10 mL of solvent).

Verification of theoretical vs experimental optimization conditions

An optimization tool from the same statistical package was utilized to determine the conditions that would maximize the concentration of betalains. To validate the accuracy of the model's predictions, a series of experiments were conducted to compare the results predicted by the model with the actual experimental results.

Influence of factors affecting the stability of betalains.

pH

This study aimed to evaluate the effect of pH on the stability of betalains in the dissolution medium. Water and ethanol solutions were adjusted to pH levels ranging from 1 to 13 using hydrochloric acid (HCl) or sodium hydroxide (NaOH) solutions at concentrations of 0.1%, 0.2%, and 1%. The experiment was carried out by taking 2 mL of each solution at different pH levels, and 20 µL of each extract. Absorption spectra were generated in the visible light range (300-700 nm) using a spectrophotometer (PerkinElmer, UV Lambda 35, USA), and absorbances were measured at 483 nm for BX (Betaxanthins) and 538 nm for BC (Betacyanins). The concentration of BX and BC was calculated using Eq. 1.

Ethanol: Water Solvent Ratio

Ethanol-to-water ratios ranging from 0 to 100 were evaluated for pH levels 4, 5, and 6. Briefly, 2 mL of each solution was taken, and 25 µL of extract was added. After that, the absorbances were measured using a spectrophotometer to quantify BX and BC as previously indicated.

Temperature Kinetics

Briefly, 2 mL of pH-adjusted solutions (pH 1-13) were taken, and 50 µL of each extract were added. They were placed in a temperature-controlled bath (Proquisur, BA50640, México) for 0.5, 1.5, and 2.5 hours at 40, 50, and 60 °C. They were transferred to an ice bath for three minutes and then left at room temperature for five minutes. Finally, absorbance was measured in a spectrophotometer to quantify BX and BC at different time intervals. Temperature kinetics were only studied with water as the solvent because precipitation began in ethanol after 30 minutes of heating at 40 °C.

Statistical Analysis

Response Surface Methodology was used for the statistical analysis of the results through Minitab software (version17.1.0, Minitab, Ltd., Coventry, UK). An Analysis of variance (ANOVA) was conducted to identify significant differences, and the Tukey Multiple Range (HSD) test was employed at a significance level of α = 0.05 to compare means. A prediction model was developed to estimate optimal conditions, and ANOVA was also used to assess the significance of factors and the coefficients within the model. Additionally, contour plots were also created using Design-Expert software (version 10, Stat-Ease, Minneapolis, MN, USA).

Results and Discussion

Optimization of the extraction process

Based on the data obtained in the Box-Behnken design (Table 2) and through multiple regression analysis, the predictive models for BX and BC concentrations were developed. The data were fitted to second-order models (Equations 2 and 3). To evaluate the quality of the models, the determination coefficient (R^2), indicates the percentage of the data variability that the model can explain. A good model is considered solid when the value of R^2 exceeds 70% [26]. In this study, it is relevant to highlight that the models for BC were 77.24 and 94.60 for BX, which indicates a significant capacity of the models to explain the data variability.

$$BX = 37.3352 + 7.3059 * \text{pH} + 0.191701 * \text{RS} + 2.37364 * \text{RM} - 0.52664 * \text{pH\^2} - 0.00225714 * \text{pH} * \text{RS} - 0.0597143 * \text{pH} * \text{RM} - 0.00101959 * \text{RS\^2} - 0.0118776 * \text{RS} * \text{RM} - 0.0660408 * \text{RM\^2} \quad (Eq.\ 2)$$

$$BC = 9.38878 + 10.3503 * \text{pH} - 0.0378592 * \text{RS} + 4.67218 * \text{RM} - 0.83952 * \text{pH\^2} - 0.0171429 * \text{pH} * \text{RS} - 0.0554286 * \text{pH} * \text{RM} + 0.00186776 * \text{RS\^2} - 0.0139592 * \text{RS} * \text{RM} - 0.24302 * \text{RM\^2} \quad (Eq.\ 3)$$

When evaluating the effect of factors (Table 3) on BX concentration, it was observed that pH, RS, and RM were significant (α=0.05) as the main effects. Additionally, an interaction effect between RS and RM was observed. This indicates that the levels of RS and RM influence the BX concentration.

On the other hand, when analyzing BC concentration, it was observed that this variable was unaffected by any of the three evaluated factors (pH, RS, RM). This suggests that BC is more stable to these factors compared to BX, at least under the evaluated levels of these factors.

The contour plots (Fig. 1a) show that the concentration of BX is maximized at pH values close to 6 and 8, RS between 40 and 80% ethanol: water, and RM above 9 mL of solvent per 1 g of fresh pulp. For BC (Fig. 1b), the pH range with higher concentrations is between 4 and 8, with RM between 6 and 8 mL of solvent per 1g of fresh pulp. These results are consistent with other studies on the optimization of betalains from cacti fruits. For example, Maran et al. [27] reported higher concentrations of betalains from *Opuntia ficus-indica* at pH 6.9. Meanwhile, Gómez-López et al. [28] and Vázquez-Espinosa et al. [29] reported higher concentrations of betalains at solvent ratios of 55% and 60% ethanol: water, respectively.

Kushwaha et al. [30] reported that a mass-solvent ratio of 1:15 improves the extraction of betalains, while higher ratios significantly decrease their concentration due to solvent dilution of the solute. Conversely, increasing the mass can lead to solvent saturation, affecting pigment release.

Finally, the optimal extraction conditions were determined through model derivation: a pH of 6, an ethanol: water solvent ratio of 30%, and a mass-solvent ratio of 1 g of pulp per 9.5 mL of solvent. Under these conditions, it is estimated that 76.44 mg kg^{-1} of fresh pulp of BX and 54.01 mg kg^{-1} of fresh pulp of BC can be obtained. Similar conditions were obtained in other studies on cactus fruits (prickly pear) [27, 28, 30]. On the other hand, in the work of Cervantes-Arista et al. [10], a higher concentration of betalains extracted from *Stenocereus* spp. is reported under pH conditions of 4.2 and ethanol: water solvent ratio of 55 %. It is important to mention that in addition to the factors above, temperature and extraction time were considered in this research. Therefore, it is relevant to identify how these factors impact both stability and the extraction of these compounds of pharmacological and nutritional relevance.

Factors affecting betalain stability

Analysis of pH

Color is estimated under three parameters or dimensions: hue, which relates to the dominant wavelength also known as tone; value or luminosity, which indicates the clarity of the color; and chroma, which corresponds to the color saturation or purity (HunterLab). This text will refer to hue and luminosity to describe color changes.

Figure 2a shows the betalain solutions from pitaya in water at pH values ranging from 1 to 13 (left to right). It was observed that at pH 1 and 2, the solutions exhibit yellow-orange hues, while at pH 10, the solutions show brown hues. The solutions at pH 11 and 12 turn yellow, with the hue disappearing entirely at pH 13. The range in which the hue remains unchanged was between 3 and 9, maintaining an orange hue. The hue is determined by the proportion of BX and BC [5], and these fruits exhibit a higher concentration of BX, which are responsible for the orange hue [14].

For the beetroot solutions in water (Fig. 2b), considering that both the type and ratio of betalains (BC and BX) are different compared to pitaya solutions, the hue changed to pink-purple (with a higher proportion of betacyanins). Changes in hue were observed at pH 1, 9, and 13, just like the pitaya solutions. In this

case, pale-pink hue was observed at pH 1, purple hue at 9 and 10, which has been previously reported [16], brown at pH 11, yellow at pH 12, and loss of hue at pH 13. The stability range of the hue was observed between 2 and 8, maintaining a bright pink hue.

A pH range between 3 and 7 has been established where betalains remain stable [31], and specific pH conditions where BX and BC are stable have also been reported. The optimal pH for betanin (betacyanin) was estimated to be between 4 and 6. However, under aerobic conditions, betanin is more stable at pH 5.5 and 5.8, and in the absence of oxygen between 4 and 5. The highest stability of BX has been reported at 5.5 [15]. Therefore, pH is a factor that is conditioned by other variables that can modify the stability of these pigments.

Figures 2c and 2d show the spectra of pitaya and beetroot in water, respectively, in order to understand the effect of pH on the profile of BX and BC, absorption spectra of pitaya and beetroot solutions in water were taken, observing peaks at 483 nm for BX and 538 nm for BC.

A shift towards longer wavelengths (bathochromic effect) for the solutions at pH 10 and 11 (Fig. 2c-d) is observed. Von Elbe et al. [16] also observed this behavior for beetroot betalains at pH 9. On the other hand, at pH 12 and 13, there was a significant decrease in the peaks of BX and BC, which may be due to the hydrolysis of betalains into colorless betalamic acid, DOPA-cycle for BC, or amines and amino acids for BX [32], furthermore a shift towards shorter wavelengths (hypsochromic effect) was observed. This behavior may be associated with the decarboxylation of betalains as mentioned by Herbach et al. [19], as well as the possible formation of dehydrogenated compounds like neobetanin, responsible for yellow hue [20].

For pitaya betalain solutions in ethanol (Fig. 3a), changes in hue were only observed at pH 1-2, presenting a yellow hue. The stability range of hue was observed between 3 and 13, although no loss of color was observed in alkaline pH in ethanol, as seen in aqueous solutions, precipitation occurred after one hour. In contrast, when analyzing beetroot betalain solutions in ethanol (Fig. 3b), a purple hue was observed for pH 1 and 2, which in turn was also noted by García Barrera et al. [20]. A slight loss of hue was observed for pH 3, 7, and 8. Precipitation was observed after a few hours, except for pH 1 and 2, similar to pitaya betalains in ethanol. Additionally, there was a greater hue intensity loss than betalain solutions in water. This could be explained by the presence of ethanol, which increases the degradation rate of betalains. According to García Barrera et al. [21], this is due to the high electron density of the oxygen atom in the alcohol group and its greater nucleophilic character compared to water. During this nucleophilic attack, betalains hydrolyze due to the formation of an unstable protonated intermediate compound that breaks conjugated bonds, promoting the hydrolysis process, which tends to be faster in neutral to alkaline solutions than in solutions with a pH between 4 and 5 [33].

Unlike the spectra of pitaya solutions in water (Fig. 2c), the spectra of pitaya in ethanol (Fig. 3c) did not show any wavelength shift, and there was no significant difference in absorbances due to pH effects. Similar behavior was observed for BX and BC of beetroots in ethanol (Fig. 3d) versus beetroots in water (Fig. 2d).

Figure 4 shows the effect of the concentration of betalains from pitaya and beetroot extracts as a function of the dissolution medium (water or ethanol). The graph shows that regardless of the dissolution medium in pitayas extract there is a higher concentration of BX than of BC (Fig. 4a), while in the case of beetroot extract, regardless of the dissolution medium, there is a higher concentration of BC than of BX (Fig. 4b). In addition, there are no significant changes in the concentration of BX in the pitaya and beetroot extracts (regardless of the solution medium and pH values evaluated), however, the concentration of BC was higher in beetroot extracts than in pitaya extracts regardless of the solution medium and pH values, except for pH 13 of the beetroot extract in an aqueous solution, where maintained the concentration equal to that of the pitaya extract in the same solution medium.

When evaluating the effect of pH on the concentrations of BX and BC in pitaya water solutions, no significant difference was observed for BX in the pH range from 1 to 11. No difference was noted for BC concentration from pH 1 to 9 (Fig. 4a). However, the concentration decreased at more alkaline pH values (10-13), which was related to the evident degradation of pigmentation (see Fig. 2a).

Meanwhile, BX and BC concentrations from pitaya extracts in ethanolic solution showed a certain degree of stability at pH 3 to 13, evidencing greater stability at alkaline pH. In contrast, the betalain concentration was lower when evaluated at acidic pHs, which is related to the pigmentation degradation that occurred at pH 1 to 2 (see Fig. 3a).

The behavior of BX and BC concentrations in the beetroot extract in aqueous solution as a function of pH was characterized by three zones of differentiation. The highest and most constant concentration of betalains occurred at pH values of 3 to 8, while the lowest concentration of betalains occurred at pH values of 1 and 2, and 9 to 13. These zones are consistent with the tones shown in Figure 2b. Meanwhile, the concentrations of BX and BC in the beetroot extract in ethanolic solution were characterized by two distinct zones. At pH values of 3 to 13, the betalain concentration was highest and constant, and at pH values of 1 to 2, the concentrations of BX and BC were lowest. Therefore, although the concentration of the beetroot extract in aqueous solutions is higher than in ethanolic solutions, the hue is more stable and constant in ethanolic solutions at very alkaline pHs (see Fig. 2c and 3c).

Significant differences in the hue of the solutions were observed in both solvents (water and ethanol). Therefore, when evaluating the stability of betalains, it is important to consider not only their concentration but also the ratio of BX to BC and the type of betalains present. Apparently, color is sensitive to small variations in betalain concentrations, as evidenced by the research of Yang et al. [23], who found that small changes in color parameters measured by instrumental techniques can be perceived by people when undergoing sensory evaluation.

Analysis of the solvent ratio effect

pHs 4, 5, and 6 were selected to evaluate the effect of the solvent ratio ethanol: water since the lowest precipitate formation in ethanolic solutions was observed at these pHs.

No changes were observed regarding the hue, with different solvent ratios (0-100% ethanol: water), on the other hand, it was possible to identify the interaction between the solvent ratio and pH on the betalain concentration. The highest concentrations of BX and BC of pitaya were obtained for the ratios of

40, 50, and 60 ethanol: water and pH of 5.

No significant difference was observed for BX of beetroot, due to the effect of pH and solvent ratio, meanwhile for BC, the highest concentrations were observed at pH 4 with ratios of 30, 40, 70, 80, and 90 ethanol: water. This is in accordance with the reports of some researchers, where the highest stability of betalains is between 4 and 5 [16, 21, 34]. Both the concentrations of betalains in beetroot and pitaya in ratios higher than 40% ethanol: water were higher than in more aqueous ratios (less than 40%), which could be due to the dehydrating effect of ethanol, which allows better extraction of the pigment found in the cellular vacuoles [33]. However, the behavior of these pigments in relation to the solvent is not yet clear, so more specific studies are opened that allow observing the structural changes of betalains under these different solvent conditions.

Evaluation of the temperature effect

For thermal stability analysis, pH values were selected where the hue of the solutions remained constant (pH 3-8). In Figure 5a, pitaya betalain solutions in water at pH values of 3 to 8 exposed to different heat treatments (40, 50, and 60 °C) are observed. It was noted that at 40 °C, the hue remained unchanged throughout the exposure period (2.5 h). However, at 50 °C and 60 °C, a change in hue from orange to orange-yellow was observable after 0.5 h of exposure, and after 1.5 h, the hue turned yellow. The greatest loss of hue was observed at 60 °C after 2.5 h of exposure. On the other hand, no difference was observed due to pH, except at pH 3, where the greatest loss of hue was observed. When evaluating beetroot betalains in water (Fig. 5b), changes in hue were observed at a temperature of 60 °C after 1.5 h, turning brown, except at pH 3 where the hue remained unchanged.

Similar to this study, it is reported that increasing the temperature leads to increased degradation of betalains [19, 21, 34, 35]. This is because when a molecule is exposed to external heat, it tends to increase its kinetic energy, which can lead to changes in bond elongation or even bond breakage [36]. Herbach et al. [15] report that temperature is one of the main degradation factors for betalains and is responsible for structural changes such as breaking the molecule into betalamic acid and its complement (amines or amino acids for betaxanthins and cyclo-dopa for betacyanins), decarboxylation at C_{17} and C_{15}, as well as dehydrogenation, responsible for yellow hues [24].

Degradation kinetics

It has been reported that the degradation of betalains due to temperature follows first-order kinetics [16, 24, 37]. This means that the degradation of betalains is proportional to the temperature change. In this research, the degradation of both pitaya and beetroot betalains also exhibited first-order kinetics.

In Figures 6a-c, the degradation kinetics of BX, and in Figures 6d-f, those of BC from pitaya in water are observed for the temperatures (40 °C, 50 °C, and 60 °C) and pH values evaluated (3-8). BX at 40 °C (Fig. 6a) and 50 °C (Fig. 6b) showed no significant difference due to pH at any exposure time. At 60 °C (Fig. 6c), they exhibited the highest degradation at pH 3 throughout the exposure time, which is evident from the loss of hue (Fig. 5a). Significant degradation was observed at pH 8 after 2.5 hours. For BC at 40 °C (Fig. 6d) and 50 °C (Fig. 6e), no significant difference was observed due to pH, except for pH 4 at 0.5 hours (lower degradation). At 60 °C (Fig. 6f), similar to BX, the highest degradation was observed at pH 3. Castellar et al. [34] reported that exposing betalains from *Opuntia* fruit, another species of cactus, to high temperatures (50 and 90 °C) showed the highest stability at pH between 4 and 5, which was not observed in this study.

The degradation kinetics of betalains (BX and BC) from beetroot in water are shown in Figures 6g-6l. Similar behavior to pitaya BX, beetroot BX at 40 °C (Fig. 6g) showed no significant difference due to pH at any exposure time. At 50 °C (Fig. 6h), a greater degradation was observed at pH 8 starting from 1.5 hours. At 60 °C (Fig. 6i), there was a tendency for preservation at acidic pH levels of 3 and 4 for beetroot BX, and greater degradation at pH 7 and 8. BC showed no significant difference due to pH at 40 °C. At 50 °C, the lowest degradation was observed at pH 3 after 2.5 hours. At 60 °C, similar behavior to BX, greater stability was observed at pH 3, 4, and 5 from 1.5 hours, and lower stability at pH 7 and 8. These results are similar to those reported by Mikolajczyk-Bator and Czapski [17], who evaluated betalain solutions from beetroot. They reported that when heating betalain solutions to 90 °C at different pH levels (4-9), the highest content of degradation pigments (neobetanin) was formed at pH 6.5 and 7.

Figures 7a and 7b showed the degradation rates for BX and BC of pitaya at different pH levels. It is evident that the degradation rates of both BX and BC are higher at pH 3. However, the opposite effect is observed for both BX (Fig. 7c) and BC (Fig. 7d) of beetroot, with lower degradation rates at pH 3 and 4. This indicates that the stability of these pigments may depend on the type of betalains.

In Table 2, the half-life ($t_{1/2}$, h), degradation rates (k, h^{-1}), determination coefficients (R^2), and activation energy (Ea, kJ/mol) for BC and BX of pitaya and beetroot are presented. The range of half-life at 40 °C for pitaya BX was found to be between 9.82 (pH=3) and 42.37 (pH=7) h, for 50 °C between 3.35 (pH=3) and 6.60 (pH=4) h, and 60°C between 1.74 (pH=3) and 3.56 (pH=6) h, and for BC 9.61 (pH=8) to 24.57 (pH=6) h, at 40 °C, between 2.20 (pH=3) to 3.36 (pH=4) h, at 50 °C, and 60 °C from 0.87 (pH=3) to 1.87 (pH=6) h. As expected, the half-life decreases with increasing temperature. On the other hand, the half-lives for beetroot BX at 40 °C ranged from 6.47 (pH=3) to 13.83 (pH=4) h, at 50 °C from 3.02 (pH=8) to 5.85 (pH=4) h, and at 60 °C from 1.71 (pH=7) to 2.82 (pH=4) h, and for BC at 40 °C from 11.62 (pH=5) to 16.88 (pH=6) h, at 50 °C from 4.02 (pH=8) to 8.81 (pH=3) h, and 60 °C from 1.48 (pH=8) to 2.33 (pH=3) h. The half-life of pitaya BX at all temperatures was higher compared to beetroot. However, the half-life of beetroot BC was higher at 50 and 60°C compared to pitaya. Cai et al. [35] in their study on betalains of *Celosia argentea*, reported similar half-life times for betaxanthins and betanin (betacyanin) when exposed to 60 and 80 °C.

Higher degradation rates were recorded at a pH of 3 for both BX and BC of pitaya. Additionally, BC exhibited higher degradation rates at all pH levels and temperatures evaluated compared to BX. On the other hand, the degradation rates BX and BC of beetroot showed similar behavior across all pH levels (3-8) and temperatures but were lower at pH levels of 3, 4, and 5. Von Elbe et al. [16] also observed that beetroot betalains, when heated (100 °C), had lower degradation rates at pH levels of 5 and 3 compared to pH 7.

It was also observed that there is a stronger relationship between the degradation rate and the temperature when the temperature increases. This was evident from observing that the determination coefficients were closer to 1 when moving from 40 to 60 °C. This trend was observed for both pitaya and beetroot BX and BC.

The effect of temperature on the degradation rate (reaction rate) is determined by the activation energy and the temperature level [38]. For all pH levels except 7 and 8, the activation energies of pitaya BX were lower than those of BC. This result was also observed by Sánchez-Chávez et al. [37] in their study on beetroot. It indicates that the degradation reactions of BC are more sensitive to temperature. This phenomenon was also observed for beetroot betalains, where BC exhibited higher activation energy values for all pH levels.

Additionally, it is important to mention that the activation energies of both beetroot BX and BC were lower than those of pitaya betalains. This aligns with a greater stability of the hue color (Fig. 5) of beetroot betalains under thermal exposure compared to pitaya betalains.

Conclusion

The differential behavior of betalains BX and BC in pitaya and beetroot has been investigated, examining the impact of pH, solvent type (water and ethanol), and temperature on their color and concentration. By analyzing the pigments from both sources, marked differences in their stability with respect to the evaluated factors have been identified, demonstrating significant variations in their concentration and color.

The detailed study of stability parameters, including half-life, degradation rates, and activation energies, has consistently corroborated the observations made through color changes, absorption spectra, and kinetics. A significant discrepancy in BX and BC stability has been observed in pitaya and beetroot. It was noted that BX in pitaya is more stable (with a longer half-life) than their BC counterparts. In contrast, the opposite phenomenon was observed for BX and BC in beetroot, particularly noticeable at 40 °C. These findings underscore the inability to generalize about stability, as there are notable differences in both the type and proportion of betalains present in various sources of these pigments.

As pitaya emerges as an alternative source for betalain extraction, optimal extraction conditions have been defined for BX and BC. These conditions include a pH of 6, a solvent ratio of 30 % ethanol, and a ratio of 1 g per 9.5 mL of solvent, resulting in concentrations of 76.45 mg/kg of BX and 52.64 mg/kg of BC in fresh fruit.

This research contributes to the existing knowledge regarding the stability of betalains, natural pigments used as colorants. It is crucial to understand how these pigments behave in response to various factors considered in the development of products in the food, pharmaceutical, and cosmetic areas. The aim is to identify their behavior under these conditions, offering relevant information to improve their application in these fields. Understanding how these pigments behave in the face of various factors considered in product development is crucial, spanning across industries such as food, pharmaceuticals, and cosmetics. The goal was to identify their behavior under these conditions, providing relevant information to enhance their application in these fields.

Declarations

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Author contributions

All authors have contributed to the conception and design of the study. Material preparation, experimental design, data collection, analysis, and writing the main part of the manuscript were performed by Daniel Trujillo-Ramírez. Clara Cervantes-Arista performed the sample measurement. Ma. Guadalupe Bustos-Vázquez supervised the experimental work. Angélica Román-Guerrero devised the manuscript’s content and supervised the writing. All authors read and approved the final version of the manuscript.

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Code availability: Not applicable

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Tables

Table 1. Design of Box Behnken experiments for the extraction of betaxanthins and betacyanins of pitaya (*Stenocereus* spp.).

Run	pH	RS	RM	BX (mg/g)	BC (mg/g)
1	3.0	30.0	6.5	68.14	46.26
2	8.0	30.0	6.5	72.74	52.07
3	3.0	100.0	6.5	67.41	49.90
4	8.0	100.0	6.5	71.22	49.71
5	3.0	65.0	3.0	65.89	46.45
6	8.0	65.0	3.0	72.51	41.02
7	3.0	65.0	10.0	69.17	48.39
8	8.0	65.0	10.0	73.70	41.02
9	5.5	30.0	3.0	70.69	47.35
10	5.5	100.0	3.0	70.46	52.92
11	5.5	30.0	10.0	77.17	54.01
12	5.5	100.0	10.0	71.12	52.74
13	5.5	65.0	6.5	74.72	53.59
14	5.5	65.0	6.5	75.62	53.35
15	5.5	65.0	6.5	72.77	48.72
16	5.5	65.0	6.5	74.49	53.35
17	5.5	65.0	6.5	74.49	53.21

Table 2. Effect of pH and temperature on half-life, degradation rate, regression, and activation energy of betalains of pitaya (*Stenocereus* spp.) and beetroot (*Beta vulgaris*).

		Half-life (t1/2, min)				Degradation constant (k, h^{-1})				Regression (R^2)				Activation energy (Ea		
		Stenocereus spp.		*Beta vulgaris*		*Stenocereus* spp.		*Beta vulgaris*		*Stenocereus* spp.		*Beta vulgaris*		*Stenocereus* spp.		
pH	Temperature (°C)	BX	BC	BX	BC	BX	BC	BX	BC	BX	BC	BX	BC	BX	BC	
	40	9.82	9.98	6.47	13.15	0.07	0.07	0.11	0.05	0.94	0.82	0.94	0.86			
3	50	3.35	2.20	5.34	8.81	0.21	0.32	0.13	0.08	0.81	0.94	0.97	0.99	75.27	106.22	
	60	1.74	0.87	2.41	2.33	0.40	0.80	0.29	0.30	0.82	0.97	0.99	0.99			
	40	20.79	16.60	13.83	15.35	0.03	0.04	0.05	0.05	0.99	0.91	0.75	0.77			
4	50	6.60	3.36	5.85	5.34	0.11	0.21	0.12	0.13	0.64	0.87	0.93	0.93	82.83	100.58	
	60	3.09	1.65	2.82	2.31	0.22	0.42	0.25	0.30	0.83	0.93	0.99	0.99			
	40	15.78	11.82	10.41	11.62	0.04	0.06	0.07	0.06	0.83	0.89	0.99	0.98			
5	50	5.62	3.30	4.71	4.63	0.12	0.21	0.15	0.15	0.69	0.87	0.76	0.91	70.32	84.75	
	60	3.13	1.68	2.52	2.14	0.22	0.41	0.27	0.32	0.89	0.97	0.99	0.99			
	40	22.13	24.57	9.59	16.88	0.03	0.03	0.07	0.04	0.44	0.43	0.91	0.91			
6	50	4.38	2.81	3.75	4.31	0.16	0.25	0.18	0.16	0.69	0.88	0.98	0.99	79.84	112.43	
	60	3.56	1.87	2.04	1.93	0.19	0.37	0.34	0.36	0.87	0.95	0.97	0.99			
	40	42.37	11.97	6.98	12.46	0.02	0.06	0.10	0.06	0.28	0.54	0.99	0.99			
7	50	5.85	3.29	4.06	5.47	0.12	0.21	0.17	0.13	0.6	0.76	0.96	0.89	109.71	83.13	
	60	3.42	1.77	1.71	1.62	0.20	0.39	0.41	0.43	0.83	0.94	0.96	0.99			
	40	16.96	9.61	7.91	15.43	0.04	0.07	0.09	0.04	0.72	0.70	0.93	0.87			
8	50	5.57	3.22	3.02	4.02	0.12	0.22	0.23	0.17	0.72	0.89	0.92	0.94	87.87	83.33	
	60	2.24	1.41	1.72	1.48	0.31	0.49	0.40	0.47	0.93	0.97	0.96	0.99			

Table 3. Analysis of Variance for betaxanthins and betacyanins.

Source	DF	Sum of squares	Mean square	F-value	*p*-value
Betaxanthins (BX)					
Model	9	142.412	15.8235	13.64	0.001
Linear	3	73.768	24.5894	21.19	0.001
pH	1	47.824	47.8242	41.22	0.000
RS	1	9.095	9.0951	7.84	0.027
RM	1	16.849	16.8490	14.52	0.007
Square	3	58.927	19.6424	16.93	0.001
pH*pH	1	45.617	45.6167	39.32	0.000
RS*RS	1	6.568	6.5684	5.66	0.049
RM*RM	1	2.756	2.7557	2.38	0.167
Interaction of 2 factors	3	9.716	3.2387	2.79	0.119
pH*RS	1	0.156	0.1560	0.13	0.725
pH*RM	1	1.092	1.0920	0.94	0.364
RS*RM	1	8.468	8.4681	7.30	0.031
Error	7	8.122	1.1603	-	-
Lack of fit	3	3.860	1.2866	1.21	0.415
Betacyanins (BC)					
Model	9	215.640	23.960	2.64	0.107
Linear	3	19.198	6.399	0.71	0.579
pH	1	6.444	6.444	0.71	0.427
RS	1	3.892	3.892	0.43	0.534
RM	1	8.862	8.862	0.98	0.356
Square	3	174.805	58.268	6.42	0.020
pH*pH	1	115.920	115.920	12.77	0.009
RS*RS	1	22.042	22.042	2.43	0.163
RM*RM	1	37.316	37.316	4.11	0.082
Interaction of 2 factors	3	21.637	7.212	0.79	0.535
pH*RS	1	9.000	9.000	0.99	0.353
pH*RM	1	0.941	0.941	0.10	0.757
RS*RM	1	11.696	11.696	1.29	0.294
Error	7	63.538	9.077	-	-
Lack of fit	3	46.129	15.376	3.53	0.127

Figures

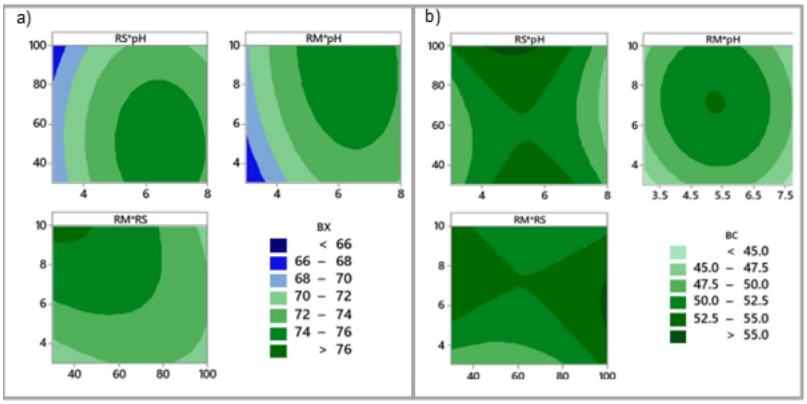

Figure 1

Contour plots for (a) Betacyanins (BX) and (b) Betaxanthins (BC) of pitaya (*Stenocereus* spp.)

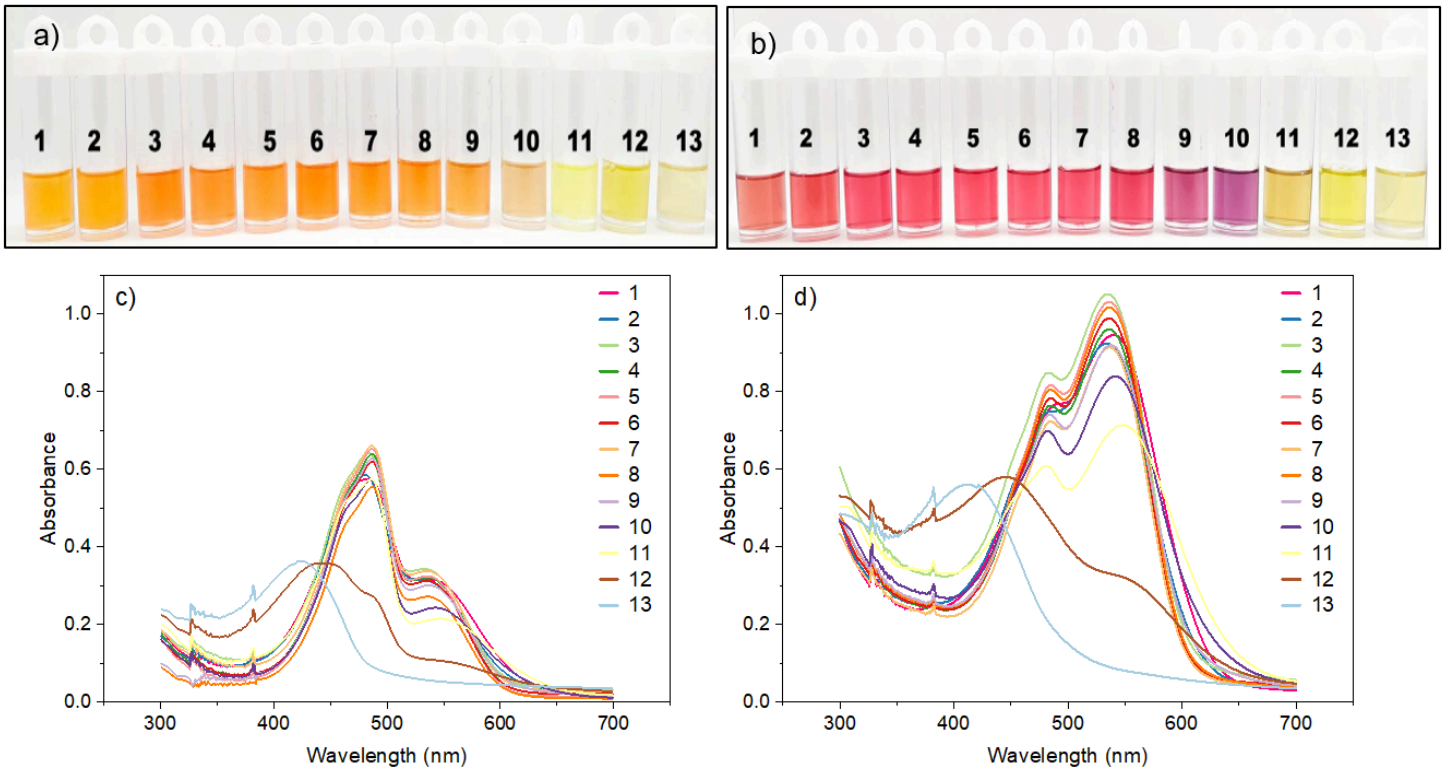

Figure 2

Effect of pH on the hue of pitaya (a) and beetroot (b) betalain and their absorption spectra (c and d, respectively) in a pH profile from 1-13 in aqueous solutions

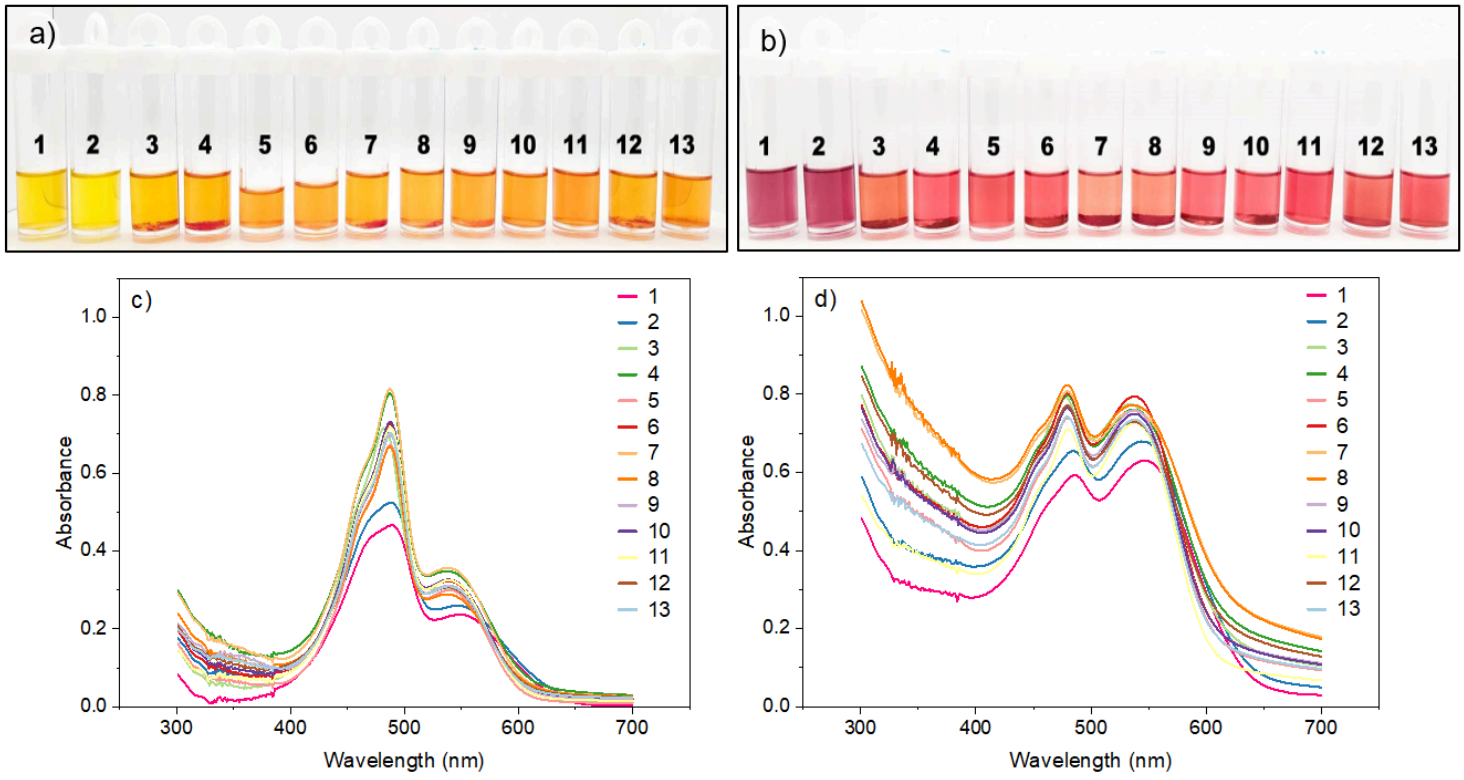

Figure 3

Effect of pH on the hue of pitaya (a) and beetroot (b) betalain and their absorption spectra (c and d, respectively) in a pH profile from 1-13 in ethanolic solutions

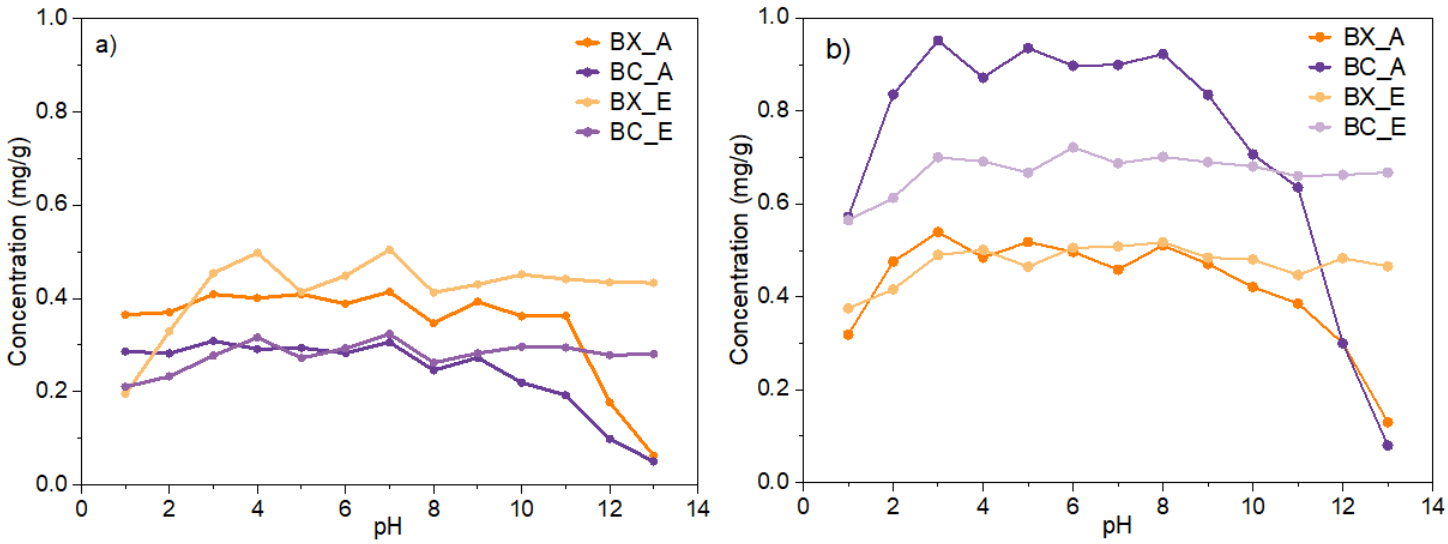

Figure 4

Effect of pH on the concentration of BX and BC of pitaya (a) and beetroot (b) as a function of the dissolution medium. Letter “A” after the underscore symbol indicates that the dissolution medium was in water, and “B” in ethanol.

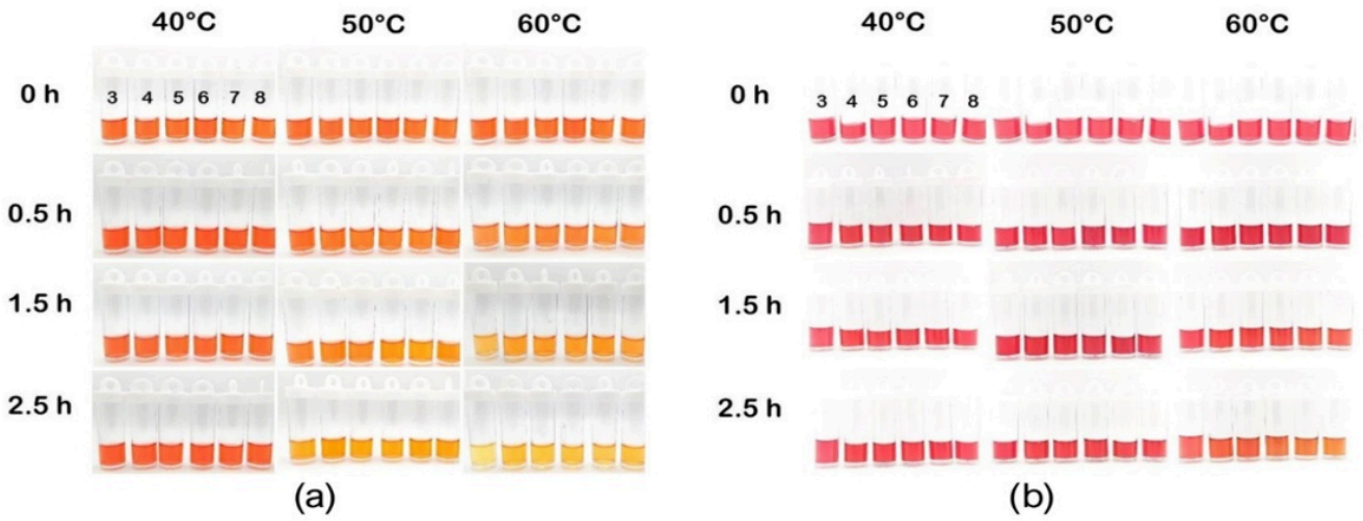

Figure 5

Aqueous solutions of pitaya (a) and beetroot (b) betalains at pH profiles of 3-8, exposed to 40 °C, 50 °C, and 60 °C for 0, 0.5, 1.5, and 2.5 hours

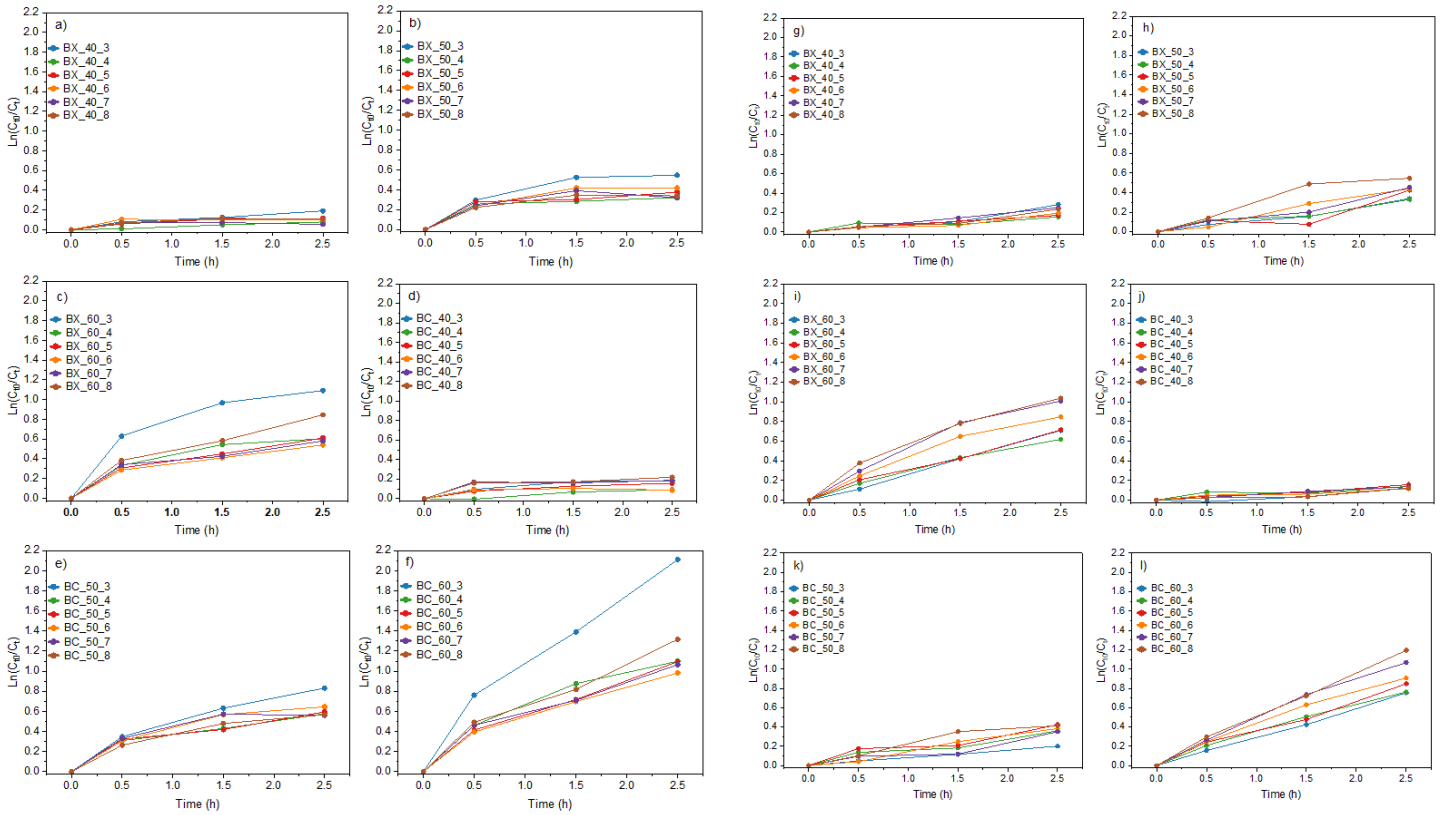

Figure 6

Degradation kinetics for BX, BC from pitaya (a-f), and for beetroot (g-l) at temperatures of 40, 50, and 60 °C and pH profiles from 3 to 8.

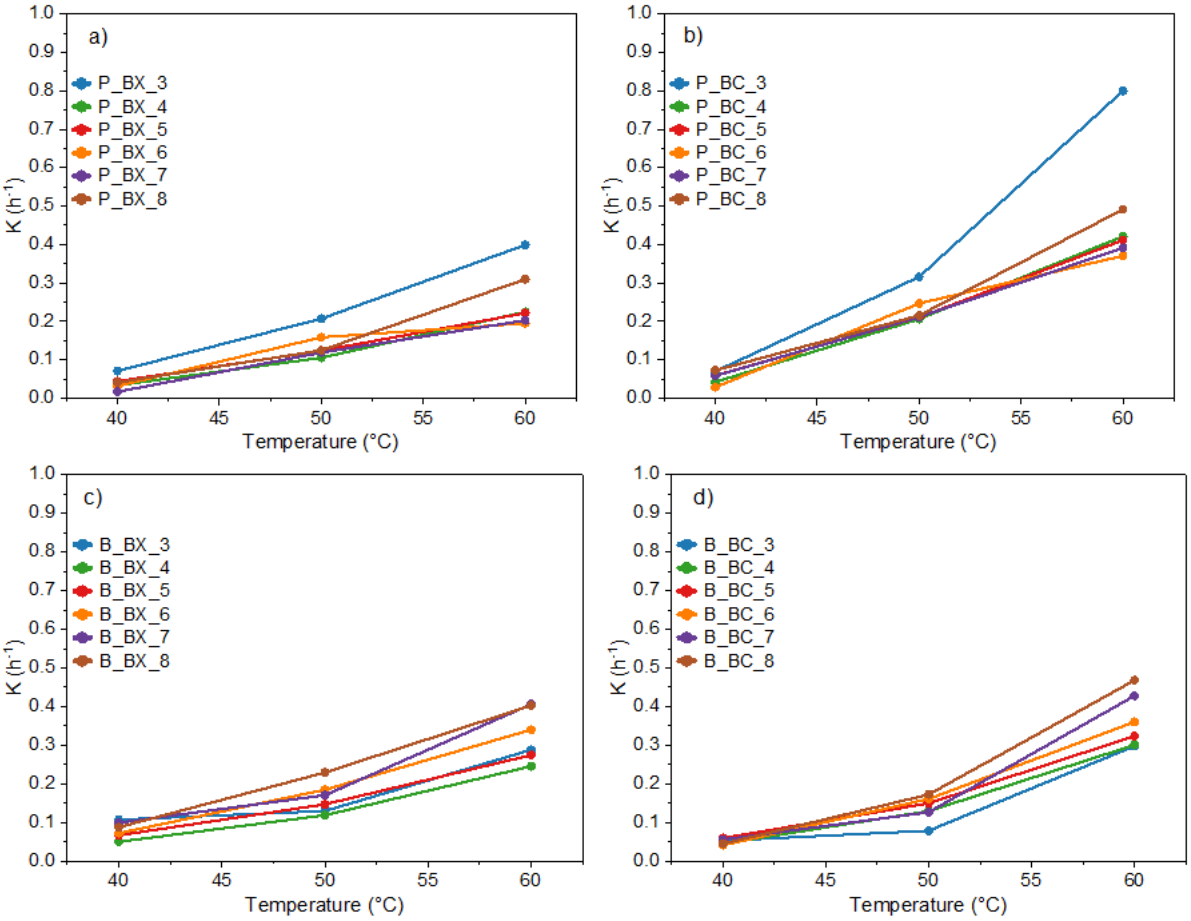

Figure 7

Degradation rates for BX (a) and BC (b) from pitaya and degradation rates for BX (c) and BC (d) from beetroot for pH profiles 3 to 8.