Evaluation of different methods for betanin quantification in pitaya (Stenocereus spp.)

KEYWORDS: Betanin, pitaya, Stenocereus, quantification, HPLC.

Abstract Spectrophotometry UV-Vis, with calibration curve (SWCC), High-performance liquid chromatography (HPLC), and Spectrophotometry with an extinction coefficient (SWEC) were used for betanin quantification in pulp and peel of pitaya (Stenocereus spp). The concentrations of pulp betanin measured by the three analytical methods did not differ significantly, as assessed by the Scheffe method (P > 0.05). The mean of pulp betanin was 2.092 mg/g dry sample. With respect to the peel samples, there was a significant difference: a lower betanin concentration was obtained by HPLC. Since the SWCC method required a standard solution, and that HPLC was time-consuming, the SWEC method represented the best choice for betanin quantification in pulp in order to reduce cost, time, and wastes.

INTRODUCTION
In the food industry, the use of synthetic dyes (especially the red and yellow ones) has been restricted due to their potential risks for human health (1). Consequently, there has been a growing interest in the development of natural additives. Besides, consumers have been lately inclined to prefer natural products because of their eco-friendliness, apparent lack of toxicity, and nutraceutical properties (2, 3). Some natural colours, as betalains, are antioxidants; these help reduce cardiovascular diseases, cancer, and disorders associated to ageing (4, 5). Betalains have been used as food colourants in gelatines, desserts, confectioneries, dry mixes, poultry, dairy, and meat products (6). Betalains are divided into two groups: betacyanins, that provide red hues and betaxanthins that provide yellow colours. Betanin is the dye that contributes from 75 to 95% to red colour in betacyanins. In order to obtain the desired hue in the product, the amount of betain to be added, calculated as betanin, does not exceed 150 ppm (7).

Betalains are water-soluble polyphenolic pigments, found in plants related to the order Caryophyllales, which includes members as carnations, leaf succulents, but most of all species that can tolerate dry environments as Cactaceae. In cacti, betalains provide the colours in flowers and fruits (8). Recently, the study of these pigments and their properties in cactus fruits, such as Opuntia (9, 10, 11) and Hylocereus (12, 13, 14), has been increasing. The Pitaya de Mayo (Stenocereus griseus) has not been studied as much, despite the fact that its peel, pulp and juice have very attractive colours that go from white, pinkish, yellow to red and even purplish, hues related to the presence of the two main types of betalains: the red violet betacyanins and the yellow betaxanthines (4). However, it is important to take into account that these pigments are susceptible to temperature, oxygen, UV light, pH, and different cofactors during processing or storage (13). The method to quantify this component should be therefore easy, quick and with a minimum sample preparation. Traditionally, spectrophotometric methods are used to quantify betacyanins and betaxanthins in food products. The former absorbs light at 540nm and the latter at 480nm (15, 16-22). Nilsson (1970) established a spectrophotometric method for fresh red beets (23). Nevertheless, Schwartz (24) reported that this method was made for small amounts of interfering substances, and that it was not either intended to measure pigment content in samples that had been subjected to heat treatments (24, 25). Schwartz proposed a time-consuming isolation of crystalline reference substances for quantification purposes.

High-performance liquid chromatography (HPLC) is a separation method widely used due to its high sensitivity, and many other advantages. The spectrophotometry UV-vis with calibration curve (SWCC) is another common technique for betain quantification. Both HPLC and SWCC, by using a calibration curve, can be helpful to determine the potential of a product from which the pigment could be extracted in order to use it as a food colourant. However, HPLC technique is expensive and can
be highly contaminant because of the solvents used as the mobile phase. A third method for betalain quantification is the spectrophotometry with the molar extinction coefficient (SWEC). In this method the concentration is obtained solving the expression of Beer’s law for concentration, dividing the measured absorbance of betalain solution by the molar extinction coefficient, a calibration curve is not required for the SWEC method, there is no need to get a chemical standard because Beer’s Law states that molar absorptivity is constant (and the absorbance is proportional to concentration) for a given substance dissolved in a given solute and measured at a given wavelength (26). Besides, it is faster to run tests with SWEC than with SWCC or HPLC. Nevertheless, the molar coefficient is an intrinsic property of the species; it has to be tested for each chemical compound. The objective of this work was to quantify betalain in both peel and pulp of pitaya fruit, using three different analytical methods (SWCC, SWEC, and HPLC) and determine which one was a more viable option.

**MATERIALS AND METHODS**

**Chemicals**
Acetic acid > 99.9% was purchased from Sigma Aldrich, USA. Acetonitrile 99.9% was obtained from TEDIA high purity solvents, USA. Betanin (red beet extract diluted with dextrin) standard was procured from ALDRICH chemistry, USA. However the concentration was not reported by the manufacturer. The betanin concentration was determined by spectrophotometry with the molar extinction coefficient (23, 2). Water was provided by a Milli-Q water purification system (Millipore, Germany).

**Pitaya preparation**
Pitaya (Stenocereus spp) was collected from a field of Ahuatlán, which belongs to the state of Puebla, México, on April 28, 2015. The pitayas were processed at the Fruit and Vegetable Processing laboratory, in the Biotechnology Centre of FEMSA at the Campus Monterrey at ITESM, on April 29, 2015. The fruits were washed and disinfected, and then the prickles were manually removed. Peel and pulp were separated and the former was processed in an extractor (Turmix, TU05, USA) in order to remove the seeds. Peel, pulp and seeds were frozen and stored at -18°C for further analyses. The pulp and peel were defrosted at 4°C one day before the analysis, moisture was determined by gravimetric method (27). Since light has been documented as a deteriorative factor for betalains (18), all extraction procedures were carried out in darkness.

**Pigment extraction from pitaya pulp**
Three betanin quantification methods were evaluated: Spectrophotometry UV-vis with calibration curve (SWCC), High-performance liquid chromatography (HPLC), and Spectrophotometry with the molar extinction coefficient (SWEC). Fifteen replicates of pigment extraction per method were prepared. The pulp was defrosted. For each sample, 5g were weighed and placed in 40mL vials; 10mL of acidified water (1% acetic acid/deionized water v/v) were added; and then the vials were agitated for 15 min. The resultant mix was filtered through a paper filter No. 4 (150mm, Whatman, China) to a 50mL volumetric flask. The filter was washed with 30mL of acidified water. Then, the volumetric flask was filled to the mark with acidified water. Finally, the samples were diluted by a factor of 2 for HPLC analysis.

**Pigment extraction from pitaya peel**
In order to optimize the betanin extraction from the peel of pitaya, a leaching system was conducted. Fifteen samples were extracted using acidified water (1% v/v acetic acid) as the solvent. The pitaya’s peel was cut into squares of approximately 1cm². The peel was weighed (20g) and brewed in 60mL of solvent. The infusion was filtered through cheesecloth and then 25mL of acidified water were used to rinse the peel through the cap. The resultant liquid was collected and centrifuged at 4°C and 10,000 g for 20 min. The supernatant was then placed in 100mL volumetric flasks, and acidified water was used to complete the volume.

**pH**
pH was determined with Thermo Fisher Scientific, Orion 3 star, U.S.A.

**Calibration curves**
A solution stock was prepared transferring 0.2g of the Betanin standard to a 10mL volumetric flask and making up to volume with acidified water [1% acetic acid/water v/v]. The concentration of betacyanin was calculated by the extinction coefficient for betanin (ε=60,000) the absorption maxima at 538, the equation used was [mg/L] = ([A*DF*MW*1000]/(ε*L)), where A is the absorption, DF is the dilution factor, MW and ε are the molecular weight and extinction coefficient and L the pathlength of the 1-cm cuvette, (25, 28). And it was 31.9mg/L. From the stock solution, 6 aliquots were taken to obtain six different concentrations: 1.595, 3.190, 5.852, 6.380, 7.975, and 12.760 mg/L.

**Estimation of betamins**
**SWCC**
The concentration of betacyanin pigment in extracts from pulp and peel of pitaya, was determined using a calibration curve (25, 28). The spectrophotometer used was HACH DR 500, Germany and Beckman Coulter DU 800, USA for spectrum. The absorbance of the samples was
measured at 540nm. The calibration curve was constructed by measuring the absorbance rate of betanin in six solutions. The linear equation derived from the calibration curve was used to determine the concentration of the pigment extraction from pulp and peel of pitaya.

**SWEC**

Only the pigment extractions from pulp and peel of pitaya were analysed in spectrophotometer (for quantifying HACH DR 500, Germany). The absorbance of the samples was measured at 540nm (2, 23). The concentrations were obtained solving the expression of Beer’s law for concentration, dividing the measured absorbances of betalain solutions by the molar extinction coefficient (60,000). The spectrophotometric method used was (25, 28).

**HPLC**

In HPLC technique the pigment extractions from pulp and peel of pitaya, as well as the standards for the calibration curve were analysed by HPLC/UV-Vis (Agilent Technologies, 1200 Series, Germany) at the same wavelength. A column Eclipse XDBC18, 5um, 150mm × 4.6mm (Agilent Technologies, Germany) was used. The method of Vergara (22) was adjusted to a mobile phase: 85% v/v A (water-acetic acid 1%), and 15% B (acetonitrile-acetic acid 1% v/v). The flow rate was 1 mL/min. The column temperature was 25°C, and the run time was 10 min.

**Experimental design and statistical analyses**

The experiment comprised two fixed factors: quantification method, with three levels (SWCC, HPLC, and SWEC), and betanin source, with two levels (peel and pulp of pitaya). A total of 90 samples were analysed (15 replicates per treatment).

The data analysis was carried out by SAS (SAS 9.3, SAS Institute Inc., Cary, NC, USA).

The results of betanin concentration were not normally distributed, according to the Kolmogorov-Smirnov test. In order to normalize it, a power transformation (λ = 0.25) was performed. The results were then subjected to an ANOVA, and differences of Least Square Means were obtained by the Scheffe method (α = 0.05), which estimates all possible contrasts among the factor level means, and not just the pairwise differences considered by the Tukey-Kramer method.

**RESULTS AND DISCUSSION**

**Betanin in pulp**

As is shown on Table 1, the concentration obtained of betanin by the SWEC analytical method, in the pitaya pulp was 2.048± 0.089mg/g dry sample, García-Cruz (16) determined that red fruits of Stenocereus pruinosus had a concentration of 2.860 ± 0.038mg/g of betacyanins in dry pulp. It has been documented (15) that the red pulp of Stenocereus griesius had a betacyanin concentration of 1.996 ± 0.024mg/g dry sample.

In this work, only betanin concentration was determined. The similarities observed between the results obtained in this study and those of betacyanins reported in literature could be attributed to the fact that betanin is the major red pigment among the group of betacyanins; it represents from 75 to 95% of the red colour (29). It has been reported (16, 11) that the pulp of beetroot (Beta vulgaris) and cactacceae fruit, such as the varieties of Rojo San Martín and Rojo Cenizo of Opuntia ficus indica, have about twice or even three times the amount of betacyanins as the ones of pulp of Stenocereus spp.

Despite the fact that there were slight differences between the mean values of betanin concentration in pitaya pulp obtained with the three different quantification techniques, they were not statistically significant (P > 0.05) (Table 1). Therefore, the choice of the best method for betanin quantification in pulp could be based on the analytical requirements. For instance, HPLC is not very convenient for industrial applications because the equipment is expensive and requires highly specialized training for its use. Besides, the solvents utilized are costly and contribute significantly to the environmental pollution. The SWCC technique is easy to implement, but it requires preparation of chemical standards and the evaluation of a calibration curve. On the other hand, the SWEC method could be the most suitable in terms of time, sample preparation and use of resources for measuring betanin in pitaya pulp.

In spite of SWEC, this method has already been used in other references (2, 15, 23, 28) but no statistical evaluation was found to support this procedure replacing the HPLC technique.

**Betanin in peel**

In pitaya peel, the SWCC, SWEC, and HPLC methods measured, respectively, betanin concentrations of 0.116 ± 0.033, 0.118 ± 0.036, and 0.056 ± 0.011mg/g dry sample. As seen on Table 1, the results obtained from the SWCC and SWEC techniques were not significantly different from each other, but the betanin concentration measured with HPLC was significantly lower (P < 0.05). The significant difference of HPLC was probably due to the fact that this method measured a specific compound at the specified wavelength. The other two methods might have measured all the similar compounds in a wavelength (total betacyanins). Furthermore, betanin concentration was higher in the pulp than in the peel, compared to other betacyanins.

The varieties Rojo San Martín and Rojo Cenizo of prickly pear (Opuntia ficus-indica) are reported to have betacyanin concentrations of 0.241 and 1.346mg/g dry sample of peel, respectively (11). Betanin concentration in prickly pear is 4-24 fold times higher than in pitaya peel estimated by the HPLC method in this work.

On the other hand, the pigment extraction from the peel showed an orange hue. The pigments that could have given such colouration to the pitaya peel are betaxanthins. García-Cruz (16) determined that the red pulp of Stenocereus pruinosus had a betaxanthin concentration of 1.996 ± 0.024mg/g dry sample.

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3.21 ± 0.56μg/g dry sample. However, betaxanthins have never been determined in the peel of pitaya. Another important thing to consider was that probably in the peel, the betaxanthins concentration was going to be larger than the betacyanin concentration, exactly like what happened in the red pulp that García-Cruz et al. (15, 16) reported. Therefore, the standard (betanin solutions), and the pulp and peel extractions were subjected to a wavelength scan (300-600 nm) in the spectrophotometer (Figure 1).

As seen on Figure 1, the chemical standard (betanin) had a signal only at 540nm, whereas both pulp and peel showed a remarkable signal at 480nm, and a slight signal at 540nm. It is known that betaxanthins absorb light at 480nm (22). So, based on this qualitative test of wavelength scan, it can be inferred that despite the attractive red colouration, the presence of betaxanthins (responsible for the orange colour) might be significant in peel of the Stenocereus spp.

Standard solution, and pulp and peel extractions were analysed in HPLC at 540nm. Figure 2 shows the chromatograms obtained. For the peel extraction, there were signals at different retention times, each representing a different type of betacyanin. They all had about the same height. This could have been the reason why the betacyanin concentration measured with HPLC was

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lower than SWCC and SWEC in peel extraction samples. Therefore, the spectrophotometric techniques might have overestimated the betanin concentration in the pitaya peel, similarly to what Schwarts [30] reported with respect to beetroot.

CONCLUSIONS
Three different methods for betanin quantification were tested in peel and pulp of pitaya (Stenocereus spp.). Spectrophotometry with a molar extinction coefficient (SWEC) was reported for this purpose for the first time in this work. The mean of betanin concentration found in pitaya pulp was 2.092 mg/g dry sample, which is within the range previously reported by other authors. The betanin concentration in the peel was 0.117 mg/g dry sample, as measured by the SWCC and SWEC methods, and 0.056 mg/g by HPLC. The SWEC method represented the best choice for betanin quantification in pulp in order to reduce cost, time, and wastes. Since betaxanthins might be important components in the colour of the pitaya peel, a recommendation for future works would be to measure those compounds with a quantitative method. For that purpose, an extract or standard of betaxanthin is required.

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REFERENCES AND NOTES
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