Characterization and Quantification of Dragon Fruit (*Hylocereus polyrhizus*) Betacyanin Pigments Extracted by Two Procedures

Nassim Naderi¹, Hasanah M. Ghazali², Anis Shobirin Meor Hussin¹, Mehrnoush Amid¹ and Mohd Yazid Abd. Manap*¹

¹Food Technology Department,  
²Food Science Department,  
Faculty of Food Science and Technology  
Universiti Putra Malaysia,  
43400 UPM Serdang, Selangor, Malaysia  
*E-mail: myazid@putra.upm.edu.my

ABSTRACT

A method for the extraction of betacyanins pigments of dragon fruit (*Hylocereus polyrhizus*) grown in Malaysia was studied. A processing scheme consisting of solvent system selection (ethanolic and aqueous ethanolic) was proposed to study the effect of water in enhancing betacyanin recovery from the pulp of *H. polyrhizus* fruit. Betacyanins, in concentrated extracts from the dragon fruit (*H. polyrhizus*), were identified as betanin, phylloactin, hylocerenin and their respective C-15 isoforms using High-performance liquid chromatographic (HPLC) analysis. Structural alteration was monitored by using selected solvent systems. As for the relative peak area ratios, some betacyanins showed a higher stability than others. Betanin, one of the main betacyanin in selected Malaysian *H. polyrhizus* cultivars, displayed the most stable structure. Comparing the peak area ratios of individual betacyanins, it was noticed that ethanolic assay might induce co-occurring of the C-15 isoforms.

Keywords: Dragon fruit, *Hylocereus polyrhizus*, Betacyanins, Betanin, Phylloactin, Hylocerenin

INTRODUCTION

Exempt (natural) colours are recognized as organic products which have gained a growing interest from health-conscious consumers and researchers (Griffiths, 2005). Food and Drug Administration (FDA) assigned “exempt colour additives” label to specify that colourings are free from the certification process (Meggos, 1995). These colorants are obtained from natural sources by solvent extractions (Sajilata & Singhal, 2006). Despite the numerous number of anthocyanin containing food colorant extracts, there is only one single betacyanin source, i.e. red beet (*Beta vulgaris L. ssp. vulgaris*), which has been approved in the market (Castellar et al., 2003). Nevertheless, a high molar absorbency index of betacyanins and their potential as colorant are equivalent for synthetic colorants (Strack et al., 2003). As betacyanins and anthocyanins are chemically related, the methods of anthocyanin extractions can therefore be applied for betacyanin (Sajilata & Singhal, 2006). Compared to polar anthocyanins, betacyanins are more hydrophilic. In more specific, they can dissolve in three common polar solvents (namely, water, methanol and ethanol) and their mixtures; to certain extent, water and organic acids are miscible (Schoefs, 2004). Thus, finding a good separation system is rather challenging. Based on their chemical structure, Betacyanins belong to alkaloids.
These pigments are also water-soluble and localized in vacuole as bis-anions (Stintzing & Carle, 2004). They are more soluble in water than in non-polar solvents and this characteristic helps extraction and separation processes (Strack et al., 2003). Methanol has generally been used to extract betacyanins (Moreno et al., 2007). Since methanol has toxic characteristics, food scientists prefer other extraction systems (Xavier et al., 2008). In this study, a processing scheme of solvent system selection (ethanolic and aqueous ethanolic) was proposed to study the effect of water in enhancing betacyanin recovery from the pulp of *Hylocereus polyrhizus* fruit in the final concentrated colour extract.

**MATERIALS AND METHODS**

**Plant Material and Chemicals**

The fruit of two-year-old plants of the dragon fruit (*Hylocereus polyrhizus*), grown on trellis system in a modern agriculture farm in Kluang, Johor, Malaysia, were used in this investigation. The clones were originally introduced as cuttings from Vietnam several years ago and the plantation has been done through a modern technology directed by the Ministry of Agriculture and Agro-Based Industry (Kluang, Johor, Malaysia). The fruit were harvested for analysis when reaching the full ripening stage, i.e. 30-35 days after pollination. The skin was separated from the pulp using a stainless steel knife. Vacuum packaging was used for a complete removal of oxygen. Subsequently, the fruit were stored at -38°C for further analysis.

**Solvents and Reference Substances**

Trifluoroacetic Acid 98% (TFA), Acetonitril and MeOH were purchased from Sigma-Aldrich (Selangor D.E., Malaysia) and they were of analytical or HPLC grade. Food grade EtOH (98%) was retrieved from Merck (Germany). For reference, authentic standard containing betanin and isobetanin was donated by Scott M. Engel (D.D. Williamson Co., WA, USA).

**Preparation of the Concentrated Betacyanin Extract**

Two extraction methods were applied to monitor the concentration of betacyanin and pigment retention, as well as their composition in concentrated colorants. This was followed by a direct extraction of the pigments by homogenization using a 1/1 (w/v) ratio of fruit/solvent. Typically, 100g of the peeled fruit (pH =4.5; TSS=10%) of watery consistency was shaken and macerated with 100 mL solvents (EtOH, aqueous ethanol 50:50) for 15 minutes under ice cooling condition. The aqueous mixture was centrifuged at 18000 rpm and 4°C for 20 min, followed by a fast filtration on nylon mesh. The extract obtained was concentrated in a vacuum at 35°C, using a rotary evaporator, to 3-4 mL. The ethanol was completely removed after the concentration process and the samples were then kept in a dark vessel. Once again, 100 gm of the peeled fruit was pressed and filtered to obtain purified juice which was immediately stored at 4°C in a dark vessel as a control. Each sample was analyzed for °Brix (which is a percentage by weight of sugar in a solution at room temperature), pH, titrable Acidity and betacyanins.

**Photometric Quantification of the Total Betacyanins**

The betacyanin content was measured in triplicate in deionised water. The extracted samples were diluted by 100-fold with deionised water to obtain the absorption values. After 20 min of equilibration, the quantification of betacyanins was carried out by applying the following equation (Cai & Corke, 1999):

\[
\text{BC (Betacyanin Concentration)} = \frac{A \times DF \times MW \times 1000}{\varepsilon \times L} 
\]

(1)

Where BC is the betacyanin concentration in milligrams per litre, A is the absorption value at the absorption maximum (\(\lambda_{\text{max}} = 540\) nm), F is the dilution factor, MW is the molecular weight of betanin (550 g/mol), \(\varepsilon\) is the molar extinction
coefficient of betanin ($\varepsilon = 60,000 \text{ L \ mol}^{-1} \text{ cm}^{-1}$ in H2O) and L is the pathlength of the cuvette.

**High-Performance Liquid Chromatography (HPLC)-UV Visible Detector Analysis**

A modified method from Wybraniec and Mizrahi (2002) and Esquivel et al. (2007) was utilized to determine the pigment patterns of betacyanins in the concentrated extracts. The HPLC analysis for identification of betacyanins was carried out with a liquid chromatographic apparatus (Waters, Ca, USA), equipped with a pump Waters 600 Controller, and a UV-Vis detector (Waters™ 486 Tunable Absorbance Detector). An analytical Lichrocart® 250×4.6 mm i.d. Purospher ® Star RP18-column, with a particle size of 5 μm (Merck, Darmstadt, Germany) was used for the pigment analyses. The separation was performed isocratically using a mixture of 90% solvent A (0.5% aqueous TFA) with 10% solvent B (Acetonitril) for 40 min at a flow rate of 1 mL/min (injection volume: 10µL); detection was carried out at a wavelength of 540 nm, while the betanin and isobetanin were identified by comparing their retention times with those of the standards. The concentrations of betacyanins were calculated from the standard curves of betanin and isobetanin, at four concentrations (namely, 0.02, 0.04, 0.06, 0.08 mg/L) using a linear regression analysis ($r > 0.99$). Later, individual betacyanin composition (%) was measured by analytical HPLC and was expressed as the percentage of the peak area. All determinations were performed in duplicate.

**Other Analytical Measurements**

Titrable acidity was calculated as the percentage of citric acid by titrating 10mL of the concentrated betacyanin extract with a solution of NaOH (0.1N) to get constant pH 8.2. The pH was measured using a pH meter. The soluble solid content was measured as °Brix using a manual refractometer (ATAGO, Japan). All the analytical measurements were repeated three times.

**Statistical Analysis**

Statistical analyses were carried out utilizing ANOVA by using Minitab (Minitab 13.1 Inc., USA). Significant differences between the values are at $P<0.05$ levels using Tukey’s test.

**RESULTS AND DISCUSSIONS**

**Isolation of the Concentrated Betacyanin Extracts by Solvent System Selection**

The extraction method for the production of concentrated betacyanin consists of three general steps, namely, extraction, centrifugation and concentration. The fruit of *Hylocereus polyrhizus* were harvested and transported to the laboratory. These fruit were subsequently peeled and placed in vacuumed plastic bags exempt of air prior to freeze (-38 °C) storage. As the pulp of fruit consists of thousands small soft seeds that were distributed homogeneously throughout the flesh, defrosted fruit flesh was pre-treated. Freeze-thawing of the fruit pulps in an air tight condition was found to damage tissues and consequently cause the pigments to leak out. Thus, the freeze-thawing method was chosen for tissue disruption instead of the mechanical method. This method was applied to prevent disruption to the seeds during the procedure. Meanwhile, it is important to note that broken seeds may contain components that degrade betacyanins or increase viscosity.

Defrosted fruit pulp was macerated with selected solvents (ethanol and ethanol:water) in an ice cooling condition. This solvent system assay was selected to reduce the concentration of mucilages that was present in *H. polyrhizus* which could increase the viscosity of the extracts, and this characteristic is certainly not desirable in concentrated extract. Although pectin could be extracted in water and afterward precipitated with organic solvents but, with such a procedure, pigments could also be precipitated, along with pectin (Castellar et al., 2006). Thus, a system consisting of a mixture of ethanol and water was selected to extract pigments from the fruit. The next step was centrifugation to discard the residue and the concentration of extracted...
betacyanin by evaporation under vacuum at low temperature (35°C). Each extract obtained from the assays was concentrated by 3-4 folds and analyzed. Table 1 shows the analysis of the concentrated betacyanin extracts obtained from each assay.

The pH value was rather low and similar for all the concentrated extracts. This pH value was slightly higher from 4.6 to 4.67 for the sample that was obtained from the ethanolic assay. This pH value is considered as favourable because it has also been reported to be the optimum pH of betacyanins in several studies (Prudencio et al., 2007; Stintzing et al., 2006; Stintzing et al., 2003; Cai & Corke, 1999). The soluble solid (°Brix) also was not different in the concentrated extract from each assay. °Brix was increased by applying both assays, and therefore, sugar was extracted using both the solvent system assays. The significant difference was observed in the concentration of betacyanin and titrable acidity. Meanwhile, the concentration of betacyanin (811 mgL⁻¹) in H. polyrhizus fruit was found to be higher than those shown for the red beet (Stintzing et al., 2006) and Opuntia stricta (Moreno et al., 2008) fruit. The highest pigment concentration (655.5 mg/L) was obtained for the aqueous ethanolic assay. The acidity expressed as citric acid (0.004%) was higher in the ethanolic assay than that obtained for the aqueous ethanolic.

**Pigment Characterization and Betacyanin Retention in Concentrated Extracts**

The analysis of betacyanins can be deduced from their chromatographic behaviour, and corroborative data may be retrieved from the analysis of their absorption spectra. Fig. 1 shows the chromatographic pattern of betacyanins of the pulp of H. polyrhizus fruit. Peaks 1 and 1' were readily identified by UV-vis chromatography with authentic standards (Fig. 1-D). Based on the HPLC elusion order (Esquivel et al., 2007; Wybraniec et al., 2002), compounds 2/2 and 3/3 were inferred as phyllocactin/isophyllocactin and hylocerenin/isohylocerenin. Phyllocactin was considered as a major betacyanin in the pigment pattern of H. polyrhizus extracts, followed by betanin. To get an insight into the impacts of selected solvent systems on structural alteration, the development of betanin and phyllocactin was monitored. To this extent, betanin isomerisation (i.e. isomerisation index and phyllocactin deacylation) was evaluated by comparing their respective peak area and peak area ratios. In the purified juice (control), five betacyanin structures were found to exist in the equilibrium, namely betanin, isobetanin, phyllocactin, isophyllocactin and hylocerenin. As a rule, it was confirmed that betacyanins were accompanied by their respective isobetacyanins (Herbach et al., 2006a).

<table>
<thead>
<tr>
<th>Extraction solvent</th>
<th>pH</th>
<th>Titratable acids°</th>
<th>Soluble solid Contents (°Brix)</th>
<th>Betacyanin concentration mgL⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtOH</td>
<td>4.67±0.01a</td>
<td>0.004 ± 0.0a</td>
<td>23.2 ± 0.00a</td>
<td>449 ± 13.58a</td>
</tr>
<tr>
<td>EtOH:Water (50:50, v/v)</td>
<td>4.6±0.05a</td>
<td>0.002 ± 0.0b</td>
<td>23 ± 0.00a</td>
<td>655.5 ± 4.38b</td>
</tr>
<tr>
<td>Control b</td>
<td>4.2± 0.02b</td>
<td>0.01 ± 0.0c</td>
<td>9.5 ± 0.7b</td>
<td>811 ± 6.48c</td>
</tr>
</tbody>
</table>

°% of citric acid

b purified juice

Means in the same column are significantly different at p<0.05, using Tukey’s HSD test.
Characterization and Quantification of Dragon Fruit (*Hylocereus polyrhizus*) Betacyanin Pigments

Fig. 1: HPLC pattern of extracted betacyanins from fruit-pulp of *H. Polyrhizus*.

(A) Purified juice (B) aqueous ethanolic assay (C) ethanolic assay (D) Standard: (1) betanin, (1') isobetanin, (1) Betanin, (1') Isobetanin, (2) Phyllocactin, (2') Isophyllocactin, (3) Hylocerenin, (3') Isohylocerenin
Meanwhile, the relative amounts of these structures at equilibrium varied with the application of two solvent system assays. As for the relative peak area ratios, some betacyanins have showed higher stability than others. Betanin, one of the main betacyanins in selected Malaysian *H. polyrhizus* cultivars, displayed the most stable structure. As shown in Table 2, selected solvent system assays in the pigment extraction did not result in betanin isomerisation but rather in the betanin/isobetanin peak area ratio. The highest peak area ratio of betanin/isobetanin was observed in the concentrated colorant which had been extracted by ethanol, whereas, phyllocactin peak area ratio was found to have declined. In all the samples, however, phyllocactin represented the predominant betacyanin. The peak area in the samples obtained from the aqueous ethanolic assay was almost twice than that of the betanin, as reflected by the betanin/phyllocactin peak area ratio presented in Table 2. As reported previously, phyllocactin is more prone to deacylation, but this reaction was only tentatively observed in the thermally treated samples (Herbach *et al.*, 2006b; 2005). The findings of the present study revealed that the ethanolic system might also provoke this reaction, revealing that phyllocactin is less stable than betanin. Isomerization was observed in hylocerenin, where isohylocerenin ratios were found to have increased in the ethanolic assay. Nevertheless, this compound was not detectable in the samples from the aqueous ethanolic assay as well as the purified juice. On the basis of the previous studies on purified betacyanins, as well as considering the retention time and elution order, the presence of isobetacyanins could not be degradation products of their parent betacyanins (2, 3) (Wybraniec & Mizrahi, 2005). Conclusion could be drawn by the observation of a more acidic condition resulted from the ethanolic assay that might induce the co-occurring of C-15 isoforms. Further analysis was carried out by calculating the content of betanin and isobetanin using calibration curves. Interestingly, the highest concentration of betanin was obtained in the ethanolic extraction method, whereby it comprised 50% of the total betacyanins in the concentrated pigments yielded.

### Table 2

Pigment characteristics of the concentrated extract using organic and aqueous organic extraction assay and Betanin/isobetanin, betanin/phyllocactin peak area ratios at 540 nm in the preparation of *H. polyrhizus* pigment

<table>
<thead>
<tr>
<th>Extraction solvent</th>
<th>BC (%) a</th>
<th>Relative concentration (%) for peaks c</th>
<th>Betanin/ Isobetanin</th>
<th>Betanin/ Phyllocactin</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtOH</td>
<td>50.39 (+9.39)</td>
<td>33.9(±5.1) 47.8(±6.1) 11.2(±0.1)</td>
<td>6.7</td>
<td>0.71</td>
</tr>
<tr>
<td>EtOH:Water (50:50,v/v)</td>
<td>24.3 (+3.16)</td>
<td>29.7(±1.5) 52.3(±0.1) 11.1(±0.01)</td>
<td>6.19</td>
<td>0.57</td>
</tr>
<tr>
<td>Control b</td>
<td>19.28 (+1.0)</td>
<td>32.3(±0.9) 51.0(±0.5) 10.6(±1.0)</td>
<td>6.0</td>
<td>0.6</td>
</tr>
</tbody>
</table>

*Betacyanin content expressed as betanin, isobetanin*  
*purified juice*  
1:Betanin, 1’:Isobetanin, 2: phyllocactin, 2’: Isophyllocactin, 3:Hylocerenin, 3’:Isohylocerenin  
*not detected*  
Mean values of duplicate measurements; (±SD)=standard deviation

CONCLUSION
As commercial production of betacyanins involves solvent extraction and that these pigments are water soluble, therefore in this work, a system based on the admixture of water with organic solvent (ethanol) was chosen for pigment extraction. Through HPLC analysis, betacyanins in the concentrated extracts from H. polyrhizus were identified as the known betanin, phylloquinone, hylocerenin and their respective C-15 isoforms. Meanwhile, an addition of water to the organic solvent (ethanol), which were partly miscible together, resulted in a better solubility of betacyanins in the organic phase. Although water extraction is simple, a highly efficient and low cost method for crude betacyanin extraction led to a difficult separation of betacyanin and water-soluble protein components (Castellar et al., 2006). The quantification of betacyanin has shown that the extraction of the pigments with aqueous ethanolic assay resulted in a higher yield. In addition, a closer inspection for the impact of selected solvent systems on structural alteration was carried out by comparing individual peak area ratio using the HPLC analysis. Since the absence of isohylocerenin was the characteristic of betacyanins in the purified juice and the aqueous ethanolic extract resulted in a higher yield. In addition, a closer inspection for the impact of selected solvent systems on structural alteration was carried out by comparing individual peak area ratio using the HPLC analysis. Since the absence of isohylocerenin was the characteristic of betacyanins in the purified juice and the aqueous ethanolic extract resulted in a higher yield. In addition, a closer inspection for the impact of selected solvent systems on structural alteration was carried out by comparing individual peak area ratio using the HPLC analysis. Since the absence of isohylocerenin was the characteristic of betacyanins in the purified juice and the aqueous ethanolic extract resulted in a higher yield.

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