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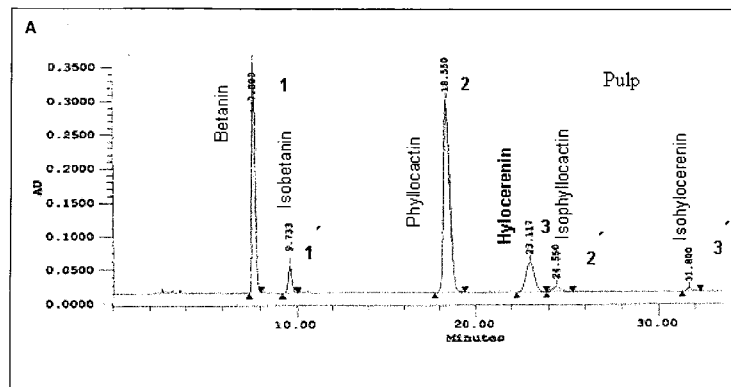
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(54) Title: STABILISED COLORANT COMPOSITION

Figure 1a



(57) Abstract: The present invention discloses a stable colorant composition of a plant extract, which has a high staining power in the purple-red range. Accordingly, the present invention provides a stable colorant composition of a plant extract that has a pigment concentration of more than 811 mg per 1000 g of fresh weight of pitaya fruit which makes the preparation of food colorant from the fruit easier and less costly. The stable betacyanin concentrate can be suspended in a food-compatible liquid, as a food colorant or food additive.

STABILISED COLORANT COMPOSITION

The present invention relates generally to a naturally derived colorant. More particularly, the present invention relates to a red colorant obtained from purple pitaya fruits as a stabilized colorant composition.

BACKGROUND TO THE INVENTION

Colorants are used to add a distinctive colour to both food and drink products. Using color enhancement in food makes certain types of food more aesthetically appealing and appetizing. Previously, research on colour enhancement primarily focused on the cosmetic value of colorants until organizations such as the FDA started denying the use of certain colorants. A number of synthetic colorants have been banned and the safety of some has been questioned. In the 1970's, the FDA denied the use of certain coal tar dyes for food coloring. In recent years, findings revealed the toxicity and carcinogenic effects of artificial red food colorants.

In order to avoid the bans placed on synthetic and manufactured colorants, the food industry started looking for natural plant pigments as a food colorant source. Some consumers now regard synthetic colorants as less acceptable than colorants derived from foodstuff, such as carrots, tomatoes, sweet potatoes, beets and the like. Thus, there is a need for suitable natural pigments as color additives.

For example, beet pigments (betalains) are a native red colorant that is useful as a food coloring. Betalain pigments include the red-violet betacyanins, and the yellow betaxanthins, which when combined produce a red hue useful in certain foods as a colorant. One source of betalains that is readily-available is the garden beet (*Beta vulgaris*). Thus, garden beets have become a source of betalains for the red pigment.

Betacyanin has been recently recognized as an important natural food coloring agent, because of its high extinction coefficient power in the red region, and because of its safety and potentially beneficial antioxidant effect. In recent years, chemoprevention roll of betacyanin pigments has been documented against lung and skin cancers. It is also recently recognized that natural food colors such as betanin can prevent the cell proliferation of a variety of human tumor cells.

Today, beetroot is the only allowed source of betacyanin approved additives for food applications in the United States (Title 1 of the Code of Federal Regulations, 21 CFR 73, 40) and in the European Union (E-162), and commercially free from batch certification. Unfortunately, earth-like flavor characteristics caused by geosmin and high nitrate concentrations that is related to development of carcinogenic nitrosamines may affect the commercial use. The other problem that needs to be highlighted is the fast browning of betanin from red beet through polyphenoloxidase activities and the reduction of the naturally high nitrate content. When the first is being controlled by heat inactivation and oxygen removal, the latter is reduced by fermentation strategies. Furthermore, beets grow underground and unfortunately, the presence of earth-bound germs is a safety issue. Lastly, red beets are suffered with a slight colour spectrum. Because of its betacyanin spectrum that is restricted mainly to betanin and consequently, colour variability will be poor. In this case, alternative betacyanin pigment sources rather than red beet have been searched for a long time

Since the beet extract contains 80% of the carbohydrates and nitrogenous compounds, and also contains free sugars, which causes fermentation and caramelization, thus removal of these compounds from beet extract is very much necessary. To this point clarification of red beet extract has the disadvantage of quality loss. Moreover, the content of solutes, such as sugars, hinders production of betacyanin in high concentrations.

U.S. Pat. No. 4,027,042 relates to recovering the pigment from beets in concentrated form. The beets are pulped to produce an insoluble phase (the pulp) and a soluble phase containing the beet pigment, protein and carbohydrates. The soluble phase is subjected to fermentation wherein the carbohydrates, nitrates, nitrites and some of the proteins are utilized as the food source. This effects a concentration of the beet pigment without destroying the pigment or causing any undesirable effect on the pigment from the standpoint of physical characteristics and colour.

U.S. Pat. No. 4,339,451 discloses a beet pigment stabilized by co-drying with a polyvalent metal salt. The preferred polyvalent metal salt is CaCl_2 that makes the composition highly hygroscopic. To counteract this hygroscopicity, those of ordinary skill typically add a low DE maltodextrin to the composition. However, due to the presence of maltodextrins, the resulting composition is not readily soluble in cold water.

With time, beets, beet pigment and beet pigment develop undesirable flavors and odors as well as discoloration. This limits the use of beet colour and causes serious problems when the beet colorant or food products containing it must be stored for prolonged periods.

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Very recently, other sources from the members of Cactaceae such as cactus fruits from *Hylocereus polyrhizus* have inspired scientists to study their possibility of use as food colorants due to the nutritional and technological issues. Purple pitaya (*Hylocereus polyrhizus*) was recognized as a promising source of betacyanins.

10 These fruits not only exhibit a higher pigment stability compared to red beet, but are also devoid of pettiness, high nitrate and oxalate levels, and earth-bound microorganisms. Interestingly, betacyanins can cover a broad color palette ranging from red to purple, which accomplished by different extraction condition that affects pigment alterations. Since existence of yellow betaxanthins tend to degrade more
15 easily and furthermore resulting in a shade of brown colour, their absence in pigment pattern of purple pitaya is technologically advantageous.

As compared to betanin from red beet, betacyanins in purple pitaya comprise acylated pigments beside betanin that exhibits improved stability of betacyanins in
20 extract from this fruit. As to date, no concentrated extract of purple pitaya fruits is known, with respect to food, pharmaceutical and cosmetic products.

In view of the foregoing, it would be highly desirable to provide a red-purple colorant of betacyanin obtained from purple pitaya fruits as a stabilized colorant composition
25 which is suitable for use in food products and convertible in cosmetic and pharmaceutical industries.

SUMMARY OF THE INVENTION

30 The present invention broadly provides a stable colorant composition of a plant extract, which has a high staining power in the red-purple range.

More specifically, the invention provides a stable colorant composition having high concentration of betacyanin pigments of at least 811 mg per 1000 g of fresh weight.

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An aspect of the invention provides a stable colorant composition of a plant extract that has a pigment concentration of more than 811 mg per 1000 g of fresh weight of pitaya fruit which makes the preparation of food colorant from the fruit easier and less costly.

5

It is an advantage of the present invention to provide a stable betacyanin concentrate that can be suspended in a food-compatible liquid, as a food colorant or food additive.

10 It is yet another advantage of the present invention to provide a natural red colorant, which does not contain added antioxidants and yet are stable towards oxidation.

BRIEF DESCRIPTION OF THE DRAWINGS

15 Figure 1a is a chromatogram that shows relative concentrations of *Hylocereus polyrhizus* pigments from the pulp;

Figure 1b is a chromatogram that shows relative concentrations of *Hylocereus polyrhizus* pigments from the skin;

20

Figure 2 is a UV/VIS spectrum of extracts made from the *Hylocereus polyrhizus*; and

Figure 3 shows differences in chromaticity of the *Hylocereus polyrhizus* extracted colorants obtained in the different extraction system compared to commercial Red

25 Beet.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a stabilized food colorant composition derived from
30 purple pitaya fruits. This colorant, which is also described herein as a pigment, is provided in a stabilized liquid pitaya extract in a red to purple liquid form.

Betacyanin is believed to be essentially responsible to providing colour in a pitaya colorant. Betacyanin yields red-violet colour.

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In the present invention, pitaya fruit extract having a pigment content of at least 811 mg per 1000 g of fresh weight of the pitaya fruit extract was developed. The pigment content of the present invention may exceed 811 mg per 1000 g of fresh weight of the fruit.

5

The high pigment pitaya extract was developed by reacting the fruit in a solvent extraction system or an enzymatic preparation.

10 In a preferred embodiment of the present invention, pulps of a plant which belongs to *Hylocereus polyrhizus* and which can produce betacyanin pigments are reacted in a solvent extraction system or incubated in an enzymatic preparation.

15 In another preferred embodiment of the present invention, peels of a plant which belongs to *Hylocereus polyrhizus* and which can produce betacyanin pigments are reacted in a solvent extraction system.

The solvent extraction system comprises ethanol or a combination of ethanol and water.

20 The enzymatic preparation contains a pectinase enzyme or a combination of pectinase enzymes that can be selected from the group consisting of dextrozym, fructozym, glucanase, pectinase and pectinex.

25 The high pigment pitaya extract or colorant of the present invention has the characteristic of being water-soluble or water miscible liquid that is advantageous, since it allows a homogeneous distribution of this color in the food to which it is added. Most food products of red colors to which this extracted betacyanin are added, as a colorant is water based, thus this natural colorant disperse uniformly in the water-based product, resulting in a red color.

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The present invention is directed to stable betacyanin formulations suspended in a liquid form to diversify it depending on the use for which the concentrate is intended, may be a food-compatible or a cosmetically-acceptable or a pharmaceutical - acceptable liquid.

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The betacyanin in the extract or colorant mainly consist of acylated (Phyllocactin, Hylocerenin and their following *iso*- forms) and non-acylated betacyanin (Betanin and isobetanin). These pigments are responsible for pitaya colour that was reflected by an almost stable purple tonality.

All the above description and advantages of the invention will be better understood through the following illustrative and non-limitative examples.

Example 1

Extraction of Betacyanins Using Solvent as an Extraction System

The juicy part of fruit or peel of fruit obtained from purple pitaya plant (*Hylocereus polyrhizus*) was macerated in solvent chosen from the food grade [water:EtOH (1:1) or EtOH] with a ratio of 1:1 for 15 minutes. This procedure is followed by fast filtration using sieve with mesh size of 0.1 mm for separation of seeds and pectin-like substance. Further washing of the pectin can be done by using the same solvent. The resultant colored solution was centrifuged at 18,000 rpm for 20 minutes at 2°C to 4°C. After careful decantation, the extract was concentrated in vacuo at 40°C until complete removal of solvent. Purity of the betacyanin pigment was examined by high performance chromatography. Concentrated extract obtained from ethanolic and aqueous ethanolic contained 550 to 600 mg L⁻¹ betacyanin, respectively.

Example 2

Extraction of Betacyanins Using Enzyme

In preliminary tests, various enzyme preparations at different dosages were studied for their applicability to degrade the mucilaginous material of purple pitaya juice. For each sample, 100 gram of homogenized pulp was taken and added in a glass beaker. Enzymatic treatment was carried out by using a range of concentration (0.1 – 2%) of Pectinex Ultra SP-L for 2 hours at 40°C. After incubation period, samples were placed in a boiling water bath for few seconds to inactivate enzyme. Samples were immediately centrifuged for 20 minutes in 4°C at 18000 rpm. The supernatants were collected carefully and stored in dark vessels. Samples immediately were analyzed for sugars, organic acids and betacyanins retention at room temperature.

Enzymatic treatment was selected as a naturally derived assay to obtain a concentrated extract with high pigment concentration and low viscosity. The pulp of *H. polyrhizus* fruits consists of many small seeds which are bounded in flesh tissue. Besides, high concentration of pectin present in fruit can increase the viscosity of final extract. Thereby, enzymatic treatment was applied to make the seed-removing task easier and improve the production yield by hydrolyzing the mucilage. Different concentration of enzyme was chosen to determine the yield of the product. As a result, for all samples under treatment degradation of the pectin-like material, sufficiently reduced viscosity. Visually, color changes were not observed for final concentrated extracts.

Purity of betacyanins was monitored by high performance liquid chromatography. The highest concentration of betacyanins was about 600 mg/L resulted from 0.3% Pectinex to about 550 mg/L resulted from 0.1% Pectinex.

For purity check of the concentrated betacyanins extracts, red color obtained from each approach was applied to a spectrophotometer for a color scan. Visible spectra (380 to 780 nm) of pitaya extracts obtained from each assay displayed one single peak in 540 nm. The maximum absorbance at 540 nm corresponds to the presence of red betacyanins. The same wavelength was used to measure the concentration of betacyanins in each concentrated pitaya extracts.

The spectral properties of the extracts were confirmed by HPLC (High performance chromatography) analyses as follows:

Sample was injected in to a LichroCart RP-18 column (250 x 4.6; 5 μ m), through which a mixture of 90% solvent A (0.5% TFA) with 10% solvent B (Acetonitril) was transmitted at a flow rate of 1 ml/min. The separation was performed isocratically. Betacyanin pigments were monitored at 540 nm. The results are shown in Table 1. Two peaks identified as betanin and isobetanin assigned as betanin and isobetanin by co-chromatography with authentic betanin from red beet. The presence of Phyllocactin and Hylocerenin and their respective 15-isoforms has been identified as compared to previous findings. The quantitative analysis revealed that betanin and phyllocactin were the predominant betacyanins in pitaya. Comparing with red beet, pitaya synthesis both acylated and non-acylated betacyanins was asserted to high pigment stability of pitaya. Further, more concentrated colorant from *H. polyrhizus* is

characterized by the glowing purple-red colour, which is attributed to the presence of phyllocactin and hylocerenin pigments in the fruit.

Example 3

5

Sample preparation and Chemical-physical analysis

Fresh *H. polyrhizus* fruits were used for following analysis. Weight of fruits was measured prior to analysis and reported as 389 ± 464 grams (n=10). All analyses were performed on ten random samples. The edible portion was separated from the skin by stainless steel knife and weight of pulp and peel separately were measured by using a digital balance. Peel and pulp from ten fruits, separately homogenized using blender for physico-chemical analysis. 100 grams of homogenized pulp and skin was taken for pigment extraction. Moisture, total ash, fat and protein content in the sample were determined. Moisture content was measured by drying samples at 105°C in a vacuum oven. Samples from used fruits were placed in a drying oven at 105°C for 72 hours. Once the weight was stabilized, the samples were weighed and ashed in covered crucibles at 600°C for 16 hours. The samples were cooled and weighed to determine the percent ash. The micro-Kjeldahl machine (Tecator Kjeltec System 1002, Sweden) and method was used to determine nitrogen. The percentages of nitrogen were converted to protein by multiplying by 6.25.

The fat content was determined by directly extracting the dried ground pitaya fruit with petroleum ether in an intermittent extraction apparatus of the Soxhlet type. The residue in the extraction flask after solvent removal represents the fat content of the sample. Titratable acidity was measured by titration with 0.1 mol equiv/L NaOH to a pH end point of 8.2, the result being expressed as mg/100 g of citric acid in the sample. Total soluble solids (TSS) were measured with a hand refractometer (Tokyo, Japan) of 0–28° range and expressed as °Brix. The pH of the clarified and concentrated juices was measured through experiments using a digital pH meter calibrated with pH 4 and 7 buffers.

35

Characterization and chemical composition

The chemical quality parameters of skin and pulp of *Hylocereus polyrhizus* are listed in Table 1 below.

5

Table 1 Chemical composition of skin and pulp of *Hylocereus polyrhizus* fruits

	Weight (%)	Bc (mg/L)	pH	Acidity (%) of citric acid)	°Brix	Moisture (%)	Protein (%)	Fat (%)	Ash (%)
Pulp	65.4 - 76	452	4.6	0.2	11	87.5	0.88	0.98	0.6
Skin	26.9 - 35.2	302	5.2	0.1	0.5	8.0	1.8	0.96	1.5

The moisture content formed the main part of tissue, expressed in fresh matter basis (100 gram), weight with the mean value of 87.5% for pulp and 8.0% for the skin. The low moisture content of the peel is an indication of the high solid matter content of the samples. The total ash mean value was 0.6 g in pulp and 1.5% for the skin and it showed that the fruit contained some minerals which could be higher in the skin part of fruit. The mean values for fat were almost the same and protein showed higher value in skin.

Both samples were low-acid products, as indicated by comparatively higher pH value in the skin. Therefore, acidification is required prior to extraction procedure to increase betacyanin stabilization. The skin of *H. polyrhizus* fruits accounts for 27% to 35%, while the pulp is 65% to 76%. The pulp is the edible part of the fruit and is composed of water (87%). The protein was higher, being 1.8% for the peel, 0.88% for the pulp. It appears that the quantity of insoluble protein in the peel is higher than the pulp. The fat (ether extract) for the peel and the pulp was measured at 0.96% and 0.98%, respectively. Another important compositional factor is the presence of pigments, which give particular attractiveness to fruit and products. Pitaya is very particular for the presence of betacyanins, widely can be used as natural colorant in the food industry.

HPLC analysis of pigments

Tentative identification of betacyanins can be deduced from their chromatographic behavior, and corroborative data may be provided by an analysis of their absorption spectra. Figures 1a and 1b show the chromatographic pattern of the ethanolic extract of peel and pulp of reddish purple fruits. At 540 nm, six major peaks can be observed for extract from the pulp when at the same wavelength four peaks monitored with the same retention time for betacyanin extract from the peel.

From the spectral properties (see Figure 1a) provided by the UV detector six different peaks were quantified in the extracts from the pulp. Peak 1 and 1' was assigned as betanin and isobetanin by co-chromatography with authentic betanin from red beet and peak 2/2' and 3/3' on the basis of their chromatographic and spectral characteristics and by comparison with the related literature data, as has been previously reported corresponded to phyllocactin/isophyllocactin and hylocerenin/isohylocerenin.

Figure 1b shows a chromatogram corresponding to an extract from skins of *H. polyrhizus* fruits. Four different peaks were quantified and were assigned to betanin/isobetanin, phyllocactin and hylocerenin. Whereas, the lack of isophyllocactin and isohylocerenin in the peel extract was surprising.

Distribution and quantification of the betacyanins in the skin of the fruits of *Hylocereus polyrhizus* have not been explained previously. From the quantitative analysis of the total peak area it was observed that betanin and phyllocactin were the major betacyanins in pulp and skin of *Hylocereus polyrhizus* (see Table 2 below). The betanin concentration, expressed as percentage of the total HPLC peak area, was calculated as 30.18% for pulp and 66.79% for skin. Large amount of betanin in peel was remarkable that was two times higher than the one in the pulp. The highest relative concentration of phyllocactin appears in peel in half the concentration found in pulp, ranged between 51.87% and 24.97% of the total area for pulp and peel, respectively. Phyllocactin and hylocerenin isoforms were not detected in the peel.

Table 2 Total contents and relative concentrations (expressed as percentage of the total HPLC peak area) of betacyanins found in analyzed skin and pulp of *Hylocereus polyrhizus*

Sample	Relative concentration (%) for peaks			Total pigment content (mg/g)
	1	2	3	
	1	2	3	
Pulp	30.18	51.87	11.03	452
	4.41	2.00	0.51	
Skin	66.64	24.91	1.05	302
	7.41	Not detected	Not detected	

- 5 Peak numbers refer to: 1,1 Betanin, Isobetanin
 2,2 Phyllocactin, Isophyllocactin
 3,3 Hylocerenin, Isohylocerenin

10 Table 3 Chromatic parameters of the extracted fruit pulp and skin obtained from *Hylocereus polyrhizus* fruits

Sample	Colour characteristics		
	(Lightness) L^*	(Chroma) C^*	(Hue Angle) h°
Pulp	73.58	48.35	336.01
Skin	50.37	27.49	332.46

15 The visually observed differences between betacyanin extracts from peel and pulp of the fruits were confirmed by the colorimetric measurements. The initial values of L^* , C^* and h° are shown in the above Table 3. L^* measures the sample's lightness; C^* is the chroma or color purity; h° refers to the hue angle of tone and indicates the sample's color (0° or 360° =red, 90° =yellow, 180° =green, and 270° =blue). Extracted betacyanin solutions at constant absorbance ($A_{\lambda\max}=1.0$) gave L^* values which is significantly different. Values for standardized extracted solutions from peel were lighter ($L^*=50.4$) than the one in pulp ($L^*=73.6$), indicating lower betacyanin concentrations in peel. Usually synthetic colorants in FD&C (Food, Drug and Cosmetics) which were used in industry, such as Red 3 and 40, cover L values about 70. Further, for the extract samples from pulp, also the chroma values showed higher

saturation. The significant differences were observed by the values of chroma: for the peel 27.5 and for pulp colorants around 48.35 indicate a more vivid color.

These values together with angle h° value indicate a slight tendency of the extracts to purple color for the skin extract samples ($h^\circ = 332.5^\circ$).

Conclusion

Fruit pulp of *H. polyrhizus* is characterized by the glowing purpled color compare to less glowing colors in the skin of fruits. The difference could be attributed to different pigments and their different concentration ratios in the pulp and the skin. The color of the fruit pulp of *H. polyrhizus* can be assigned to the presence of phyllocactin in the pulp whereas color of the skin of fruits can be assigned to the higher concentration ratio of betanin.

Pulp of fruits from *H. polyrhizus* showed much higher contents of phyllocactin, hylocerenin and their respective isoforms. Hylocerenin and isohylocerenin, present in the extracted betacyanins from the pulp at 11.03% and 0.51%, whereas lower concentration ratio was observed in the extracts from the skin. Thus, lower concentration of these pigments resulted in lower value of chroma (C^*) which was obtained through colorimetry analysis.

H. polyrhizus fruits pulp have a much higher content of phyllocactin, which is almost two times higher than the betanin content. It is interesting to note that the skin of fruits had a content of red pigments similar to that of the pulp of fruits, but the ratio among the pigments was different.

The results of analysis for betacyanin concentration in fruits of *Hylocereus polyrhizus* revealed that these fruits are the richest source of betacyanin as s food colorant agents after *Beta Vulgaris* and *Amaranthus* species.

This is the first report on pigment profile of skin and pulp of these new fruits compare together and how profiles and hues are related.

Example 4Matrices evaluation of betacyanin colored extracts5 HPLC conditions for organic acids (citric, tartaric and ascorbic acids)

The diluted samples were analyzed with the liquid chromatographic apparatus (Shimadzu LC 6A) consisted of a pump and controller coupled to a UV-Spectrophotometric detector (Shimadzu SPC 6A). Separations were performed on an
10 Aminex HPX-87H 300 x 7.8 mm column with particle size of 8 μm (Bio-Rad Laboratories, Ca, USA) operating at 36°C with a flow rate of 0.6 mL min⁻¹. Elution was effected using an isocratic elution of the solvent 0.008 N H₂SO₄. Detection was carried out at 210 nm. The different organic acids were identified by comparison of their retention times with those of pure standards. The concentrations of these
15 compounds were calculated from standard curves of the organic acids.

For vitamin C (ascorbic acid) the liquid chromatographic apparatus (Shimadzu LC 6A) consisted of a pump and controller coupled to a UV-Spectrophotometric detector (Shimadzu SPC 6A). Separations were performed on a Licro CART® 250 x 4.6 mm
20 i.d. with particle size of 5 μm (Merck, Darmstadt, Germany) operating at 36°C with a flow rate of 0.6 mL min⁻¹. Detection was carried out at wavelengths of 254 nm. Elution was done using an isocratic elution of the solvent ACN: Acetic acid: H₂O (20:5:75 v/v). Component was identified by comparison of retention time to authentic standard under analysis condition and quantified by external standard method.

25

Determination of sugars by HPLC analysis

Analysis of sugars was performed using an HPLC system (Jasco Co., Tokyo, Japan) equipped with refractive index (RI) detector (Jasco RI-1530), a pump (Jasco PU-
30 1580), a mixer (Jasco LG-1580-04), and a degasser (Jasco DG-1580-054). HPLC was run by a Jasco Borwin GPC (Tokyo, Japan, 2000) chromatography manager system. Separation of sugars was carried out using a SUPLECOSIL™ LC-NH₂ HPLC column (250 x 4.6 mm) with a particle size of 5 μm (Sigma-Aldrich Co., PA, USA) at room temperature. The flow rate was 1 mL min⁻¹. The samples were eluted
35 isocratically by using 80% ACN as solvent. The different sugars were identified by

comparison of their retention times with those of pure standards. The concentrations of these compounds were calculated from standard curves of the respective sugars.

Quantitative and statistical analysis

5 Stock solution preparation, standards of organic acids (ascorbic and tartaric acid) were dissolved in water. Standard of ascorbic acid was prepared fresh in 10% meta-phosphoric acid. Sugar standards, glucose, fructose and sucrose, were dissolved in water. All the samples and standards were injected three times each and mean
10 values were used (see Table 4.1). The results were statistically evaluated by one way analysis of variance (ANOVA). Statistical differences with p-values under 0.05 were considered significant.

15 Table 4.1. Concentration ranges and calibration equations of reference compounds used for calibration of the HPLC analysis^a

	Organic acids		Sugars		
	Citric acid	Ascorbic acid	Fructose	Glucose	Sucrose
Concentration range (mg L ⁻¹)	0.1 – 0.4	0.5 – 2.5	5 - 20	5 - 20	5 - 20
Calibration equation	y - 4851753 x - 60419	y - 950010x - 305087	y - 103766x + 33405	y - 120054x + 3608.3	y - 102350x + 16214

^a All calibration coefficients >0.99

Conclusion

- Enzymes are natural tools that can be used to obtain a better extraction of the color and improving extraction yield. Pectinases was introduced as a natural enzyme in breaking down the residual pectins and decreasing the viscosity resulted in improving extraction yield (Thereby, an enzymatic processing method proposed for the production of the concentrated betacyanin extract from *H. polyrhizus* fruits. Pectinex Ultra SP-L enzyme preparations at different dosages were studied to identify their applicability to degrade the mucilage in pulp of *H. polyrhizus* fruit.
- The presence of matrix compounds (sugars and organic acids) was monitored by using HPLC analysis (see Table 4.2). Different concentration of enzyme did not effectively change the matrix fractions, which are desirable property in improving pigment stability. The results of this study indicate that during enzymatic treatments sugars and acids did not formed as ester. Glucose/fructose and sugar acid ratio was at equilibrium level in enzyme-treated samples with 0.3% dosage of enzyme. The sugar-acid ratio as flavor indicator with ratio of 16:1 to 21:1 showed the characteristic of a pleasant sour-sweet taste for concentrated extracts.
- In order to optimize enzyme concentration to obtain a concentrated betacyanin extract with high pigment concentration, betacyanin retention was monitored by spectrophotometry and HPLC analysis. Individual and total peak area changes in concentrated betacyanin extracts was monitored. The total peak area retention was higher as compared to the individual betacyanins. Betanin and isobetanin retention was greater compared to phyllocactin and isophyllocactin. Moreover, the betanin/phyllocactin peak area ratio increases. Apparently, phyllocactin showed less stability than other betacyanins presented in *H. polyrhizus*. Requiring enzyme dosages at 0.3% resulted highest concentration of pigments. Enhancing enzyme dosage did not result in further concentration of betacyanins. Phyllocactin and betanin presented the predominant betacyanins.

Table 4.2. HPLC qualitative and quantitative data of sugars and organic acids in concentrated betacyanin extracts from *H. polyrhizus* fruit pulp, applying different extraction methods

Sample	Fructose (g/L)	Glucose (g/L)	Glucose/ Fructose	Sucrose (g/L)	Citric Acid (mg/L)	Ascorbic Acid (mg/L)	Sugar- acid ratio
Concentrated extract by enzyme treatment	20.2	52.6	2.6	20.1	678	0.7	21:1
Selected solvent system:							
EtOH	42.5	106.1	2.5	35.9	476.5	2.3	56:1
EtOH:H ₂ O	26.6	56.5	2.1	24.6	441.5	1.0	38:1
Control ^c	19.6	50.1	2.5	19.5	444	0.5	16:1

5

Example 5

Spectra Characteristic of each colorant

- 10 The CIEL*a*b* system (International Commission on Illumination, Vienna) that has been accepted by the USA food industry for measuring color of food products was used to investigate color quality (visual appearance) of colorants.

Figure 2 displays the UV-Vis spectra of concentrated colorant preparation of *H.*
 15 *polyrhizus* pigments determined spectrophotometrically. The UV-VIS spectrum was recorded from each sample in visible light absorption spectra (380-780 nm) in a quartz cuvette with path length of 10 mm. The concentrated pigments extracted from *H. polyrhizus* fruit (pulp and skin) absorb light as strongly as red beet colorant with only one maximum absorbance peak (530-540 nm) at the range of visible light. The
 20 result is quite similar to previously reported data for the visible spectra absorption maxima (λ_{max}) for betacyanin pigments at pH 4. The presence of one single maximum wavelengths (540 nm) suggesting the presence of Betacyanins in extracted colorant obtained from each assay. It should be noticed that red colorant from *H. polyrhizus* fruits displayed the most symmetrical spectrum according to the
 25 red beet colorant. It is interesting to note that the *H. polyrhizus* spectrum is

characteristic of a simple-composition of betacyanin colorant devoid of betaxanthins in contrast to betanin from red beet. Pigment composition of *H. polyrhizus* fruits are devoid of betaxanthins, therefore prior betaxanthin removal will not be necessary; in other word these fruits offer high color purity of juices.

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Color characteristic of concentrated natural extracts

The CIELAB color measurements was used to determine quality parameters, and L^* , a^* , b^* , C^* , h° and ΔE^* were calculated for each sample (see Table 5). Lightness
 10 ranged from the $L^* = 84.35$ of concentrated extract from the pulp of fruit obtained through enzymatic treatment to the $L^* = 50.1$ of skin extract. *H. polyrhizus* displayed higher value of lightness compare to red beet. Positive a^* values for all the colorants explained by their red color. Concentrated extracts obtained through solvent assays showed the highest a^* values, $a^* = 48.98$ and $a^* = 44.17$ for aqueous ethanolic and
 15 ethanolic assay, respectively.

Table 5 Chromatic parameters in concentrated betacyanin from *H. polyrhizus* using three different solvent systems

Concentrated <i>H. polyrhizus</i> extract		L^*	a^*	b^*	C^*	h°	Color strength	ΔE
Extractin system assays								
Aqueos								
ethanolic (pulp)	S_1	71.38	48.98	-23.71	54.42	334.17	88.5	20.29
Ethanolic (pulp)	S_2	73.57	44.17	-19.66	48.35	336.01	30.7	20.64
Enzymatic								
treatment (pulp)	S_3	84.35	20.18	-10.51	22.75	332.478	13.5	42.18
Ethanolic (skin)	S_4	50.1	24.28	-12.7	27.4	332.39	33.25	35.58
Red beet colorant	S_5	63.59	56.70	-6.63	57.09	353.33	73.5	0

S₁: concentrated betacyanin from pulp of *H. polyrhizus* using aqueous ethanol as solvent, S₂: concentrated betacyanin from pulp of *H. polyrhizus* using EtOH as solvent S₃: concentrated betacyanin from pulp of *H. polyrhizus* by Enzymatic extraction, S₄ : concentrated betacyanin from the skin of *H. polyrhizus* fruits using
5 EtOH as solvent S₅ :Standard (betacyanin from Red Beet)

Greater diffusion was observed in the b^* parameter (blueness–yellowness) ranging in the negative region and higher values exhibited purple color, devoid of yellowish shade. The significant difference was attributed to different pigments and their
10 different concentration ratios in the concentrated extracts from pulp and skin obtained through extraction systems. The color of the fruit pulp of *H. polyrhizus* was attributed to the presence of phyllocactin and hylocerenin pigments in the fruit. High-performance liquid chromatography analysis revealed pigment alterations and different contents of hylocerenin, phyllocactin and their isoforms. This can be a
15 reason for variability among resulted b^* values.

Chroma, was significantly affected by the type of extractant and solvent. Since this parameter is the quantitative expression of colorfulness, selected extraction systems would improve C^* values. Higher C^* values of the concentrated extract samples from
20 aqueous ethanolic assay (54.42) indicated a more vivid red color than the other samples with lower C^* values.

The hue angle, which indicates the tonality, visually showing that they varied in color from purplish-red for *H. polyrhizus* concentrated extracts compare to red in red beet.
25 Lower h° indicated a more purple shade of red. Differences in hue angle values may depend on the individual pigment patterns of betacyanins. ΔE represents the total color difference between two samples, in this case from Table 5 it was generally found that S₁ ($\Delta E = 20.29$) and S₂ ($\Delta E = 20.64$) were the most similar in color. ΔE showed largest differences among S₃, S₄ and concentrated colors obtained through
30 solvent extraction. Taking in to account that more than 5 units of ΔE is needed to distinguish samples visually. In the present work, ΔE values of more than 5 were observed when comparing extracted colors by enzymatic treatments with samples produced by solvent assays, which indicates that the differences in the color parameters determined instrumentally may also be perceived visually.

Color strength (tinctorial power) is an indicator to determine which color is suitable for commercial applications. Extracted color from aqueous ethanolic assay displayed higher value compare to red beet colorant. Colorants obtained by solvent system did not differ significantly. Concentrated colorant produced by enzymatic treatment showed lowest value. In general, therefore, color strength was considered suitable for commercial application by comparing the obtained values with other red colorants under investigation. In natural colorant with low color strength more of them have been liable to use to increase the color strength.

10 Color changes with selected extraction conditions assayed

To test the effect of extraction condition on the color characteristic of concentrated colorants, color differences can be clearly depicted in a chromaticity diagram, as shown in Figure 3. The color attributes of *H. polyrhizus* colorants were also compared to the red beet.

The two extracts obtained from the pulp of fruits by solvent extraction assay provided color closest to the red beet color. According to their hue value they were more red-purple colorant compare to red beet. Tonality of color extracts from the skin of fruit and the one extracted by enzymatic treatment shifted into an intense purple-red color and due to their chroma value imparted a more purplish color. In relation to the color properties of the different extracts, lightness (L^*) was the attribute related with the transmission of light observed, and for concentrated natural colors under study it ranged from 50.1 to 84.35. Chroma (C^*) is the quantitative component of chromaticity and hue (h°) is the qualitative expression of colorants. Although differences in total betacyanin content did not result in color shades (h°), hue angle values may depend on the individual betacyanin patterns of each concentrated extract. Differences between chroma might be dependent on the chemical nature of the color extracts since C^* is a combination of a^* and b^* and therefore introduces the corresponding distortion due to the b^* coordinate.

Hylocereus polyrhizus (purple pitaya with red flesh) fruits are a valuable source of betacyanins. The present work provides stable concentrated extracts of high color strength that cover a wide spectrum from red to purple with high pigment content. Betacyanin ratios varying between the concentrated pitaya extracts which are resulting in different color shades. Additionally, matrix compounds were found to

have a positive effect on betalain stability. Taking in to consideration the previous studies on positive effect of matrix compounds in increasing betalain stability, matrix compounds (sugars and organic acids) were evaluated in each concentrated extract and for the first time the presence of sucrose in *Hylocereus polyrhizus* fruits was revealed according to the data obtained by High performance liquid chromatography analysis. The sugar-acid ratio was observed harmonic for all the concentrated extracts.

Therefore, concentrated betacyanin extracts from the fruits of *Hylocereus polyrhizus* (purple pitaya) with its pleasant taste and flavor could present new opportunities for the use of betacyanin as a food colorant.

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CLAIMS

1. A colorant composition of a plant extract, which has a high staining power in the red-purple range, characterized in that the colorant having a pigment
5 concentration of at least 811 mg per 1000 g of fresh weight of the fruit.

2. A colorant composition according to claim 1, wherein the plant is *Hylocereus polyrhizus*.
10

3. A colorant composition according to claim 1, wherein the composition is a pH 4 composition.
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4. A colorant composition according to claim 1, wherein the pigment is mixed betacyanins.

20 5. A colorant composition according to claim 4, wherein the betacyanin pigment consists of acylated and non-acylated betacyanin.

25 6. A colorant composition according to claim 5, wherein the acylated betacyanin includes phyllocactin, hylocerenin and *iso*-forms thereof.

7. A colorant composition according to claim 5, wherein the non-acylated betacyanin includes betanin and isobetanin.
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8. A colorant composition according to any one of the preceding claims having a stable betacyanin concentrate that is capable of being suspended in a food-compatible liquid, as a food colorant or food additive.
35

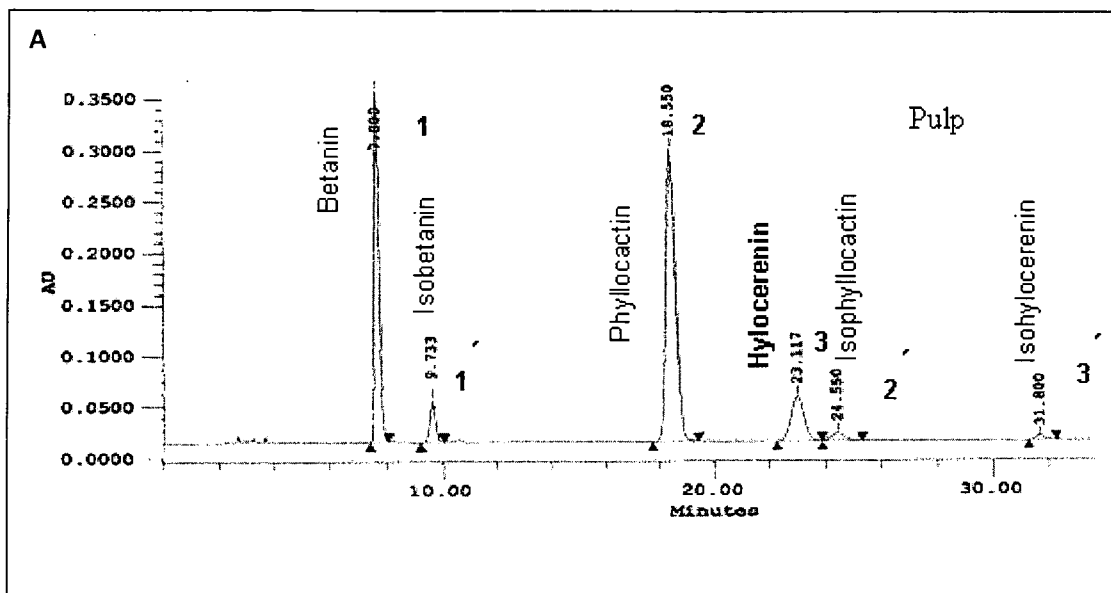


Figure 1a

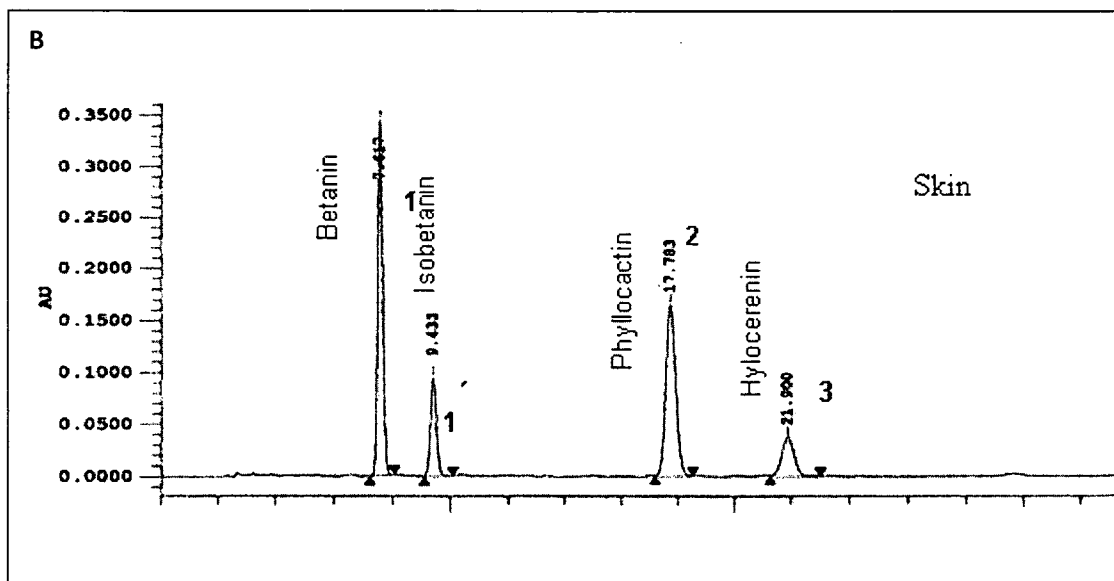
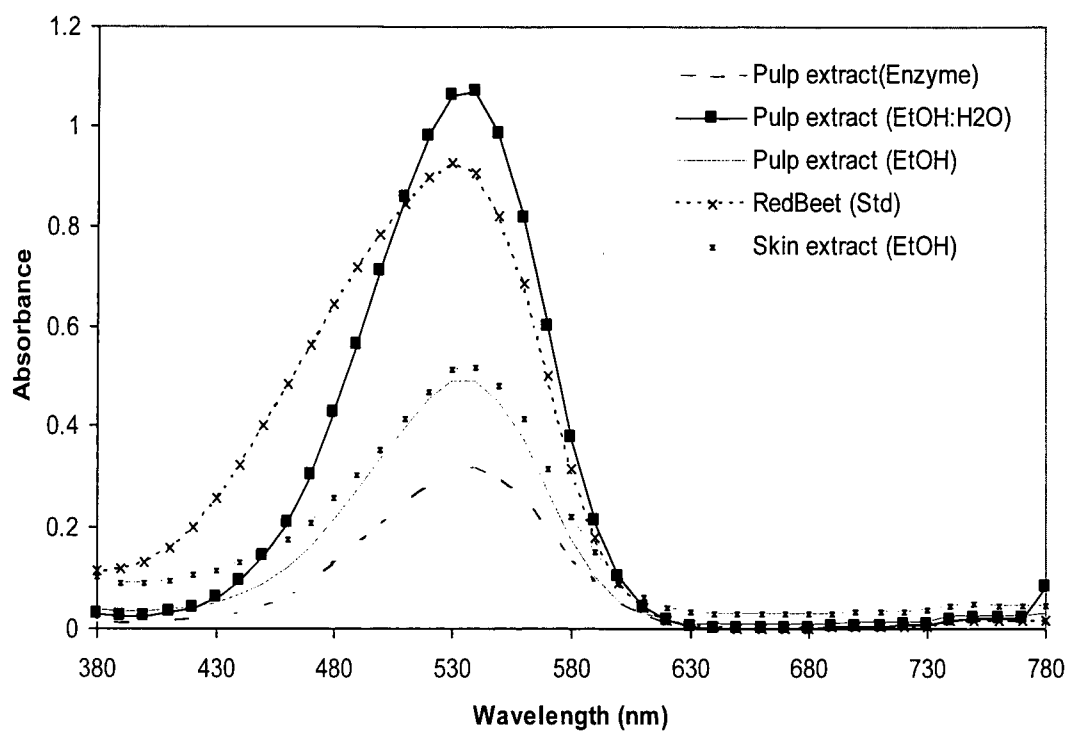
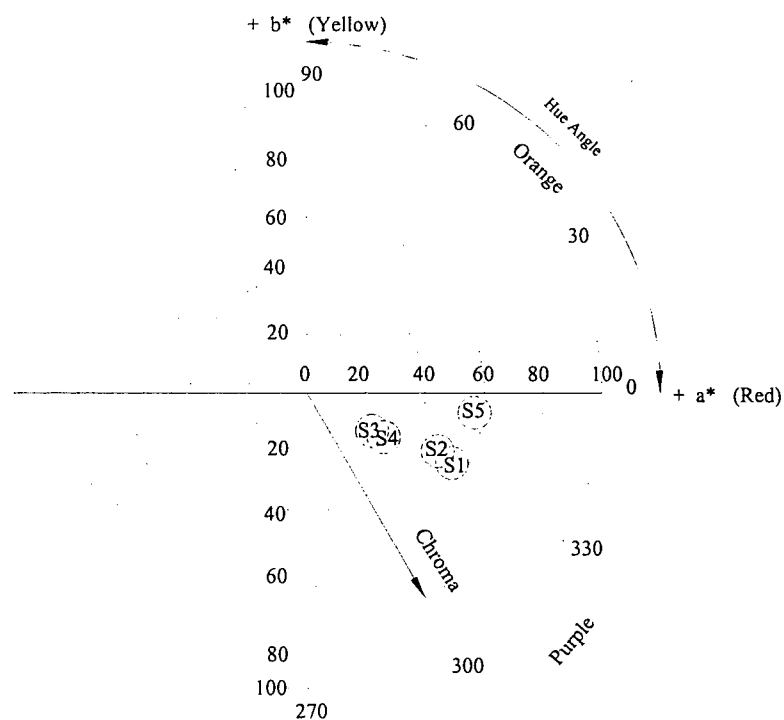


Figure 1b

**Figure 2****Figure 3**

INTERNATIONAL SEARCH REPORT

International application No.
PCT/MY2009/000180

A. CLASSIFICATION OF SUBJECT MATTER

C09B 61/00(2006.01)i, A23L 1/27(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C09B 61/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKOMPASS(KIPO internal) & Keywords: pitaya, Hylocereus polyrhizus, betacyanin

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FLORIAN C. STINTZING et al., Food Chemistry, Vol. 77, Issue 1, May 2002, page 101-106	1-8
X	KIRSTEN M. HERBACH et al., Food Research International, Vol. 39, Issue 6, July 2006, page 667-677	1-8
X	FLORIAN C. STINTZING et al., Phytochemistry, Vol. 65, Issue 4, Feb. 2004, page 415-422	1-8
X	KIRSTEN M. HERBACH et al., European Food Research Journal, Vol. 219, No. 4, Sept. 2004, page 377-385	1-8
X	PATRICIA ESQUIVEL et al., Innovative Food Sci., Vol. 8, Issue 3, Sept. 2007, page 451-457	1-8
X	JP 2003-253147 A (JAAFURIKKU CORPORATION:KK) 10 September 2003 See Abstract, Claims, Paragraph [2],[26]-[30]	1-8



Further documents are listed in the continuation of Box C.



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INTERNATIONAL SEARCH REPORT

Information on patent family members

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
JP 2003-253147 A	10.09.2003	None	