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Characterization of Anthocyanins from the Fruits of Baguaçu (*Eugenia umbelliflora* Berg)

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Anthocyanin pigments in the berries of baguaçu (*Eugenia umbelliflora* Berg), a tropical fruit from Brazil, were extracted with 0.1% HCl in ethanol, and the crude anthocyanin extract was purified by Amberlite XAD-7 open-column chromatography. Six major anthocyanins were isolated by preparative HPLC, and their chemical structures were identified by spectroscopic methods (TLC, UV−vis, MS, and ^{1}H NMR). Delphinidin 3-*O*-β-glucopyranoside, cyanidin 3-*O*-β-glucopyranoside, petunidin 3-*O*-β-glucopyranoside, pelargonidin 3-*O*-β-glucopyranoside, peonidin 3-*O*-β-glucopyranoside, and malvidin 3-*O*-β-glucopyranoside were identified. On the basis of chromatographic data the total anthocyanin content was 342 mg/100 g of fresh baguaçu berries. Therefore, the concomitant presence of six anthocyanins in a single plant species makes this product promising as a new pigment source.

KEYWORDS: ***Eugenia umbelliflora* Berg; baguaçu; anthocyanins; natural colorants; ^{1}H NMR**

INTRODUCTION

The most important and widespread group of pigments in nature are the anthocyanins, which are present in various plant species (*1*), especially in berries such as blackberries, raspberries, huckleberries, and highbush blueberries. Interest in this kind of pigments has increased substantially because of their attractive colors, easy incorporation into aqueous systems, and possible beneficial health effects, that is, antioxidant properties (*2*, *3*).

The genus *Eugenia* is one of 75 genera (~3000 species) belonging to the family Myrtaceae, which are native in the tropics (*4*), mainly tropical America and Australia. The genus *Eugenia* has been shown to possess anti-inflammatory, analgesic, antipyretic (*5*), and antifungal properties (*6*). Baguaçu (*Eugenia umbelliflora* Berg) is the name given to a tree and its fruits that grow wild in the southern regions of Brazil. The fruits are similar to sweet cherries (1 cm diameter), with a dark-red skin and green translucent flesh. Although it is an edible fruit, so far as we know there are no references available concerning this plant, except that reported by Kuskoski et al. (*7*), which demonstrated antibacterial activity in an extract of baguaçu leaves. Therefore, the present study was undertaken with the purpose of identifying the structure and ascertaining the content of the anthocyanin pigments in the fruits of baguaçu.

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MATERIALS AND METHODS

Materials and Solvents. All were of analytical grade and obtained from Merck-Clevenot Corp. (Darmstadt, Germany). The berries of baguaçu (*E. umbelliflora* Berg) were collected in October 2000 at Florianópolis, Santa Catarina, in southern Brazil (48° 35′ W and 27° 55′ S), placed in polyethylene bags, and stored at −10 °C until they were used.

Extraction of Anthocyanins. The pigments were extracted twice by maceration of 500 g of the berries with 0.1% HCl in ethanol (*8*) at 5 °C for 48 h, because this mixture has proved to be the best solvent. The berries were blended with ~2 L of solvent/kg and filtered on a Büchner funnel. The filter residue was re-extracted until a clear solution was obtained. Filtrates were then combined and shaken in a separatory funnel with 3 × 50 mL of methanol/petroleum ether (1:2 v/v). The aqueous portion was collected and placed on a Büchi rotovapor at 35 °C until all alcoholic residue was evaporated.

Anthocyanin Purification. The pigment extracts were filtered and fixed on a 300 × 20 mm i.d. nonionic polymeric absorbent, Amberlite XAD-7 HP (Supelco, Bellefonte, PA) column prewashed with 0.01% HCl/H_2O. The pigments were then eluted by using gradient elution from MeOH/H_2O (8:92 v/v) to MeOH/H_2O (65:35 v/v). The eluate was concentrated and passed through a C_{18} Sep-Pak cartridge (Waters Corp., Milford, MA), which had been previously activated with methanol. Anthocyanins and other phenolics were adsorbed onto the Sep-Pak, whereas sugars, acids, and other water-soluble compounds were eluted with 2 × 5 mL of 1% aqueous acetic acid. The pigments were finally eluted with methanol/water/acetic acid (89:10:1). The methanolic extracts were concentrated using a Büchi rotovapor at 35 °C, and the pigments were dissolved in ultrapure water (Milli-Q, Millipore, Bedford, MA).

Thin-Layer Chromatography (TLC) Method. TLC was carried out using microcrystalline cellulose F flexible TLC plates (layer thickness = 0.1 mm) with ethyl acetate, *n*-butanol/HOAc/H_2O (4:1:5

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upper phase) and acetic acid/concentrated HCl/water (15:3:82) as solvents. The sample spots on the chromatogram were detected by using a UV lamp (254 and 365 nm) and the naked eye.

Acid Hydrolysis of Anthocyanins and GC Conditions. Purified pigments (~1 mg) were hydrolyzed in a screw-cap test tube with 1 mL of 2 N HCl for 45 min at 100 °C and then cooled in an ice bath (*9*, *10*). The resulting anthocyanidins were separated by the addition of 1-pentanol, and both phases were evaporated to dryness. The pigments were dissolved in methanol and passed through a C_{18} Sep-Pak cartridge (Waters Corp.) as previously described for subsequent MS analysis. The sugar residues were dissolved in ultrapure water and filtered through a 0.45 μm filter prior to injection into the HPLC and GC-MS. The residues were taken for silylation in *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (MSTFA) and heated in a sealed vial at 60 °C for 45 min. The silylated derivatives of sugar residues were analyzed by GC-MS by using a 30 mm × 0.25 mm i.d., 0.25 μm DB-5 MS fused silica capillary (Chrompack, Middelburg, Belgium).

HPLC Analysis. *Apparatus.* A Hewlett-Packard series 1050 liquid chromatograph was used, equipped with a Hewlett-Packard 1040A photodiode array detector and a Hewlett-Packard 9000 computer system with Hewlett-Packard HPLC ChemStation software. Detection was carried out simultaneously at 280, 320, and 525 nm.

Analytical HPLC for Anthocyanins. A 250 × 4.6 mm i.d., 5 μm ODS-Superspher 100 RP-18 column (Merck Chemical Co., Darmdstadt, Germany) was used for identification of anthocyanins and anthocyanidins in analytical systems. The solvents used were (A) 100% HPLC grade acetonitrile and (B) 1% phosphoric acid, 10% acetic acid (glacial), and 5% acetonitrile (v/v/v) and water (Milli-Q, Millipore) (*10*). The program followed a linear gradient from 0 to 22% A in 25 min. The flow rate was 0.8 mL/min and the injection volume 20 μL. Solvents and samples were previously filtered through a 0.45 μm filter (Millipore Corp.).

Semipreparative HPLC of Anthocyanins. A 250 × 21.2 mm i.d., 12 μm Supelcosil LC-18 column (Supelco) was used for the separation and isolation of anthocyanins on a semipreparative scale. The spectra were recorded for all peaks. The solvents used were (A) 100% HPLC grade acetonitrile and (B) 1% phosphoric acid, 5% acetic acid (glacial), 10% acetonitrile, 5% methanol (v/v/v/v), and water (Milli-Q, Millipore). The program followed a linear gradient from 0 to 22% A in 35 min. The flow rate was 10 mL/min and the injection volume 200 μL. Solvents and samples were previously filtered through a 0.45 μm filter (Millipore Corp.).

Analytical HPLC for Sugar. Sugars were analyzed according to the method of Pérez et al. (*11*). Isocratic separations of the compounds were made on a 300 × 7.8 mm, 10 μm ICSep ICE-ION300 (Interaction, San Jose, CA) column, containing a cation-exchange polymer in the ionic hydrogen form, with an IonGuard GC-801 guard column (Interaction) and thermostated at 23 °C. The mobile phase utilized for the elution was 0.0085 N H_2SO_4 with a flow rate of 0.4 mL/min. The refractive index detector was used at 16× sensitivity, and the injection volume was 20 μL. The sugar residues were identified using cochromatography with sugar standards: glucose, fructose, and xylose.

Spectroscopic Analysis. *UV−Vis Spectroscopy.* A Hewlett-Packard HP 8452 UV−vis spectrophotometer and 1 cm path length silica cells were used for spectroscopic measurements at 420, 510, and 700 nm of purified anthocyanins in 0.1% HCl/CH_3OH.

Electrospray Mass Spectrometry (ESI-MS). Low-resolution MS analysis were performed on a Finnigan Mat95′s mass spectrometer (Finnigan Mat, Bremen, Germany), equipped with an ESI−II interface. The electrospray spray voltage applied was 3.5 kV, and the capillary temperature was set to 220 °C. Isolated anthocyanins from semipreparative HPLC were injected directly into the system by means of an infusion pump at a rate of 2 μL/min, and nitrogen sheath gas was used when necessary. Full accelerating voltage was used and the scan range set from 100 to 2000 Da; scan speed was 1 s/100 amu in profile mode at a resolution value of 1500. Samples were dissolved in a mixture of water/methanol/acetic acid (50:50:1 v/v/v).

NMR Analysis. ^{1}H NMR spectral data were recorded on a model AC-200 instrument operating at 200 MHz (Bruker, Karlsruhe, Germany) in CD_3SOCD_3/CD_3OD (9:1 v/v) with TMS as internal standard. Sample temperatures were stabilized at 25 °C.

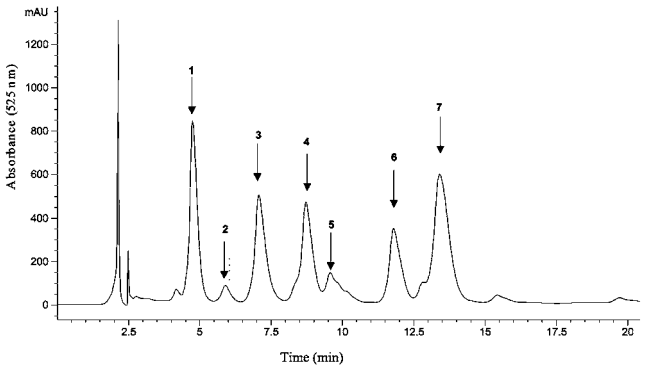

Figure 1. Semipreparative HPLC separation of anthocyanins from baguaçu. See **Table 1** for identification.

Table 1. Peak Assignment for Anthocyanin Pigments from *E. umbelliflora* Berg

anthocyanin		HPLC (semipreparative)		ESI-MS
peak	peak assignment[a]	retention time (min)	peak area (%)	$[M + H]^+$ ion (*m*/*z*)
1	dp 3-glu	4.7	17.9	465
2	NI	5.9	1.8	453
3	cy 3-glu	7.0	15.9	449
4	pt 3-glu	8.7	15.9	479
5	pg 3-glu	9.5	6.1	433
6	pn 3-glu	11.8	11.7	463
7	mv 3-glu	13.4	27.1	493

[a] Tentative identification: cy, cyanidin; dp, delphinidin; pt, petunidin; pg, pelargonidin; pn, peonidin; mv, malvidin; NI, not identified.

Monomeric Anthocyanin Content. The extract obtained was analyzed for the levels of monomeric anthocyanins using chromatographic data (*12*). Pigment contents were calculated by using extinction coefficients and molecular weights, respectively, as follows: 34300 and 502.5 g for cyanidin 3-glucoside (cy 3-glu) (*13*); 29000 and 518.5 g for delphinidin 3-glucoside (dp 3-glu) (*14*); 12900 and 532.5 g for petunidin 3-glucoside (pt 3-glu) (*15*); 31620 and 433 g for pelargonidin 3-glucoside (pg 3-glu) (*16*); 11300 and 516.5 g for peonidin 3-glucoside (pn 3-glu) (*15*); and 29500 and 562.5 g for malvidin 3-glucoside (mv 3-glu) (*17*). Results were expressed as milligrams of anthocyanin per 100 g of fresh berries.

RESULTS AND DISCUSSION

HPLC Analysis. Anthocyanins from baguaçu were isolated and separated by HPLC preparative columns (**Figure 1**). All isolated pigments were present in the crude methanol extract as confirmed by comparison of the HPLC profiles of a crude methanol extract prepared without HCl; a possible hydrolysis during the extraction steps in the presence of HCl was thus discarded. The HPLC chromatogram obtained by analytical columns revealed seven anthocyanin peaks. Peaks 1 and 3−7 represented ~89% of the total area at 525 nm. One minor peak was also detected with poor resolution (peak 2), accountable for <2% (**Table 1**). The typical order of elution of the different anthocyanins with similar glycosylation patterns is determined by the polarity of the respective aglycons. Therefore, the expected elution order would be as follows: first delphinidin, followed by cyanidin, petunidin, pelargonidin, peonidin, and finally malvidin. The presence of hexose was also confirmed by GC-MS analysis after derivatization with MSTFA. The hydrolysis of purified pigments using HCl yielded sugar, the principal sugar in baguaçu being glucose (t_R = 15.0 min) confirmed by HPLC analysis.

Spectroscopic and TLC Analysis. Further characterization of anthocyanins in the fruits of baguaçu, based on TLC and

Table 2. Chromatographic and UV–Vis Absorption Data of Anthocyanin Pigments from *E. umbelliflora* Berg

anthocyanin	UV–vis characterization[b] (in 0.1% HCl–CH_3OH)				TLC ($R_f \times 100$) anthocyanidin		TLC ($R_f \times 100$) sugar
peak	E_{UV}/E_{vis} (%)	E_{acyl}/E_{vis} (%)	E_{440}/E_{vis} (%)	λ_{max} (nm)	BAW[a]	AHW[a]	ethyl acetate
1	84	19	20	278, 542	26	17	16
2	89	18	22	286, 534			
3	91	21	23	284, 528	38	26	15
4	81	20	20	280, 544	28	17	16
5	86	20	23	278, 518			16
6	87	25	26	278, 532	31	22	16
7	77	10	19	280, 536	33	24	16

[a] Solvent systems: BAW, 1-butanol/acetic acid/water (4:1:5 upper phase); AHW, acetic acid/concentrated HCl/water (15:3:82). [b] E_{UV}, extinction coefficient of maximum absorption peak in UV region; E_{vis}, extinction coefficient of maximum absorption peak in visible region; E_{acyl}, extinction coefficient at 330 nm; E_{440}, extinction coefficient at 440 nm.

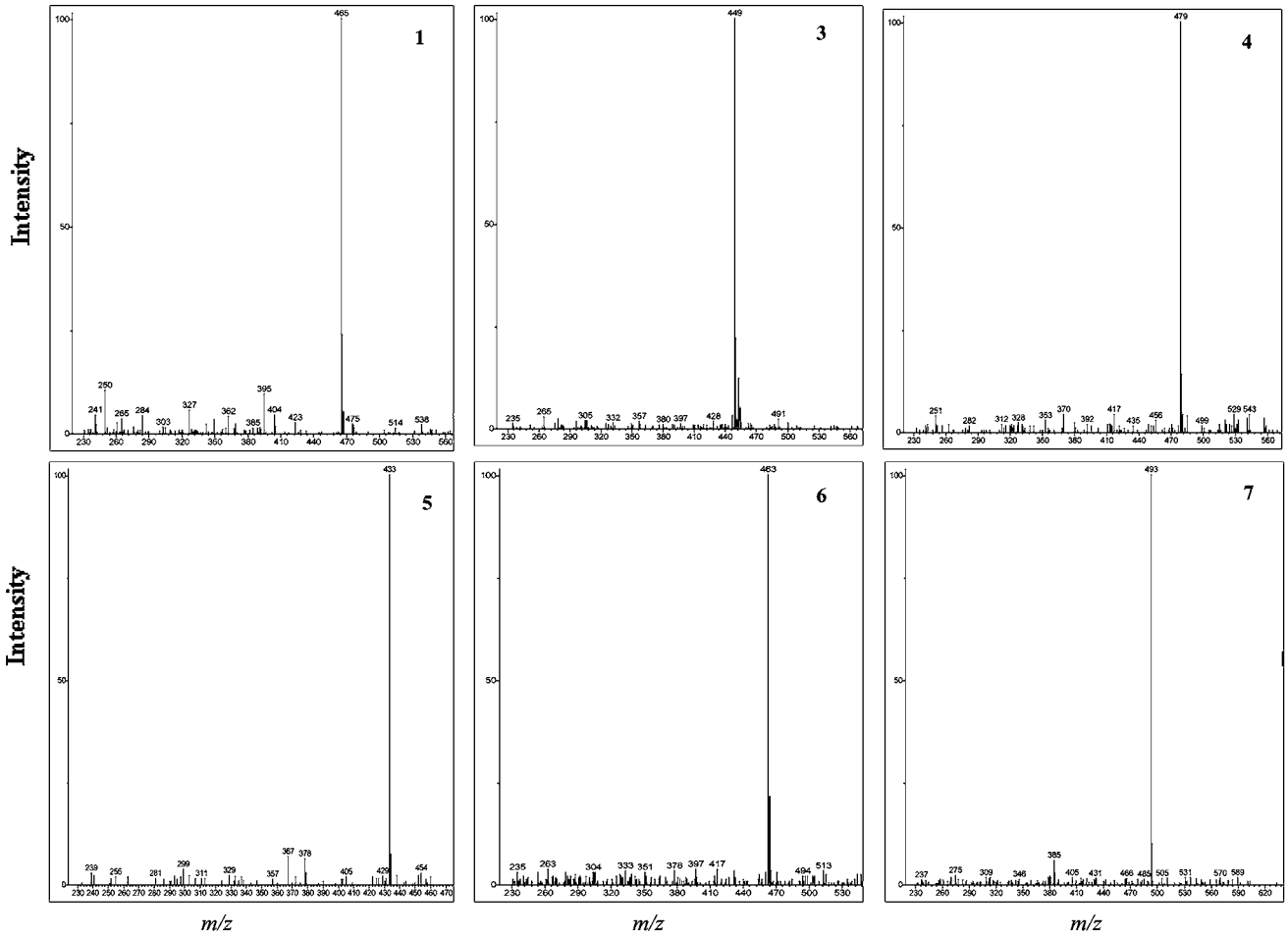

Figure 2. Mass spectra of anthocyanins detected in baguaçu. Peaks: (1) delphinidin 3-*O*-β-glucopyranoside; (3) cyanidin 3-*O*-β-glucopyranoside; (4) petunidin 3-*O*-β-glucopyranoside; (5) pelargonidin 3-*O*-β-glucopyranoside; (6) peonidin 3-*O*-β-glucopyranoside; (7) malvidin 3-*O*-β-glucopyranoside.

UV−vis analysis, is presented in **Table 2**. The results of UV−vis characterization using purified anthocyanins showed a maximum absorbance (λ_{max}) at 518−550 nm with E_{440}/E_{vis} of 19−26%, indicating a substitution in the C-3 position of an anthocyanidin. An anthocyanin with glycosidic substituents in the C-3 position exhibits a ratio of absorbance at 400−440 nm to the absorbance at the visible λ_{max}, almost twice as much as that of anthocyanins with glycosidic substitution at position 5 or both positions 3 and 5 (*18*−*21*). The obtained ratios of E_{UV}/E_{vis} (91−81%) and E_{acyl}/E_{vis} (10−21%) suggested that the purified anthocyanins were simple anthocyanin structures without complex acylation (*22*). The results of UV−vis analysis of the baguaçu were confirmed by HPLC, ESI-MS, and ^{1}H NMR analysis.

Mass Spectrometry Analysis. Electrospray ionization mass spectrometry analysis of anthocyanins produced, primarily, intact protonated molecular ions $[M + H]^+$ at *m*/*z* 465, 449, 479, 433, 463, and 493 corresponding with glycosides derivatives of delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin (**Figure 2**). The mass spectra of baguaçu anthocyanidins show protonated molecular ions $[M + H]^+$ at *m*/*z* 304, 288, 318, 272, 302, and 332 corresponding with molecular ions of delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin (*21*−*23*). The joint presence in a single product of the six most common anthocyanidins is of great interest.

NMR Analysis. The chemical shifts (δ) obtained from the ^{1}H NMR analysis of baguaçu anthocyanins (**Table 3**) confirmed the identity of the pigments, the proposed structures of which

Table 3. ^{1}H NMR Spectroscopic Data of Anthocyanins [δ in CD_3SOCD_3/CD_3OD (9:1)]

	δ (*J* in Hz)				
anthocyanin	**1**, delphinidin	**3**, cyanidin	**4**, petunidin	**6**, peonidin	**7**, malvidin
aglycon					
H-4	8.81 s	8.85 s	8.87 s	8.92 s	8.91 s
H-6	6.65 d (1.0)	6.67 d (2.1)	6.67 d (1.9)	6.70 d (2.0)	6.70 d (1.9)
H-8	6.88 d (1.0)	6.88 brs	6.94 d (1.3)	7.09 d (2.0)	6.86 d (1.6)
H-2′	7.70 s	7.98 d (2.3)	7.91 d (2.2)	8.24 d (2.3)	7.95 s
H-5′		7.05 d (8.7)		7.07 d (8.8)	
H-6′	7.70 s	8.10 dd (2.3; 8.7)	7.74 d (2.1)	8.40 dd (2.3; 8.8)	7.95 s
O–CH_3			3.89 s	3.91 s	3.90 s
					3.89 s
3-*O*-β-glucose					
H-1″	5.34 d (7.6)	5.33 d (7.4)	5.40 d (7.6)	5.38 d (7.6)	5.43 d (7.7)
H-2″,H-3″, H-4″, H-5″, -6a″ and H-6b″	3.23–3.69	3.20–3.52	3.21–3.69	3.20–3.80	3.41–3.75

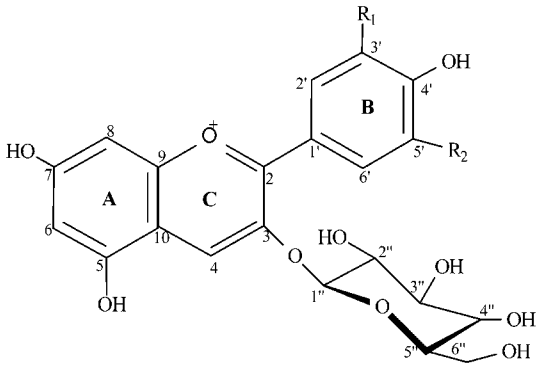

Figure 3. Chemical structures of anthocyanins in baguaçu: **1**, delphinidin 3-glucoside (R_1 = OH, R_2 = OH); **3**, cyanidin 3-glucoside (R_1 = OH, R_2 = H); **4**, petunidin 3-glucoside (R_1 = OCH_3, R_2 = OH); **5**, pelargonidin 3-glucoside (R_1 = H, R_2 = H); **6**, peonidin 3-glucoside (R_1 = OCH_3, R_2 = H); **7**, malvidin 3- glucoside (R_1 = OCH_3, R_2 = OCH_3).

are shown in **Figure 3**. Signals in the downfield of the ^{1}H NMR spectrum between δ 6.6 and 9.0 were clearly attributable to the aromatic protons (A and B rings) of the aglycon molecule as has been previously reported (*24*, *25*) for similar compounds. The signal doublets between δ 5.33 and 5.43 correspond to the protons on the anomeric carbon from the glucose residues, revealing that they were in position C-3 (**Table 3**) as indicated also by the E_{440}/E_{vis} ratio values (*26*) (**Table 2**). The β-configuration of this moiety in all anthocyanins was confirmed from the magnitude (J = 7.58 Hz) of the $J_{1''2''}$ coupling constant in the ^{1}H NMR spectra (*25*, *26*).

The ^{1}H NMR spectra of pigments **4** and **6** showed three-proton singlets at δ 3.89 and 3.91 (OCH_3), respectively, in accordance with petunidin (**4**, R_1 = OCH_3, R_2 = OH) and peonidin (**6**, R_1 = OCH_3, R_2 = H) structures. The spectrum of pigment **7** showed two three-proton singlets at δ 3.89 and 3.90, respectively (**7**, R_1 = OCH_3, R_2 = OCH_3), thus confirming malvidin. Compounds **1** (R_1 = OH, R_2 = OH) and **3** (R_1 = OH, R_2 = H) were identified as delphinidin and cyanidin, respectively, so that they do not present reference methoxy singlets around δ 3.89. It was not possible to identify the anthocyanins corresponding to pigment **5**. This was due to its low concentration compared to the other anthocyanins, shown by the low intensity detected during NMR analysis. The complete data, which have been only partially reported until now, are listed in **Table 3**.

Monomeric Anthocyanin Content. Anthocyanin content was calculated by using the extinction coefficient given for each anthocyanin. The total anthocyanin content of baguaçu was 342 ± 14.6 mg/100 g of fresh weight (40.79 ± 5.2 mg for dp 3-glu; 33.94 ± 2.3 mg for cy 3-glu; 88.76 ± 5.5 mg for pt 3-glu; 12.17 ± 0.5 mg for pg 3-glu; 77.91 ± 3.9 mg for pn 3-glu; and 88.87 ± 5.2 mg for mv 3-glu). This pigment content is higher than the reported values of 99.9 mg of anthocyanins/100 g of fresh weight for highbush blueberries (*27*), 111 mg/100 g of fresh weight for blueberries (*28*), and 250 mg/100 g of dry weight for banana bracts (*9*).

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LITERATURE CITED

(1) Mazza, G.; Miniati, E. *Anthocyanins in Fruits, Vegetables and Grains*; CRC Press: Boca Raton, FL, 1993.
(2) Wang, H.; Cao, G.; Prior, R. L. Oxygen radical absorbing capacity of anthocyanins. *J. Agric. Food Chem.* **1997**, *45*, 304−309.
(3) Moyer, R. A.; Hummer, K. E.; Finn, C. E.; Frei, C. E.; Wrolstad, R. E. Anthocyanins, phenolics and antioxidant capacity in diverse small fruits: *Vaccinium*, *Rubus* and *Ribes*. *J. Agric. Food Chem.* **2002**, *50*, 519−525.
(4) Hutchinson, J. *The Families of Flowering Plants*, 2nd ed.; Clarendon: Oxford, U.K., 1960.
(5) Karla, S.; Monica, S.; Emilia, C.; Angel, V. Flavonoid glycosides from *Eugenia jambos*. *Phytochemistry* **1994**, *37*, 225−258.
(6) Rahhal, M. M. Antifungal activity of some plant oils. *Alexandria Sci. Exch.* **1997**, *18*, 225−230.
(7) Kuskoski, E. M.; Cruz, A. B.; Gonzaga, L. V.; Dutra, P. P.; Philippi, J. M. S.; Moretto, E. Caracterização química e atividade antibacteriana de *E. umbelliflora* Berg. *ENEF−II Congr. Cientif. Estud. Farm.* **1995**, *6*, 13−15.
(8) Kuskoski, E. M.; Fett, R.; Luiz, M. T. B. Antocianinas: estudo de estabilidade em alimentos processados. *Rev. Bras. Cor. Natur.* **2000**, *4*, 63−66.
(9) Pazmiño-Durán, E. A.; Giusti, M. M.; Wrolstad, R. E.; Gloria, B. A. Anthocyanins from banana bracts (*Musa* × *paradisiaca)* as potential food colorants. *Food Chem.* **2001**, *73*, 327−332.
(10) Giusti, M. M.; Wrolstad, R. E. Characterization of red radish anthocyanins. *J. Food Sci.* **1996**, *61*, 322−326.
(11) Pérez, A. G.; Olías, R.; Espada, J.; Olías, J. M.; Sanz, C. Rapid determination of sugars, nonvolatile acids, and ascorbic acid in strawberry and other fruits. *J. Agric. Food Chem.* **1997**, *45*, 3545−3549.
(12) Giusti, M. M.; Rodriguez-Saona, L.; Wrolstad, R. E. Molar absorptivity and color characteristics of acylated and nonacylated pelargonidin-based anthocyanins. *J. Agric. Food Chem.* **1999**, *47*, 4631−4637.

(13) Siegelman, H. W.; Hendricks, S. B. Photocontrol of alcohol, aldehyde and anthocyanin production in apple skin. *Plant Physiol.* **1958**, *33*, 409−413.
(14) Asen, S.; Stuart, N. W.; Seigelman, H. W. Effect of various concentrations of nitrogen, phosphorus and potassium on sepal color of *Hydrangea macrophylla*. *Am. Soc. Hortic. Sci.* **1959**, *73*, 495−502.
(15) Somers, T. C. Grape phenolics: The anthocyanins of *Vitis vinifera* var. Shiraz. *J. Sci. Food Agric.* **1966**, *17*, 215−219.
(16) Swain, T. Analytical methods for flavonoids. In *Chemistry and Biochemistry of Plant Pigments*; Goodwin, T. W., Ed.; Academic Press: London, U.K., 1965; Chapter 19.
(17) Koeppen, B. H.; Basson, D. S. The anthocyanin pigments of Barlinka grapes. *Phytochemistry* **1966**, *5*, 183−187.
(18) Harborne, J. B. *Comparative Biochemistry of the Flavonoids*; Academic Press: New York, 1967; pp 1−36.
(19) Hong, V.; Wrolstad, R. E. Characterization of anthocyanin-containing colorants and fruit juices by HPLC/photodiode array detection. *J. Agric. Food Chem.* **1990**, *38*, 698−708.
(20) Harborne, J. B. The chromatographic identification of anthocyanin pigments. *J. Chromatogr.* **1958**, *1*, 473−488.
(21) Giusti, M. M.; Rodriguez-Saona, L.; Griffin, D.; Wrolstad, R. E. Electrospray and tandem mass spectroscopy as tools for anthocyanin characterization. *J. Agric. Food Chem.* **1999**, *47*, 4657−4664.
(22) Choung, M.-G.; Baek, I.-Y.; Kang, S.-T.; Han, W.-Y.; Shin, D.-C.; Moon, H.-P.; Kang K−H. Isolation and determination of anthocyanins in seed coats of black soybean (*Glycine max* (L.) Merr.). *J. Agric. Food Chem.* **2001**, *49*, 5848−5851.
(23) Dougall, D. K.; Baker, D. C.; Gakh, E. G.; Redus, M. A.; Whittemore, N. A. Anthocyanins from wild carrot suspension cultures acylated with supplied carboxylic acids. *Carbohydr. Res.* **1998**, *310*, 177−189.
(24) Giusti, M. M.; Ghanadan, L.; Wrolstad, R. E. Elucidation of the structure and conformation of red radish (*Raphanus sativus*) anthocyanins using one- and two-dimensional nuclear magnetic resonance techniques. *J. Agric. Food Chem.* **1998**, *46*, 4858−4863.
(25) Pale, E.; Nacro, M.; Vanhaelen, M.; Vanhaelen-Fastré, R. Anthocyanins from bambara groundnut (*Vigna subterranea*). *J. Agric. Food Chem.* **1997**, *45*, 3359−3361.
(26) Gonnet, J. F.; Fenet, B. "Cyclamen Red" colors based on a macrocyclic anthocyanins in carnation flowers. *J. Agric. Food Chem.* **2000**, *48*, 22−26.
(27) Skrede, G.; Wrolstad, R. E.; Durst, R. W. Changes in anthocyanins and polyphenolics during juice processing of highbush blueberries (*Vaccinium corymbosum* L.). *J. Food Sci.* **2000**, *65*, 357−364.
(28) Gao, L.; Mazza, G. Quantification and distribution of simple and acylated anthocyanins and other phenolics in blueberries. *J. Food Sci.* **1994**, *59*, 1057−1059.

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