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Determination of Anthocyanins from Camu-camu (*Myrciaria dubia*) by HPLC−PDA, HPLC−MS, and NMR

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Camu-camu [*Myrciaria dubia* (HBK) McVaugh] is a small fruit native to the Amazonian rain forest. Its anthocyanin profile has now been investigated for the first time. Fruits from two different regions of the São Paulo state, Brazil, were analyzed. The major anthocyanins were isolated by high-speed countercurrent chromatography. HPLC−PDA, HPLC−MS/MS, and ^{1}H NMR were used to confirm the identity of the main anthocyanins of camu-camu. Cyanidin-3-glucoside was identified as the major pigment in the fruits from both regions, representing 89.5% in the fruits produced in Iguape and 88.0% in those from Mirandópolis, followed by the delphinidin-3-glucoside, ranging between 4.2 and 5.1%, respectively. Higher total anthocyanin contents were detected in the fruits from Iguape (54.0 ± 25.9 mg/100 g) compared to those from Mirandópolis (30.3 ± 6.8 mg/100 g), most likely because of the lower temperatures in the Iguape region.

KEYWORDS: Anthocyanins; camu-camu; *Myrciaria dubia*; HPLC−PDA; HPLC−MS; high-speed countercurrent chromatography; climatic effects

INTRODUCTION

The camu-camu [*Myrciaria dubia* (HBK) McVaugh], belonging to the family Myrtaceae, is a bush native to the Amazonian rainforest with round berries averaging 2.5 cm in diameter (**Figure 1**). According to the Amazon Research National Institute (INPA), the camu-camu fruit is considered to have high nutritional value, especially because of its high levels of potassium and ascorbic acid (1380−1490 mg/100 g of pulp and 2050 mg/100 g of peel) (*1*, unpublished results). The fruit is considered to be one of the richest sources of vitamin C in Brazil, with contents higher than those found in acerola (1125−1790 mg/100 g of fresh pulp) (*2*, *3*) and in different varieties of cashew apple fruits from Northern and Southern Brazilian States (106−121 mg/100 g) (*4*).

When immature, the camu-camu fruit has a green color, and during the ripening process, the color changes from green to hues varying from red to purple, as a result of the presence of anthocyanins. Apart from their colorant properties, anthocyanins can promote many positive health benefits, such as reduction of the risk of coronary heart disease and diabetes and antioxidant and anticancer activities, and can also act on obesity control (*5*−*9*).

A large number of anthocyanins are found in foods, and high-performance liquid chromatography (HPLC) with a photodiode array detector (PDA) has been proven to be a useful tool for the characterization of food anthocyanins (*10*, *11*). However, identification of anthocyanins based solely on HPLC−PDA requires authentic standards, which are often difficult to obtain (*11*). Electrospray ionization mass spectrometry (MS) coupled to HPLC has emerged as a powerful technique for the characterization of biomolecules (*12*−*14*). Notwithstanding, nuclear magnetic resonance (NMR) spectroscopy represents the most powerful technique for structure elucidation, although it requires pure anthocyanin in the milligram scale. Because of the lack of anthocyanin standards, in many studies, HPLC quantification of anthocyanins has been carried out using only

Figure 1. Camu-camu (*Myrciaria dubia*) fruit.

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one reference compound (*15*, *16*). In some studies, the total anthocyanin content was quantified by a spectrophotometer using the absorption coefficient of the main anthocyanin and the proportion of each one was calculated from the HPLC areas (*17*−*19*).

Many factors are known to influence the anthocyanin composition. Different cultivars can be distinguished either by qualitative or quantitative differences, whereas the ripening process can be followed by increasing the amount of pigments (*12*, *20*). Some environmental factors, such as light and temperature, also affect the anthocyanin profile, as reported for red radishes (*21*). To our knowledge, there is no available survey regarding the anthocyanin composition of camu-camu. Because the São Paulo state is the main Brazilian consumer center, the composition of anthocyanins from camu-camu cultivated in two regions located in the São Paulo state was evaluated in this study.

MATERIALS AND METHODS

Samples. Fruits of camu-camu from two different plantations located in Mirandópolis and Iguape regions of the São Paulo state, Brazil, were analyzed. From each region, three batches, consisting of about 2 kg each, were collected in the harvesting season, from May to July 2003. The ripe camu-camu was manually peeled, and the obtained peel was further homogenized in a blender.

Camu-camu Characterization. The fruits from both regions were weighed and measured, and at the same time, the pH and Brix were verified in the pulp. The fruits from Mirandópolis were smaller (21.7 ± 3.0 mm) and lighter (7.6 ± 1.9 g) than the camu-camu produced in Iguape (24.0 ± 2.1 mm; 8.7 ± 2.1 g). The contents of soluble solids (°Brix) varied from 6.5 to 8.5 for the fruits from Mirandópolis and from 6.0 to 6.5 for the fruits from Iguape. The pH ranged from 2.6 to 2.9 and from 2.4 to 2.8 for the fruits from Mirandópolis and Iguape, respectively. These values are similar to those found for fruits of camu-camu produced in Peru (*22*).

Total Anthocyanin Determination. The anthocyanins were exhaustively extracted from 10 to 25 g of fresh peel of camu-camu, in duplicate, with 95% ethanol/1.5 N HCl (85:15, v:v), according to the procedure described by Francis (*23*). Absorbance was measured in a UV−vis spectrophotometer at a λ_{max} of 520 nm, and the total anthocyanin content was calculated by using the absorption coefficient of 982, corresponding to cyanidin-3-glucoside in ethanol/1.5 N HCl (*24*, *25*).

Extraction, Purification, and Isolation of Anthocyanins. The crushed peel was previously freeze-dried, followed by the extraction of 100 g of the lyophilized material with 1000 mL of 0.5% HCl in methanol overnight at 5 °C, in darkness. The slurry was filtered, and the solids were washed with additional 0.5% HCl in methanol. The methanol extracts were combined and concentrated in a rotary evaporator (T < 38 °C) to obtain the crude extract.

A total of 30 mL of the crude extract was dissolved up to 50 mL with distilled water and partially purified by chromatography (CC) on a 60 × 3 cm Amberlite XAD-7 resin open column, according to the procedure described by Degenhardt et al. (*26*). After CC, the removal of free sugars from the partially purified extract was confirmed by paper chromatography. HPLC−PDA analysis showed that large amounts of nonanthocyanin flavonoids were also removed by CC. The partially purified extract was freeze-dried, and 500 mg of the lyophilized extract was dissolved in water/acetonitrile/formic acid (87:3:10, v/v/v) and fractionated by high-speed countercurrent chromatography (HSCCC).

HPLC−PDA. The crude extract (250 μL) was diluted in 1.5 mL of 4% phosphoric acid/acetonitrile (85:15, v:v) immediately before analysis by HPLC−PDA to obtain the relative anthocyanin distribution in the samples. The HPLC−PDA was equipped with a model 600 quaternary solvent delivery system (Waters, Milford, MA), an on-line degasser, Rheodyne injection valve with a 20 μL loop, and an external oven coupled to the model 996 PDA detector (Waters). The data acquisition and processing were performed by the Millenium Waters software. For all samples, anthocyanins separations were carried out on a 250 × 4.6 mm i.d., 5 μm C_{18} Shim-pack CLC-ODS column (Shimadzu, Canby, OR) using as mobile phase a linear gradient of acetonitrile/4% phosphoric acid from 85:15 to 20:85 (v/v) in 25 min at a flow rate of 1 mL/min and a column temperature set at 25 °C. The chromatograms were processed at 520 nm, and the spectra were obtained between 250 and 600 nm.

HSCCC. A high-speed model CCC-1000 manufactured by Pharma-Tech Research Corp. (Baltimore, MD) was equipped with three preparative coils, connected in series (tubing diameter of 2.6 mm and total volume of 850 mL). The separation was run at a speed of 890 rpm. A solvent system consisting of *tert*-butyl methyl ether (MTBE)/*n*-butanol/acetonitrile/water (2:2:1:5, v/v/v/v) acidified with 0.1% trifluoroacetic acid (TFA) was used. The elution mode was head to tail, with the less dense layer being the stationary phase. The flow rate was set at 3.5 mL/min and delivered by a BT 3020 HPLC pump (Jasco, Gross-Umstadt, Germany). Freeze-dried XAD-7 extract (500 mg) was dissolved in a 1:1 mixture of light and heavy phases (20 mL) and injected into the system by a loop injection. Fractions of 10 mL were collected with a fraction collector. Elution was monitored with a K-2501 UV/vis detector (Knauer, Berlin, Germany) at 520 nm.

HPLC−MS/MS. The two major fractions separated by HSCCC (ca. 5 mg) were dissolved in 1 mL of water/acetonitrile/formic acid (87:3:10, v/v/v) and analyzed by an HPLC system consisted of a model G1328A binary pump (Agilent, Palo Alto, CA) equipped with a Rheodyne injection valve with a 20 μL loop, coupled to a model L 4000 UV/vis detector (Merck) monitored by a Chromatopac C-R6A integrator (Shimadzu) and to a Bruker Esquire mass spectrometer with electrospray ionization, and data-processed by Esquire NT 4.0 software (Bruker). The MS/MS parameters were as follows: positive mode; capillary voltage, 2500 V; end plate offset, 2000 V; capillary exit, 110 V; skimmer 1, 20 V; skimmer 2, 10 V; dry gas (N_2) temperature, 325 °C; flow, 11 L/min; nebulizer, 60 psi; and scan range m/z, 200−2500. The separations were performed on a 250 × 4.6 mm, 5 μm, RP-12 Synergi MaxRO column (Phenomenex, Germany). Two different solvent systems were used as a mobile phase: system I, consisting of solvent A (87:3:10 water/acetonitrile/formic acid, v/v/v) and solvent B (40:50:10 water/acetonitrile/formic acid, v/v/v), at a flow rate of 0.5 mL/min and a linear gradient of A/B from 94:6 to 80:20 in 20 min, to 60:40 in 15 min, to 40:60 in 5 min, to 10:90 in 5 min, and then back to the initial conditions.

Preparative HPLC. The fractions eluted from HSCCC were purified by HPLC using a model 64 binary pump (Knauer) equipped with a Rheodyne injection valve with a 500 μL loop, coupled to a Knauer UV/vis detector. The separation was carried out on a 250 × 10 mm, 5 μm RP-18 Luna (18/2) column, with water/acetonitrile/formic acid (15:80:5, v/v/v) as the mobile phase, at a flow rate of 4 mL/min to afford a purified fraction 4 (3 mg) and fraction 5 (7 mg).

Proton NMR. All spectra experiments were recorded on a Bruker AMX 300 spectrometer (300.13 MHz) at 302.9 K in a mixture of methanol-d_4/TFA-d_1 (19:1, v/v). Assignments were made on the basis of spectroscopy data published in the literature (*27*−*29*).

Statistical Analysis. To evaluate the differences in the anthocyanin contents between the regions and within the batches, analysis of variance was conducted using one-way ANOVA (Origin 5.0).

RESULTS AND DISCUSSION

Identification of Anthocyanins. The anthocyanin profiles from camu-camu fruits collected in Mirandópolis and Iguape regions were very similar, as shown by the HPLC−PDA chromatograms in **Figure 2**. Differences were only found in the presence of minor peaks (the sum corresponds to less than 1% of total area), which could not be identified. The anthocyanin peaks were identified by their HPLC elution order, UV/vis, and mass spectrometric characteristics (**Table 1**) from a comparison with the literature data (*10*, *12*, *13*, *26*).

The partially purified extract was fractionated by HSCCC, resulting in five fractions (F1−F5). However, only the two last fractions (F4 and F5) were further analyzed by HPLC−MS/

Table 1. Chromatographic, Spectroscopic, and Mass Spectrometric Characteristics of the Anthocyanins from Camu-camu, Obtained by HPLC–PDA and HPLC–MS

peak number[a]	t_R HPLC–PDA (min)	t_R HPLC–MS/MS (min)	λ_{max} (nm)	A_{440}/A_{max} (%)	M^+ (*m*/*z*)	M^+–X (*m*/*z*)	compounds
1	4.8	15.3	282, 340, 526	28	465	303	delphidinin-3-glucoside
2	6.4	19.4	278, 326, 517	32	449	287	cyanidin-3-glucoside
3	7.7	31.9	277, 380, 520	31	477	287	cyanidin based
4	9.4		277, 334, 528	27			not identified 1
5	10.9		273, 322, 527	25			not identified 2

[a] Numbered according to the chromatograms shown in **Figure 2**.

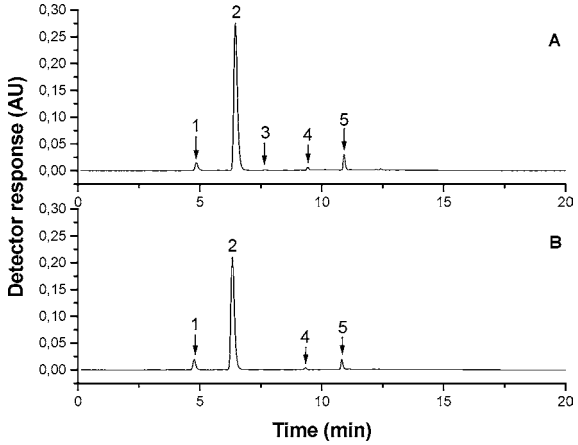


Figure 2. Chromatograms, obtained by HPLC–PDA, of the crude extract of anthocyanins from camu-camu produced in Mirandópolis (A) and Iguape (B) regions. Peak identification is given in **Table 1**.

MS, because of the very low concentration of anthocyanins observed in fractions F1−F3, as can be seen in **Figure 3**.

Fraction 4 was composed of two main compounds (**Figure 4A**); one eluted at 15.3 min, and the other one that eluted at 37.6 min did not show absorbance in the visible region. The peak eluted at 15.3 min had an apparent molecular ion $[M]^+$ at *m*/*z* 465 and a fragment ion with 303 amu, which corresponds to the molecular ion of the delphinidin aglycone, as a result of the loss of a hexose (162 amu). The visible maximum absorbance wavelength (λ_{max}) at 526 nm (peak 1 in **Table 1**) supported the identity of the anthocyanidin and the absence of acylated cinnamic acids. The NMR spectra unequivocally confirmed the identity of peak 1 as delphinidin-3-glucoside, compared to the chemical shifts already published for this anthocyanin (*28*).

The HPLC−MS chromatogram (**Figure 4B**) showed that F5 was composed of two anthocyanins. Although these pigments had different molecular ions, at *m*/*z* 449 for the peak eluted at 19.4 min and at *m*/*z* 477 for the one eluted at 31.9 min, a fragment ion with 287 amu was observed for both compounds, which corresponds to the molecular ion of the cyanidin moiety. According to the λ_{max}, the major peak at t_R of 19.4 min, corresponds to peak 2 in **Table 1** and **Figure 2**, whereas the peak at 31.9 min in **Figure 4B** is probably the very minor compound corresponding to peak 3 in **Figure 2A**, which showed λ_{max} at 520 nm. The presence of a hexose in peak 2 was also indicated by the loss of 162 amu from its molecular ion, and the identity was confirmed by NMR as cyanidin-3-glucoside by comparison with literature data for this anthocyanin (*29*). On the other hand, the loss of 190 amu from the molecular ion of peak 3 showed the presence of a compound not commonly found in anthocyanins. Owing to the small amount available for ^{1}H NMR measurement, the structure of peak 3 could not be identified.

The amount of compounds **4** and **5** separated by HPLC−PDA were not sufficient for HPLC−MS/MS analysis nor for chemical reactions. Their λ_{max} at 527−8 nm indicates that the aglycone might be delphinidin, petunidin, or malvidin. The A_{440}/A_{max} ratio suggests glycosylation only at C-3, and the low

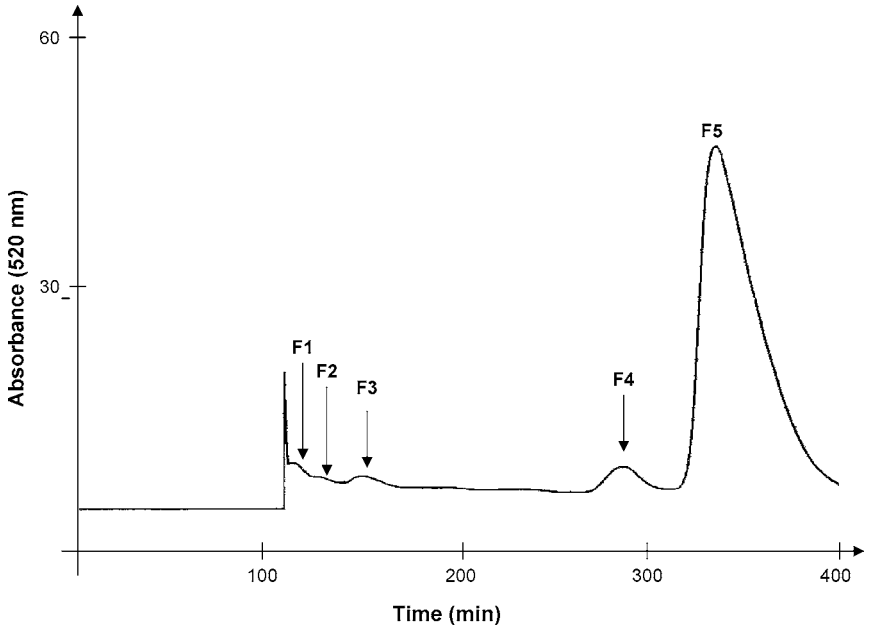


Figure 3. HSCCC separation of the partially purified camu-camu extract, obtained from the XAD-7 column.

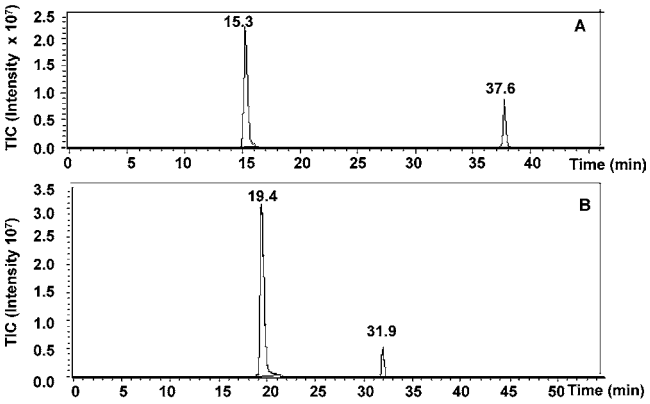
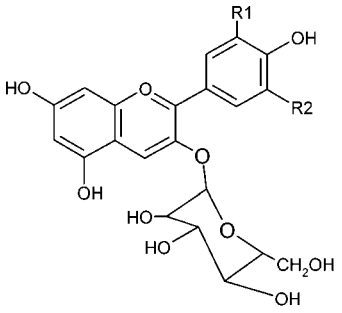


Figure 4. HPLC–MS TIC chromatograms of (A) F4 and (B) F5.



cyanidin-3-glucoside: R_1 = OH, R_2 = H
delphinidin-3-glucoside: R_1 = R_2 = OH

Figure 5. Chemical structures of the main anthocyanins of camu-camu.

Table 2. Total Anthocyanin Content and Relative Composition of Anthocyanins from Camu-camu

anthocyanin	peak area (%)[a] Iguape	Mirandópolis
delphidinin-3-glucoside	4.2 ± 1.5	5.1 ± 1.0
cyanidin-3-glucoside	89.5 ± 1.7	88.0± 1.0
cyanidin based	not detected	0.2 ± 0.2
not identified 1	0.6 ± 0.2	1.2 ± 0.3
not identified 2	5.7 ± 0.5	5.5 ± 0.1
total content (mg/100 g)[a]	54.0 ± 25.9	30.3 ± 6.8

[a] Mean and standard deviation of three batches.

relative absorbance at the 310−320 nm range indicates that these anthocyanins were not acylated with cinnamic acids.

In fact, the amount of nonanthocyanic flavonoids present in camu-camu was too high, not allowing the isolation of the minor anthocyanins.

Influence of Climatic Conditions on the Anthocyanin Composition. Cyanidin-3-glucoside was the major anthocyanin in camu-camu fruits from both regions, representing 89.5% of the total area at 520 nm in the fruits from Iguape and 88.0% in the fruits from Mirandópolis, whereas, delphinidin-3-glucoside was 4.2% in the fruits from Iguape and 5.1% in those produced in Mirandópolis (**Figure 5**). The other minor anthocyanins (peaks 4 and 5) represented together about 6.3 and 6.7%, respectively, for those regions. The cyanidin derivative (peak 3) was only found in small amounts in two samples from Mirandópolis (**Table 2**). Cyanidin-3-glucoside was also reported as the major anthocyanin of jabuticaba (*Myrciaria cauliflora*) (*30*), a tropical fruit that belongs to the same botanical family as camu-camu.

The total anthocyanin content in the camu-camu fresh peel was found to be 54.0 ± 25.9 and 30.3 ± 6.8 mg/100 g for fruits from Iguape and Mirandópolis, respectively (**Table 2**). Although the anthocyanins levels found in the fruits from Iguape were higher than those observed in the camu-camu from Mirandópolis, this difference was not significant ($p = 0.0866$). In contrast, when the variability within lots was considered, p values became statistically significant, because anthocyanin concentrations varied from 23.4 to 86.7 mg/100 g in fruits from Iguape ($p = 0.0008$) and from 22.8 to 36.0 mg/100 g ($p = 0.0072$) for the camu-camu produced in the Mirandópolis region. This variation is probably due to maturity differences between the batches. In other berries, for instance, raspberries, the total anthocyanin content has also increased during ripeness (*21*).

During the month of May, which preceded the harvest, higher maximum and minimum average temperatures were registered in Mirandópolis (31.5 and 17.2 °C) in comparison to those in Iguape (25.9 and 14.5 °C). Besides, the month of May presented higher rainfall in Iguape (22.5 mm) than in Mirandópolis (12.5 mm), indicating higher sun incidence in Mirandópolis, considering that Iguape presented more cloudy days. These weather conditions indicate that the lower temperature or higher rainfall might be the main factors responsible for the higher anthocyanin contents found in the camu-camu from Iguape. A correlation between low temperatures and increased anthocyanin contents was previously reported by Giusti et al. (*21*).

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