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Anthocyanin Identification, Vitamin C Content, and Antioxidant Capacity of Acerola (*Malpighia emarginata* DC) Juices

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The main objectives of this study were to identify and quantify anthocyanin content in acerola juices and to determine the antioxidant capacity of the juices. Anthocyanin content was analyzed by a high-performance liquid chromatograph connected to a diode array and mass spectrometry detectors (HPLC–DAD–MS/MS). The antioxidant capacity was determined by the 2,2´-diphenyl picryl hydrazyl (DPPH) and the oxygen radical absorbance capacity (ORAC) assays. The ascorbic acid content was determined by HPLC. The frozen single-strength acerola juice presented 53.60 mg total anthocyanin for 100 mL with predominance of cyanidin 3-rhamnoside as individual anthocyanin. The frozen single-strength juice showed 306.77 and 85.29 mmole Trolox equivalent for 100 mL juice respectively for DPPH and ORAC antioxidant values. The ascorbic acid (vitamin C) content was found to be 4440 mg for 100 mL single-strength juice. Anthocyanin and ascorbic acid seemed to be the major contributors of the antioxidant capacity of the juice. Overall, this study shows that acerola juice is good source of anthocyanin pigment, an outstanding source of ascorbic acid and a potential good source of antioxidant.

Acerola (*Malpighia emarginata* DC. Syn. *Malpighia punicifolia*, L.; *Malpighia glabra* L.) is a plant originating in the West Indies that has spread to South America, including Brazil, and Central America due to its good adaptation to soil and climate. The perennial shrub bears a red fruit known by the common names Barbados cherry or West Indian cherry, especially in English-speaking Caribbean countries. However, the name acerola, as it is called in Puerto Rico, is becoming more and more popular (Sean-Carrington and King, 2002).

Acerola trees may reach an average height of 3–5 m (9.84–16.40 ft) with a short slender trunk that is 0.5–1 m (1.64–3.28 ft) high, and 7–10 cm (2.75–3.94 inches) in diameter. The fruits become mature 3–4 weeks after flowering. The mature fruit is very perishable and last only 2–3 d at room temperature (Vendramini and Trugo, 2000). The fruit is climacteric with a very high respiratory rate (900 mL CO_2 $kg^{-1}{\cdot}h^{-1}$) but with a low rate of peak ethylene production (3 μL C_2H_4 $kg^{-1}{\cdot}h^{-1}$); the high respiration rate is thought to be in part responsible for its perishable nature (Sean-Carrington and King, 2002). Fruit matures from green to red but can be a yellowish-red at complete maturity. The sweetness of the fruit depends on the variety; some varieties are tart and acid, while others are considerably sweet. The sweeter varieties tend to be more popular in the fresh markets and juicing operations (Johnson, 2003).

Acerola had long been grown as an ornamental plant in subtropical areas, until the publication in 1946 of the first report of its very high vitamin C content (Asenjo, 1980). It is written in a publication of Johnson (2003) that: "a 180-mL glass of fresh acerola juice with a potency of 35 mg/mL ascorbic acid can contain as much vitamin C as 14 L of orange juice." Matta et al. (2004) added that "the consumption of three fruits per day satisfies the vitamin C recommended dietary allowance for an adult." The current recommendation in the United States for vitamin C daily is 60 mg based on a caloric intake of 2,000 calories, for adults and children four or more years of age (FDA, 2008). In addition to its very high vitamin C content, acerola contains anthocyanins and non-anthocyanin phenolic compounds (De Rosso and Mercadante, 2005; Vendramini and Trugo, 2000, 2004). Because vitamin C, anthocyanins, and non-anthocyanin phenolics are good antioxidants, these attributes make the fruit very attractive for the world's health food market and for domestic consumption, especially in a very diverse state like Florida. The problem is that the antioxidant capacity, the anthocyanin profile, as well as the quantification of the individual anthocyanin are not well investigated.

Recently, the anthocyanins in acerola have been characterized. However, there are some discrepancies in the results. De Rosso et al. (2008) extracted anthocyanins from two different acerola varieties (Waldy and Olivier) with 0.5% HCl in methanol and analyzed them by HPLC–PDA–MS/MS. They found that cyanidin-3-rhamnoside with 76% to 78% of the total anthocyanin represented the major anthocyanin in acerola followed by pelargonidin-3-rhamnoside (13% to 16%), cyanidin (6% to 8%), and pelargonidin (2% to 3%). While those results seem to be more or less consistent with the results of Hanamura et al. (2005), who identified cyanidin-3-α-O-rhamnoside and pelargonidin-3-α-O-rhamoside in acerola by NMR, there was less qualitative agreement with another study that utilized different extraction and analytical methods. Vendramini and Trugo (2004) identified three types of anthocyanins in acerola by means of chromatographic and spectral data, finding only malvidin 3,5-diglucoside, cyanidin-3-glucoside, and pelargonidin. The objectives of this research were to identify anthocyanin, to determine the antioxidant capacity, and to quantify ascorbic acid and dehydroascorbic acid (vitamin C) in acerola juices.

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Materials and Methods

Juices. Frozen single-strength and frozen concentrated acerola juices were purchased from ITI Tropicals (Lawrenceville, NJ) and stored at –20 °C until needed.

Chemicals. Cyanidin chloride and pelargonidin chloride were purchased from Sigma-Aldrich (St. Louis, MO). AAPH [2,2´-azobis (2-amidinopropane)] was a product of Wako Chemicals Inc.

Anthocyanin extraction. The anthocyanin extraction and analysis was performed according to Wu et al. (2004) with some modifications. One gram of freeze-dried powder was weighed and placed into a 50-mL centrifuge tube and extracted with 15 mL of methanol : water : formic acid (MWF) 85:15:0.5 (v/v). The tube was vortexed for 30 s, sonicated for 5 min, and shaken twice during sonication to resuspend the sample. The tube was kept at room temperature for 10 min, being vortexed for 30 s after 5 min, centrifuged at 4550 g_n for 10 min and the supernatant removed. The sample was extracted two more times with 10 mL of MWF each time using the same procedure. The supernatants were combined and filtered using a 0.45-µm Teflon syringe filter (Fisher Scientific) prior to injection (30 µL) to the HPLC system.

HPLC–DAD–ESI–MS^n analysis. Chromatographic analyses were performed on an Agilent 1200 series HPLC system (Agilent, Palo Alto, CA) equipped with an autosampler/injector and diode array detector (DAD). A Zorbax Stablebond Analytical SB-C18 column (4.6 mm × 250 mm, 5 µm, Agilent Technologies, Rising Sun, MD) was used for the separation of anthocyanin. Elution was performed using mobile phase A (0.5% formic acid aqueous solution) and mobile phase B (methanol). UV-VIS spectra were scanned from 220 to700 nm on a diode array detector (DA) with detection wavelengths of 280, 360, and 520 nm. The flow rate was 1 mL·min^{-1}, and the following linear gradient was used: 0–2 min, 5% B; 2–10 min, 5% to 20% B; 10–15 min, 20% B; 15–30 min, 20% to 30% B; 30–35 min, 35% B; 35–50 min, 35% to 45% B; 50–55 min, 45%; 55–60 min, 45% to 5% B, and 65–68 min, 5% B. Electrospray mass spectrometry was performed with a HCT ion trap mass spectrometer (Bruker Daltonics, Billerica, MA). Column effluent was monitored in the positive ion mode of the instrument. Other experimental conditions for the mass spectrometer were as follows: nebulizer, 45 psi; dry gas, 11.0 L·min^{-1}; dry temperature, 350 °C; ion trap, scan from m/z 100 to 2200; smart parameter setting (SPS), compound stability, 50%; trap drive level, 60%. The mass spectrometer was operated in Auto MS^2 mode. MS^2 was used to capture and fragment the most abundant ion in full scan mass spectra.

Determination of Total Anthocyanin

The determination of total anthocyanin was done according to an AOAC method (Lee et al., 2005). Briefly, 1 mL of sample was placed in a 25-mL volumetric flask and diluted to volume with pH 1 buffer (0.2 M HCl + 0.2 M KCL). One milliliter of sample was placed in another 25-mL of volumetric flask and diluted to volume with pH 4.5 acetate buffer (0.1 M acetic acid + 0.1 M sodium acetate trihydrate). The flasks were allowed to stand at room temperature for 30 min, and then centrifuged. After the centrifugation the supernatant was removed and the absorbance was measured at 510 nm. To correct the turbidity of the samples, the absorbance at 700 nm was also measured. Results were calculated with the following formulas and expressed as cyanidin-3-glucoside equivalent:

$$A = (A_{510} - A_{700})_{pH\ 1.0} - (A_{510} - A_{700})_{pH\ 4.5}$$

Monomeric anthocyanins = (A × molecular weight × dilution factor)/(ε × l), molecular weight = 449.2 g·mol^{-1}, ε = molar extinction coefficient: 26900 M^{-1}·cm^{-1}, l = optical path of cuvette (cm). Final anthocyanin concentration was expressed as milligrams cyanidin-3-glucoside per liter of juice.

Determination of total phenolics by the Folin-Ciocalteu assay. The juice was diluted to the appropriate concentration prior to the analysis. The total phenolic content was determined by using a modified method of Singleton and Rossi (1965). The samples were mixed with diluted Folin-Ciocalteu reagent and 15% sodium carbonate. Absorption at 765 nm was measured in a microplate reader (SPECTRAmax 190, Sunnyvale, CA, USA) after incubation for 30 min at room temperature. The results were expressed as milligrams of gallic acid (GAE) equivalents per 100 mL of juice using a standard curve generated with 500–1000 mg L^{-1} GA

Oxygen radical absorbance capacity (ORAC). The ORAC assay was conducted according to a modified method of Sandhu and Gu (2010). The assay was conducted on a Spectra XMS Gemini plate reader (Molecular Devices, Sunnyvale, CA). In summary, 50 µL of standard and samples were added to the designated wells of a 96-well black plate. This was followed by the addition of 100 µL of fluorescein (20 nM). The mixture was incubated at 37 °C for 7 min before the addition of 50 µL of the free radical (AAPH). The fluorescence was monitored using 485 nm excitation and 530 nm emission at 1 min intervals for 40 min. Trolox was used to generate a standard curve. The antioxidant capacity of the juices was expressed as micromole Trolox equivalent (TE) per liter.

DPPH (2-2´-Diphenyl picryl Hydrazyl) Assay. The DPPH assay was conducted according to Sandhu and Gu (2010). Briefly 50 µL sample was mixed with 950 µL DPPH solution. The mixture was incubated for 60 min in dark. Fifty microliters of Trolox solution was added to 950 µL DPPH solution to generate a standard curve. Fifty microliters of Methanol were mixed with 950 µL DPPH working solution and used as a control. After the incubation, 200 µL of mixture was pipetted into a 96-well plate and the plate was read in a spectrophotomer at 515 nm. The result was expressed as micromole Trolox equivalent (TE) for 100 mL of juice.

Determination of Ascorbic Acid (AA) and Dehydroascorbic Acid (DHAA)

Sample preparation. The sample preparation was done according to Lee and Coates (1999) and Gokmën et al. (2000) with necessary modifications. Twenty-five milliliters of juice were placed into a 50-mL centrifuge tube and centrifuged for 5 min at 2000 g_n. After centrifugation, 10 µL of the supernatant (clear juice) was diluted with 9990 µL deionized water (DF 1000). This diluted juice was filtered through a 0.45-µm disposable filter (Whatman). This clarified and filtered sample was divided into two parts. One part was synchronously analyzed for AA content by injecting 50 µL into the HPLC system. Dithiothreitol (DDT) was added to the other part at a concentration of 0.2 mg·mL^{-1}. The solution was allowed to stay away from light for 90 min. According to Gokmën et al. (2000), a concentration as low as 0.1 mg of DDT per milliter of test solution is sufficient to convert all DHAA to AA in fruits like lemon, orange, grapefruit, kiwi or strawberry. Since acerola contains more ascorbic acid than those fruits, 0.2 mg mL^{-1} was considered. After conversion of DHAA was achieved, the sample was analyzed for its total AA (TAC) by HPLC.

The frozen acerola concentrate (FCA) (39 °Brix) was first diluted to single-strength (6.5 °Brix) and the preparation was performed as described above for frozen single strength (FSA).

HPLC procedure. A Diomex model P680 liquid chromatograph (Sunnyvale, CA) was used. It was equipped with a Diomex model AS-100 automated sample injector, and a Diomex model 100 photodiode array (PDA) detector set at 210 and 254 nm. A Diomex model Acclaim® 120, 250 × 4.6 mm I.D, C18 (5 µm) column operated at ambient temperature was used. A 0.2 M potassium phosphate monobasic (KH_2PO_4) (Merck) in deionized water solution was used as the mobile phase with a flow rate of 1.0 mL/min. The pH of the mobile phase was adjusted to 2.4 with phosphoric acid (H_3PO_4).

Ascorbic acid and dehydroascorbic acid standard curve. A stock solution of ascorbic (AA) acid was prepared daily by dissolving 10 mg of AA in 100 mL deionized water which gives a concentration of 100 µg·mL^{-1}. This stock was further diluted with deionized water to obtain final concentrations of 20, 30, 40, 60, 70, and 80 µg·mL^{-1}.

Statistical analysis. Each experiment was conducted in triplicate; correlation analysis was performed to explain potential contribution of ascorbic acid and anthocyanin to the antioxidant capacity. The correlation analysis was performed using the Statistical Analysis System (SAS) software.

Results and Discussion

Identification of anthocyanin. Two peaks were detected in the crude extracts of anthocyanin from acerola juice measured by HPLC-DAD (Fig. 1). The characteristics of the separated peaks are presented in Table 1. The molecular ion of peak 1 was found at m/z 433 and a fragment with m/z 287 indicated that the aglycon cyanidin was glycosylated with a deoxyhexose due to the loss of 146 units of mass, then peak 1 was identified as cyanidin 3-rhamnoside. The confirmation was done by the HPLC co-elution of peak 1 with pure standard. Peak 2 was identified as pelargonidin 3-rhamnoside, taking in consideration the molecular ion at m/z 417 and a fragment ion at m/z 271 ([M146]$^+$) corresponding to the aglycon pelargonidin, by the loss of one molecule of a deoxyhexose. The identification of these two anthocyanin hexosides (cyanidin-3-rhamnoside and pelargonidin-3) partly agreed with Hanamura et al. (2005). In contrast, no free anthocyanin aglycons (anthocyanidin) were identified under our experimental conditions.

One of the objectives of this study was to quantify the identified anthocyanins. With 2829 mg·g^{-1} fresh weight, cyanidin-3-rhamoside is by far the most predominant kind of anthocyanin in acerola juice under the conditions of this study (Table 1). Total free anthocyanin was also determined in the frozen single-strength

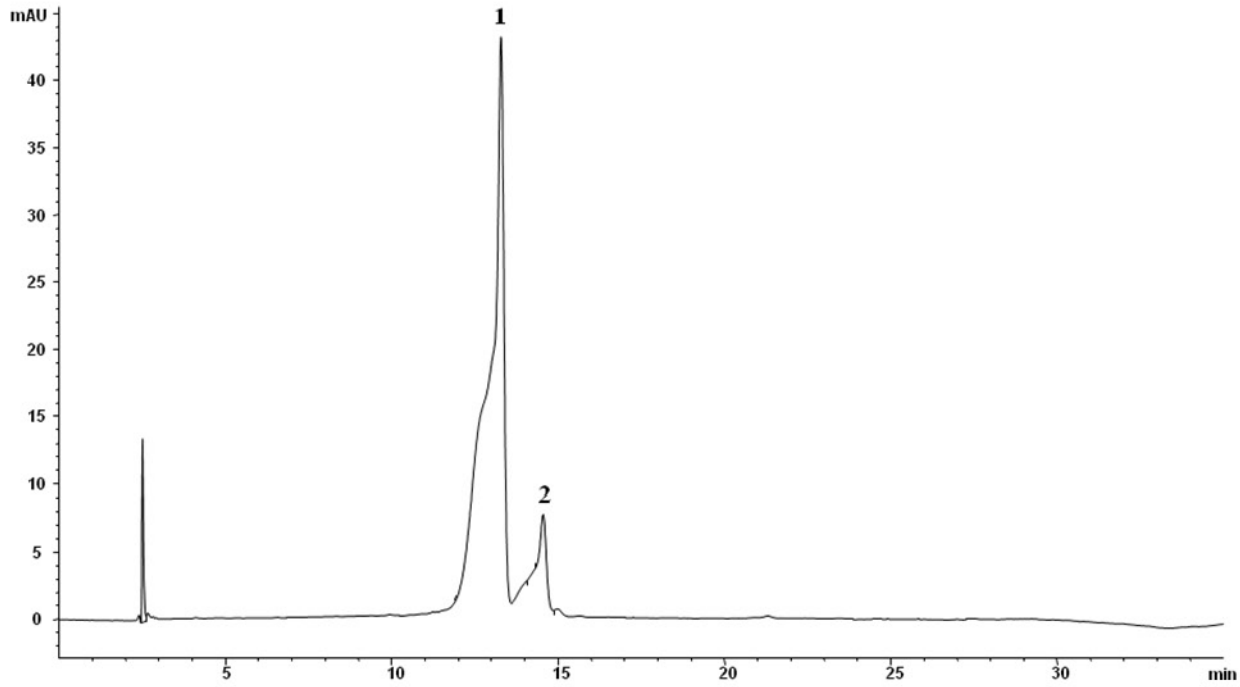

Fig. 1. HPLC-DDA chromatogram from the crude extract of anthocyanin from acerola juice at 520 nm. Peak identification is given in Table 1.

Table 1. Chromatographic UV-VIS and mass spectroscopy characteristics of the anthocyanin from acerola, obtained by HPLC-DDA-MS/MS.

Peak	t_R (min)	M^+ (m/z)[z]	MS^2 (m/z)[y]	Content[x]	Compound
1	13.281	433	287 (433–146)	2829	Cyanidin-3-rhamnoside
2	14.553	417	271 (417–146)	48.08	Pelargonidin-3-rhamnoside

[z]M^+ (m/z): Molecular ion/parent ion.
[y]MS^2: Fragment/daughter ion.
[x]Content in microgram anthocyanin per gram fresh weight acerola.

Table 2. Antioxidant capacity, total phenolics, and total anthocyanins in the acerola juices.

Assays and units	Frozen SS juice
DPPH (µmole TE/100 mL)	306.77 ± 15 (274)[z]
Total phenolics (mg GAE/100 mL)	256.24 ± 24 (140)
ORAC (µmole TE/L)	8529 ± 25
Total anthocyanin (mg/L)	53.60 ± 5

[z]Information in parentheses is from Medzadri et al. (2008).

acerola juice using a standardized AOAC method (Lee et al., 2005). The result shows that 1 L of acerola juice contains 53.50 mg anthocyanin (Table 2). This amount of total free anthocyanin is higher than that found in plum (20 mg/L) (Pazmiño-Durán, 2001). This indicates that acerola could be a used as a good source of natural anthocyanin pigment.

Ascorbic acid and dehydroascorbic acid contents. Ascorbic acid (AA) was determined according an HPLC method developed by Gokmën et al. (2000) with some modifications. Dehydroascorbic (DHAA) acid was calculated by subtraction of the initial AA content from the total after conversion of DHAA to AA using dithiothreitol as a chemical reductant. The result shows that100 mL of frozen single strength acerola juice contains 4440 mg AA and 41 mg DHAA. This suggests that acerola is an outstanding source of vitamin C. The frozen concentrate was diluted to single strength prior to AA and DHAA analysis. The result shows that the concentrated juice diluted to single strength contains 921 mg dehydoascorbic acid (Table 3), nearly four times as less as the amount found in the single strength juice. However, the amount of vitamin C in the diluted concentrate is much higher than the amount of vitamin C found in fruit juices traditionally used as sources of vitamin C such as orange juice.

Antioxidant capacity. The DPPH scavenging capacity of the frozen single-strength acerola juice was found to be 306.77 mmol TE for 100 mL juice (Table 2). This value was higher than that reported by Medzadri et al. (2008). The oxygen radical absorbance capacity (ORAC) value was also investigated. Only the result obtained for the single-strength juice is reported in this paper. The ORAC value was found to be 8529 µMTE per liter of juice. This relatively high ORAC value coupled with the DPPH scavenging power suggest that acerola may be a good source of antioxidants.

Correlation analysis. To understand the contribution of ascorbic and anthocyanin to the overall antioxidant capacity of acerola juice which was the third objective of this study, a correlation analysis was performed. A high positive correlation was found between the ascorbic acid (vitamin C) content and ORAC as well as a high positive correlation between total anthocyanin content and ORAC (Table 4) suggesting that both ascorbic acid and anthocyanin have a significant contribution to the antioxidant capacity of the juice. Table 4 also shows that the correlation between the DPPH and total anthocyanins as well as the correlation between DPPH value and total phenolics were rather low (0.68 and 0.69, respectively). This suggests that besides anthocyanin, the non anthocyanin phenolic compounds may also have a significant contribution to the overall antioxidant power of acerola juice. The highly positive correlation between DPPH and ORAC suggests that both methods are suitable to measure the antioxidant capacity of acerola juice.

Table 3. Ascorbic acid and dehydroascorbic acid contents of acerola juices.

	Vit. C content (mg per 100 mL)	
Samples	AA[z]	DHAA[y]
Frozen SS[x] juice	4440 (944–1370)	41
Frozen concentrate, diluted to SS[w]	921	25

[z]AA: Ascorbic acid.
[y]DHAA: Dehydroascorbic acid.
[x]SS: Single-strength; information in parentheses is from Medzadri et al. (2008).
[w]Concentrate diluted to 7 °Brix.

Table 4. Correlation analysis among different parameters.

	TACY[z]	Vit C[y]	ORAC[x]	DPPH[w]	TP[v]
TACY	1.00				
Vit C	0.90	1.00			
ORAC	0.90	0.99	1.00		
DPPH	0.68	0.83	0.98	1.00	
TP	0.69	–0.65	0.75	0.81	1.00

[z]TACY: Total free anthocyanins.
[y]VitC: Vitamin C content.
[x]ORAC: Oxygen radical absorbance capacity assay.
[w]DPPH: 2,2´-diphenyl, picryl hydrazyl.
[v]TP: Total phenol.

Summary Conclusion

In summary, two anthocyanin hexosides (cyanidin-3-rhamnoside and pelargonidin-3-rhamnoside) were identified in acerola juice; no free aglycone was found in this study. Statistically, ascorbic acid and anthocyanin appear to have significant contribution to the antioxidant capacity of acerola juice. However, the contribution of the non anthocyanin phenolic compounds to the total antioxidant capacity needs to be taken into consideration. Overall, this study shows that acerola juice is a good source of anthocyanin pigment, an outstanding source of ascorbic acid and a potential good source of antioxidant.

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