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Antioxidant Metabolism during Fruit Development of Different Acerola (*Malpighia emarginata* D.C) Clones

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ABSTRACT: The present research work describes the major changes in the antioxidant properties during development of acerola from five different clones. Ripening improved fruit physicochemical quality parameters; however, total vitamin C and total soluble phenols (TSP) contents declined during development, which resulted in a lower total antioxidant activity (TAA). Despite the decline in TSP, at ripening, the anthocyanin and yellow flavonoid content increased and was mainly constituted of cyanidin 3-rhamnoside and quercetin 3-rhamnoside, respectively. The activities of oxygen-scavenging enzymes also decreased with ripening; furthermore, the reduction in vitamin C was inversely correlated to membrane lipid peroxidation, indicating that acerola ripening is characterized by a progressive oxidative stress. Among the studied clones, II47/1, BRS 237, and BRS 236 presented outstanding results for vitamin C, phenols, and antioxidant enzyme activity. If antioxidants were to be used in the food supplement industry, immature green would be the most suitable harvest stage; for the consumer's market, fruit should be eaten ripe.

KEYWORDS: *acerola, antioxidant, vitamin C, enzymes, ripening*

INTRODUCTION

Originating in Central America, acerola or Babados cherry (*Malpighia emarginata* D.C) is best known as an extremely rich source of vitamin C. Thereby, acerola became popular as a remarkable functional food among health-conscious consumers that prefer vitamin C from natural sources, which is absorbed better by the human organism than synthetic ascorbic acid.[1] However, in addition to vitamin C, acerola contains other functional constituents that are beneficial to human health such as phenols.[2] Therefore, acerola has a great potential in the food industry as a source of vitamin C and phenol to be used as nutritional supplements or as additives to increase the nutritional value of other products.[3,4]

Throughout development, fruit undergo several biochemical, physiological, and structural changes that will determine their antioxidant properties. Studies evidenced reactions involving reactive oxygen species (ROS) are intrinsic to fruit ripening and senescence because they promote an oxidative process that contributes to a general deterioration of cellular metabolism.[5,6] ROS are reduced and rendered harmless by antioxidants, which play an essential role in not only keeping production and scavenging of ROS in equilibrium during nonstressed conditions but also determining the level of oxidative stress.[6] Therefore, the increase in ROS levels during fruit ripening can be attributed to changes in antioxidant component levels. The antioxidant defense system is constituted of a complex array of enzymatic and nonenzymatic compounds, which should not be studied individually due to probable synergistic and antagonistic interactions between them.

The beneficial properties of fruit for human health maintenance and illness prevention have been previously attributed to antioxidants.[7−9] However, according to Menichini et al.,[10] the content of phytochemicals as antioxidants is influenced by several factors: climate conditions, genotype, cultivation techniques, and harvest maturity stage.[11,12] According to Hanamura et al.,[2] tropical fruits are considered to be outstanding because of the quality and quantity of bioactive compounds such as antioxidants due to their exposure to strong sunlight.

When ripe, acerola is consumed mainly as juice and, like other tropical climacteric fruits, has a short postharvest life of a maximum 4 days due to its accelerated metabolism and fragile structure.[13,14] Therefore, other strategies of use have been developed to ensure its marketability.[15] Acerola has proven to be useful for improving the quality of fruit products or producing pharmacological and nutritional products; however, few studies have been published about the phytochemical contents and chemical changes during the maturation of acerola.[3,4,15] Thus, information on antioxidant properties at different ontological stages would help producers and food technologists to identify which cultivars or maturity stages are abundant with antioxidants. Thereby, this study investigated the changes in the antioxidant metabolism during fruit development of different acerola clones aiming to substantiate a database for both in natura consumption and industrial use.

MATERIALS AND METHODS

Fruit Material. Acerola clones BRS 235 (Apodi), BRS 236 (Cereja), BRS 237 (Roxinha), BRS 238 (Frutacor), and II 47/1 were

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obtained from Frutacor Farm in Limoeiro do Norte, CE, Brazil, where they were harvested at different stages according to skin color and size: immature green (IG), mature green with maximum size (MG), breaker-turning red (BR), and full red ripe (R). After harvest, fruits were selected on the basis of homogeneity in color/size and absence of defects, washed in tap water, and divided in samples that were evaluated as four replications of 500 g each. Fruit mass ranged from 3 to 8 g. Fruit samples were then processed using a domestic blender, and the processed pulp was stored at −20 °C and, within a 30 day period, was further analyzed as following.

Chemicals. 2,6-Dichloroindophenol (DFI), Folin−Ciocalteu reagent, formic acid, acetonitrile, cyanidin 3-rhamnoside, quercetin 3-rhamnoside, 2,2-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), trichloroacetic acid (TCA), thiobarbituric acid (TBA), ethylenediaminetetraacetic acid (EDTA), nitroblue tetrazolium chloride (NBT), and hydrogen peroxide (H_2O_2) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Quality Parameters and Nonenzymatic Antioxidants. Titratable acidity of acerola pulp was evaluated as determined by AOAC method[16] using an automatic titrator (Mettler-Toledo DL12, Columbus, OH, USA), and results were expressed as percent of malic acid. The pH was measured using an automatic pH-meter (Labmeter PHS-3B, São Paulo, Brazil) as recommended by AOAC.[16] Soluble solids content was determined by refractometry as described by AOAC[16] using a digital refractometer (ATAGO N1, Kirkland, WA, USA) with an automatic temperature compensation. The results were expressed in degrees Brix (concentration of sucrose w/w).

The total vitamin C was determined by titration with 0.02% DFI.[17] One gram of pulp was diluted to 100 mL of 0.5% oxalic acid and homogenized. Then, 5 mL of this solution was diluted to 50 mL with distilled water and titrated, and results were expressed as milligrams per 100 g fresh weight (FW). Anthocyanins and yellow flavonoids were extracted and determined as described by Francis.[18] One gram of pulp was extracted with a 95% ethanol/1.5 N HCl (85:15) solution, vortexed for 2 min, and, then, brought to 50 mL with the extracting solution. Protected from the light, the mixture was refrigerated at 4 °C for 12 h and then filtered on Whatman no. 1 paper, and the filtrate was gathered. The absorbance of the filtrate was measured at 535 nm for the total anthocyanin content using an absorption coefficient of 98.2 mol/cm and at 374 nm for the total yellow flavonoid content using an absorption coefficient of 76.6 mol/cm. Both results were expressed as milligrams per 100 g FW.

The total phenol content of acerola was measured by a colorimetric assay using Folin−Ciocalteu reagent as described by Obanda and Owuor.[19] Before the colorimetric assay, the samples were subjected to extraction in 50% methanol and 70% acetone as described by Larrauri et al.[20] Extracts were added to 1 mL of Folin−Ciocalteau reagent (1 N), 2 mL of Na_2CO_3 at 20%, and 2 mL of distilled water. Results were expressed as gallic acid equivalent (GAE), milligrams per 100 g FW.

Phenols were also determined by liquid chromatography (LC) coupled to mass spectrometry (MS). For extraction, 300 mg of freeze-dried acerola pulp was suspended in 5 mL of 40% methanolic solution, vortex-mixed for 1 min, and sonicated for 60 min prior to centrifugation at 2500*g* for 10 min at 20 °C. The supernatant was filtered (0.45 μm) and then submitted to chromatographic analysis. The LC-DAD-ESI/MS was a Varian ProStar system HPLC (Walnut Creek, CA) coupled with a diode array detector (DAD) and a 500-MS IT mass spectrometer (Varian). A Symmetry C18 column (5.0 μm, 250 × 4.6 mm) was used at a flow rate of 400 μL/min. The column oven temperature was set at 30 °C. The mobile phase consisted of a combination of A (0.1% formic acid in Milli-Q water) and B (0.1% formic acid in acetonitrile). The gradient varied linearly from 10 to 26% B (v/v) in 40 min, to 65% B at 60 min, and, finally, to 100% B at 70 min and then held at 100% B for 75 min. The DAD was set at 340, 270, and 512 nm for real-time read-out, and UV−vis spectra from 190 to 650 nm were continuously collected. Mass spectra were simultaneously acquired using electrospray ionization in the positive and negative ionization modes (PI and NI) at a fragmentation voltage of 80 V for the mass range of 200−1000 (m/z). A drying gas pressure of 35 psi, a nebulizer gas pressure of 40 psi, a drying gas temperature of 370 °C, capillary voltages of 3500 V for PI and NI, and spray shield voltages of 600 V were used. The LC system was directly coupled to the MS without stream splitting. Compound identification was primarily based on mass spectrometric data for molecular ions and MS-MS product ions and on published observations for phenolics in fruits and vegetables. Quantification was performed on the basis of UV−vis data. The UV−vis detector was set to collect the signal at 512 nm for cyanidin 3-rhamnoside and that at 340 nm for quercetin. External standard curves of cyanidin and quercetin were used, and concentrations were expressed as cyanidin equivalents (mg) and as quercetin equivalents (mg) for 100 g of dry weight (DW).

Total Antioxidant Activity (TAA) and Lipid Peroxidation Degree. The TAA was determined using the 2,2-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) method as described by Rufino et al.[21] Before the colorimetric assay, the samples were subjected to a procedure of extraction in 50% methanol and 70% acetone.[20] Once the radical was formed, the reaction was started by adding 30 μL of extract in 3 mL of radical solution, absorbance was measured (734 nm) after 6 min, and the decrease in absorption was used to calculate the TAA. A calibration curve was prepared, and different Trolox concentrations (standard trolox solutions ranging from 100 to 2000 μM) were also evaluated against the radical. Antioxidant activity was expressed as Trolox equivalent antioxidant capacity (TEAC), micromoles of Trolox per gram FW.

Lipid peroxidation was measured by the formation of malondialdehyde (MDA) based on the method from Zhu et al.[22] Pulp samples (0.5 g) were homogenized in 5 mL of 0.1% TCA and centrifuged at 3300*g* at 4 °C for 20 min. The supernatant (750 μL) was collected and added to 3 mL of 0.5% TBA in 20% TCA and incubated at 95 °C for 30 min. Following incubation, the tubes were immediately cooled in an ice bath and centrifuged at 3000*g* for 10 min. Measurements of absorbance at 532 nm were corrected for unspecific turbidity by subtraction from the absorbance at 600 nm. The content of MDA was calculated using an extinction coefficient of 155 nmol/cm[23] and expressed as nanomoles per gram FW.

Activity of Antioxidant Enzymes. Two grams of fruit pulp was homogenized in 10 mL of 0.1 M potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA for 1 min, followed by centrifugation at 3248*g* for 40 min at 4 °C.[24] The supernatant fraction was used as a crude extract for the enzyme activity assays, and all procedures were performed at 4 °C. The total protein content was determined according to the method of Bradford [25] using bovine serum albumin (BSA) as a standard.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined spectrophotometrically on the basis of the inhibition of the photochemical reduction of NBT.[26] The reaction mixture absorbance was measured by a Spectrum SP 2000UV spectrophotometer at 560 nm, and 1 unit of SOD activity (UA) was defined as the amount of enzyme required to cause a 50% reduction in the NBT photoreduction rate. Thus, results were expressed as UA per milligram of protein (P).

Catalase (CAT, EC 1.11.1.6) activity was measured according to the method of Beers and Sizer.[27] The reaction started by adding the enzyme extract, and then the decrease in hydrogen peroxide (H_2O_2) was monitored through absorbance at 240 nm and quantified by its molar extinction coefficient (36 M/cm). One unit of CAT activity (UA) was defined as the amount of enzyme required to decompose H_2O_2 (μmol H_2O_2/min) and the results were expressed as UA per milligram of P.

Ascorbate peroxidase (APX, EC 1.11.1.1) activity was assayed according to the method of Nakano and Asada.[28] Enzyme activity was measured using the molar extinction coefficient for ascorbate (2.8 mM/cm), considering that 1 mol of ascorbate is required for a reduction of 1 mol of H_2O_2. Results expressed as UA per milligram of P, when a unit of enzyme activity (UA) is micromoles of H_2O_2 per minute.

Statistical Analysis. The experimental design was completely randomized in factorial 5 × 4 (cultivars × harvest stage) with four replications of 500 g each. The data obtained were subjected to analysis of variance (ANOVA) using a computer program (SISVAR

Table 1. Changes in Postharvest Quality during Fruit Development of Five Acerola Clones[a]

clone	stage	soluble solids (°Brix)	titratable acidity (% malic acid)	soluble solids/titratable acidity	pH	total vitamin C (mg/100 g FW)
II 47/1	IG	7.83 ± 0.09 Ca	1.59 ± 0.03 Db	4.93 ± 0.06 ABa	3.38 ± 0.04 Cb	2864 ± 0.41 Cc
	MG	8.35 ± 0.08 Db	1.36 ± 0.05 Ba	6.14 ± 0.20 Db	3.13 ± 0.02 Ba	2355 ± 0.20 Cb
	BR	8.55 ± 0.08 Db	1.74 ± 0.03 Cc	4.92 ± 0.09 BCa	3.16 ± 0.04 Ca	2340 ± 0.30 Bb
	R	9.28 ± 0.06 Dc	1.81 ± 0.01 Dc	5.12 ± 0.03 Aa	3.38 ± 0.00 Ab	1820 ± 0.04 Da
BRS 235	IG	7.12 ± 0.17 Ba	1.37 ± 0.02 Ba	5.19 ± 0.07 Ba	3.10 ± 0.01 Aa	3243 ± 0.43 Dd
	MG	7.50 ± 0.21 Cb	1.47 ± 0.02 Cb	5.09 ± 0.09 Ba	3.22 ± 0.05 Cb	2793 ± 0.53 Dc
	BR	7.75 ± 0.08 Cb	1.39 ± 0.03 Aa	5.56 ± 0.08 Db	3.36 ± 0.05 Dc	2313 ± 0.20 Bb
	R	8.22 ± 0.14 Cc	1.61 ± 0.05 Cc	5.11 ± 0.19 Aa	3.44 ± 0.02 ABd	1578 ± 0.16 Ca
BRS 236	IG	7.20 ± 0.16 Bb	1.54 ± 0.02 CDb	4.67 ± 0.06 Aa	3.12 ± 0.02 Ab	3756 ± 0.14 Ed
	MG	6.67 ± 0.10 Aa	1.52 ± 0.01 CDb	4.39 ± 0.07 Aa	2.91 ± 0.01 Aa	2719 ± 0.29 Dc
	BR	6.75 ± 0.19 **Aa**	1.50 ± 0.01 **Bb**	4.50 ± 0.12 **Aa**	2.92 ± 0.01 **Aa**	2495 ± 0.44 **Cb**
	R	7.20 ± 0.04 Ab	1.40 ± 0.03 Ba	5.15 ± 0.13 ABb	3.44 ± 0.01 ABc	1642 ± 0.08 Ca
BRS 237	IG	6.42 ± 0.12 Aa	1.11 ± 0.05 Aa	5.79 ± 0.31 Cb	3.28 ± 0.03 Bc	2453 ± 0.50 Ad
	MG	7.15 ± 0.11 Bb	1.26 ± 0.03 Ab	5.68 ± 0.08 Cb	3.16 ± 0.01 BCb	1921 ± 0.14 Ac
	BR	7.25 ± 0.04 Bb	1.37 ± 0.01 Ac	5.28 ± 0.01 CDa	3.03 ± 0.01 Ba	1782 ± 0.34 Ab
	R	7.78 ± 0.12 Bc	1.42 ± 0.01 Bc	5.49 ± 0.04 Bab	3.50 ± 0.01 Bd	1293 ± 0.14 Ba
BRS 238	IG	7.20 ± 0.08 Ba	1.50 ± 0.02 Cb	4.82 ± 0.11 Aa	3.03 ± 0.03 Aa	2713 ± 0.51 Bd
	MG	7.15 ± 0.11 Ba	1.59 ± 0.03 Dc	4.91 ± 0.11 Ba	3.12 ± 0.01 BCb	2107 ± 0.18 Bc
	BR	7.25 ± 0.04 Ba	1.45 ± 0.04 ABb	4.79 ± 0.12 ABa	3.03 ± 0.01 Bb	1770 ± 0.23 Ab
	R	7.78 ± 0.12 Bb	1.24 ± 0.03 Aa	5.98 ± 0.19 Cb	3.50 ± 0.01 Bc	1201 ± 0.12 Aa

[a]For each parameter and cultivar, different capital letters indicate significant differences at $P < 0.05$ between harvest dates. For each parameter and harvest date, different lower case letters indicate significant differences at $P < 0.05$ among cultivars. IG, immature green; MG, mature green; BR, breaker; R, red ripe stage.

Table 2. Changes in Antioxidant Phenolic Compounds during Fruit Development of Five Acerola Clones[a]

clone	stage	total soluble phenols (mg GAE/100 g FW)	yellow flavonoids (mg/100 g FW)	quercetin (mg/100 g DW)	total anthocyanins (mg/100 g FW)	cyanidin (mg/100 g DW)
II 47/1	IG	4202 ± 2.15 CDb	6.02 ± 0.74 ABb	19.83 ± 1.49 BCab	0.72 ± 0.17 Aa	
	MG	1310 ± 1.00 Aa	6.35 ± 0.31 Bb	12.67 ± 1.81 Aba	1.39 ± 0.23 Ba	28.23 ± 7.90 Aa
	BR	1684 ± 0.48 Ba	4.13 ± 0.30 Aa	16.70 ± 0.33 Bab	2.66 ± 0.24 Bb	57.00 ± 12.09 Bb
	R	1679 ± 1.59 Ba	7.84 ± 0.45 Bc	24.80 ± 1.10 BCb	17.72 ± 0.91 Cc	169.45 ± 12.52 Dc
BRS 235	IG	3116 ± 1.69 Bb	6.68 ± 0.66 Bb	17.87 ± 0.76 Aba	0.45 ± 0.31 Aab	
	MG	1351 ± 0.29 Aa	5.00 ± 0.11 Aa	22.24 ± 6.18 Ca	0.30 ± 0.04 Aa	22.71 ± 1.66 Aa
	BR	1215 ± 0.74 Aba	4.43 ± 0.14 ABa	19.25 ± 4.98 Ba	1.31 ± 0.16 Ab	27.84 ± 1.61 Aa
	R	969 ± 0.74 ABa	6.46 ± 0.26 Ab	21.77 ± 3.50 Ba	6.49 ± 0.28 Ac	58.52 ± 1.19 Ab
BRS 236	IG	4371 ± 9.92 Db	5.12 ± 0.15 Aa	28.18 ± 3.63 Ca	0.19 ± 0.10 Aa	
	MG	1641 ± 1.09 Aa	4.70 ± 0.21 Aa	21.54 ± 2.97 Bca	0.65 ± 0.10 Aa	24.30 ± 4.96 Aa
	BR	1294 ± 2.15 ABa	5.70 ± 0.35 Ba	19.57 ± 2.92 Ba	1.88 ± 0.21 Aa	46.07 ± 6.59 ABb
	R	1099 ± 0.89 ABa	9.28 ± 0.27 CDb	23.59 ± 0.05 Ba	9.12 ± 0.46 ABb	148.03 ± 9.53 Cc
BRS 237	IG	2131 ± 2.77 Ab	8.75 ± 1.10 Cb	9.91 ± 5.50 Aa	1.82 ± 0.29 Ba	
	MG	949 ± 0.99 Aa	5.35 ± 0.70 ABa	13.71 ± 3.84 ABCa	1.64 ± 0.12 Ba	32.75 ± 5.32 Aa
	BR	888 ± 0.39 Aa	5.23 ± 0.37 ABa	24.80 ± 3.98 Bb	4.21 ± 0.37 Cb	92.07 ± 9.43 Cb
	R	1021 ± 0.57 ABa	10.06 ± 0.69 Dc	33.49 ± 3.61 Cb	17.30 ± 1.01 Cc	241.11 ± 11.11 Ec
BRS 238	IG	3630 ± 1.10 BCb	5.40 ± 0.19 ABa	8.55 ± 1.22 Aa	0.79 ± 0.04 ABa	
	MG	1501 ± 0.24 Aa	4.94 ± 0.26 Aa	6.23 ± 2.22 Aa	1.16 ± 0.15 ABab	41.83 ± 8.46 Aa
	BR	1237 ± 0.35 ABa	4.87 ± 0.25 ABa	6.57 ± 2.11 Aa	2.14 ± 0.20 ABb	42.71 ± 2.66 ABa
	R	931 ± 0.45 Aa	8.10 ± 0.28 BCb	12.81 ± 3.85 ABa	7.42 ± 0.18 Ac	104.87 ± 15.00 Bb

[a]For each parameter and cultivar, different capital letters indicate significant differences at $P < 0.05$ between harvest dates. For each parameter and harvest date, different lower case letters indicate significant differences at $P < 0.05$ among cultivars. IG, immature green; MG, mature green; BR, breaker; R, red ripe stage.

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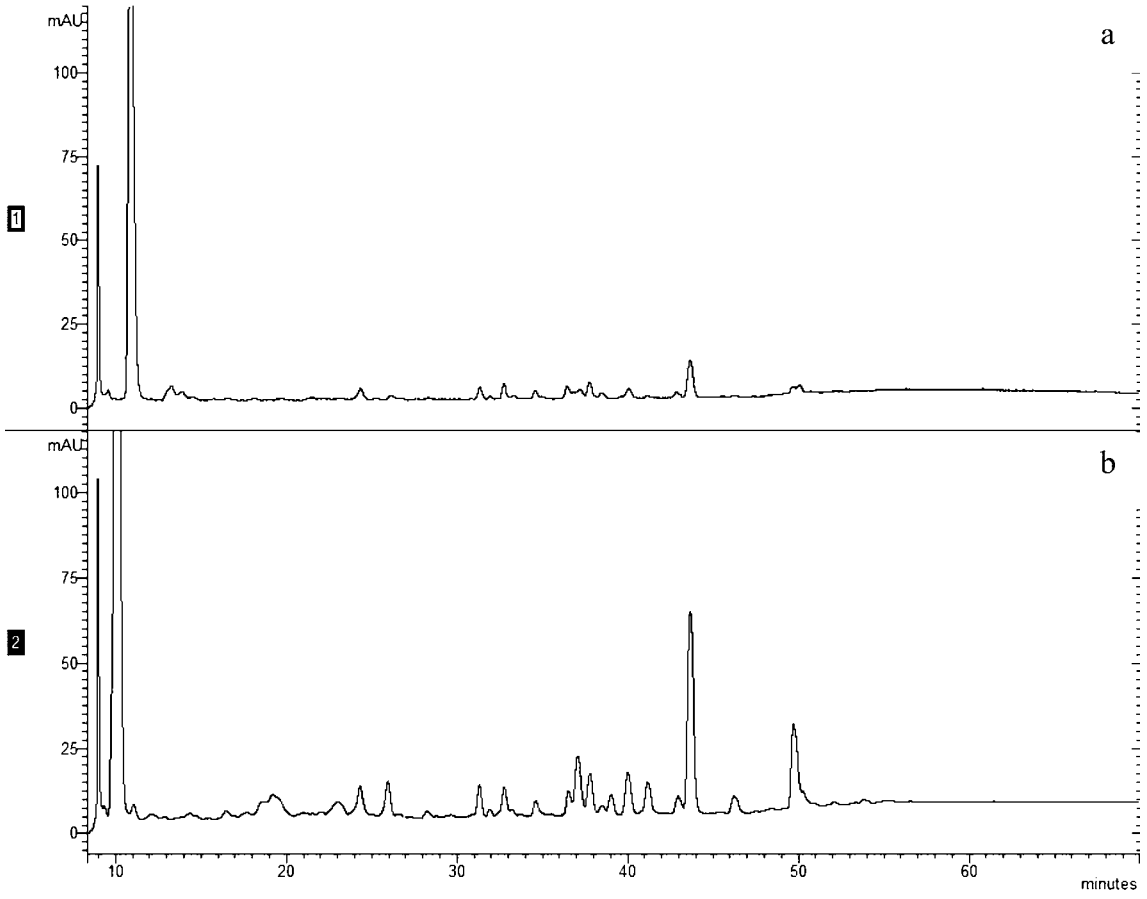

Figure 1. Chromatogram of BRS 237 acerola clone at immature green (IG) (a) and ripe (R) (b) stages at 340 nm.

3.01), and the averages were compared by the Tukey test at 5% probability.[29]

RESULTS AND DISCUSSION

Quality and Nonenzymatic Antioxidants during Development. Changes related to physicochemical parameters during the development of acerola are shown in Table 1. During ripening, the soluble solids (SS) content increased, with the greatest value (9.28 °Brix) for clone II 47/1 and the lowest (7.20 °Brix) for BRS 236. For most fruits, the increase in SS is explained by gluconeogenesis and/or hydrolysis of polysaccharides as starch;[30] however, there are only traces of starch in acerola.[31] Therefore, sugar is probably synthesized from gluconeogenese, although other compounds will also contribute to the increase in SS in ripe acerola as it has been reported that it was positively correlated to vitamin C, polyphenols, and anthocyanin content.[32]

Titratable acidity (TA) was also evaluated, and acerola clones exhibited different patterns as development proceeded; clones II 47/1, BRS 235, and BRS 237 increased their acidity, whereas clones BRS 238 and BRS 236 showed a decrease. The unusual increase in TA is attributed to organic acid formation during acerola maturation and was also reported by Vendramini and Trugo;[31] meanwhile, the decrease in acidity resulted from organic acid consumption as respiratory substrate.[30] Although there were controversial results with TA, pH values increased slightly during acerola maturation, with little variation among clones. These results suggest a net balance was created due to differences in the dissociation degree of ascorbic acid, which decreased (Table 1), and of other organic acids produced during fruit maturation. The slightly acid flavor of acerolas is evidenced by the low SS/TA ratios, observed as ripe BRS 238 fruit reached the highest value of 5.98, when compared to sapodilla, 111.17.[33]

Acerola clones also showed a decline in total vitamin C content, more abrupt during ripening. BRS 236 vitamin C declined from 3756.47 mg/100 g FW in immature green fruit (IG) to 1642 mg/100 g FW when ripe (R). Among the evaluated clones, BRS 236 and II 47/1 had the highest vitamin C content at IG and R stages, respectively. Despite the reported decrease, the five studied acerola clones still presented extremely high vitamin C contents when compared to tomato, 20 mg/100 g FW,[6] to strawberry, 57 mg/100 g FW,[9] and to kiwi, 52 mg/100 g, and oranges, 36 mg/100 g FW.[34] Vendramini and Trugo[31] also observed a reduction in vitamin C during acerola ripening from 2160 to 1070 mg/100 g FW and associated the reductions with biochemical oxidation, because 3-hydroxy-2-pyrone, a product of the oxidative breakdown of ascorbic acid, was found in mature acerola.

Phenolic Antioxidants during Development. The changes in phenolic antioxidants during the development of acerola clones are shown in Table 2. The content of total soluble phenols (TSP) decreased statistically during fruit development, and the steepest fall occurred during growth between immature and mature green stages. At IG stage, clones BRS 236 and II 47/1 had the greatest phenol content, and at R stage, clone II 47/1 was the highest with 1679 g GAE/100 g FW. The decline in TSP may be due to polymerization of soluble phenols or oxidation of phenolic content by polyphenol oxidase enzyme.[35]

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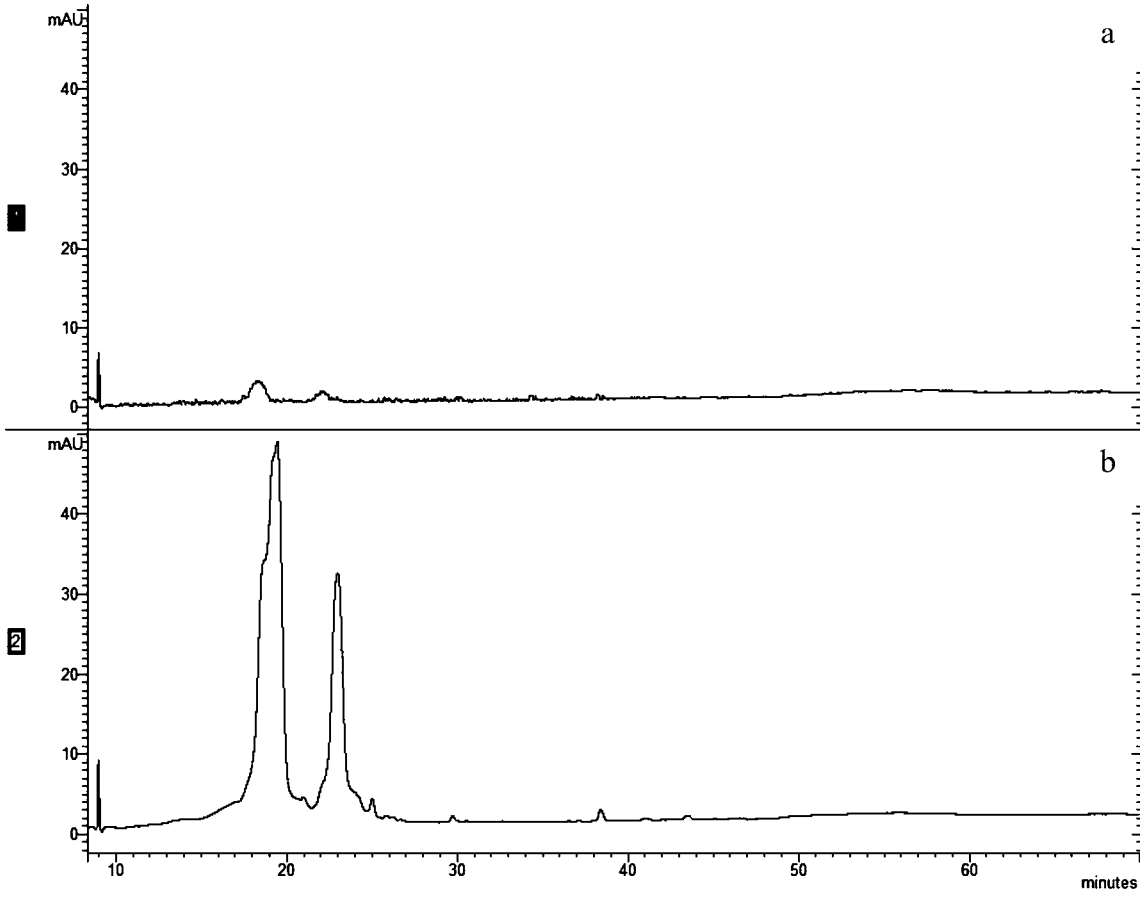

Figure 2. Chromatogram of BRS 237 acerola clone at mature green (MG) (a) and ripe (R) (b) stages at 512 nm.

Despite the decline in TSP, the yellow flavonoid and total anthocyanin contents showed an evident increase at acerola ripening (Table 2), which is probably associated with fruit coloring. Flavonoids represent a large group of phenolic compounds with physiological roles in plant reproduction and defense mechanisms against microorganisms and photo-oxidation. Among the flavonoids, anthocyanins and yellow flavonoids are mainly responsible for flower and fruit pigmentation to attract pollinators and seed disseminators.[36] Besides their role in plant physiology, flavonoids are associated with health-promoting qualities as studies show that consumption leads to prevention of cardiovascular problems and cancer in humans,[37−39] which is strongly related to their high antioxidant potential.[36] According to Kalt et al.,[40] during ripening there is a shift in the pool of total phenolics toward anthocyanin synthesis and an overall decline in the content of other phenolic components. Furthermore, a decrease in total phenolic may contribute to the biosynthesis of the flavylium ring of anthocyanins.

When ripe, acerola presented an orange-colored pulp except for clones II 47/1 and BRS 237, which were dark red (data not shown); nevertheless, the latter clone had the highest yellow flavonoid content (10.06 mg/100 g FW). Ripe fruits from clone BRS 237 also showed the highest quercetin content (33.49 mg/100 g DW) (Table 2), justifying its high yellow flavonoid content. Quercetin is a yellow flavonol well-known as the strongest antioxidant flavonoid[36] with potent anti-inflammatory activity[41] and, more specifically, acerola quercetin is an excellent antidiabetic agent.[2] Quercetin was ubiquitous in acerola development and was detected as different derivates (see Table 5). However, it increased during ripening and, therefore, as acerola ripened and the vitamin C content declined (Table 1), quercetin accumulated, contributing to its antioxidant properties. The increase in quercetin derivatives was also observed at 340 nm (Figure 1).

The intense and characteristic red skin color, one of the most important indicators of acerola ripeness and edible quality, is due to synthesis of anthocyanin (Table 2). The anthocyanin contents were low until breaker (BR) and increased rapidly to ripe (R) stage. Clones with the highest anthocyanin contents were II 47/1 and BRS 237 with 17.12 and 17.30 mg/100 g FW, respectively, and therefore exhibited a characteristic dark red skin and, furthermore, a red pulp, at ripe stage. During ripening of climacteric fruit such as acerola, ethylene regulates anthocyanin synthesis as a homeostatic imbalance takes place with the increment of ROS signaling the regulation of key enzymes for anthocyanin synthesis.[42,43]

In acerola, two major anthocyanins were found as cyanidin 3-rhamnoside, taking into consideration the m/z 433 and a fragment with m/z 287, and pelargonidin 3-rhaminoside, with m/z 417 and 271 (see Table 5). In the final stages of development, cyanidin accumulated, and the greatest values found for ripe fruit were directly related to total anthocyanin content (Figure 2). Ripe fruit of clones II 47/1 and BRS 237 presented highest cyanidin contents with 169.45 and 241.11 mg/100 g DW, respectively. Clones II 47/1 and BRS 236 also presented cyanidin 3-rhamnoside and cyanidin 3-glucoside (m/z 449 and fragment with m/z 287) at mature green (MG) stage. Hanamura et al.[2] also identified cyanidin 3-rhamnoside as the main anthocyanin in ripe acerola and reported that its

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Table 3. Changes in Activity of Antioxidant Enzymes, Lipid Peroxidation, and Total Antioxidant Activity during Fruit Development of Five Acerola Clones[a]

clone	stage	SOD (10^2 UA/mg P)	CAT (10^2 UA/mg P)	APX (UA/mg P)	lipid peroxidation (nmol MDA/g FW)	TAA (μM Trolox/g FW)
II 47/1	IG	129.33 ± 4.17 Dc	456.82 ± 37.87 Bc	183.91 ± 15.57 Bb	28.79 ± 3.13 ABab	148.64 ± 10.04 Bc
	MG	49.87 ± 4.70 Ab	444.82 ± 43.11 ABb	183.41 ± 29.52 ABb	20.67 ± 1.19 Aa	96.67 ± 15.16 Aa
	BR	28.77 ± 1.14 Aa	332.40 ± 19.29 ABab	130.25 ± 14.32 ABab	32.98 ± 1.06 ABb	125.43 ± 4.76 Cb
	R	22.96 ± 1.37 Aa	227.95 ± 25.67 ABa	96.51 ± 13.37 Aa	73.68 ± 1.50 Cc	119.97 ± 1.06 Db
BRS 235	IG	61.73 ± 0.84 Ba	224.83 ± 7.80 Aa	110.66 ± 5.93 Aa	36.64 ± 1.01 Ba	148.13 ± 9.41 Bc
	MG	65.82 ± 0.40 Ba	351.60 ± 16.69 Aab	158.84 ± 20.95 Aab	35.54 ± 1.70 Ba	120.20 ± 4.86 Bb
	BR	64.55 ± 1.62 Ca	455.51 ± 22.22 Bb	179.72 ± 40.08 Bb	40.05 ± 3.73 BCab	108.16 ± 13.63 BCab
	R	62.65 ± 2.19 Ca	291.72 ± 24.34 ABab	141.96 ± 9.86 ABab	46.10 ± 2.27 Bb	91.44 ± 5.75 BCa
BRS 236	IG	47.80 ± 5.52 Aa	310.99 ± 19.65 Aa	98.25 ± 10.42 Aa	36.72 ± 2.13 Ba	160.46 ± 4.87 Bc
	MG	85.82 ± 3.56 Cc	618.19 ± 46.52 Cb	235.68 ± 19.71 Bc	34.76 ± 4.38 Ba	124.60 ± 8.53 Bb
	BR	64.50 ± 7.32 Cb	532.48 ± 14.96 Cb	147.82 ± 29.24 ABab	33.28 ± 2.82 ABa	99.75 ± 10.66 Ba
	R	50.28 ± 3.14 Ba	349.05 ± 27.47 Ba	161.82 ± 49.56 Bb	48.76 ± 1.07 Bb	105.61 ± 2.26 CDab
BRS 237	IG	181.61 ± 3.47 Ed	917.61 ± 49.70 Cc	365.38 ± 21.29 Cc	36.96 ± 7.00 Ba	111.42 ± 1.89 Ab
	MG	100.79 ± 4.27 Dc	510.59 ± 23.70 BCb	225.41 ± 24.00 Bb	39.43 ± 6.50 Ba	87.13 ± 4.84 Aa
	BR	81.27 ± 3.84 Db	457.57 ± 35.30 BCb	174.11 ± 3.46 Bab	43.23 ± 1.80 Ca	91.70 ± 9.52 ABab
	R	61.25 ± 1.61 Ca	290.25 ± 13.06 ABa	125.40 ± 6.42 ABa	79.89 ± 7.04 Cb	75.59 ± 3.96 ABa
BRS 238	IG	75.22 ± 3.39 Cb	637.63 ± 25.28 Bc	198.95 ± 30.26 Bb	26.67 ± 2.15 Aa	123.00 ± 8.44 Ab
	MG	43.15 ± 1.40 Aa	398.40 ± 38.03 ABb	123.54 ± 4.42 Aa	22.45 ± 0.89 Aa	101.43 ± 10.60 ABb
	BR	43.78 ± 5.36 Ba	216.45 ± 11.72 Aa	98.52 ± 9.85 Aa	24.11 ± 1.51 Aa	76.11 ± 10.12 Aa
	R	43.15 ± 0.73 Ba	170.66 ± 5.06 Aa	94.59 ± 2.57 Aa	34.27 ± 0.46 Ab	59.75 ± 0.26 Aa

[a]For each parameter and cultivar, different capital letters indicate significant differences at $P < 0.05$ between harvest dates. For each parameter and harvest date, different lower case letters indicate significant differences at $P < 0.05$ among cultivars. IG, immature green; MG, mature green; BR, breaker; R, red ripe stage; SOD, superoxide dismutase; CAT, catalase; APX, ascorbate peroxidase; TAA, total antioxidant activity.

ability to neutralize superoxide radical was similar to that of quercetin. The authors also explained that antioxidant activity is strongly correlated with the number of hydroxyl groups of the polyphenol structure.

Activity of Antioxidant Enzymes during Acerola Development. Because fruit ripening was described as an oxidative phenomenon, questions were raised as to whether the increasing oxidative stress accompanying ripening was associated with a reduced ability to catabolize ROS by either nonenzymatic or enzymatic antioxidants. Thus, the activities of antioxidant enzymes were determined during the development of acerola clones (Table 3).

SOD, a class of metal-containing proteins, catalyzes the breakdown of O_2^- to H_2O_2 and O_2 and has been implicated as an essential defense against the potential toxicity of oxygen.[26] As acerola developed, SOD activity declined, although most dramatically in clones II 47/1 and BRS 237, from 129.33×10^2 to 22.96×10^2 UA/mg P and from 181.61×10^2 to 61.25×10^2 UA/mg P, respectively. For these two clones, the decline in SOD activity is concomitant with the increase in lipid peroxidation (Table 3).

CAT and APX are involved with H_2O_2 scavenging in plant cells. CAT activity differed greatly between clones with BRS 235 and BRS 236 presenting an increase during fruit development; meanwhile, II 47/1, BRS 238, and BRS 237 showed a decline. Clones BRS 237 and BRS 238 showed progressive significant 3-fold decrease from 917.61×10^2 (IG) to 290.25×10^2 (R) UA/mg P and from 637.63×10^2 (IG) to 170.66×10^2 (R) UA/mg P, respectively. As observed for SOD, the decline in CAT activity of clones II 47/1 and BRS 237 may also be associated with the increase in lipid peroxidation (Table 3). Clone BRS 236 showed the highest CAT activity at ripe stage, 349.05×10^2 UA/mg P, although not statistically different from those of clones BRS 237, BRS 235, and II 47/1.

APX uses ascorbate as the electron donor for H_2O_2 neutralization, and it declined during the development of fruit from clones BRS 238 and II 47/1 and more dramatically in BRS 237 from 365.38 (IG) to 125.40 (R) UA/mg P. Clones BRS 235 and BRS 236 showed an increase in APX activity during development. As also reported in a previous work,[32] APX activity was much lower than CAT, suggesting that the latter is the key enzyme in H_2O_2 catabolism and together with SOD is the most important antioxidant enzyme in acerola. These results are well related to the progressive increase in oxidative stress during acerola development and ripening as evidenced by lipid peroxidation (Table 3). Thus, the increasing oxidative stress probably related to the lower enzyme activities is needed to facilitate the metabolic changes associated with ripening.

Total Antioxidant Activity and Lipid Peroxidation during Acerola Development. The TAA significantly declined during acerola development (Table 3) and was highest at IG stage. At ripening, clone II 47/1 had the greatest TAA with 119.97 μM Trolox/g FW, which may be associated with the high vitamin C (Table 1) and phenol (Table 2) contents. A previous study with acerola clone II 47/1 showed TAA was strongly correlated to SS content, as also were the phenols and vitamin C; meanwhile, for clone BRS 235, TAA and vitamin C were negatively correlated to SS, phenols, and anthocyanin contents.[32] Therefore, parameters may contribute differently to TAA with regard to each acerola clone.

Table 4. Pearson Correlation Coefficients of Lipid Peroxidation Degree versus Antioxidant Parameters during Development of Fruit from Five Acerola Clones

	lipid peroxidation[a]				
parameter[b]	II 47/1	BRS 235	BRS 236	BRS 237	BRS 238
CAT (10^2 UA/mg P)	−0.674*		−0.585*	−0.684**	
APX (UA/mg P)	−0.788**			−0.647*	
SOD (10^2 UA/mg P)				−0.627*	
anthocyanins (mg/100 g FW)	0.977**	0.853**	0.837**	0.934**	0.867**
yellow flavonoids (mg/100 g FW)	0.605*		0.833**	0.637*	0.934**
total phenols (mg GAE/100 g FW)					
total vitamin C (mg/100 g FW)	−0.773**	−0.800**	−0.566*	−0.802**	−0.569*
TAA (μM Trolox/g FW)		−0.726**		−0.690**	−0.578*

[a]Lipid peroxidation (nmol MDA g^{-1}FW) vs parameters. *, $P \leq 0.05$; **, $P \leq 0.01$. [b]Antioxidant properties evaluated during fruit development. SOD, superoxide dismutase; CAT, catalase; APX, ascorbate peroxidase; TAA, total antioxidant activity.

Cell membrane lipids are highly susceptible to oxidation by ROS and, thus, oxidative stress would induce the accumulation of lipid peroxidation products such as MDA.[44] Therefore, the lipid peroxidation degree may be used as an oxidative stress indicator and would be expected to rise as fruit ripens, for this developmental process is considered an oxidative phenomenon that would result in the loss of membrane integrity. The lipid peroxidation degree increased from breaker (BR) to ripe (R) stage, indicating that an oxidative imbalance increased substantially throughout the acerola ripening process (Table 3). The highest lipid peroxidation degrees were observed in ripe BRS 237 and II 47/1 fruits, 79.89 and 73.68 nmol MDA/g FW, respectively. However, the latter clone presented the highest TAA; therefore, it may be concluded that nonenzymatic antioxidants associated with TAA are not the main contributors to the redox balance and fruit protection.

When lipid peroxidation values were correlated to enzymatic and nonenzymatic antioxidants (Table 4), for clone BRS 237 there was a negative correlation ($P < 0.01$) to CAT activity (−0.684), total vitamin C content (−0.802), and TAA (−0.690) and a positive correlation ($P < 0.01$) to total anthocyanin content (0.934). For clone II 47/1, lipid peroxidation was negatively correlated ($P < 0.01$) to APX activity (−0.788) and total vitamin C content (−0.773) and was strongly correlated ($P < 0.01$) to total anthocyanin content (0.977). Thus, these two clones were evidently dependent on antioxidant enzyme protection against cell membrane peroxidation and loss of integrity, besides depending on vitamin C. Moreover, for all five clones, the total vitamin C content was negatively correlated; meanwhile, the anthocyanin content was positively ($P < 0.01$) correlated to lipid peroxidation. These results suggest vitamin C is the ubiquitous antioxidant protection against cellular oxidative damage in acerola and, moreover, that anthocyanin accumulation that results in color changes occurs at the same ontological moment as cell membrane lipid peroxidation increases.

Table 5. Major Phenolics Mass for the Different Acerola Clones at Ripe Stage (R)

time (min)	$[M]^+$ (m/z)	MS/MS (m/z)	compound
17.99	433 (677)	287 (1092)	cyanidin 3-rhamnoside
22.95	417 (533)	271 (1037)	pelargonidin 3-rhamnoside
37.28	487 (119)	303 (341)	quercetin derivative
44.36	447 (173)	303 (987)	quercetin derivative

This work demonstrated that acerola ripening was characterized by a progressive oxidative stress as membrane lipids underwent peroxidation, probably due to a reduction in vitamin C content and, to a lesser degree, in antioxidant enzyme activity. Vitamin C was the major antioxidant protection against cellular oxidative damage; meanwhile, anthocyanin and flavonoids accumulated, leading to changes in color, at the same ontological moment as cell membrane lipid peroxidation increases. Cyanidin 3-rhamnoside and quercetin 3-rhamnoside were identified as the main anthocyanin and yellow flavonoid, respectively. Among the studied acerola clones, II 47/1, BRS 237, and BRS 236 presented outstanding results, and if antioxidants were to be extracted for the food supplement industry, immature fruit would be the most suitable, whereas, for marketing, fruit should be eaten ripe.

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Notes

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