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Original Article

Antioxidant compounds and antioxidant activity in acerola (*Malpighia emarginata* DC.) fruits and derivatives

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ABSTRACT

Acerola (*Malpighia emarginata* DC.) is a wild plant from Central America. This fruit is well known as an excellent food source of vitamin C, and it also contains phytochemicals such as carotenoids and polyphenols. The present work evaluates the antioxidant capacity of hydrophilic extracts of acerola pulps and juices by 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ORAC and 1,1-diphenyl-2-picrylhydrazyl (DPPH) methods. Antioxidant activity values obtained for acerola juice were higher than those reported for other fruit juices particularly rich in polyphenols such as strawberry, grape and apple juices, among others. Vitamin C, total phenol index (TPI), total anthocyanins and polyphenolic compounds by high performance liquid chromatography (HPLC), as main factors responsible for antioxidant activity, were determined. Contents in total ascorbic acid ranged from 6.32 to 9.20 g kg^{-1} of pulp and 9.44 to 17.97 g L^{-1} of juice. Five different polyphenolic compounds were identified in the samples by means of HPLC and diode-array detection: chlorogenic acid, (−)-epigallocatechin gallate, (−)-epicatechin, procyanidin B1 and rutin, being the two last predominant. By means of solid phase extraction (SPE) three soluble polyphenolic fractions (phenolic acids, anthocyanins and flavonoids) were separated from the different sample extracts, and their respective antioxidant activities calculated. Among them, phenolic acids are the main contributors to the antioxidant activity.

1. Introduction

Acerola (*Malpighia emarginata* DC.) comprises 30 species of shrubs native to the West Indies. It also grows in Central and South America including Brazil due to its good adaptation to soil and climate. It belongs to the Malpighiaceae family (Asenjo, 1980). Until recently, the plant has been known by the synonymous *Malpighia glabra* L. and *Malpighia punicifolia* L. but recent taxonomic work has resulted in the acceptance of *M. emarginata* DC. as the current scientific name for this plant (Howard, 1988). The plant bears a small trilobate cherry-like red fruit. The pulp is very juicy and refreshing with a sweet, fruity flavour. It is principally known for its high vitamin C content, varying between 3 and 46 g kg^{-1} of pulp, being one of the most important natural sources for this vitamin (Vendramini and Trugo, 2000; Itoo et al., 1990). The consumption of three fruit units per day satisfies the vitamin C recommended dietary allowance for an adult. In addition, it presents amounts of thiamine, riboflavin, niacin, proteins, and mineral salts, mainly iron, calcium and phosphorous (Mezquita and Vigoa, 2000). The components of acerola fruit (per kg), as well as the concentration ranges found are as follows (Mezadri et al., 2006; FAO, 2007): carbohydrates (35.7–78 g), proteins (2.1–8 g), lipids (2.3–8 g), phosphor (171 mg), calcium (117 mg), iron (2.4 mg), pyridoxine (87 mg), riboflavin (0.7 mg), thiamine (0.2 mg), water (906–920 g) and dietetic fibre (30 g).

Recently, much attention has been paid to their content in carotenoids and bioflavonoids for their antioxidant properties (Mezquita and Vigoa, 2000). Carotenoids are present at levels between 3.2 and 406 mg kg^{-1} (Lima et al., 2005). Mezadri et al. (2005) identified by high performance liquid chromatography (HPLC) 17 carotenoid pigments in acerola fruit: neoxanthin, neoxanthin isomer, neochrome, neochrome isomer, violaxanthin, violaxanthin isomer, luteoxanthin, auroxanthin, antheraxanthin, mutatoxanthin, lutein, *cis*-lutein, β-cryptoxanthin 5-8-epoxide, β-cryptoxanthin 5-6-epoxide, β-cryptoxanthin, β-carotene and *cis*-β-carotene. Among identified carotenoids, β-carotene presents the highest content (40–60% of total carotenoids).

In relation to flavonoids, the main components of acerola fruit are anthocyanins (37.9–597.4 mg kg^{-1}) (Lima et al., 2003) and flavonols (70–185 mg kg^{-1}). Hanamura et al. (2005a) identified two anthocyanins (cyanidin-3-rhamnoside and pelargonidin-3-rhamnoside) and one flavonol (quercetrin) in the fruits.

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The composition of the fruit depends on some factors like climatic conditions, treatment of the culture, geographic location, application of pesticides, stage of maturation or processing and storage.

Until now, the most commercialized acerola products in Brazil have been the fruit itself, the frozen pulp and the pasteurized single strength juice. However, new products appear in the market, such as juice blends (acerola and cashew, acerola and orange), blends with "guarana", powdered refreshments and concentrated juice (Martinelli, 1998).

The association between the consumption of fruit and vegetables and a decreased risk of cardiovascular disease and cancer is supported by considerable epidemiological evidence (Hertog et al., 1995; WHO, 2003). This beneficial effect is due to the action of antioxidant compounds, which are capable of neutralizing free radicals and reduce oxidative damage in the body (Clifford, 1995).

For this reason, the interest in the evaluation of antioxidant activity of fruits and vegetables has substantially increased and numerous studies have been performed (Wada and Ou, 2002; Kolayli et al., 2003; Chinnici et al., 2004; Silva et al., 2004; Roesler et al., 2006). In this sense, antioxidant activity of acerola is a topic of interest but few data have been reported yet. A wide variety of analytical methods have been set up to evaluate antioxidant activity of fruits and vegetables. The antioxidant activity of juices from acerola has been evaluated by the linoleate method, yielding high values (Righetto et al., 2005). Frozen pulps were analysed by the liposome oxidation and β-carotene bleaching methods and results were poorly correlated with vitamin C content (Hassimotto et al., 2005).

The aim of this work was to evaluate the antioxidant activity of hydrophilic extracts of acerola fruits and commercial pulps and to explore the contribution of vitamin C and polyphenols to the antioxidant activity, as well as to study the polyphenolic profile of this fruit.

2. Material and methods

2.1. Samples and sample preparation

Acerola fruits at mature stage were randomly harvested from plants grown in the Itajaí District (Santa Catarina, Brazil) during the months of January, March and April. Acerola berries were divided in two lots to perform two different treatments: squeeze processing or press crushing. Care was taken to have homogeneity in each lot. Once harvested, the fruits were frozen and stored at −18 °C until analysis was performed. Then they were unfrozen and immediately processed. The squeeze treatment was performed in a home food processor for 3 min. A manual press was used for the second lot. In both cases, the pulp was vacuum filtered through No. 1 Whatman paper into a Büchner flask to remove the seeds and skin. The clear juice was stored at −18 °C until analysis.

Commercial juices (normal and concentrated) as well as ready-made pulp were purchased from local markets. The commercial frozen pulp was centrifuged at 4000 rpm for 10 min and kept at −18 °C prior to analysis.

For further antioxidant activity determinations, two solvents (water and methanol) were tested as extractants as referenced by authors (Pulido et al., 2000; Monagas et al., 2005; Vinson et al., 2005).

Samples were taken in triplicate. Table 1 shows the codification of the samples.

2.2. Chemicals

2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) in the crystallized diammonium salt form, horseradish peroxidase type VI-A, hydrogen peroxide (30%, v/v) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) as free radical were obtained from Sigma-Aldrich Quimica (Alcobendas, Spain). Folin–Ciocalteu reagent, methanol, glycine, ethanol and hydrochloric acid (32%) were provided by Merck (Mollet del Vallés, Spain). β-Phycoerithrin (β-PE) from *Porphydium cruentum* was obtained from Sigma (St. Louis, MO, USA). Before using this compound, we tested its suitability for the assay by verifying the loss of more than 90% fluorescence within 30 min in the presence of 160 mM of 2,2′-azobis(2-amidino-propane) dihydrochloride (AAPH). AAPH and 6-hydroxy-2,5,7,8 tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Wako Chemicals and Aldrich, respectively. All reagents were of analytical grade. Double-distilled water (Millipore Co.) was used throughout.

A total of 37 phenolic compounds were studied, including phenolic acids, flavonoids and furanic derivatives. Among them furfural, procyanidin B1, chlorogenic acid, epigallocatechin gallate, epicatechin and rutin were identified in the samples. Standards were purchased from Fluka (furfural, epicatechin), Chromadex (procyanidin B1, chlorogenic acid) and Sigma (epigallocatechin gallate, rutin).

2.3. Instruments

Absorbance measurements (ABTS, DPPH assays) were recorded on a UV-2800® Spectrophotometer (Hitachi), thermostatized with

Table 1
Contents of ascorbic acid (AA), dehydroascorbic acid (DHAA) and total ascorbic acid (AAT) in acerola samples

Sample	Code	AA ($g\,kg^{-1}$; $g\,L^{-1}$)	DHAA ($g\,kg^{-1}$; $g\,L^{-1}$)	AAT ($g\,kg^{-1}$; $g\,L^{-1}$)
Commercial frozen pulp	P1	9.04 ± 0.002	0.16 ± 0.001	9.20
Commercial frozen pulp	P2	8.16 ± 0.001	0.40 ± 0.002	8.56
Commercial frozen pulp	P3	7.68 ± 0.002	0.08 ± 0.000	7.76
Commercial frozen pulp	P4	5.65 ± 0.001	1.02 ± 0.005	6.67
Commercial frozen pulp	P5	4.78 ± 0.003	1.64 ± 0.010	6.32
Commercial frozen pulp	P6	5.31 ± 0.001	1.10 ± 0.009	6.41
Crushed fruit—March	A1	8.24 ± 0.012	1.20 ± 0.013	9.44
Squeezed fruit—March	A2	10.0 ± 0.015	0.48 ± 0.004	10.5
Crushed fruit—April	B1	10.0 ± 0.019	0.40 ± 0.003	10.4
Squeezed fruit—April	B2	8.56 ± 0.005	0.96 ± 0.005	9.52
Crushed fruit—January	C1	13.4 ± 0.014	4.30 ± 0.022	17.7
Squeezed fruit—January	C2	14.5 ± 0.020	3.48 ± 0.014	18.0
Commercial juice	D1	0.42 ± 0.000	0.01 ± 0.000	0.43
Commercial concentrate juice	D2	4.44 ± 0.002	0.19 ± 0.000	4.25

a Peltier system at 25 °C. Fluorimetric measurements (ORAC assay) were recorded in an F-2500 Hitachi Fluorimeter connected to a device which maintained the temperature of 37 °C.

2.4. Total phenol index

Total phenol index (TPI) was determined by the Folin–Ciocalteu method (Singleton and Rossi, 1965) and results were expressed as gallic acid equivalents (GAE, mg L^{-1} for the juice, mg kg^{-1} for the pulp).

2.5. Total anthocyanin content

Total anthocyanin content was determined by using a pH-differential method (Giusti and Wrolstad, 2001). For the extraction of anthocyanins, pulp samples were filtered (Whatman1®) and centrifuged at 4000 rpm for 10 min. Extraction was performed on acetonitrile with 4% acetic acid. Extracts were 10-fold diluted in buffers KCl (0.025 M, pH 1.0) and CH_3COONa (0.4 M, pH 4.5). Absorbance was measured at 510 and 700 nm and results were calculated with the following formulas and expressed as cyanidin-3-glucoside:

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

Monomeric anthocyanins = ($A \times$ molecular weight $\times$ dilution factor $\times$ 100)/($\varepsilon \times l$), 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 is expressed as mg kg^{-1} (pulp) or mg L^{-1} (juice) of cyanidin-3-glucoside.

2.6. Determination of antioxidant activity

2.6.1. ABTS method

The method used is based on Cano et al. (1998) with slight modifications (Villaño et al., 2004).

Once the radical was formed, the reaction started by adding 100 μL of the test sample to 2 mL of $ABTS^+$ radical cation and absorbance at 414 nm was measured for 15 min. All measurements were performed in triplicate. Standard Trolox solutions (40–200 μM) were also evaluated against the radical.

2.6.2. DPPH method

The DPPH free radical is commercially available and it was prepared at a 0.1 mM concentration (25 mg L^{-1}) in methanol, following the procedure described by Sánchez-Moreno et al. (1998) and Larrauri et al. (1999). In the two scavenging methods the radical was prepared daily and protected from the light. Absorbance was recorded to check the stability of the radical throughout the time of analysis.

Antioxidant activity was expressed as Trolox equivalent antioxidant capacity (TEAC) as defined by Van den Berg et al. (1999) for food items. TEAC value expresses the concentration of a Trolox solution whose antioxidant activity is identical to that of the food itself. For calculations, a calibration curve was plotted for Trolox, for each method and both methanol and water extracts.

TEAC value is obtained by interpolating the decrease in absorbance (corresponding to a diluted sample) on the calibration curves, thus giving a concentration of Trolox (Villaño et al., 2007). Appropriate corrections were made taking into account the dilution. Each sample value is the mean of five or six determinations of different dilutions of pulp or juice within the linear range.

2.6.3. ORAC assay

This procedure is based on a previously reported method (Cao and Prior, 1999). Samples were prepared in concentrations 0.3 g L^{-1} (pulp) and 0.5 mL L^{-1} (juice). Results are calculated as ORAC values using the differences of areas under the β-PE decay curve between the blank and the sample and are expressed as Trolox equivalents. Trolox fluorescence decay curves are registered for every new solution of β-PE.

In previous studies, authors have tested the repeatability of the antioxidants methods as calculated for their respective standards and results are as follows (Fernández-Pachón et al., 2004). ABTS method: (−)-epicatechin = 1.0% ($n = 6$); (−)-epigallocatechingallate = 2.6% ($n = 6$); DPPH method: (−)-epicatechin = 5.9% ($n = 6$); (−)-epigallocatechingallate = 5.8% ($n = 6$); rutin 3.2% ($n = 6$); ORAC (−)-epicatechin = 13.9% ($n = 6$); (−)-epigallocatechingallate = 30.5% ($n = 6$); rutin 8.7% ($n = 6$).

2.7. Liquid chromatographic analysis of vitamin C

The liquid chromatographic equipment used was a Waters 600E System Controller connected in series to a Waters 996 Photodiode Array Detector. Samples were injected onto a Merck LichroCart® (250 × 4 mm) Superspher 100 RP-18100RP-18 column, 5 μm particle size, protected by a LiChroCART 4-4 guard cartridge (Merck) and eluted with sulphuric acid 18 mM (pH 2.6) at a flow rate of 0.9 mL min^{-1}. Ascorbic acid (AA) elution was monitored at 254 nm. The volume injected was 50 μL.

The two biologically active forms of vitamin C were identified: AA and dehydroascorbic acid (DHAA).

Samples were prepared in an extractive HPO_3 solution, of widespread use in food analysis, to preserve vitamin C content avoiding its oxidation. Concentration of HPO_3 was set at 3% w/v as pH values are below 3. AA stability and its complete extraction are thus accomplished (Watada, 1982). About 1.25 mL of sample (pulp or juice) previously filtered is diluted with HPO_3 solution up to 1 L volume. Then samples were injected directly after filtration through a Millex-GV13 0.22 μm filter. DHAA is reduced to AA with 0.2% α-dithiotreitol (DTT), thus determining total AA content. DHAA content is obtained by the difference of the two determinations. Results are expressed as mg mL^{-1} AA (Sánchez-Mata et al., 2000). Assays were performed to get the maximum reduction of DHAA in the minor time.

To quantify total AA, an aliquot of 1 mL of diluted sample in the extraction solution (HPO_3 3% with 1 mM EDTA) is mixed with 0.2 mL of reduction solution. This reduction solution consists of DTT 0.2% w/v in phosphate buffer, pH 7. Afterwards, 0.1 mL K_2PO_4 45% are added. This mixture is placed for 10 min in darkness until the reduction of DHAA is complete and 0.2 mL H_3PO_4 2 M were added. The final solution was filtered and injected onto chromatograph.

The identification of AA was carried out thanks to retention time and spectra matching, while quantification was performed by external calibration with the standard.

Recovery was tested in one pulp sample and two juice samples. As AA is the faster to react and be degraded, we chose it as reference antioxidant compound to express recovery. About 0.05 mL of a solution and 5 mg mL^{-1} of standard compound were added to 1 mL of sample. We obtained recoveries ranging from 90% to 95% for AA in these matrices.

2.8. Liquid chromatographic determination of phenolic compounds

Phenolic acids and flavonoids of acerola samples were determined by HPLC adapted from Betés-Saura et al. (1996). Samples were not purified but filtered through Millex-GV13

0.22 μm filter before injection. Pulp and concentrated juice were previously centrifuged at 4000 rpm for 10 min. Volume injected was 50 μL.

The column was a Merck Superspher 100 RP-18 (250 × 4 mm) protected by a guard cartridge LiChroCart® 4-4 (Merck). The flow rate was 0.9 mL min^{-1} and the temperature was set at 40 °C. Time of analysis was 80 min. The mobile phase consisted of two solvents A (acetic acid in water adjusted to pH = 2.65) and B (20% A+80% acetonitrile) programmed as follows:

Time (min)	Mobile phase A	Mobile phase B
0	100	0
5	98	2
10	96	4
20	90	10
25	86	14
35	80	20
45	75	25
55	70	30
60	0	100
70	100	0
80	100	0

Duplicate analyses have been performed for each sample. The identification was carried out thanks to retention time and spectra matching. Once spectra matching succeeded, results were confirmed by spiking with respective standards to achieve a complete identification by means of the so-called peak purity test. Those peaks not fulfilling these requirements were not quantified. Quantification was performed by external calibration with standards.

Calibration graphs, for linearity determination, were established with standard solutions containing a mixture of the six compounds identified in the samples. Concentration range for each compound was as follows: furfural (0.1–2 mg L^{-1}), procyanidin B1 (0.2–10 mg L^{-1}), chlorogenic acid (0.1–0.67 mg L^{-1}), rutin (0.43–3.58 mg L^{-1}), Epigallocatechingallate (0.5–3 mg L^{-1}). Accuracy was determined by adding three known quantities (80%, 100% and 120% of expected value) of each compound to the sample. Three sample replicates were prepared for each case, and percentage of recovery was calculated for each polyphenol.

2.9. Solid phase extraction (SPE) of polyphenolic compounds

The aim of this step is to ascertain the contribution of the different polyphenolic families to the antioxidant activity. The procedure involves a SPE using Waters C18 cartridges as described by Brenneman and Ebeler (1999). Initially, the sample (juice or pulp) extracts are adjusted to pH 7 and passed through the cartridge, followed by double-distilled water. The most hydrosoluble phenolic acids are not retained by the stationary phase and are collected in fraction 1. Fraction 2 (flavanols and anthocyanins) and 3 (flavonols) are then adsorbed onto the column. Their separation is accomplished by modifying the polarity of the mobile phase, eluting the former with an aqueous solution 16% acetonitrile (pH 2), followed by the latter with ethyl acetate.

The three extracts from each sample were vacuum-concentrated until solvent disappearance and reconstituted to original volume with water.

2.10. Statistical analysis

All data were reported as means ± standard deviation of three samples. Statistical analysis was performed with Statistica 7.0 software (Statsoft, Tulsa, USA). Differences were tested for significance by using the ANOVA procedure, using a significance level of $P \leqslant 0.05$.

3. Results and discussion

3.1. Vitamin C content

Table 1 shows the values obtained for AA and its reduced form DHAA in pulp and juice samples of acerola. Contents in total AA ranged from 6.32 to 9.20 g kg^{-1} of pulp and 9.44–17.97 g L^{-1} of juice, within the current values found in literature (Vendramini and Trugo, 2000; Marques et al., 2007). These values are sensibly higher than those found in other fruit juices such as orange juice (0.516 g L^{-1}), grapefruit juice (0.274 g L^{-1}), or lemon juice (0.327 g L^{-1}) (Ashoor et al., 1984). The processing techniques may explain the low values obtained in commercial juice samples. According to the literature, the state of maturity of the fruit in the period of harvest influences in this content, being higher in unripe fruits (Vendramini and Trugo, 2000). DHAA content is low in relation to AA (0.080–1.640 g kg^{-1} for pulp; 0.01–4.30 g L^{-1} for juice), as AA is the predominant form of vitamin C, contributing up to 85% of total vitamin C content in acerola (Carvalho and Manica, 1993). In our case, it accounted for a mean value of 90% of the total AA present in samples.

3.2. Polyphenolic composition of acerola samples

As can be seen in Table 2, pulp, squeezed and pressed samples show TPI values higher than caqui (14.5 mg kg^{-1}), pineapple (13.4 mg kg^{-1}), mango (16.4 mg kg^{-1}), guayaba (49.5 mg kg^{-1}) (Gorinstein et al., 1999), virgin olive oil (3000 mg kg^{-1}) (Gallina-Toschi et al., 2005), honey (3500 mg kg^{-1}) (Gheldof et al., 2002) or Cuban oregano extract (3400 mg kg^{-1}) (Zheng and Wang, 2001).

With respect to anthocyanin content, values obtained (Table 2) are lower than those of other fruit juices, as strawberries (557 mg L^{-1}) (Torreggiani et al., 1999) or blood oranges (128–217 mg L^{-1}) (Mondello et al., 2000). Lima et al. (2003) found values varying from 37.9 to 597.4 mg kg^{-1} in acerola pulps. We obtained for juices values two-fold higher than pulps, probably due to the skin contact time, allowing a higher release of anthocyanins compounds, which are found on the peels.

Table 2
TPI and total anthocyanin content of acerola samples

Sample	TPI[a]	Total anthocyanin content[b]
P1	7510 ± 140	28.2 ± 1.1
P2	5260 ± 120	27.2 ± 2.1
P3	6430 ± 100	27.0 ± 1.7
P4	4520 ± 70	ND
P5	4870 ± 80	ND
P6	5010 ± 50	ND
A1	8050 ± 80	52.3 ± 0.2
A2	9730 ± 110	46.9 ± 0.0
B1	10500 ± 110	49.7 ± 0.1
B2	10600 ± 100	50.3 ± 0.2
C1	11500 ± 120	ND
C2	10400 ± 170	ND
D1	1400 ± 20	ND
D2	3770 ± 50	ND

ND: not detected.
[a] mg kg^{-1}, gallic acid equiv. (pulp); mg L^{-1}, gallic acid equiv. (juice); mean ± standard deviation of triplicate.
[b] mg kg^{-1}, cyanidin-3-glucoside (pulp); mg L^{-1}, cyanidin-3-glucoside (juice); mean ± standard deviation of triplicate.

By means of HPLC and diode-array detection five polyphenolic compounds (procyanidin B1, chlorogenic acid, (−)-epigallocatechin gallate, (−)-epicatechin and rutin) have been identified in the samples analysed (Table 3), as well as furfural. Fig. 1 shows a chromatogram of an acerola juice containing numerous peaks. We attempted to identify other phenolic compounds as we observed peaks with similar spectra to p-coumaric acid, ferulic acid or (+)-catechin. Therefore, we spiked samples with these standards and concluded that these compounds were absent in our samples, as opposed to other authors findings, reporting the presence of ferulic acid in acerola juices (Righetto et al., 2005). Then we tried an acid hydrolysis (1:1 HCl pH 2 for 30 min and 1 h ultrasound treatment) in order to ascertain whether some unidentified peaks were derivatives of known phenolics (such as esters), but it yielded no results. Therefore, four peaks with a similar spectrum to flavan-3-ol dimers have been quantified as procyanidin B1. The same was applied to five peaks similar to rutin and one peak similar to chlorogenic acid. Presence of furfural in the commercial juice sample (D1) could be explained by caramelization during the pasteurization process. Procyanidin B1 and rutin are the most abundant of the identified compounds in pulps and juices. Chlorogenic acid is present in juices together with epigallocatechingallate whilst they are absent in pulps. In general, samples collected in January account for the highest concentrations in these compounds.

3.3. In vitro antioxidant activity of acerola samples

Different solvents are used as extractants to determine the antioxidant activity of fruits. Hence water has been used by some authors with ABTS (García-Alonso et al., 2004; Chen and Yen, 2007; Pellegrini et al., 2003), DPPH (Banerjee et al., 2005; Dasgupta and De, 2007) and ORAC methods (Cao et al., 1996; Wang et al., 1996; Al-Farsi et al., 2005; Kurilich et al., 2002).

Other authors use methanol with ABTS (Thaipong et al., 2006; Bhandari and Kawabata, 2004), DPPH (Silva et al., 2004; Thaipong et al., 2006; Jayaprakasha and Patil, 2007) and ORAC (Thaipong et al., 2006; Kalt et al., 1999) methods. Methanolic extracts were formerly used to determine the antioxidant activity of acerola by the methyl-linoleate method (Righetto et al., 2005).

Eight samples (three pulps and five juices) were extracted using water and methanol. Linearity was only obtained in

Table 4
Optimum sample dilution ranges to obtain linear responses in the different methods for antioxidant activity

	Pulp	Juice
ABTS method	1:1000– 1:200	1:1000– 0.3:100
DPPH method	1:200– 1.5:100	0.3:100– 1.2:100
ORAC method	1:2500– 1:3000	1:2000– 1:4000

Table 3
Polyphenolic composition (mg L^{-1}) of acerola samples

Sample	Furfural	Procyanidin B1	Chlorogenic acid	(−)-Epigallocatechin gallate	(−)-Epicatechin	Rutin
P1	ND	2.20 ± 0.08	ND	ND	ND	0.58 ± 0.06
P2	ND	3.19 ± 0.30	ND	0.89 ± 0.05	ND	1.43 ± 0.37
P3	ND	1.38 ± 0.03	ND	ND	ND	0.70 ± 0.05
P4	ND	1.58 ± 0.10	ND	ND	ND	0.60 ± 0.01
P5	ND	2.17 ± 0.21	ND	ND	ND	1.02 ± 0.07
P6	ND	1.77 ± 0.30	ND	ND	ND	1.09 ± 0.23
A1	ND	1.86 ± 0.24	ND	0.79 ± 0.06	ND	0.47 ± 0.04
A2	ND	1.44 ± 0.33	0.07 ± 0.00	0.74 ± 0.01	ND	0.59 ± 0.10
B1	ND	1.03 ± 0.19	ND	ND	ND	1.05 ± 0.10
B2	ND	1.23 ± 0.08	0.48 ± 0.00	0.78 ± 0.04	ND	0.90 ± 0.15
C1	ND	8.53 ± 0.15	ND	1.40 ± 0.03	ND	0.96 ± 0.02
C2	ND	5.59 ± 0.32	0.16 ± 0.00	0.53 ± 0.00	ND	1.75 ± 0.06
D1	0.15 ± 0.02		0.18 ± 0.00	ND	ND	1.71 ± 0.02
D2	ND		ND	3.19 ± 0.10	1.38 ± 0.06	3.01 ± 0.13
Retention time (min)	20.5	25.7	34.0	37.8	40.2	52.1
λ_{max} (nm)	278.8	281.2	326.3	276.5	278.8	257.5/355

ND: not detected.

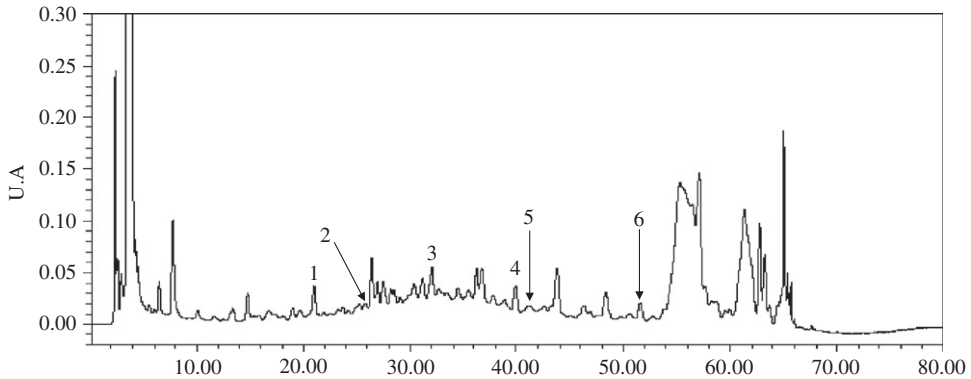

Fig. 1. Chromatogram of an acerola juice: (1) furfural, (2) procyanidin B1, (3) chlorogenic acid, (4) EPCG (epigallocatechin gallate), (5) (+)-epicatechin and (6) rutin.

a particular range of dilution, with both extractants, above which the results are not valid. Table 4 shows the appropriate dilution range found for each acerola sample under study, which has been used to calculate the antioxidant activity values of samples (Table 5). On the other hand, these dilution ranges ensure that the absorbance of samples at the wavelengths of measurement (414 nm for ABTS and 515 nm for DPPH) are avoided or at least minimized.

ANOVA did not show significant differences between antioxidant activities obtained for both extractants (water and methanol) as determined with all methods under study. Thus, water was selected as extractant for all samples.

Antioxidant activity values of the samples as determined by ABTS, DPPH and ORAC are displayed in Table 5. We explored if different treatments applied to acerola fruits (crushed and squeezed) yielded significant differences in antioxidant activity. Taking mean values for crushed and squeezed fruits and applying *t*-test, only ORAC values are significantly different ($P \leqslant 0.05$) for both groups.

TEAC values obtained for all the samples under study with ABTS method (Table 5) are higher than those reported for other juices or vegetables as orange (5.8 mM) (Proteggente et al., 2003), gazpacho (1.2 mM), blueberries (38.3 mM) and blackberries (26.5 mM) (Sellapan et al., 2002).

Table 5
Antioxidant activity values (TEAC, ORAC) of acerola samples

Sample	$TEAC_{ABTS}$[a]	$TEAC_{DPPH}$[a]	ORAC[b]
P1	64.76 ± 5.05	85.11 ± 3.30	58.83 ± 0.18
P2	51.11 ± 2.10	77.99 ± 3.28	47.42 ± 0.27
P3	46.16 ± 1.78	74.36 ± 6.21	46.75 ± 0.23
P4	36.95 ± 1.18	39.89 ± 0.10	34.58 ± 0.05
P5	38.80 ± 1.00	38.31 ± 0.70	34.87 ± 1.97
P6	37.84 ± 0.71	39.81 ± 1.00	40.40 ± 1.17
A1	90.28 ± 6.24	122.69 ± 8.15	85.39 ± 0.36
A2	76.13 ± 2.42	95.70 ± 4.91	50.57 ± 2.28
B1	91.76 ± 6.24	125.66 ± 8.37	76.71 ± 1.34
B2	85.21 ± 8.27	112.98 ± 1.77	57.52 ± 2.89
C1	81.50 ± 2.26	82.33 ± 1.82	77.14 ± 5.59
C2	74.30 ± 3.14	91.80 ± 1.35	83.03 ± 2.25
D1	2.03 ± 0.28	2.74 ± 0.36	1.26 ± 0.20
D2	32.16 ± 0.71	36.56 ± 3.08	22.37 ± 0.73

Each value is the mean ± standard deviation of the triplicate within the linear interval of response.
[a] TEAC values expressed as mM (mmol kg^{-1} pulp, mmol L^{-1} juice).
[b] ORAC values expressed as mM (mmol kg^{-1} pulp, mmol L^{-1} juice).

TEAC values for DPPH method are higher than wine (6–8 mM) (Fogliano et al., 1999), green tea infusions (8.3 mM) (Prior and Cao, 2000) and pomegranate fruit (28 mM) (Gil et al., 2000).

ORAC values of acerola samples were higher than those found in literature for cauliflower (17.7 mM), strawberry (15.4 mM) and spinach (12.6 mM) (Cao et al., 1996), and similar to bilberries (Howard et al., 2003).

3.4. Relationship between chemical composition of acerola and antioxidant activity

Antioxidant activity of acerola juices depends on the synergistic action of the constituents of the different fractions, with the most important components being phenolic compounds and vitamin C (Righetto et al., 2005). The contribution of AA to the total antioxidant activity of the samples, was evaluated by determining the antioxidant activity of AA solutions with equal concentrations as the assayed samples. It accounted for a 40–83% of the overall activity, similar to that of citric fruits (65–100%) and sensibly higher than apple (0.8%) or pineapple (0.8%) (Gardner et al., 2000).

Hence other phytochemicals, mainly polyphenols, would be responsible of the remaining antioxidant activity. Total polyphenols ranged from 6430 to 7510 mg kg^{-1} of pulp and 8050–10590 mg L^{-1} of juice, explaining the high figures obtained for the antioxidant activity. Antioxidant activity of the different phenolic families can be estimated by fractionating the samples by means of SPE. Figs. 2–4 show the percentage of contribution of vitamin C and the sum of phenolic fractions ($F1+F2+F3$) to the total antioxidant activity.

The relative contribution of the three phenolic fractions to the total antioxidant activity was as follows: fraction 1 (phenolic acids) > fraction 2 (anthocyanins and flavanols) > fraction 3 (flavonols). These results are obtained for all the methods under consideration (ABTS, DPPH, ORAC). Table 6 shows values obtained for DPPH method.

The remaining activity is due to other antioxidant compounds, as carotenoids. The carotenoid profile of acerola has been determined (Mezadri et al., 2005) and this fraction possesses mean ORAC values of 0.14 mM (pulp) and 0.10 mM (juice), accounting for a contribution between 0.4–0.2% and 0.5% to the total antioxidant activity of the samples.

The sum of the three phenolic fractions values accounted for 10–30% of the antioxidant values determined by ABTS and DPPH methods, being the highest contribution on pulp samples. If we

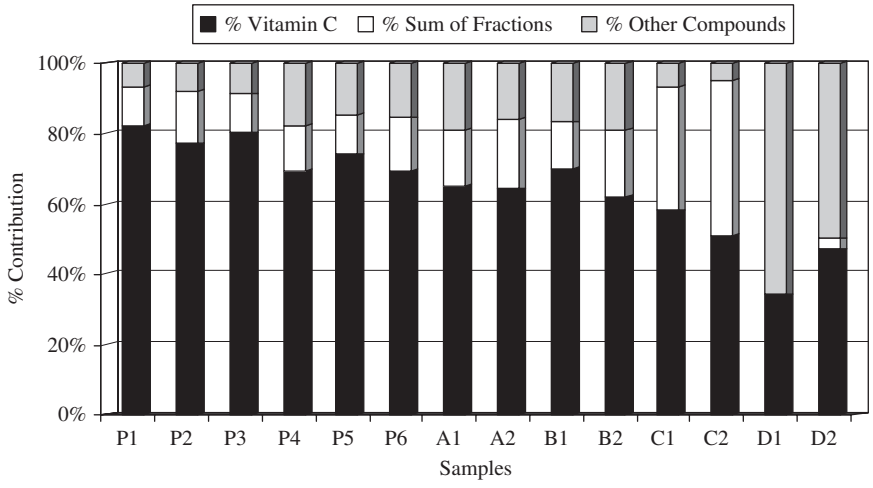

Fig. 2. Contribution percentage of different compounds to antioxidant activity of acerola measured with ABTS method.

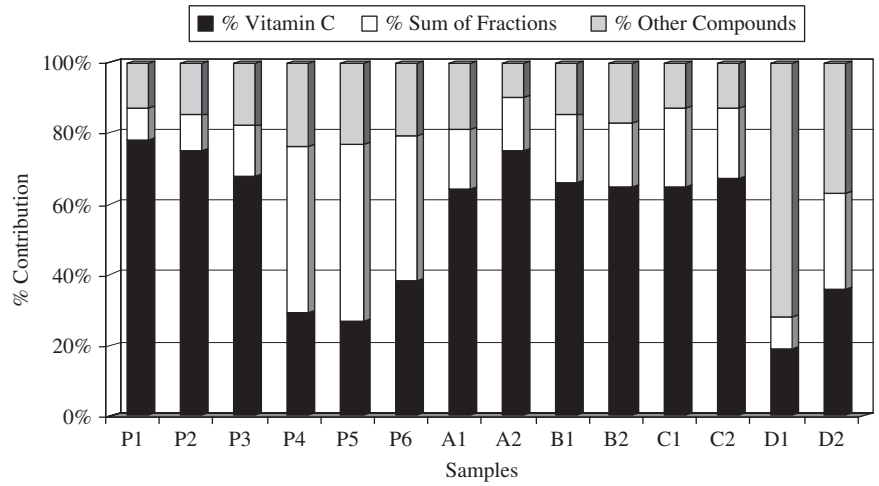

Fig. 3. Contribution percentage of different compounds to antioxidant activity of acerola measured with DPPH method.

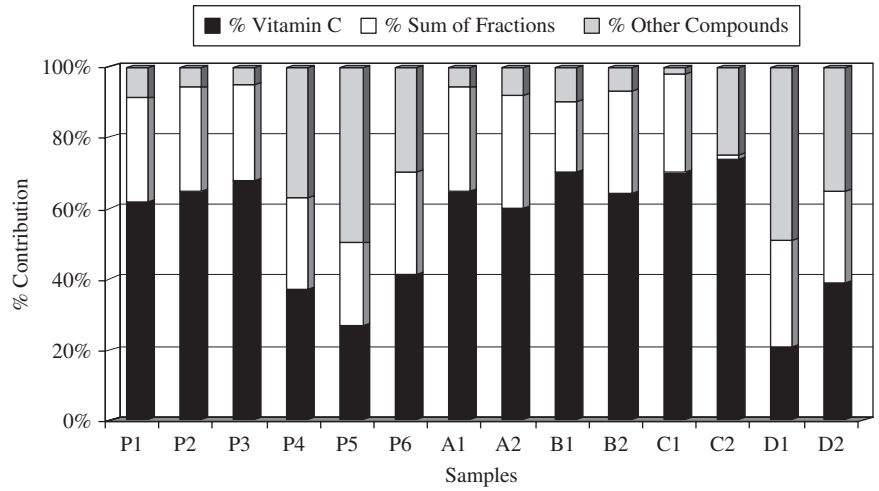

Fig. 4. Contribution percentage of different compounds to antioxidant activity of acerola measured with ORAC method.

Table 6
TEAC values[a]

Code	*F*1	*F*2	*F*3	Σ*F*	Sample	% Contribution
P1	6.24±0.32	1.65±0.36	0.22±0.00	8.11	85.1±3.30	9.55
P2	3.52±0.21	2.65±0.39	0.29±0.01	7.50	78.0±3.28	9.62
P3	8.81±0.24	1.10±0.18	0.31±0.01	10.2	74.4±6.21	13.7
P4	17.0±0.13	1.63±0.35	0.07±0.00	18.7	39.9±0.10	46.8
P5	16.4±0.16	2.58±0.39	0.11±0.00	19.1	38.3±0.70	49.5
P6	13.7±0.13	2.50±0.31	0.14±0.00	16.4	39.8±1.00	41.1
A1	17.2±0.63	3.06±0.16	0.19±0.02	20.4	123±8.15	16.5
A2	11.1±0.58	2.71±0.47	0.32±0.00	14.2	95.7±4.91	14.8
B1	20.2±0.59	3.34±0.48	0.28±0.02	23.8	126±8.37	18.9
B2	18.6±0.24	1.47±0.23	0.20±0.01	20.3	113±11.77	18.0
C1	14.7±1.17	3.12±0.53	0.06±0.00	17.9	82.3±1.82	21.8
C2	12.4±1.09	2.86±0.72	0.01±0.00	18.1	91.8±1.35	19.7
D1	–	–	0.25±0.09	0.25	2.74±0.36	9.12
D2	6.66±1.15	3.01±0.21	0.05±0.00	9.72	36.6±3.08	26.6

Each value is the mean±standard deviation of the triplicate within the linear interval of response.

[a] TEAC values expressed as mM (mmol kg^{-1} pulp, mmol L^{-1} juice).

consider ORAC method these values were of 25–30%. High correlation coefficients were for TPI and antioxidant activity measured by ABTS, DPPH and ORAC methods when the whole set of samples is considered (Table 7).

Table 7
Correlation coefficients

	Vitamin C concentration (g kg^{-1}; g L^{-1})	TPI (GAE; mg kg^{-1}; mg L^{-1})	Vitamin C antioxidant activity and TPI
ABTS	0.591	0.898	0.962
DPPH	0.415	0.784	0.959
ORAC	0.752	0.780	0.980

Attempts were made to correlate the single concentrations of the major phenolic compounds identified in the samples with the antioxidant activities observed, but poor correlations were found. The antioxidant activities of pure standards of procyanidin B1, chlorogenic acid and rutin were assessed in the laboratory (Villaño et al., 2005) and multiplied by their respective concentrations, thus calculating the theoretical contribution of each phenolic compound to AA of acerola samples but results obtained were similar. In addition, the inclusion of two variables in the statistical model was considered jointly: TPI and antioxidant activity of AA. The values of correlation are higher for the set of the samples analysed in all the methods, ABTS ($r = 0.962$), DPPH ($r = 0.959$) and ORAC ($r = 0.980$). It can be concluded that

antioxidant activity of acerola does not rely on one single phytochemical compound, but on the sum of them.

4. Conclusions

Acerola is a fruit with a high content on phytochemicals with proven antioxidant activities. Acerola fruits, commercial pulps and juices present high *in vitro* antioxidant activity, demonstrated with ABTS, DPPH and ORAC methods. The contribution of AA to the hydrophilic antioxidant activity ranges between 40% and 83%, the remaining due to polyphenols, mainly phenolic acids. These results therefore indicate that acerola fruit and derivatives are potent antioxidant foods and might have potential value as functional food ingredients.

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