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Phenolic Compounds in Acerola Fruit (*Malpighia punicifolia, L.*)

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Agliconas de antocianinas e outros compostos fenólicos foram identificados na fruta acerola (*Malpighia punicifolia, L).* As antocianinas, os flavonóides e os ácidos fenólicos foram fracionados e caracterizados através de dados cromatográficos e espectrais. O teor de antocianinas na casca da acerola madura foi 37,5 mg·100g^{-1}. Os pigmentos fenólicos identificados foram a pelargonidina, a malvidina 3,5-diglicosilada e a cianidina 3-glicosilada. Foram também identificados a quercetina, o kaempferol, e os ácidos p-cumárico, ferúlico, cafeico e clorogênico.

Anthocyanin aglycons and other phenolic compounds were identified in acerola (*Malpighia punicifolia, L.*). Anthocyanins, flavonoids and phenolic acids were fractionated and characterized by means of chromatographic and spectral data. The total content of anthocyanin pigments was 37.5 mg per 100 g of ripe acerola skin. The phenolic pigments identified were pelargonidin, malvidin 3,5-diglycoside and cyanidin 3-glycoside. Quercetin, kaempferol and the phenolic acids p-coumaric acid, ferulic, caffeic and chlorogenic were also identified.

Keywords: flavonoids, anthocyanins, phenolic acids, acerola, *Malpighia punicifolia L.*

Introduction

Phenolic compounds are important for plant metabolism and have also becoming important for humans due to their health characteristics, particularly related to their antioxidant properties.[1]

Anthocyanins are one of the most attractive plant phenolic pigments of the group of flavonoids. Their visual impact allied to their health properties make them potentially useful as natural food colorants.

One possible source of anthocyanin pigments is the fruit of acerola (*Malpighia punicifolia, L.*), a highly productive plant, which provides fruits during long periods of the year and is extensively cultivated in Brazil. The attractive red colour in acerola skin is mainly due to anthocyanins and the abundance of this fruit in Brazil represents a potential source of anthocyanin pigments.

Flavonoids are polyphenolic compounds that occur ubiquitously in food of plant origin and more than 2000 chemically distinct flavonoids have been already reported.[2]

Phenolic compounds are also important because of their contribution to the sensory quality of fruits (colour and flavour, including astringency and bitterness) which may be affected during the technological processes used for production of juice and other derived products.[3] In addition, anthocyanins and other phenolic compounds have been used successfully in the characterization of fruits and juices.[4]

Due to the importance of flavonoids, many techniques have been used to identify and quantify these compounds. In the early 1990s, high-performance liquid chromatography (HPLC), with photodiode array detection was used to isolation and quantification. ^{1}H and ^{13}C NMR spectroscopy are the most powerful techniques for determination of molecular structure. The coupling of HPLC and mass spectrometry methods, such as electrospray, thermospray, or fast-atom bombardment, have been widely used to provide molecular weight and characteristic fragment ions for structural elucidation[2], but these are very expensive techniques which are not readily available.[5] Due to simplicity of methodology and low cost, techniques like paper chromatography and thin-layer chormatography have been applied routinely in many laboratories.[2,6-8]

Acerola skin is a byproduct of acerola processing usually discarded in juice and pulp industries and due to its relatively high levels of anthocyanins it could be exploited as a potential source of these pigments. The interest in identifying sources of anthocyanin pigments to

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be used in food, pharmaceutical and cosmetic industries is based on consumer demand for natural colorants, and on the nutraceutical properties frequently reported for flavonoids.[9] However, as far as we know, no studies on characterization of acerola phenolics have been published. Thus, the objective of the present work was to isolate and characterize the phenolic compounds present in acerola and to estimate the amount of anthocyanin pigments found in the acerola skin which is usually a by product from the production of acerola pulp.

Material and Methods

Fruit

Acerola fruits (*M. punicifolia, L.*) were chosen by intensive red colour, firmness and a pleasant acid flavour, which are indicative of an ideal stage of ripeness. The fruits were harvested during June 2000 in a commercial plantation in Rio de Janeiro city (Brazil). After harversting, the fruits were washed, packed in polyethylene bags and stored in the dark at –18 °C for two days before anthocyanin extraction.

Chemicals

Caffeic, ferulic, p-coumaric and chlorogenic acids and quercetin, kaempferol, myricetin, and pelargonidin chloride, were obtained from Sigma Chemical Co (USA). The solvents used were of HPLC grade obtained from Tedia Co (USA). All other chemicals were of analytical grade.

HPLC equipment

A Pharmacia (Switzerland) liquid chromatographic system, equipped with two pumps (LKB model 2248), a controller (LKB model LCC 2252), and an injector motor valve (model PMV-7) with 20 μL loop was used. The system was attached to a UV-VIS detector SPD 10 AV (Shimadzu – Japan). An integrator model C-R6A (Shimadzu - Japan) was used for data processing.

Characterization of anthocyanin aglycones

Anthocyanins were analyzed in the extract from acerola skin where these pigments are concentrated. The skin of ripe acerola fruit (500g) was extracted with 600 mL of acidified methanol (0.1% citric acid, v/v) in the dark at about 15 °C and the extract concentrated under vacuum. Due to the non-availability of standards, anthocyanin characterization was carried out based on several physico-

chemical information such as mobility in HPLC, separation by paper chromatography and spectroscopic characteristics. Paper chromatographic separation was performed in the dark using Whatman paper 3 MM Chr (23 x 57 cm) according to the method described by Francis.[10] Analysis of HPLC was performed in a Lichrospher-100 RP-18 column (250 x 4.6 mm, Merck, Germany) using methanol-water (1:1, v/v) acidified with 2 mol L^{-1} HCl as mobile phase until pH 2.5 and flow rate of 1.3 mL min^{-1}. Detection was carried out at 530 nm.[11] UV-Vis spectroscopy was carried out in the fractions separated by paper chromatography that were dissolved in methanolic 0.01% HCl. UV-Vis spectra were obtained with a Beckman DU 650 spectrophotometer (Fullerton, USA), in the range of 200 – 600 nm, with the absorption spectra registered before and after the addition of 3 drops of a solution of the aluminum chloride salt in absolute ethanol (5% m/v).[8,12-14] The position of the sugar in the anthocyanin molecule was assigned based on the spectral shifts by calculation of the ratio of absorption at 440 nm to the specific absorption maximum for each pigment.[8,13,14] The amount of total anthocyanin in the acerola skin was estimated based on the calculation described by Lees and Francis[15] after measurement of the absorbance of the acidified ethanol extract at 535 nm.

Characterization of flavonols and phenolic acids

Flavonols and phenolic acids are distributed both in the fruit skin and pulp. Consequently, in order to obtain a higher yield, the whole fruit was used for extraction. Frozen acerola fruit (260 g) was blended with 250 mL of cooled distilled water and centrifuged at 3000 x g for 20 min at 4 °C. The extraction and centrifugation procedures were repeated twice. The combined supernatants were filtered through Whatman No.1 filter paper. Selective solvent extractions were then carried out to obtain one fraction containing the flavonol aglycons and other containing the phenolic acids, following the procedure described by Kader *et al.*[16] Characterization was then carried out using HPLC for separation and identification was done by comparison and spiking with external standards. For the analysis of flavonol aglycons, the mobile phase was methanol containing 0.5% orthophosphoric acid (1:1 v/v) with a flow rate of 1.2 mL/min., and detection at 370 nm.[17] For the analysis of the phenolic acids, the mobile phase consisted of a gradient elution with 0.01 mol L^{-1} tri-sodium citrate solution and 20% methanol adjusted to pH 2.5 with 6 mol L^{-1} HCl (solvent A) and methanol (solvent B). Elution was performed at a flow rate of 1.0 mL/min with the gradient of 5 min for B, increasing to 19% in 15 min, 20% in 5 min, 30% in 10 min and 50% in 10 min. Detection was

at 325 nm.[18] In both cases, a Lichrospher-100 RP-18 column (250 x 4.6 mm, 5 μm, Merck, Germany) was used.

Results and Discussion

The main objective of the present study was to characterize the phenolic compounds present in acerola, including the aglycones of phenolic glycosides. The structures of the compounds studied are shown in Figure 1. Phenolic pigments such as anthocyanins are usually found as glycosides in plants, however, when they are ingested as food the sugars are easily hydrolyzed from the
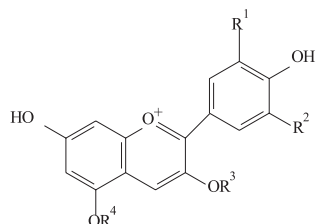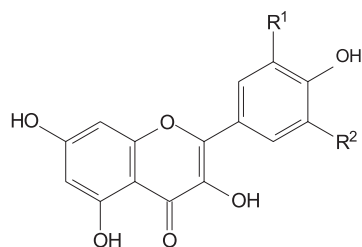
a) Acerola anthocyanins and anthocyanidin:
1 Malvidin 3,5-diglucoside ($R^1 = R^2 = OCH_3$, $R^3 = R^4 =$ glucose)
2 Cyanidin 3-glucoside ($R^1 = OH$, $R^2 = R^4 = H$, $R^3 =$ glucose)
3 Pelargonidin ($R^1 = R^2 = R^3 = R^4 = H$)
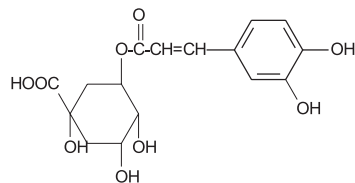
b) Acerola phenolic acids
4 clorogenic acid
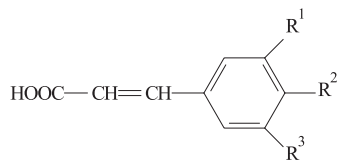
c) Acerola phenolic acids
5 caffeic acid ($R^1 = H$, $R^2 = R^3 = OH$)
6 p-coumaric acid ($R^1 = R^3 = H$, $R^2 = OH$)
7 ferulic acid ($R^1 = H$, $R^2 = OH$, $R^3 = OCH_3$)

d) Acerola flavonols:
8 Quercetin ($R^1 = OH$, $R^2 = H$)
9 Kaempferol ($R^1 = R^2 = H$)

Figure 1. Chemical structure of phenolic compounds identified in acerola.

aglycones. HPLC analysis of the anthocyanin fraction showed three peaks with retention times of 5.7 min, 14.9 min and 22.1 min (Figure 2). Similarly, paper chromatography revealed three bands with pink, magenta and orange colours, respectively. The typical order of chromatographic elution in reverse phase HPLC of different anthocyanins with similar glycosylation patterns is determined by the polarity of the aglycone. The three peaks observed in the HPLC chromatogram were correspondent to the three bands observed by descending paper chromatography. Peak 1 corresponded to the pink band (Rf = 0.43); peak 2 corresponded to the magenta band (Rf = 0.12) and peak 3 to the orange band (Rf = 0.56). The different colours of anthocyanin pigments reflect the nature of their hydroxylation and methoxylation patterns. An increase in hydroxylation is accompanied by an increase in blue colour while methoxylation enhances the red colour.[1] This would indicate that peak 1 may correspond to a malvidin with one methoxylation and peak 2 to a cianidin with two hydroxylations. Peak 3 indicates one hydroxylation in the molecule which confirms the assignment of pelargonidin that was determined by comparison and spiking with the external standard.
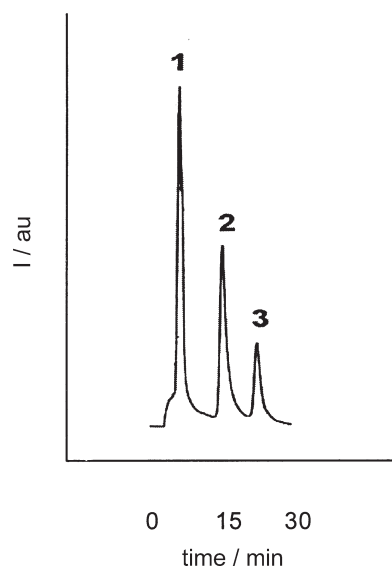
Figure 2. HPLC chromatogram of the methanolic extract of acerola. Column: Lichrospher – 100 RP 18 (250 x 4.6 mm); eluent: methanol/water (1:1 v/v) pH 2.5; flow-rate: 1.3 mL min^{-1}; detection: 530 nm; Peaks: 1, malvidin 3,5-diglycoside (5.7 min); 2, cyanidin 3-glycoside (14.9 min); 3, pelargonidin (22.1 min).

Additional qualitative information was obtained with the aid of the spectral characteristics of the chromatographic bands. The spectral data presented in Table 1 show the maximum absorptions for the chromatographic peaks as 277 and 536 nm for peak 1 (5.7 min); 281 and 527 nm

Table 1. Data of anthocyanin pigments from acerola

Rf (%) of bands from paper chromatography	λ_{max} (nm)	$Abs_{\lambda 440nm}/Abs_{\lambda vis\ max}$	AlCl$_3$ shift	HPLC t_R (min.)	Relative amount (% of peak area)	Peak Assignment
43	277; 536	11	–	5.7	68	Malvidin 3,5-diglycoside
12	281; 527	24	+	14.9	21	Cyanidin 3-glycoside
56	266; 523	35	–	22.1	11	Pelargonidin

for peak 2 (14.9 min) and 266 and 523 nm for peak 3 (22.1 min). These data are correspondent to spectral data of malvidin, cyanidin and pelargonidin.[7,10-12,19]

No change in colour was observed after addition of aluminum chloride in the fractions corresponding to peaks 1 and 3, but the one corresponding to peak 2 changed from red to blue as a consequence of a shift in the maximum of absorption. This is an indication that peak 2 is an *ortho*-dihydroxy anthocyanin belonging to the groups of pelargonidin, peonidin or malvidin.[19] This test enables to make a broad differentiation between the derivatives of the cyanidin, delphinidin and petunidin groups, which show a positive colour change, and those of the pelargonidin, peonidin and malvidin groups, which show no colour change.[19] These results further support the assignment of peak 3 as pelargonidin and indicate that peak 1 does not belong to the cianidin, delphinidin or petunidin groups.

The ratios of $Abs_{440}/Abs_{\lambda max}$ calculated for peaks 1, 2 and 3 were 11, 24 and 35, respectively. The ratio of absorption at 440nm to the maximum is useful in distinguishing anthocyanins when glycosilated at the 3- and 5- positions.[11,13] The $Abs_{440}/Abs_{\lambda max}$ ratio is usually about 11 for malvidin, corresponding to 3,5-diglycosides. The $Abs_{440}/Abs_{\lambda max}$ ratio is 24 for cyanidin derivatives substituted at position 3 and is 35 for pelargonidin which is not glycosilated.[11,13]

The results obtained in the present work are in agreement with literature data[7,13,19,20] for the assignment of a malvidin 3,5-diglycoside as peak 1 and a cyanidin 3-monoglycoside as peak 2.

Absence of absorption in the 308–328 nm region of the spectra indicated that none of the acerola pigments were acylated with aromatic organic acid.[20]

The content of anthocyanins was estimated as 37.5 mg per 100 g of ripe acerola skin. This anthocyanin content is higher than that found in red cabbage (25 mg), plum (2 – 25 mg), strawberries (15 – 35 mg) and banana bracts (32 mg) per 100g fresh weight.[14,21] This indicates that acerola skin which is a by product of the acerola pulp production may be used as a commercial source of natural pigment.

Qualitative HPLC analysis and identification by means of spiking and comparison with external standards were carried out in the fraction containing phenolic acids. Two major peaks were identified as p-coumaric and ferulic acid (peaks 6 and 7). Smaller peaks corresponding to chlorogenic acid (5-caffeoylquinic acid), and caffeic acid (peaks 4 and 5, respectively) were also detected in the fraction (Figure 3).
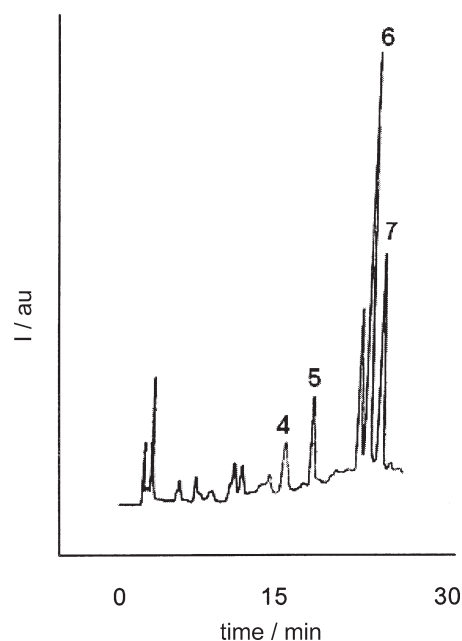

Figure 3. HPLC chromatogram of the phenolic acids extract of acerola. Column: Lichrospher – 100 RP 18 (250 x 4.6 mm); eluent: 0.01 mol L^{-1} tri-sodium citrate solution and 20% methanol (solvent A) and methanol (solvent B); flow-rate: 1.0 mL min^{-1}; gradient elution: 5 min for B, increasing to 19% in 15 min, 20% in 5 min, 30% in 10 min and 50% in 10 min; detection: 325 nm. Peaks: 4, chlorogenic acid (17.5 min); 5, caffeic acid (20.2 min); 6, p-coumaric acid (25.9 min); 7, ferulic acid (27.2 min).

Caracterization of flavonol aglycons is also important due to their biological activities particularly in relation to their antioxidant properties.[9,22-24] The fraction containing flavonol aglycons showed as expected maximum absorbance at 370 nm. Individual peaks obtained by HPLC were assigned after comparison of retention times and coelution with flavonol standards showing the presence of quercetin (Rt 8.2 min) and kaempferol (Rt 13.0 min) (Figure 4).
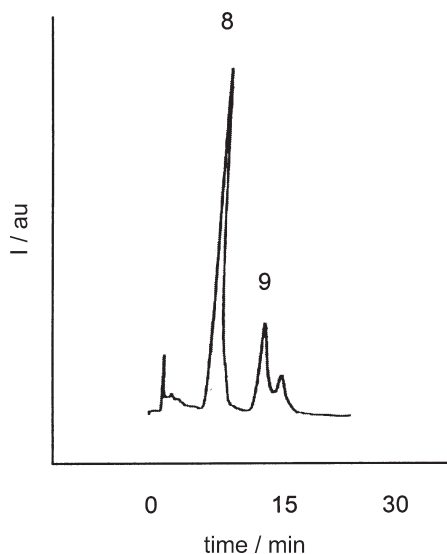
Figure 4. HPLC chromatogram of the flavonol aglycons extract of acerola. Column: Lichrospher – 100 RP 18 (250 x 4.6 mm); eluent: methanol/ 0.5% orthophosphoric acid (1:1 v/v); flow-rate: 1.2 mL min^{-1}; detection: 370 nm. Peaks: 8, quercetin (8.2 min); 9, kaempferol (13.0 min).

In conclusion, the phenolic compounds detected in acerola may be classified in two categories; phenolic anthocyanin pigments and non-anthocyanin phenolics. The pigments detected were a 3,5-diglycosilated malvidin, a 3-monoglycosilated cyanidin and pelargonidin. Non-anthocyanin phenolic compounds identified were p-coumaric acid, ferulic acid, caffeic acid, chlorogenic acid, kaempferol and quercetin.

Acknowledgements

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