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Anthocyanins from *Oxalis triangularis* as potential food colorants

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Abstract

Anthocyanin pigment content and profile from *Oxalis triangularis* (oxalis) was investigated for the first time. The pigments were extracted, purified using C-18 resin and characterized by HPLC, UV-visible spectral analysis, physicochemical reactions, and mass spectrometry before and after alkaline and acid hydrolysis. Monomeric anthocyanin content was 195 mg/100 g leaves on malvidin-3,5-diglucoside basis. Colour characteristics of a pH 3.5 oxalis anthocyanin extract (optical density of 0.3, 520 nm) were 86.0, 9.8 and 8.0 for Hunter CIE L^*, hue angle and chroma, respectively. Three anthocyanins represented 90% of the total peak area obtained by HPLC. The major anthocyanin was malvidin-3-rutinoside-5-glucoside and the others were acylated derivatives containing one and two molecules of malonic acid esterified to the main structure. The attractive hue, high anthocyanin content and edible character of *O. triangularis* make it a potential desirable source of natural colorant.

Keywords: *Oxalis triangularis*; Anthocyanins; Natural colorant; Pigments

1. Introduction

Colour is an important factor in the acceptability of a food product. The safety of synthetic colorants has previously been questioned, leading to a reduction in the number of permitted colorants. Due to this limitation and to the worldwide tendency towards the consumption of natural products, the interest in natural colorants has increased significantly (Huck & Wilkes, 1996). Of special interest to the food industry, is the limited availability of red pigments, mainly due to the prohibition of Red No. 2 and the continued scrutiny of Red No. 40 and Red No. 3 (Hallagan, 1991; Lauro, 1991).

Anthocyanins provide attractive colours such as orange, red and blue. They are water-soluble, which facilitates their incorporation into aqueous food systems. They are widespread in nature and are innocuous. These qualities make anthocyanins attractive natural colorants. However, some factors, such as the low stability when compared with synthetic dyes have limited their use (Markakis, 1982). Research on new viable sources of anthocyanins is required to find alternatives with desirable stability, low cost and high tinctorial strength.

Besides the colour attributes, anthocyanins have been reported to be beneficial to health, with potential physiological effects such as antineoplastic, radiation-protective, vasotonic, vasoprotective, anti-inflammatory, chemo- and hepato-protective (Kamei et al., 1995; Mazza & Miniati, 1993; Minkova, Drenska, Pantev, & Ovcharov, 1990; Wang, Cao, & Prior, 1997). Anthocyanins have also been observed to possess potent antioxidant properties (Cao, Sofic, & Prior, 1997; Wang et al., 1997).

Oxalis triangularis is popularly known as 'trevo roxo' — purple clover in Brazil, wood sorrel or Irish shamrock in England, vinagrillo in Cuba and vinagrillo rastrero in Argentina. It will be referred to as oxalis in this manuscript. It is a perennial plant from the Oxalidaceae family, possesses high germination power and is

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easily cultivated. It grows well in humid and low fields, near rivers, up to 1100 m of altitude (Lourteig, 1983). It has intensely purple-coloured leaves. Zurlo and Brandão (1990) have described its use for culinary purposes: raw in salads or as a complement for meat, either cooked or in gravies. The leaves are especially appreciated because of their sour and exotic taste. Preliminary studies showed that the pigments responsible for the purple coloration of the leaves are pink-reddish anthocyanins (Glória & Pazmiño-Dúran, 1996). The colour characteristics of the plant, combined with its traditional use in cuisine and the ease of cultivation, make Oxalis leaves a promising pigment source for food applications.

The objective of this study was to determine the anthocyanin pigment content and profile of the leaves of *O. triangularis*, for their potentital application as a natural food colorant.

2. Material and methods

2.1. Materials

Oxalis plants were grown in pots in a green house. The plant was classified at the Department of Botany at Universidade Católica de Minas Gerais, Belo Horizonte, MG, Brazil, as *Oxalis triangularis*. The leaves were separated from the stem, washed, dried and kept at −18°C until use. Concord grape (*Vitis labrusca*) and grape peel (*Vitis vinifera*) concentrates were used as standard sources of anthocyanins and anthocyanidins for characterization of the pigments. Succinic and malonic acids were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Extraction of anthocyanins

The anthocyanins from oxalis were extracted with 0.15% HCl in methanol (Francis, 1982). The leaves were blended with ca. 2 l of solvent/kg of leaves and filtered on a Buchner funnel. The filter cake residue was re-extracted until a clear solution was obtained. The extracts were combined and concentrated using a Büchi rotavapor at 35°C. The aqueous extract was made up to a known volume with distilled water.

2.3. Monomeric anthocyanin content

The extract obtained was analyzed for monomeric anthocyanins using a pH differential method (Wrolstad, Culbertson, Cornwell, & Mattick, 1982). A Shimadzu 160A UV spectrophotometer and 1-cm pathlength silica cells were used for spectral measurements at 420, 510 and 700 nm. Pigment content was calculated as malvidin-3,5-diglucoside, using an extinction coefficient of 37,700 l cm^{-1} mg^{-1} and molecular weight of 655.5 (Giusti, Rodriguez-Saona, & Wrolstad, 1999).

2.4. Colour characteristics

Hunter CIE L^* a^* b^* characteristics of anthocyanin-containing solutions (pH 3.5, optical density of 0.3 at 520 nm) were determined using a ColorQuest Hunter colorimeter. Samples were placed in 1-cm pathlength optical glass cells (Hellma, Germany) and colour parameters were measured in duplicate under total transmission mode, using illuminant C and 10° observer angle. The chroma (c) and hue angle (h) were calculated by the formulas $c=(a^{*2}+b^{*2})^{1/2}$ and $h=(\tan^{-1} a^*/b^*)$.

2.5. Anthocyanin purification

The aqueous extract was passed through a C-18 Sep-Pak cartridge (Water Associates, Milford, MA), prewashed with methanol followed by 0.01% aqueous HCl (Giusti & Wrolstad, 1996). Anthocyanins (and other phenolics) were adsorbed onto the Sep-Pak and washed with 2 volumes of 0.01% aqueous HCl. Anthocyanins were recovered with methanol containing 0.01% HCl (v/v). The methanolic extract was concentrated and the pigments were dissolved in ultrapure water (Milli-Q, Millipore, Bedford, MA) containing 0.01% HCl.

2.6. Alkaline hydrolysis of anthocyanins

Purified pigment (ca. 10 mg) was saponified in a screw-cap test tube with 10 ml of 10% aqueous KOH for 8 min at room temperature (ca. 23°C) in the dark, as described by Hong and Wrolstad (1990a). The solution was neutralized with 2 M HCl, the saponified anthocyanins were purified using C-18 Sep-Pak cartridge (Waters Assoc., Milford, MA) and concentrated.

2.7. Acid hydrolysis of anthocyanins

Fifteen millilitres of 2 M HCl were added to ca. 1 mg of purified saponified pigment in a screw-cap test tube, flushed with nitrogen and capped. The pigment was hydrolyzed for 45 min at 100°C, then cooled in an ice bath (Hong & Wrolstad, 1990b). The anthocyanidins were purified using C-18 Sep-Pak cartridge.

2.8. HPLC separation of anthocyanins, saponified anthocyanins and anthocyanidins

A Perkin-Elmer Series 400 liquid chromatograph, equipped with a Hewlett-Packard 1040A photodiode array detector and a Hewlett-Packard 9000 computer system was used. Detection was operated at 280, 320 and 520 nm, simultaneously. The spectra from 250 to 600 nm were recorded for all peaks.

For the separation of anthocyanins, saponified anthocyanins and anthocyanidins, a Supelcosil LC-18 column (5 μm), 250×5 mm i.d. (Supelco, Bellefonte, PA) was used, along with a ODS-10, 40×4.6 mm i.d., Micro-Guard column (Bio-Rad Laboratories). Solvent A was 100% acetonitrile, and B was 1% phosphoric acid, 10% acetic acid, 5% acetonitrile and water (Milli-Q, Millipore, Bedford, MA). The program followed a linear gradient from 0 to 30% A in 30 min. The flow rate was 1 ml/min and the injection volume 50 μl (Giusti & Wrolstad, 1996).

2.9. Electrospray mass spectroscopy of anthocyanins

Low-resolution mass spectroscopy (MS) was performed using electrospray MS. The instrument was a Perkin-Elmer SCIEX API III+ Mass Spectrometer, equipped with an Ion Spray source (ISV = 4700, orifice voltage of 80) and loop injection. 5 μl of partially-purified anthocyanin, saponified anthocyanin solution and anthocyanidin extracts were injected directly into the system (Giusti & Wrolstad, 1996).

2.10. Organic acid analysis

Organic acids, obtained from the saponification of oxalis anthocyanins, were separated by HPLC. A Perkin-Elmer Series 400 liquid chromatograph, equipped with a Hewlett-Packard 1040A photodiode array detector and a Hewlett-Packard 9000 computer system, was used. Detection was at 214 and 224 nm, simultaneously. Spherisorb ODS-2 and Spherisorb ODS (Supelco, Bellefonte, PA), both of 5 μm and 250×4.6 mm i.d., were connected in series. The mobile phase used was phosphate buffer (27.2 g KH_2PO_4/l with water), pH-adjusted to 2.4 with concentrated phosphoric acid. The flow rate was 0.7 ml/min and the injection volume 50 μl (Giusti & Wrolstad, 1996). The spectra and retention times of the acids were compared with those of pure standards.

2.11. Physico-chemical reactions

The reaction with aluminium chloride was used to verify the existence of vicinal hydroxyl-groups. The absorption spectra of a methanolic solution of the pigment was registered before and after addition of 5% methanolic $AlCl_3$ (Gross, 1987; Jackman & Smith, 1996; Markakis, 1982).

The presence of fluorescence under ultraviolet radiation was checked for anthocyanins separated by paper chromatography using 1% HCl and BAW as mobile phases to investigate the existence of substitute at C-5 (Francis, 1982).

2.12. Confirmation of identity of the anthocyanins

For the confirmation of the identity of the anthocyanins from oxalis, the purified pigment was submitted to partial hydrolysis. This was performed as indicated for acid hydrolysis of anthocyanins (pH 2.7); however, hydrolysis was interrupted within 25 min.

3. Results and discussion

3.1. Monomeric anthocyanin content and colour characteristics of the extract

O. triangularis proved to be a rich source of anthocyanin pigments, with a monomeric anthocyanin content of 195 mg/100 g leaves (2.42 g/100 g of dry leaves). This is considered a good yield compared with some natural pigments reported in the literature, such as in *Tradescantia pallida*, with 120 mg/100 g leaves (Shi, Lin, & Francis, 1992) and some types of berries, such as blackberries and cranberries, with 83–326 and 60–200 mg/100 g berries, respectively (Mazza & Miniati, 1993). A solution of oxalis' anthocyanins, with absorbance of 0.3 at 520 nm and pH 3.5, showed an attractive purplish hue and Hunter CIE colour characteristics of $L^* = 86.0$, $a^* = +7.9$ and $b^* = +1.4$. The chroma was 8.0 and the hue angle was 9.8°.

3.2. HPLC analysis

Three major anthocyanins were separated by HPLC, representing 90% of the total peak area (Fig. 1a). Several other minor anthocyanins were also detected, but each represented less than 2% of the total peak area. The spectra were recorded for the peaks. No significant absorbance at 310 nm was observed, on any of the peaks, suggesting the absence of hydroxylated aromatic organic acid acylation of the anthocyanins (Hong & Wrolstad, 1990a). The percent ratios $Abs_{440}/Abs_{\lambda max}$ were 12.0, 10.5 and 10.5%, respectively, indicating that all of the anthocyanins contain glycosides at the 3 and 5 positions. The presence of substituents at the positions 3 and 5 was confirmed by the presence of fluorescence, under UV light, of the three peaks separated by paper chromatography (Harborne, 1967; Markakis, 1982).

Two of the major peaks and most of the minor peaks disappeared after saponification of the pigments (Fig. 1b); therefore these pigments are acylated or sensitive to alkali (Hong & Wrolstad, 1990b). This information, combined with the low absorption at 310 nm, suggests the presence of aliphatic acids as acylating groups.

Acid hydrolysis of the saponified anthocyanins produced one major anthocyanidin corresponding to 96% of the total area at 520 nm. The retention time of this anthocyanidin and its visible absorption spectra, matched those obtained for malvidin from Concord grape extract after saponification and acid hydrolysis (Hong & Wrolstad, 1990a; Ribereau-Gayon, 1982), indicating the presence of malvidin as the major anthocyanidin present in oxalis.

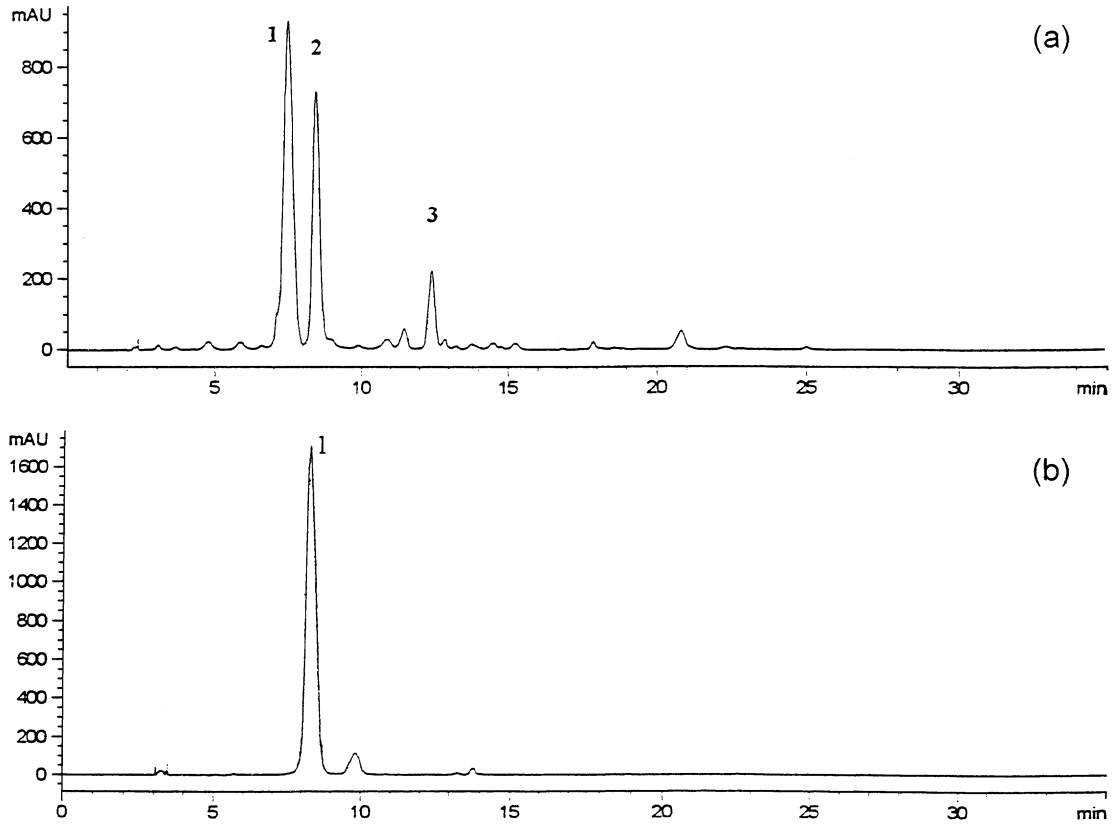

Fig. 1. HPLC separation of (a) anthocyanins and (b) saponified anthocyanins from oxalis. Supelcosil LC-18, 5 μm, 250×5 mm. Solvent A: 100% acetonitrile; B: 1% phosphoric acid, 10% acetic acid, 5% acetonitrile and water. Linear gradient of 0–30% A in 30 min. Flow rate: 1 ml/min. Injection volume: 50 μL. Detection at 520 nm.

3.3. Electrospray mass spectroscopy of anthocyanidins and anthocyanins

The low resolution mass spectrum of oxalis anthocyanidins showed a major peak at m/z 330.8, which corresponded to the molecular mass of malvidin (331), confirming the presence of this anthocyanidin in oxalis. The mass spectrum of oxalis anthocyanins showed major peaks at m/z of 800.8, 886.8 and 972. The m/z of 800.0 matched the molecular mass of malvidin glycosylated with rutinose and a hexose (801.4). The other m/z values matched the molecular mass of the same anthocyanin with either one (887.5) or two (973.6) malonic acid acylations. These peaks were not present in the ES-MS spectra of saponified oxalis anthocyanins, in agreement with HPLC results, showing these peaks are acylated.

3.4. Organic acid analysis

The saponified anthocyanin extract was submitted to HPLC analysis of aliphatic organic acids in an attempt to determine the acylating group esterified to the sugars of anthocyanin peaks 2 and 3. HPLC analysis revealed the presence of malonic acid, which confirmed results of low resolution mass spectroscopy of anthocyanins.

3.5. Peak identification

The combined information from HPLC separation of anthocyanins before and after hydrolysis, spectral and ES-MS information (Table 1) suggested that peak 1 in Fig. 1a was malvidin glycosylated with rutinose and a hexose, and that peaks 2 and 3 were acylated derivatives of the same anthocyanin. The $Abs_{440}/Abs_{\lambda max}$ ratio suggested the presence of glycosidic substitutions at both positions 3 and 5 of the anthocyanidin (Harborne, 1967). The presence of a glycosidic substituent at position 5 was also confirmed by fluorescence under UV light (Francis, 1982). According to Harborne (1967), the sugar in the positions 5 or 7 is always glucose, and never rhamnose. This information pointed to the identification of the pigments as malvidin with rutinose at position 3 and glucose at position 5. Therefore, peak 1 was identified as malvidin-3-rutinoside-5-glucoside and peaks 2 and 3 as the mono- or di-acylated derivatives of this anthocyanin, with or and two molecules of malonic. acid, respectively.

3.6. Confirmation of the identity

In order to confirm the proposed identity for the anthocyanin, partial hydrolysis of the saponified anthocyanin

was performed, since there are no known sources of this anthocyanin. According to Francis (1982), such a procedure provides valuable information about the chemical structure of a polyglycosylated anthocyanin. The partial hydrolysis of malvidin-3-rutinoside-5-glucoside would produce six products: the intact anthocyanin (malvidin-3-rutinoside-5-glucoside) and five different hydrolysis products, malvidin-3-glucoside-5-glucoside, malvidin-3-rutinoside, malvidin-3-glucoside, malvidin-5-glucoside and the aglycone malvidin.

Six peaks were separated after 25 min of hydrolysis (Fig. 2). Under reverse phase chromatography the elution order depends on the polarity of the compounds; triglycosides of any given anthocyanin would elute before their corresponding diglycosides, which would elute before their monoglycosides (Wrolstad, Hong, Boyles, & Durst, 1993). According to this criterion, peak 1 would be malvidin-3-rutinoside-5-glucoside; peak 2 a diglycoside-malvidin-3-glucoside-5-glucoside or malvidin-3-rutinoside. However, 3-rutinoside derivatives show longer retention times than the corresponding 3-glycosides, due to the hydrophobicity of the methyl group present in C-6 of rhamnose (Hong & Wrolstad, 1990a; Spanos & Wrolstad, 1987; Wrolstad et al., 1993). Therefore, peak 2 should correspond to malvidin-3-glucoside-5-glucoside. Following the same elution order criteria, Peak 3 should correspond to malvidin-3-glucoside. Its identity was confirmed by comparison of retention times and spectra of malvidin-3-glucoside obtained from the peel of *V. vinifera* grapes. Peak 4 would correspond to malvidin-3-rutinoside, peak 5 to malvidin-5-glucoside and peak 6 to malvidin, which is the least polar. Comparison of retention time and spectra of malvidin obtained from hydrolyzed grape anthocyanins also confirmed the identity of this last peak. Therefore, partial hydrolysis confirms that peak 1 is maldivin-3-rutinoside-5-glucoside.

4. Conclusions

Anthocyanin pigments from *O. triangularis* were investigated for the first time. The monomeric anthocyanin content was 195 mg/100 g of leaves on

Table 1
Peak assignment for anthocyanin pigments from Oxalis triangularis

Peak number[a]	Peak area (%)	Retention time (min)	λ max (nm)	$A_{440}/A_{\lambda\ max}$	Molecular ion (m/z)	Peak assignment [b]
1	63	7.4	520	12.0	800.8	mvd-3-rut-5-glu
2	29	8.3	525	10.5	886.8	mvd-3-rut-5-glu + malonic acid
3	14	12.4	525	10.5	972	mvd-3-rut-5-glu + 2 malonic acid

[a] As indicated on Fig. 1a.
[b] mvd, Malvidin; rut, rutinoside; glu, glucoside.

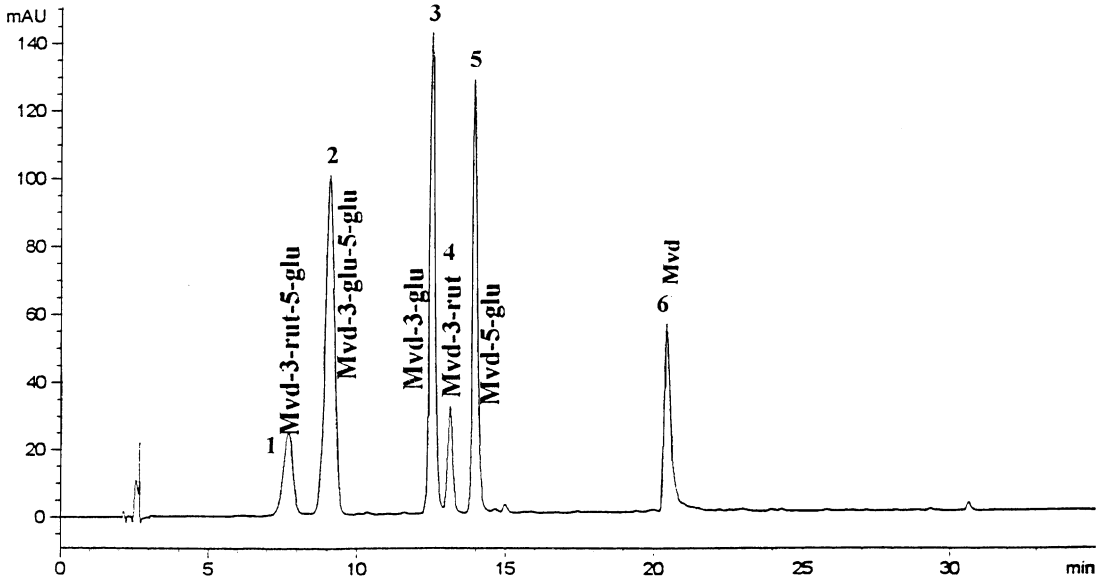

Fig. 2. HPLC separation of partially hydrolyzed non-acylated malvidin derivative from oxalis. Supelcosil LC-18, 5 μm, 250×5 mm. Solvent A: 100% acetonitrile; B: 1% phosphoric acid, 10% acetic acid, 5% acetonitrile and water. Linear gradient of 0–30% A in 30 min. Flow rate: 1 ml/min. Injection volume: 50 μL. Detection at 520 nm.

malvidin-3,5-diglucoside basis. Three major anthocyanins were separated, all of them sharing the same basic structure — malvidin-3-rutinoside-5-glucoside. Two were acylated with one and two molecules of malonic acid. Oxalis anthocyanin extract provided a purplish hue to pH 3.5 solutions. The attractive colour of oxalis anthocyanin extract, as well as the edible character of the plant, suggests that *O. triangularis* could be a good source of natural colorant. However, stability studies should be performed to investigate its potential use as a natural colorant.

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