PHYTOCHEMICAL ANALYSIS
Phytochem. Anal. **13**, 354–357 (2002)
Published online in Wiley InterScience (www.interscience.wiley.com). **DOI**: 10.1002/pca.667

Anthocyanin Composition of the Fruit of *Coriaria myrtifolia* L.

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The anthocyanin composition of the fruit of *Coriaria myrtifolia* L. and the changes which occur during ripening were studied using HPLC-PAD and LC-MS. Ten anthocyanins were detected and identified by their absorption and mass spectra as the 3-glucoside and 3-galactoside derivatives of delphinidin, cyanidin, petunidin, peonidin and malvidin. Fruit ripening was accompanied by substantial changes in the anthocyanin profile, with methoxylated anthocyanins, i.e. malvidin and peonidin, predominating in the final stages of ripening, and the trihydroxylated anthocyanin, delphinidin, during the earlier stages. Furthermore, galactoside derivatives were more abundant than glucosides in the ripe fruit. At full maturity, the fruits of *C. myrtifolia* were very rich in anthocyanins with a content of 10.7% (on a dry weight basis), a level which is higher than that found in most fruits usually considered to be anthocyanin-rich. The ability to grow *C. myrtifolia* in damaged and nitrogen poor soils, together with the possibility of using this plant for the extraction of anthocyanin, makes it ideal for consolidating soils and repopulating semi-desert or fire-damaged areas. Copyright © 2002 John Wiley & Sons, Ltd.

Keywords: Anthocyanins; flavonoids; polyphenols; *Coriaria myrtifolia*; Coriariaceae.

INTRODUCTION

The anthocyanins, naturally occurring pigments responsible for the blue, purple, violet, magenta and red colours of many fruits and vegetables, are glycosides of the anthocyanidins which consist of variously substituted 2-phenylbenzopyrylium (flavylium) salts. Differences between the anthocyanins are the number of hydroxy and methoxy groups, the nature and number of sugars in the molecule and their position of attachment, and the nature and number of aliphatic or aromatic acids that may be attached to the sugar residues (Mazza and Miniati, 1993).

There is increasing interest in plants from which anthocyanins can be extracted not only because of their use as food colorants (Francis, 1992, 1993) but also for their potential health-promoting properties. A number of studies have pointed to the ability of the anthocyanins to reduce capillary fragility, to protect against inflammatory diseases, and to increase the production of prostacyclin PGI_2 thus inhibiting the initial step in thrombus formation (Kühnau, 1976; Clifford, 2000).

Coriaria myrtifolia L. is a woody plant from the Mediterranean region, especially the western zone (Font-Quer, 1973). Its fruits are polydrupe, ranging in colour from red to violet according to the degree of ripeness. Although no bibliographical references to its anthocyanin composition have been found, the plant has certain characteristics which make it interesting as a source of anthocyanins. It readily adapts to different soil types and climates and has a particular capacity to re-establish itself in soils which have suffered fires (Fleck *et al.*, 1995). It can also colonise nitrogen-poor soils since its roots contain nitrogen-fixing nodules (Bond and Montserrat, 1958). The stems and leaves are rich in tannins and this has led to their use in the tanning industry, whilst the fruits are known to contain the sesquiterpene lactones coriamyrtin and corianin (Gastaldo *et al.*, 1987), picrotoxane analogues with potent neurochemical properties (Krische and Trost, 1998), which are also present in other species of *Coriaria* (Chang *et al.*, 1996; Valencia *et al.*, 2001).

The aim of the present work was to study the anthocyanin composition and its changes during the ripening of the fruits of *C. myrtifolia*, a plant which we consider of interest not only from an industrial point of view but also for its possible use for ameliorating environmental damage.

EXPERIMENTAL

Plant material. Fruits of *Coriaria myrtifolia* L. were collected at different times in 1997 in the province of Albacete, south-eastern Spain, at different stages of ripeness. Fruits were collected on 13 June, 1 July, 9 July and 16 July so that they ranged from fully formed (green with reddish markings) to intensely violet overripe berries. Immediately after picking fruits were kept in a desiccator containing silica gel and maintained under vacuum at 25°C until they were dry. Dried fruits were ground, passed through a 0.5 mm mesh and stored in the dark at 10–11°C in hermetically closed containers until required for analysis.

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Received 11 October 2001
Revised 26 December 2001
Accepted 24 January 2002

Extraction of fruit material. The desiccated samples were homogenised in triplicate in methanol containing 0.1% hydrochloric acid and then centrifuged. This process was continued until all of the colour had been extracted from the fruit material. A small volume of water was added to the bulked methanolic phases which were then evaporated (30°C) in a rotary evaporator until the methanol had been eliminated. The aqueous extract obtained was purified in a glass column (30 × 2 cm i.d.) filled to a height of 20 cm with a mixed stationary phase composed of 20% Polyclar AT (PVP) and 80% silica gel G60 which had previously been boiled under reflux in hydrochloric acid for 1 h and thoroughly washed with water after cooling. The extract was carefully added to the column which was then rinsed with water to remove sugars and acids and then with methanol containing 0.1% hydrochloric acid to elute the anthocyanins. After adding water to the methanolic eluent, the methanol was eliminated in a rotary evaporator and the residue made up to a known volume with water.

HPLC analysis. The anthocyanin composition of the extracts was determined by HPLC according to the method described by Wulf and Nagel (1978), using a C_{18} Nova Pak (Waters, Milford, MA, USA) column (250 × 4.5 mm i.d.; 5 μm) and acetonitrile (A) and 10% formic acid (B) as mobile phase. The gradient employed was: initially 5% A, increasing to 9% A at 5 min, to 11% A at 15 min, to 15% A at 40 min, to 20% A at 50 min, to 30% A at 65 min, and to 40% A at 70 min. The flow-rate was 1 mL/min. A photodiode array detector (PAD) was employed set at 520 nm as the preferred wavelength. Quantification of anthocyanins was performed from the peak areas recorded at 520 nm by reference to calibration curves obtained using malvidin-3-monoglucoside. The variation coefficients for each compound quantified using this method of extraction and HPLC analysis were typically lower than 8%.

Acid hydrolysis of the anthocyanins. The purified extract of anthocyanins was dissolved in 2 M trifluoroacetic acid and placed in a tube which was purged with nitrogen, closed and heated to 100°C for 90 min. After this time the tube was cooled and the contents were extracted with 3-methyl-1-butanol. The aqueous and organic phases were concentrated separately under vacuum, redissolved in 0.01 M hydrochloric acid, and analysed for the identification of sugars and anthocyanidins. The analysis of sugars was carried out by GC-FID after their transformation into the corresponding alditol-acetates using the method described by Albersheim *et al.* (1967). An ECNSS column (1/4″ OD × 2 mm ID × 2 m length) with 3% Gas Chrom P (Teknokroma, Barcelona, Spain) maintained at a constant temperature of 250°C was used for the separation. The detector and injector temperatures were 280 and 260°C, respectively, and nitrogen was used as the carrier gas. Sugars were identified from their retention times compared with those of standards. Anthocyanidins were analysed using the same method as for anthocyanins. Identifications were based on retention times and UV–vis spectra as compared with those available in an in-house library.

LC-MS analysis. A Waters Spherisorb ODS2 column (150 × 4.6 mm; 3 μm) was used with the solvents 1% formic acid (A) and acetonitrile (B). The mobile phase gradient was: initially 5% B, increasing to 15% B at 7 min, held isocratically at 15% B until 15 min, increasing to 25% B at 30 min, to 30% B at 40 min, to 40% B at 49 min, to 50% B at 54 min, to 60% B at 59 min, and to 70% B at 65 min. The flow-rate was 0.5 mL/min. Double on-line detection was carried out by PAD and MS. The mass spectrometer was a Finnigan LCQ (Thermoquest, San Jose, CA, USA) equipped with an API source, using an electrospray ionisation (ESI) interface. The HPLC system was connected to the probe of the mass spectrometer via the UV cell outlet. Both the auxiliary and the sheath gas were a mixture of nitrogen and helium. The capillary voltage was 3 V and the capillary temperature 195°C. Spectra were recorded in the positive ion mode between *m/z* 120 and 1500 and a series of three scans was made. A full mass scan, a zoom scan of the most abundant ion in the first scan, and an MS-MS of the most abundant ion using a relative collision energy of 20 eV.

RESULTS AND DISCUSSION

Identification of anthocyanins in fruits of *C. myrtifolia*

For the characterisation of the anthocyanin composition of fruits of *Coriaria myrtifolia* a methodological approach has been employed involving HPLC-PAD, GC-MS and LC-MS, thus allowing the complete identification of the anthocyanins in the fruit without requiring their previous isolation. Figure 1 shows an HPLC chromatogram measured at 520 nm of a purified extract of ripe fruits of *C. myrtifolia* in which 10 peaks corresponding to anthocyanins could be detected. Acid hydrolysis of the extract yielded five anthocyanidins which were identified as delphinidin, cyanidin, petunidin, peonidin and malvidin according to their chromatographic and spectral characteristics. The comparison of the retention times of the peaks in the chromatogram of Fig. 1 and the UV–vis spectra of the associated components with those of anthocyanin standards available in-house confirmed the presence in the extract of the 3-glucoside derivatives of delphinidin, cyanidin, petunidin, peonidin and malvidin (peaks 2, 4, 6, 8 and 10, respectively). Peaks 1, 3, 5, 7 and 9 showed UV–vis spectra identical to those of the compound eluting immediately afterwards, suggesting that each corresponded to an anthocyanin derived from the same respective aglycone. GC-FID analysis of the aqueous extract obtained after acid hydrolysis revealed the presence of only two sugars, glucose and galactose, which, therefore, must be the only sugars making up the structure of anthocyanins. LC-MS analysis revealed the molecular ions of the 10 anthocyanins detected in fruits of *C. myrtifolia* and their respective MS/MS spectra, in which the fragment corresponding to the aglycone was always evident. The results obtained are shown in Table 1, together with the wavelength of maximum absorption in the visible region of each compound as obtained by PAD.

The molecular ions of the 10 anthocyanins corresponded to monohexosides, with two compounds derived from each of the five aglycones detected. When only one sugar is present in an anthocyanin molecule it is assumed

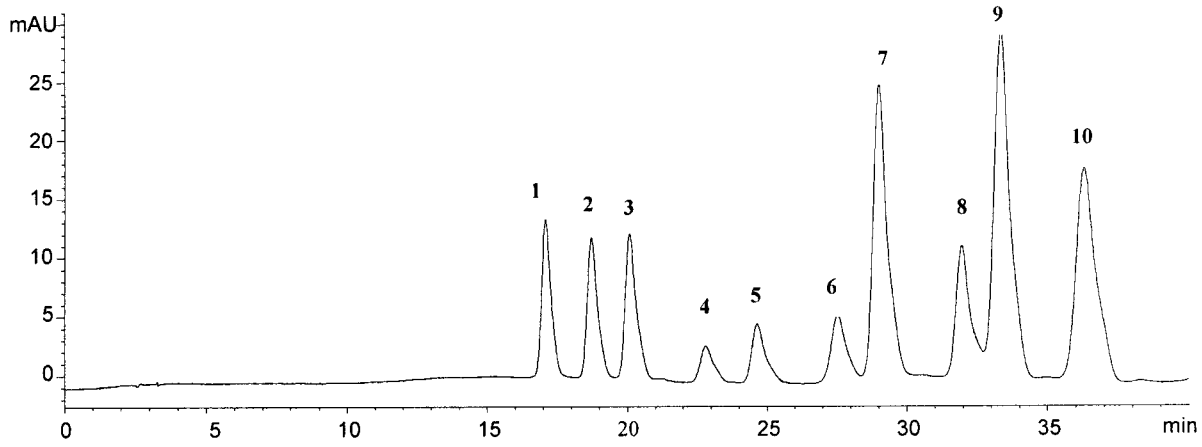

Figure 1. HPLC chromatogram recorded at 520 nm of an extract of ripe fruits of *Coriaria myrtifolia* L. (for chromatographic protocol see the Experimental section). Key to peak identities: **1**, delphinidin 3-galactoside; **2**, delphinidin 3-glucoside; **3**, cyanidin 3-galactoside; **4**, cyanidin 3-glucoside; **5**, petunidin 3-galactoside; **6**, petunidin 3-glucoside; **7**, peonidin 3-galactoside; **8**, peonidin 3-glucoside; **9**, malvidin 3-galactoside; **10**, malvidin 3-glucoside.

Table 1. Wavelengths of absorption maxima, molecular ions, MS/MS fragmentations and identities of the anthocyanins detected in fruits of *Coriaria myrtifolia* L.

Peak	λ_{max} (nm)	Molecular ion [M^+] (m/z)	MS/MS of [M^+] (*m/z*)	Anthocyanin
1	524	465	303	Delphinidin 3-galactoside
2	524	465	303	Delphinidin 3-glucoside
3	516	449	287	Cyanidin 3-galactoside
4	516	449	287	Cyanidin 3-glucoside
5	528	479	317	Petunidin 3-galactoside
6	528	479	317	Petunidin 3-glucoside
7	520	463	301	Peonidin 3-galactoside
8	520	463	301	Peonidin 3-glucoside
9	528	493	331	Malvidin 3-galactoside
10	528	493	331	Malvidin 3-glucoside

to be located at position 3 (Mazza and Miniati, 1993). Hence, the identification of glucose and galactose as the only sugars released after acid hydrolysis strongly suggests that the anthocyanins detected correspond to the 3-glucoside and 3-galactoside derivatives of each of the five anthocyanidins. Since the even numbered peaks in Fig. 1 correspond to the anthocyanidin 3-glucosides, the odd numbered peaks must correspond to the 3-galactoside derivatives. The elution of the galactosides before the glucosides coincides with the pattern of elution in reversed-phase columns described for these anthocyanins in the literature (Andersen, 1987; Oszmianski and Sapis, 1988).

Evolution of anthocyanins during fruit ripening in *C. myrtifolia*

Table 2 shows the changes which occurred in the anthocyanin content (expressed in terms of malvidin 3-glucoside equivalents on a dry weight basis) from their first appearance in the fruit until the time when fruit were fully ripe. As expected, total anthocyanin content

Table 2. Concentrations of anthocyanins during fruit-ripening in *Coriaria myrtifolia* L.

	Concentration of anthocyanin (mg malvidin 3-glucoside equivalents/100 g dry weight)			
Component	15 June	1 July	9 July	16 July
Delphinidin 3-galactoside	12.0	115.9	768.1	902.8
Delphinidin 3-glucoside	11.6	124.8	856.5	642.1
Cyanidin 3-galactoside	26.0	21.1	174.1	607.3
Cyanidin 3-glucoside	4.0	8.9	142.9	323.4
Petunidin 3-galactoside	3.7	16.1	148.9	554.0
Petunidin 3-glucoside	3.3	17.5	132.6	456.6
Peonidin 3-galactoside	13.8	5.6	63.3	1559.3
Peonidin 3-glucoside	9.1	2.7	30.5	1215.5
Malvidin 3-galactoside	26.6	51.6	125.0	3037.6
Malvidin 3-glucoside	20.7	27.1	86.6	1420.7
Total anthocyanins	130.7	391.3	2528.6	10719.3

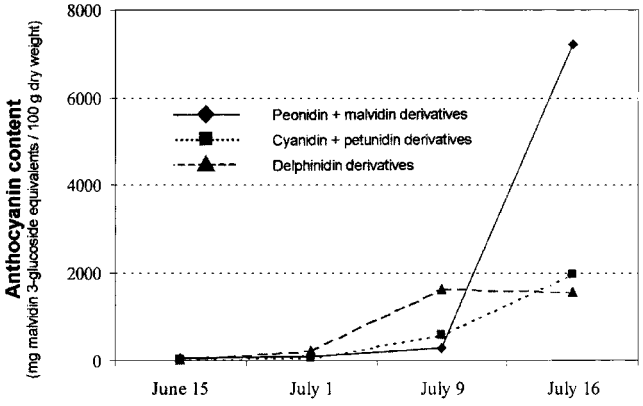

Figure 2. Changes in the concentrations of anthocyanins during the ripening of the fruits of *Coriaria myrtifolia* L.

increased as ripening progressed, especially during the last stages. There were also substantial changes in the qualitative profile with anthocyanins possessing a single hydroxy group on the B ring (peonidin and malvidin) predominating during the final stages of ripening, and trihydroxylated (delphinidin) and dihydroxylated (cyanidin and petunidin) anthocyanins being the most abundant during earlier stages (Fig. 2). This indicates an increase in the degree of methoxylation in the B ring as ripening progresses, with the formation of peonidin and malvidin at the expense of delphinidin (with petunidin as intermediary) and cyanidin, respectively. The successive accumulation of anthocyanins with increasing degree of substitution in the B ring during ripening was also noted for "Syrah" grapes (Roggero *et al.*, 1986). It is also worth noting that during the final stages of ripening there is an accumulation of galactoside derivatives, which outweigh the glucosides in the ripe fruit (62 and 38% of total anthocyanins, respectively). These findings indicate the importance of choosing the correct moment for collecting fruit since this will decide not only the final yield of anthocyanins but also the type of compounds present. Early harvesting would result in a lower total anthocyanin content but a larger proportion of delphinidin derivates, while a late harvest would provide greater quantities of malvidin and peonidin derivatives

Another aspect to emphasise is the high anthocyanin content of these fruit, which at full maturity represents 10.7% of the dry matter. Grapes, berries and currants are considered to be fruit sources most rich in anthocyanins, although literature data concerning content are generally given on a fresh weight basis (Macheix *et al.*, 1990; Mazza and Miniati, 1993; Bridle and Timberlake, 1997; Clifford, 2000). Taking into account the water content of these fruits, their anthocyanin concentration would typically be well below 10% of the dry matter. Such a consideration leads us to consider the fruit of *C. myrtifolia* as a very rich source for anthocyanin extraction. The value of the plant is even greater when one considers how undemanding it is and its ability to grow in damaged and/or nitrogen-poor soils, making it ideal for consolidating soils and repopulating semi-desert or fire-damaged areas. The environmental value of this species must be considered extremely high.

Acknowledgement

The authors wish to thank Cándida Lorenzo Tendero for technical assistance.

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