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Extraction and identification of anthocyanins from *Smilax aspera* L. berries

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Abstract

Anthocyanins, present in the berries of *Smilax aspera* L., a creeping shrub typical of the Mediterranean region, were extracted and identified for the first time. The pigments were extracted from the skin of the berries with of 0.1% HCl in methanol solution, purified on a C-18 solid-phase cartridge and characterized by means of HPLC-DAD-MS analysis. From spectral analysis before and after acid and alkaline hydrolysis, it was noted that pelargonidin 3-*O*-rutinoside represented about 83% of the total anthocyanin content in the skin of *Smilax aspera* berries. A low quantity of cyanidin 3-*O*-rutinoside (13%) was also found. The attractive colour and the great abundance of the plant in the south of Italy make *Smilax aspera* berries a new and very good source of natural pigments.

Keywords: Anthocyanins; *Smilax aspera*; HPLC-DAD; Mass spectrometry; Natural colorants

1. Introduction

Anthocyanins are flavonoid phenolic compounds, widely distributed among fruits, berries and flowers (Cooper-Driver, 2001), providing attractive colours, such as orange, red and blue. These pigments are water-soluble and this property facilitates their incorporation into numerous aqueous food systems. These qualities make anthocyanins attractive natural colorants. Moreover, it has been demonstrated that, in addition to their colourful characteristics (Pazmino-Duran, Giusti, Wrolstad, & Gloria, 2001), anthocyanins possess some positive therapeutic effects, mainly associated with their antioxidant properties (Tamura & Yamaganci, 1994; Wang et al., 1999; Wang & Jiao, 2000). Anthocyanins have recently received increasing attention as natural colorants in food systems, as a consequence of the social trend toward the consumption of natural products instead of synthetic ones. Thus, new sources of pigments, such as anthocyanins, with high colorant power, stability and low cost are nowadays desired.

Smilax aspera L., familiarly known as sarsaparilla, is an evergreen, creeping, extremely tough shrub of the family Liliaceae, typical of the Mediterranean region. It grows in bushy places and in light woodland, forming frequently hedgerows. The leaves are glossy, heart-shaped and are accompanied by a pair of tendrils at the base of the petiole. Flowers, as branched clusters, are dioecious (with male and female on separated plants); the berries, whose dimensions are between 2–4 mm, take on a red colour when ripe (Fig. 1). Leaves and root of this plant have edible use: the roots are used, for example, as an ingredient of soft drinks (Holtom & Hylton, 1979) and young shoots can be cooked and used as an asparagus substitute (Facciola, 1990; Polunin, 1969; Polunin & Huxley, 1987). The tendrils also can be simply eaten (Niebuhr, 1970). *Smilax aspera* L. also has medicinal use: the root has depurative, diaphoretic,

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Fig. 1. *Smilax aspera L.* berries.

diuretic, stimulant and tonic properties (Chiej, 1984; Holtom & Hylton, 1979). Flavonoids and tannins have been studied in the foliage and rhizome of *Smilax aspera* (Ayengar & Rangaswami, 1967; Bruno, De Laurentis, Amico, & Stefanizzi, 1985; Karuza, Petricic, Pitarevic, & Blekic, 1993; Tschesche, Harz, & Petricic, 1974). However, to our knowledge, the anthocyanin composition of *Smilax aspera* berries has never been described. So the objective of this study is to determine anthocyanin pigments in the skin of *Smilax aspera* L. berries. The colour characteristics of these berries, combined with the great abundance of the plant in the south of Italy, make *Smilax aspera* berries an interesting source of anthocyanins for use in food, pharmaceutical and cosmetics industries.

2. Material and methods

2.1. Materials

Wild-grown *Smilax aspera* berries were hand-harvested in the wood of "Parco Regionale Bosco e Paludi di Rauccio", Lecce, Italy, during May, 2004, placed in polyethylene bags and stored at −20 °C prior to use. The plant was classified at the Systematic Botanic and Vegetable Ecology Laboratory of the Department of Biological and Environmental Science and Technology, University of Lecce, Italy, as *Smilax aspera* L.

Trifluoroacetic acid (TFA) was purchased from Romil Ltd. (Cambridge, UK). Hydrochloric acid (36–38%), acetonitrile and water of HPLC grade and methanol of analytical grade were provided by J.T. Baker (Deventer, Holland). Deionised water (Easypure II RF ultrapure water system, Barnstead, USA) was used to prepare all solutions. Cyanidin 3-*O*-glucoside was purchased from Extrasynthese (Genay, France).

2.2. Procedure for the anthocyanin extraction

Anthocyanins were extracted from hand-peeled skins of *Smilax aspera* berries with 0.1% HCl (v/v) in methanol for 20 h at room temperature, in darkness. The mixture was filtered on a Buchner funnel and the remaining solids were washed with 0.1% HCl in methanol until a clear solution was obtained. The combined filtrates were dried using a rotary evaporator at 30 °C. The concentrate was dissolved in 0.01% HCl (v/v) in deionised water and the solution obtained was used for further purification.

2.3. Procedure for the anthocyanin purification

The aqueous solution obtained from the extraction procedure described above was passed through a C-18 Sep-Pak cartridge (Waters Corporation, Milford, MA), previously activated with methanol, followed by 0.01% HCl (v/v) in deionised water. Anthocyanins and polyphenolics were adsorbed onto the Sep-Pak column while sugars, acids, and other water-soluble compounds were removed by washing the minicolumn with 2 vol. of 0.01% HCl in deionised water. Polyphenolics were subsequently eluted with ethyl acetate. Then anthocyanins were recovered with methanol containing 0.01% HCl (v/v). This acidified methanol fraction was evaporated using a rotary evaporator at 30 °C and the remaining solid dissolved in 0.01% aqueous HCl (v/v) and immediately analyzed. This solution, containing the purified anthocyanins, was stored at −20 °C prior to further analyses after acid and alkaline hydrolyses.

2.4. Acid hydrolysis of anthocyanins

Five millilitres of 2 N HCl were added to the solution of the purified anthocyanins (0.5 ml) in a screw-cap test tube, flushed with nitrogen, and capped, as described by Chaovanalikit, Thompson, and Wrolstad (2004). The hydrolysis was carried out for 1 h at 100 °C and then the solution was immediately cooled in an ice bath. The hydrolysate was purified by using a C-18 Sep-Pak cartridge (Waters Corporation, Milford, MA), following the procedure previously described in the purification section.

2.5. Alkaline hydrolysis of anthocyanins

The solution of the purified anthocyanins (0.5 ml) was saponified in a screw-cap test tube with 5 ml of 10% KOH for 8 min in the dark at room temperature, as described by Chaovanalikit et al. (2004). Then, the

solution obtained was neutralized with 2 N HCl and the hydrolysate purified by using a C-18 Sep-Pak cartridge (Waters Corporation, Milford, MA), following the procedure previously described.

2.6. Analytical method

The HPLC-DAD-MS analyses were performed using an Agilent 1100 Series LC/MSD system with a DAD (diode array detector) coupled to a mass spectrometer (quadrupole analyzer) equipped with ESI (electrospray ionization) interfaces (Agilent). Chromatographic separation was carried out using a 150×4.6 mm i.d., 5 μm SS wakosil C18 column with a 4×3 mm i.d. phenomenex C18 guard cartridge both thermostatted at 32 °C. The mobile phase was composed of 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B) at a flow rate of 1 ml/min. The following gradient was utilized: 0′ 10% B; 2′ 10% B; 30′ 20% B; 40′ STOP. Absorbance spectra were recorded every 2 s, between 200 and 600 nm, with a bandwidth of 4 nm, and chromatograms were acquired at 520, 440 and 310 nm. MS parameters were as follows: capillary voltage, 4000 V; fragmentor, 160 V; drying gas temperature, 350 °C; gas flow (N_2), 10 l/min; nebulizer pressure, 50 psig. The instrument was operated in positive ion mode scanning from *m/z* 100 to 800 at a scan rate of 1.43 s/cycle.

The wavelength used for quantification was 520 nm. The calibration curve was produced by the integration of absorption peaks generated from analysis of a dilution series of cyanidin-3-glucoside.

3. Results and discussion

The anthocyanin composition of the purified extract from the skin of *Smilax aspera* berries was determined by means HPLC-DAD-MS analysis. The chromatogram obtained at 520 nm is shown in Fig. 2. As can be seen, four peaks are present in the chromatogram but the minor peaks 1 and 2, having an area percentage of less than 3% and with a poor resolution were not considered. The two major anthocyanins, corresponding to peaks 3 and 4, the structures of which are shown in Fig. 3, represented about 13% and 83%, respectively, of the total peak area revealed at 520 nm. These pigments were identified by their HPLC retention times, elution order, spectroscopic characteristics and fragmentation pattern (Table 1) by comparison with the literature values (Giusti, Rodrìguez-Saona, & Wrolstad, 1999; Hong & Wrolstad, 1990; Mullen et al., 2002) and our anthocyanin library. Electrospray ionization mass spectrometry analysis of the detected anthocyanins produced intact molecular ions (M^+) at *m/z* 595 (peak 3) and 579 (peak 4), corresponding to rutinoside derivatives of cyanidin and pelargonidin, respectively. The mass spectra of these compounds are shown in Fig. 4: they presented the molecular ions M^+ and the fragments resulting from the loss of the rutinose molecule, $M^+ - 308$ Da, corresponding to the aglycones: 287 (peak 3) and 271 (peak

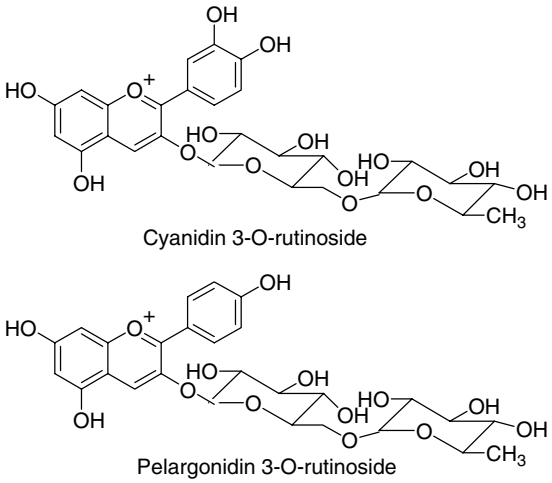

Fig. 3. Structures of the anthocyanins identified in the skin of *Smilax aspera* L. berries.

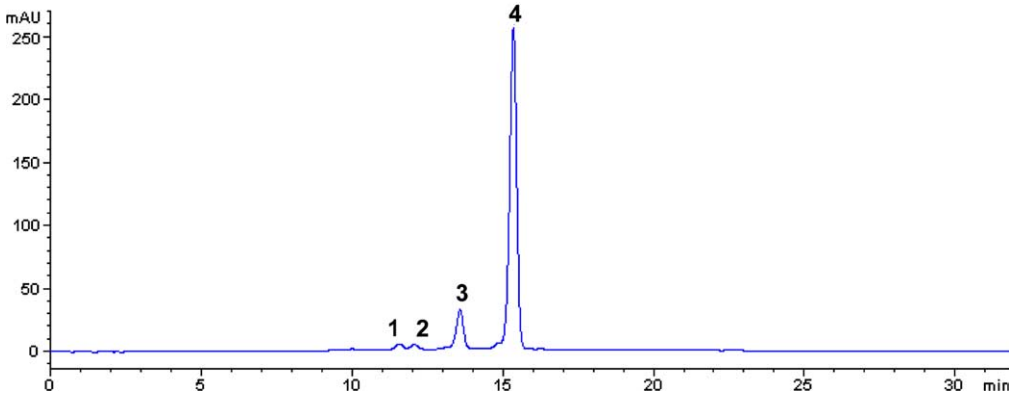

Fig. 2. Chromatogram recorded at 520 nm corresponding to the purified extract of *Smilax aspera* L. berries.

Table 1
Chromatographic and spectral characteristics of the anthocyanins found in the skin of *Smilax aspera* L. berries

Peak no. (Fig. 2)	Peak area (%)	t_R (min)	Vis λ_{max} (nm)	M^+ (*m*/*z*)	$M^+ - X$ (*m*/*z*)	Peak assignment
3	13.18	13.55	520	595	287 (M^+-rut)	Cyanidin 3-*O*-rutinoside
4	83.12	15.33	504	579	271 (M^+-rut)	Pelargonidin 3-*O*-rutinoside

4), corresponding to the molecular ions of cyanidin and pelargonidin, respectively.

The UV–Vis spectra of these compounds, shown in Fig. 5, confirmed their identity. Peaks 3 and 4 (Fig. 2) exhibited visible maximum wavelengths (λ_{max}) at 520 and 504 nm, respectively. The maximum wavelength in the visible range is closely related to the hydroxylation pattern of the anthocyanin and it has been reported that pelargonidin 3-glucoside exhibits a visible λ_{max} at about 505 nm while cyanidin 3-glucoside has a visible λ_{max} at 520–526 nm (Hong & Wrolstad, 1990). Because the nature of the sugar substitution has no effect on absorbance spectra, similar spectral characteristics can be supposed for corresponding rutinose-substituted anthocyanins. As expected, pelargonidin derivative (peak 4) had a lower visible λ_{max} than had the cyanidin derivative (peak 3) but exhibited the highest $Abs_{440}/Abs_{\lambda\ max}$ ratio (Hong & Wrolstad, 1990). Moreover, peak 4 exhibited a pronounced shoulder in the 400–450 nm region, typical of the pelargonidin–anthocyanidin-type skeleton (Hong & Wrolstad, 1990).

As reported by Harborne (1976), anthocyanins with glycosidic substitutions in the C-3 position exhibit a ratio of the absorbance at 440 nm to the absorbance at the visible maximum wavelength (520 nm) almost twice these of anthocyanins with glycosidic substitutions at position 5 or both 3 and 5. Thus, the $Abs_{440}/Abs_{\lambda\ max}$ ratios of 36.8% and 51.8%, found for peaks 3 and 4, respectively, indicated glycosidic substitution in the C-3 position of the anthocyanidin (Giusti et al., 1999; Hong & Wrolstad, 1990). The absorbance spectra of anthocyanins provide further

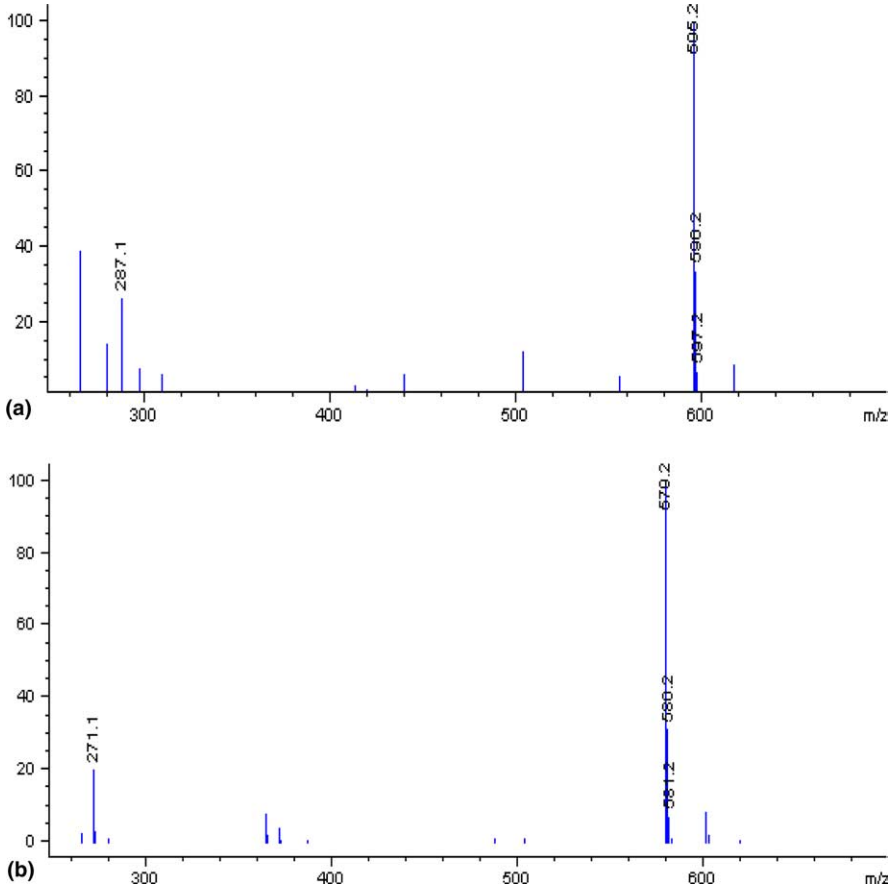

Fig. 4. (a) Mass spectrum of cyanidin 3-*O*-rutinoside, recorded at 13.55 min. (b) Mass spectrum of pelargonidin 3-*O*-rutinoside, recorded at 15.33 min.

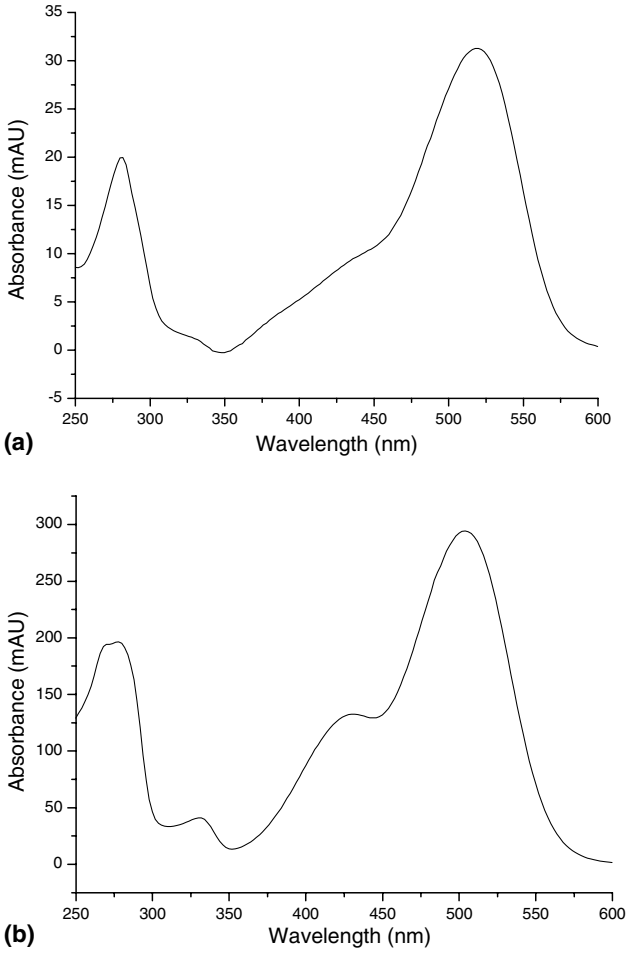

Fig. 5. (a) UV–Vis spectrum of cyanidin 3-*O*-rutinoside, recorded at 13.55 min. (b) UV–Vis spectrum of pelargonidin 3-*O*-rutinoside, recorded at 15.34 min.

information about the presence of acylating groups. In fact, the ratio of the absorbance at the acyl maximum (310 nm) to the absorbance at the anthocyanin maximum wavelength (520 nm) is a measure of the molar relation of the cinnamic acid to the anthocyanidin (Harborne, 1976). The $Abs_{310}/Abs_{\lambda max}$ ratios found for anthocyanins contained in *Smilax* berries were 43.7% and 16.4% for peaks 3 and 4 (Fig. 2), respectively. These ratios, and the low absorbance in the 310–320 nm region, suggested that *Smilax* anthocyanins were not acylated with cinnamic acids.

Alkaline hydrolysis of *Smilax* anthocyanins produced a chromatographic profile similar to that shown in Fig. 2, confirming that *Smilax* anthocyanins were not acylated (Hong & Wrolstad, 1990).

The solution of *Smilax* anthocyanins, after acid hydrolysis, yielded four peaks, as shown in the chromatogram of Fig. 6. The mass spectra of peaks 3 and 4 showed molecular ions (M^+) at *m/z* 287 (peak 3) and 271 (peak 4), corresponding to cyanidin and pelargonidin, respectively. The absorbance spectra of these compounds confirmed their identity: peaks 3 and 4 exhibited λ_{max} at 526 and 514 nm, respectively, and the UV–Vis spectrum of peak 4 had a pronounced shoulder in the 400–450 nm region, typical of the pelargonidin structure. Two new peaks (peaks 1 and 2, Fig. 6) appeared after acid hydrolysis. Peak 1 was identified as cyanidin 3-*O*-glucoside on the basis of its λ_{max} of 518 nm and a mass spectrum comprising an M^+ at *m/z* 449 and a fragment ion at *m/z* 287 (M^+ – 162). Peak 2 had an absorbance spectrum with a λ_{max} of 504 nm and a pronounced shoulder in the 400–450 nm region; its mass spectrum contained an M^+ at *m/z* 433 and a fragment ion at *m/z* 271 (M^+ – 162). Peak 2 was therefore identified as pelargonidin 3-*O*-glucoside. We believe that peaks 1 and 2 (Fig. 6) formed after partial acid hydrolysis of cyanidin 3-*O*-rutinoside and pelargonidin 3-*O*-rutinoside, respectively, under the acid hydrolysis conditions utilized.

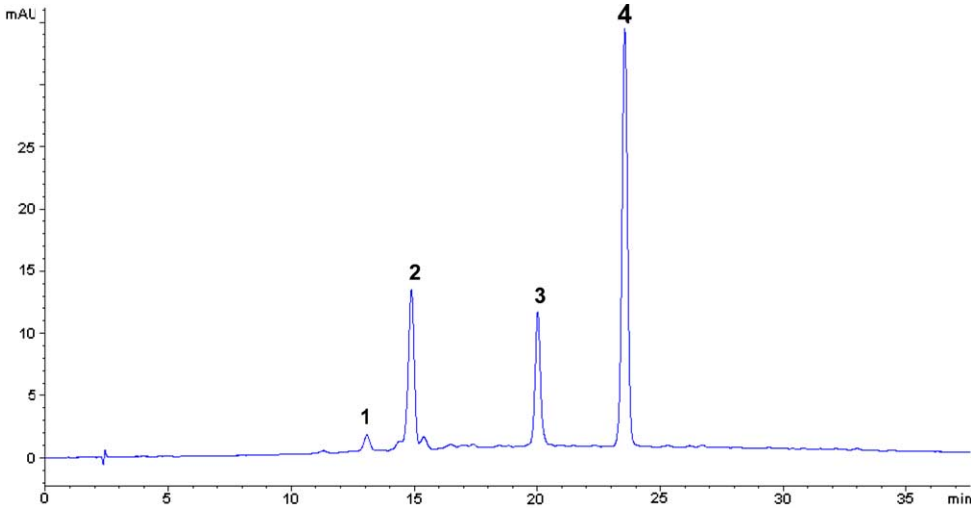

Fig. 6. Chromatogram recorded at 520 nm corresponding to the purified extract of *Smilax aspera* L. berries after acid hydrolysis.

4. Conclusion

As far as we know, this is the first time that the anthocyanin composition of *Smilax aspera* L. berries has been studied. The anthocyanin content, determined on a cyanidin 3-*O*-glucoside basis, was 23.7 mg g^{-1} of skin. Two different anthocyanins were identified in *Smilax* berries extract, by HPLC-DAD-MS. The major anthocyanin (20.4 mg g^{-1}) was identified as pelargonidin 3-*O*-rutinoside. A low quantity (1.67 mg g^{-1}) of cyanidin 3-*O*-rutinoside was also detected. The attractive colour of *Smilax* berries, the presence in their skin of only one major anthocyanin, and the great abundance of this plant in the south of Italy make *Smilax aspera* berries a new and good source of natural colorants.

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