JOURNAL OF
AGRICULTURAL AND
FOOD CHEMISTRY

Identification of Anthocyanins in *Rhamnus alaternus* L. Berries

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Anthocyanin composition in berries of *Rhamnus alaternus* L., a perennial wild shrub typical of the Mediterranean area, was determined for the first time. The pigments were extracted from the berries with 0.1% HCl in methanol and purified using a C-18 solid-phase cartridge. High-performance liquid chromatography diode array detection−mass spectrometry analysis showed that delphinidin 3-*O*-rutinoside represented about 62.4% of the total pigments. Other anthocyanins were 3-*O*-rutinoside derivatives of cyanidin (8.4%), petunidin (15.8%), pelargonidin (4.7%), and peonidin and malvidin (8.7%). The concomitant presence of the six most common anthocyanidins suggested that *R. alaternus* berries, besides being a good pigment source, could also be a useful tool for anthocyanin identification.

KEYWORDS: Anthocyanins; *Rhamnus alaternus*; Mediterranean buckthorn; berries; HPLC; diode array; mass spectrometry; hydrolysis; natural pigments

INTRODUCTION

Anthocyanins are naturally occurring phenolic compounds responsible for the color of many flowers, fruits, and berries (*1*). They are glycosylated polyhydroxy and polymethoxy derivatives of flavylium salts and have a wide distribution in the plant kingdom. Interest in this kind of pigment has increased substantially because of their possible utilization as natural food colorants (*2*, *3*), easy incorporation into aqueous systems, and potential beneficial health effects as antioxidants and antiinflammatory agents (*4*−*6*). Thus, new sources of anthocyanins with high tinctorial power, stability, and low cost are desired.

Rhamnus alaternus L., familiarly known as Mediterranean buckthorn, is a perennial shrub or tree (up to 5 m tall) of the family Rhamnaceae, typical of the Mediterranean area (*7*, *8*). It grows wild in evergreen scrub, especially in a climate with summer drought and intermittent rain during winter. It is shade tolerant and can be found on coastal areas, bare rock, and stream and forest margins. The leaves are ovate, alternate, coriaceous, with slanting teeth at the margin, and green on both sides. Yellow-green flowers in racemes occur from January to April. The fruit is a three-seed red berry, black in maturity (July−August), from 4 to 6 mm in diameter (**Figure 1**).

It was reported that *R. alaternus* is a satisfactory source of tannin and anthraquinone compounds (*9*). Different anthraquinone aglycons, such as emodin, alaternin, chrysophanol, and physcion, were isolated from the stem bark of *R. alaternus*, with emodin being the most abundant aglycon; sugar portions of these compounds generally consist of glucose and rhamnose (*10*, *11*).

Figure 1. *R. alaternus* L. berries.

Emodin was also isolated from other parts of the plant such as the leaves and fruits (*11*, *12*).

To our knowledge, the anthocyanin composition of *R. alaternus* berries has never been described. The objective of this study was to determine the anthocyanin profile in *R. alaternus* berries. The color characteristics of these berries combined with the great abundance of the plant in the south of Italy make *R. alaternus* berries a promising anthocyanin pigment source for use by the food, pharmaceutical, and cosmetic industries.

MATERIALS AND METHODS

Reagents and Standards. Trifluoroacetic acid (TFA) (99.9%) was purchased from Romil Ltd. (Cambridge, U.K.) and sodium tetraborate (99%) from Aldrich (Steinheim, Germany). Hydrochloric acid (36−

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10.1021/jf048253p CCC: $30.25 © 2005 American Chemical Society
Published on Web 02/10/2005

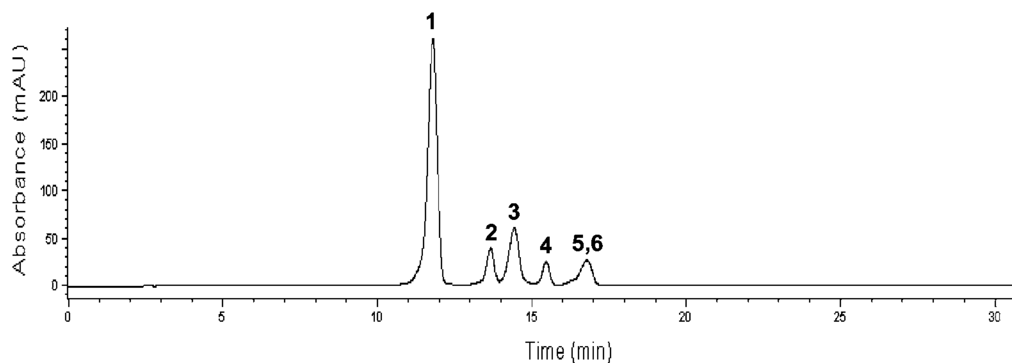

Figure 2. HPLC-DAD chromatogram recorded at 520 nm corresponding to the purified extract of *R. alaternus* berries.

38%), acetonitrile and water of high-performance liquid chromatography (HPLC) grade, and methanol of analytical grade were provided by J. T. Baker (Deventer, Holland). Cyanidin 3-*O*-glucoside was purchased from Extrasynthese (Genay, France). Deionized water was used to prepare all solutions.

Samples. Wild-grown *R. alaternus* L. berries were hand harvested in the wood of "Parco Regionale Bosco e Paludi di Rauccio", Lecce, Italy, during August 2004, placed in polyethylene bags, and stored at −20 °C until they were used. 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 *R. alaternus* L.

Anthocyanin Extraction. The berries (23 g) were crushed using a pestle and extracted in the dark by stirring with 100 mL of 0.1% HCl (v/v) in methanol for 20 h at room temperature. The samples were filtered on a Buchner funnel, and the solid residue was washed with an additional 50 mL of 0.1% HCl (v/v) in methanol. The filtrates were combined and dried using a rotary evaporator at 30 °C. The remaining solid was dissolved in 0.01% HCl (v/v) in deionized water and successively purified.

Anthocyanin Purification. The anthocyanin aqueous solution obtained from the extraction procedure described before was passed through a 1 g sorbent mass C-18 Sep-Pak cartridge (Waters Corp., Milford, MA), previously activated with methanol followed by 0.01% aqueous HCl (v/v). Anthocyanins and other polyphenolics were adsorbed onto the Sep-Pak column while sugars, acids and other water-soluble compounds were removed by washing the cartridge with 2 volumes of 0.01% aqueous HCl (v/v). Less polar polyphenolics were subsequently eluted with 2 volumes of ethyl acetate. Then, anthocyanins were eluted with methanol containing 0.01% HCl (v/v). The acidified methanol solution was evaporated using a rotary evaporator at 30 °C. The anthocyanins were then dissolved in 0.01% HCl (v/v) aqueous solution to a known concentration (3 mg/mL) and immediately analyzed. This solution was stored at −20 °C until used for successive acid and alkaline hydrolyses.

Acid Hydrolysis of Anthocyanins. A 5 mL sample of 2 N HCl was added to the above solution of purified anthocyanins (1 mL) in a screw-cap test tube, flushed with nitrogen, and capped. The pigments were hydrolyzed for different times, ranging from 1 to 5 h at 100 °C; then, the solution was immediately cooled in an ice bath (*13*). The hydrolysate was purified by using a 500 mg sorbent mass C-18 Sep-Pak cartridge (Waters) as previously described.

Alkaline Hydrolysis of Anthocyanins. The solution containing the purified anthocyanins (1 mL) was saponified in a screw-cap test tube with 5 mL of 10% KOH for 8 min in the dark at room temperature (*13*). The solution after neutralization with 2 N HCl was purified by using a 500 mg sorbent mass C-18 Sep-Pak cartridge (Waters) as previously described.

Selective Elution of Anthocyanins with Alkaline Borate Buffer. The purified anthocyanins were adsorbed onto a 500 mg sorbent mass C-18 Sep-Pak cartridge (Waters), previously activated with methanol followed by 0.01% aqueous HCl (v/v). Alkaline borate buffer (0.1 N sodium tetraborate, pH ca. 9) was passed through the column until the eluant was colorless. The anthocyanins were reconverted into their red oxonium salt form by passing 2 N HCl through the column (*14*). Washing with borate buffer and HCl was repeated one additional time. The anthocyanins remaining on the cartridge were subsequently eluted with 0.01% HCl (v/v) in methanol. The acidified methanol solution was evaporated using a rotary evaporator at 30 °C. The anthocyanins were then dissolved in 0.01% HCl (v/v) aqueous solution and immediately analyzed.

Analytical Methods. The HPLC diode array detection (DAD)−mass spectrometry (MS) analyses were performed using an Agilent 1100 Series LC-MSD system with a diode array detector (DAD) coupled to a mass spectrometer (quadrupole analyzer) equipped with an electrospray ionization (ESI) interface (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 thermostated 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 min, 10% B; 0−2 min, 10% B; 30 min, 20% B; 40 min, 30% B; 50 min, 30% B. Absorbance spectra were recorded every 2 s, between 250 and 600 nm, with a bandwidth of 4 nm, and chromatograms were acquired at 520, 440, 310, and 280 nm. MS parameters were as follows: capillary voltage, 4000 V; fragmentor voltage, 160 V; drying gas temperature, 350 °C; gas flow (N_2) rate, 10 L/min; nebulizer pressure, 50 psig. The instrument was operated in positive ion mode scanning from m/z 100 to m/z 800 at a scan rate of 1.43 s/cycle.

Quantification of Anthocyanins. 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.

RESULTS AND DISCUSSION

Anthocyanin Profile. The anthocyanin composition of *R. alaternus* berries was determined by means of HPLC-DAD−MS analysis. Due to the lack of commercially available reference compounds for the anthocyanins and since UV/vis spectroscopic characteristics do not allow unambiguous identification of individual pigments, coupling of HPLC-DAD to mass spectrometry (MS) seems to be extremely helpful for peak assignment. ESI techniques especially have been shown to be highly suitable for anthocyanin characterization due to their "soft" ionization, producing intact molecular ions and the corresponding anthocyanidin fragments (*14*−*16*).

The HPLC-DAD chromatogram of the purified anthocyanins extracted from *R. alaternus* berries, acquired at 520 nm, is shown in **Figure 2**. As can be seen, there are five peaks in the chromatogram, but the MS analysis revealed that the last peak corresponded to two different pigments (compounds **5** and **6**) that coeluted under the conditions employed in this work, indicating the presence of six different anthocyanins in *R. alaternus* berries. These six anthocyanins, the structures of which are shown in **Figure 3**, were identified by comparison of HPLC retention times, elution order, and photodiode array UV/vis spectroscopic and ESI-MS spectrometric data (**Table 1**) with

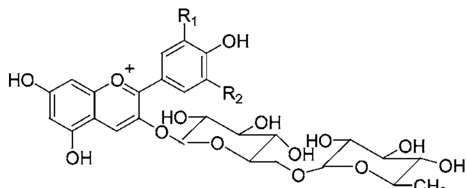

Figure 3. Chemical structures of anthocyanins identified in *R. alaternus* berries: **1**, delphinidin 3-*O*-rutinoside (R_1 = OH, R_2 = OH); **2**, cyanidin 3-*O*-rutinoside (R_1 = OH, R_2 = H); **3**, petunidin 3-*O*-rutinoside (R_1 = OCH_3, R_2 = OH); **4**, pelargonidin 3-*O*-rutinoside (R_1 = H, R_2 = H); **5**, peonidin 3-*O*-rutinoside (R_1 = OCH_3, R_2 = H); **6**, malvidin 3-*O*-rutinoside (R_1 = OCH_3, R_2 = OCH_3).

Table 1. Chromatographic, Spectroscopic, and Spectrometric Characteristics of Anthocyanins Found in *R. alaternus* Berries

peak no. (**Figure 2**)	t_R (min)	vis λ_{max} (nm)	M^+ (*m*/*z*)	M^+ – X (*m*/*z*)	peak assignment
1	11.8	528	611	303 (M^+ – rut)	delphinidin 3-rutinoside
2	13.7	520	595	287 (M^+ – rut)	cyanidin 3-rutinoside
3	14.4	528	625	317 (M^+ – rut)	petunidin 3-rutinoside
4	15.5	504	579	271 (M^+ – rut)	pelargonidin 3-rutinoside
5	16.3	526	609	301 (M^+ – rut)	peonidin 3-rutinoside
6	16.8	530	639	331 (M^+ – rut)	malvidin 3-rutinoside

published data (*14*, *17*, *18*) and our anthocyanin library. Chromatographic and spectroscopic characteristics of the coeluted anthocyanins **5** and **6** were determined by analyzing the initial and the end parts of the corresponding peak, respectively.

Delphinidin 3-*O*-rutinoside (**1**) was the major anthocyanin present in *R. alaternus* berries, representing about 62% of the total peak area. Cyanidin 3-*O*-rutinoside (**2**), petunidin 3-*O*-rutinoside (**3**), pelargonidin 3-*O*-rutinoside (**4**), peonidin 3-*O*-rutinoside (**5**), and malvidin 3-*O*-rutinoside (**6**) were found in minor amounts.

The ESI-MS profiles of these compounds presented the molecular ions M^+ and the fragments resulting from the loss of the sugar molecule (*m*/*z* 308) and corresponding to the aglycons. Their elution order and absorbance spectra confirmed the identity of the supposed anthocyanins. As known, the polarity of the aglycon (anthocyanidin) is the most important factor affecting the HPLC retention time, and the typical order of elution of the different anthocyanins with similar glycosylation patterns is as follows: first delphinidin derivatives, followed by cyanidin, petunidin, pelargonidin, peonidin, and finally malvidin derivatives (*19*, *20*).

The UV/vis spectroscopic characteristics of *R. alaternus* anthocyanins were also determined. The $Abs_{440}/Abs_{\lambda_{max}}$ ratio values calculated for each anthocyanin and ranging from 27% to 49% indicated a substitution in the C-3 position of the flavylium ring (*17*). It is well-known that anthocyanins with glycosidic substitutions at position 3 exhibit a ratio of the absorbance at 400−440 nm to the absorbance at the visible maximum wavelength (520 nm) that is almost twice as large as for the anthocyanins with glycosidic substitution at position 5 or both positions 3 and 5 (*21*). In addition, the obtained $Abs_{280}/Abs_{\lambda_{max}}$ (63−75%) and $Abs_{310}/Abs_{\lambda_{max}}$ (11−38%) ratios confirmed that *R. alaternus* anthocyanins were simple anthocyanin structures without acylation of the glycoside with aromatic acids (*14*, *22*, *23*).

Alkaline hydrolysis of the purified anthocyanins produced a chromatographic profile similar to that reported in **Figure 2**, confirming that *R. alaternus* anthocyanins were not acylated (*14*).

As can be seen in the chromatogram of **Figure 4A**, acid hydrolysis of the purified anthocyanins, after 1 h at 100 °C, yielded nine peaks, but also in this case, some individual peaks corresponded to several compounds that coeluted under the conditions employed, and the presence of twelve different compounds (compounds **7**−**18**) was actually found. They were identified by comparison of HPLC retention times, elution order, and photodiode array UV/vis spectroscopic and ESI-MS spectrometric data (**Table 2**) with published data (*14*, *17*, *18*) and our anthocyanin library. Also, in this case, chromatographic and

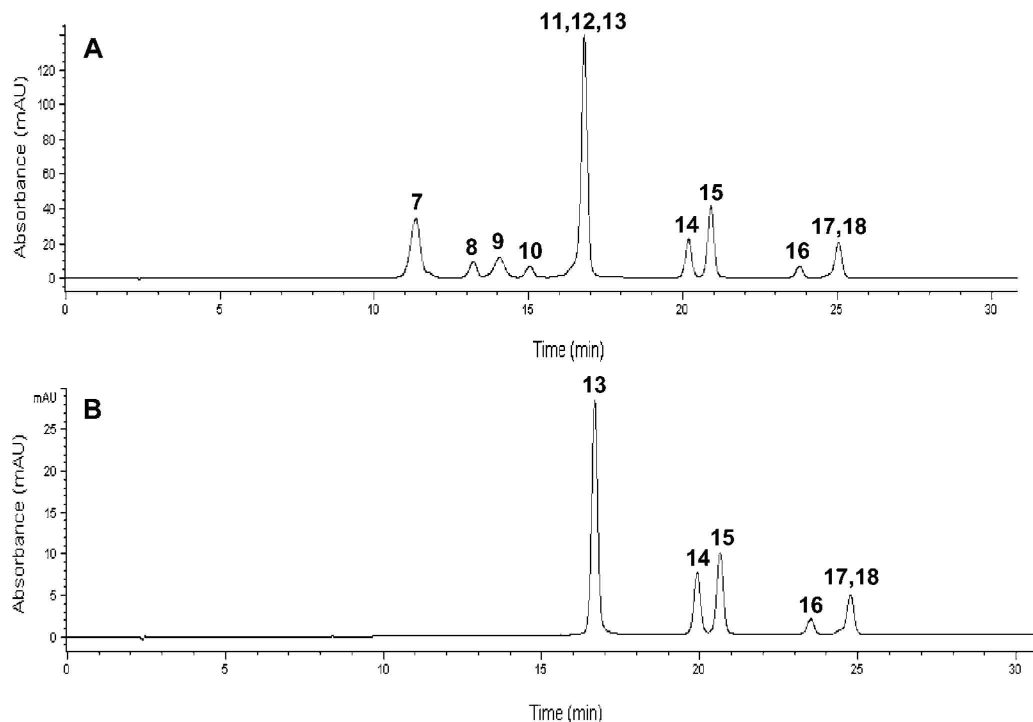

Figure 4. HPLC-DAD chromatogram recorded at 520 nm corresponding to acid-hydrolyzed anthocyanins of *R. alaternus* berries (**Table 2**) after 1 h (A) and 5 h (B) of hydrolysis.

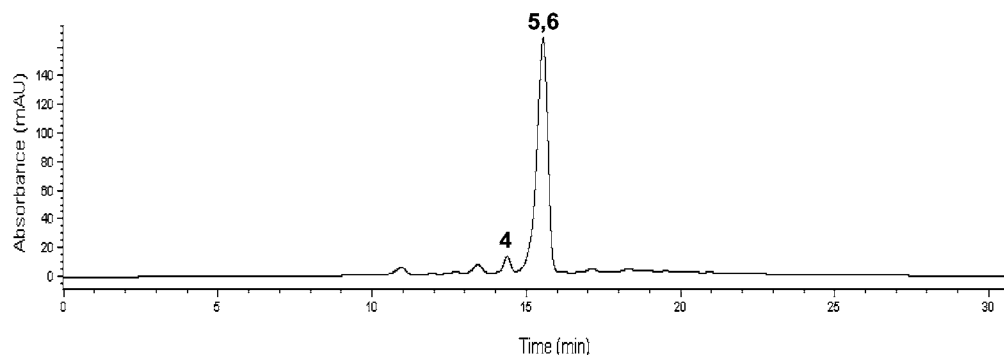

Figure 5. HPLC-DAD chromatogram recorded at 520 nm corresponding to the fraction of anthocyanins of *R. alaternus* berries (**Table 1**) remaining on a C-18 Sep-Pack cartridge after elution with alkaline borate buffer.

Table 2. Chromatographic, Spectroscopic, and Spectrometric Characteristics of Anthocyanins and Anthocyanidins Resulting from Acid Hydrolysis of *R. alaternus* Berry Extract

peak no. (**Figure 4**)	t_R (min)	vis λ_{max} (nm)	M^+ (*m/z*)	$M^+ - X$ (*m/z*)	peak assignment
7	11.3	526	465	303 (M^+ – glu)	delphinidin 3-glucoside
8	13.2	518	449	287 (M^+ – glu)	cyanidin 3-glucoside
9	14.1	528	479	317 (M^+ – glu)	petunidin 3-glucoside
10	15.0	502	433	271 (M^+ – glu)	pelargonidin 3-glucoside
11	16.0	534	463	301 (M^+ – glu)	peonidin 3-glucoside
12	16.3	530	493	331 (M^+ – glu)	malvidin 3-glucoside
13	16.8	532	303		delphindin
14	20.2	526	287		cyandin
15	20.9	534	317		petunidin
16	23.8	512	271		pelagonidin
17	24.7	532	301		peonidin
18	25.0	536	331		malvidin

spectroscopic characteristics of the coeluted anthocyanins were determined by analyzing different parts of the corresponding peaks.

Peaks **7−12** (**Figure 4A**) were identified as 3-glucoside derivatives of delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin, respectively, which have been formed owing to the loss of the rhamnosyl unit from the rutinoside derivatives. The mass spectra of these compounds presented the molecular ions M^+ and the fragments resulting from the loss of the glucose moiety (*m/z* 162) and corresponding to the aglycons. The mass spectra of peaks **13−18** (**Figure 4A**) presented the molecular ions M^+ corresponding to the aglycons delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin, respectively. The elution order and absorbance spectra of these compounds confirmed their identity.

Acid hydrolysis of the purified anthocyanins performed using longer hydrolysis times (5 h) produced only peaks **13−18** (**Figure 4B**) corresponding to the anthocyanin aglycons. The joint presence in a single product of the six most common anthocyanidins is of great interest.

Figure 5 shows the HPLC-DAD chromatogram of the fraction of *R. alaternus* anthocyanins which remained on the C-18 Sep-Pak cartridge after elution with alkaline borate solution. We have observed that nonacylated anthocyanins containing *o*-dihydroxy groups (delphinidin, cyanidin, petunidin) were preferentially eluted while those anthocyanins not containing *o*-dihydroxy groups (pelargonidin, peonidin, malvidin) were concentrated on the cartridge. It is known that borate ions react with phenols possessing *o*-dihydroxy groups, forming negatively charged complexes. For that reason, alkaline borate solutions have been used as buffers in the electrophoretic separation of phenols to enhance electrophoretic mobility (*24*). In the treatment of anthocyanins with borate the compounds possessing *o*-dihydroxy groups formed a charged borate complex, resulting in more hydrophilic species which were preferentially eluted from the C-18 cartridge, while anthocyanins not complexing with borate ions remained in the cartridge (*14*). This technique provided an additional method for characterization of *R. alaternus* anthocyanins.

Quantification of Anthocyanins. The total amount of anthocyanins in *R. alaternus* berries, determined on the cyanidin 3-glucoside basis, was 85 mg/g of fresh berries. Delphinidin 3-*O*-rutinoside was the most predominant anthocyanin (60 mg/g), followed by petunidin 3-*O*-rutinoside (12.7 mg/g) and cyanidin 3-*O*-rutinoside (5 mg/g). The total amount of peonidin 3-*O*-rutinoside and malvidin 3-*O*-rutinoside was 5.4 mg/g. Pelargonidin 3-*O*-rutinoside was the least abundant anthocyanin (1.4 mg/g).

To our knowledge, this is the first time that the anthocyanin composition of *R. alaternus* berries has been described. The attractive color of *R. alaternus* berries and the great abundance of this plant in the south of Italy make the berries of *R. alaternus* a new and good source of natural pigments. Furthermore, the concomitant presence in these berries of the six most common anthocyanidins makes them a useful tool in anthocyanin identification.

ACKNOWLEDGMENT

We acknowledge Concetta Mele of the Systematic Botanic and Vegetable Ecology Laboratory of the Department of Biological and Environmental Science and Technology, University of Lecce, Italy, for useful advice on plant classification.

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Received for review October 21, 2004. Revised manuscript received January 4, 2005. Accepted January 5, 2005. We thank MIUR (Rome, Italy) for financial support (progetto PON "Estrazione di antocianine e altri principi attivi mediante CO_2 supercritica da substrati vegetali"). We also thank PIERRE CHIMICA S.r.l. (Galatina, Lecce, Italy) and Consorzio INCA (Venice, Italy).

JF048253P