JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Determination of Anthocyanins in *Ruscus aculeatus* L. Berries

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Red berries of *Ruscus aculeatus* L., a wild shrub typical of Mediterranean Europe and Africa, were investigated for the first time in order to determine the profile of anthocyanins. The pigments were extracted from the skins of the berries with 0.1% HCl in methanol, purified using a C-18 solid phase cartridge, and identified by means of high-performance liquid chromatography (HPLC)−diode array detection−mass spectrometry analysis. Information from HPLC profiles, saponification, and acid hydrolysis of the anthocyanins showed that the major anthocyanins were pelargonidin 3-*O*-rutinoside (64%), pelargonidin 3-*O*-glucoside (16%), and pelargonidin 3-*O*-*trans*-*p*-coumarylglucoside (13%). The attractive color of *R. aculeatus* berries and the great abundance of the plant in the south of Italy make these berries a new and promising source of natural colorants.

KEYWORDS: Anthocyanins; *Ruscus aculeatus*; berries; HPLC; diode array; mass spectrometry; hydrolysis; natural colorants; antioxidants

INTRODUCTION

Anthocyanins are natural pigments belonging to the flavonoid family and are responsible for the red, blue, and purple colors of many fruits, berries, and flowers (*1*). Recent and renewed interest in anthocyanins is due not only to their potential health benefits as antioxidants and antiinflammatory agents (*2*−*4*) but mainly to their utilization as natural food colorants (*5*). In fact, anthocyanins may be used as natural colorants in the food industry, to replace the original colors lost during the processing of foods (*6*) or to enhance the shelf life of foods to which they have been added. Anthocyanins are also water soluble, and this facilitates their incorporation into aqueous foods. For this reason, new sources of pigments such as anthocyanins with high tinctorial power, stability, and low cost are now desired.

Ruscus aculeatus L., familiarly known as butcher's broom, is a short evergreen shrub of the family Liliaceae typical of Mediterranean Europe and Africa (*7*, *8*). It grows wild in woodlands but can be easily cultivated. It has false thorny leaves called cladophylles. The most outstanding appealing part of the plant is the fruit, a round red berry, from 10 to 12 mm in diameter (**Figure 1**).

In European tradition, both the aerial parts (leaves) and the rhizome of *R. aculeatus* are considered to be diuretic and mildly laxative. Today, they are used in Europe for disorders involving the venous system, including venous fragility or varicose veins, and clinical data also revealed positive effects on circulation. In particular, the extracts are used in the prevention and treatment of venous insufficiency (*9*−*11*). Moreover, because of their antielastase activity, they are components of drugs administered as antiinflammatory and vasoconstrictor agents (*12*). Their antiedematous effects have been also demonstrated (*13*, *14*). The pharmacologically active steroid glycosides of *R. aculeatus* were established and are mainly present in the rhizome and root of the plant (*15*, *16*).

Figure 1. *R. aculeatus* L. berries.

Continuing our research on anthocyanins, we have hypothesized that the red coloration of *R. aculeatus* berries may be due to the presence of anthocyanins in the skin of the berry. The color characteristics of these berries combined with the great abundance of the plant in the south of Italy could make *R. aculeatus* berries a promising source of anthocyanin pigments. To our knowledge, the anthocyanin composition of *R. aculeatus* berries has not been yet described. The objective of this study was therefore to determine anthocyanin profile in the skin of *R. aculeatus* L. berries.

MATERIALS AND METHODS

Reagents and Standards. Trifluoroacetic acid (TFA) was purchased from Romil ltd. (Cambridge, United Kingdom). Hydrochloric acid (36−38%), acetonitrile, and water of high-performance liquid chromatog-

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10.1021/jf0487250 CCC: $30.25
Published on Web 12/22/2004

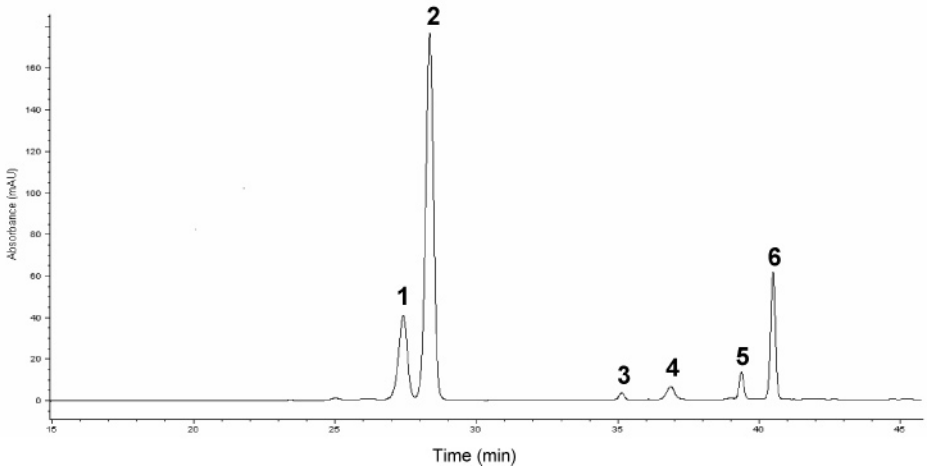

Figure 2. Chromatogram recorded at 520 nm corresponding to the purified extract of *R. aculeatus* berries.

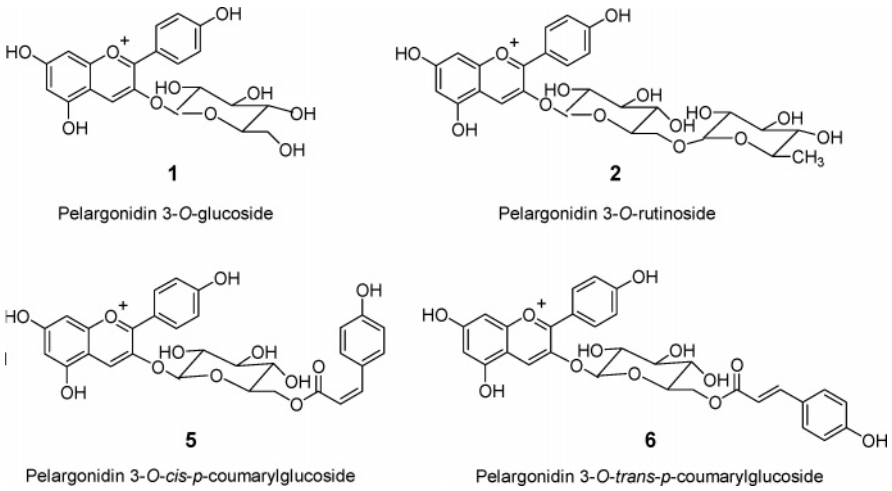

Figure 3. Structures of the anthocyanins identified in the skin of *R. aculeatus* L. berries.

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

peak no. (**Figure 2**)	peak area (%)	t_R (min)	vis λ_{max} (nm)	M^+ (*m*/*z*)	$M^+ - X$ (*m*/*z*)	peak assignment
1	16.73	27.40	502	433	271 (M^+ – glu)	pelargonidin-3-glucoside
2	63.80	28.34	504	579	271 (M^+ – rut)	pelargonidin-3-rutinoside
5	2.77	39.36	508	579	271 (M^+ – coumarylglu)	pelargonidin-3-*cis*-*p*-coumarylglucoside
6	13.44	40.47	510	579	271 (M^+ – coumarylglu)	pelargonidin-3-*trans*-*p*-coumarylglucoside

raphy (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 (Easypure II RF ultrapure water system), (Barnstead, United States) was used to prepare all solutions.

Samples. Wild-grown *R. aculeatus* L. berries were hand harvested in the woods of "Parco Regionale Bosco e Paludi di Rauccio", Lecce, Italy, during December 2003, 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. aculeatus* L.

Anthocyanin Extraction. Hand-peeled skins from partially thawed berries were extracted with 0.1% HCl (v/v) in methanol for 20 h at room temperature, in darkness. The samples were filtered on a Buchner funnel, and the solid residue was washed with 0.1% HCl in methanol. The filtrate was dried using a rotary evaporator at 30 °C. The remaining solid was redissolved in 0.01% HCl (v/v) in deionized water and successively purified.

Anthocyanin Purification. The aqueous solution obtained from the extraction procedure described before was passed through a C-18 Sep-Pak cartridge (Waters Corporation, Milford, MA), previously activated with methanol followed by 0.01% aqueous HCl (v/v). 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 volumes of 0.01% aqueous HCl. Polyphenolics were subsequently eluted with 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 remaining solid containing the anthocyanins was then redissolved in 0.01% HCl aqueous solution to a known volume and immediately analyzed. This solution can be stored at −20 °C until used for successive acid and alkaline hydrolyses.

Acid Hydrolysis of Anthocyanins. Five milliliters of 2 N HCl was added to the above solution (1 mL) containing the purified anthocyanins in a screw-cap test tube, flushed with nitrogen, and capped. The pigments were hydrolyzed for 1 h at 100 °C; then, the solution was immediately cooled in an ice bath (*17*). The hydrolysate was purified by using a C-18 Sep-Pak cartridge (Waters Corporation) 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 (*17*). The solution after neutralization with 2 N HCl was purified by using a C-18 Sep-Pak cartridge (Waters Corporation) as previously described.

Analytical Methods. The HPLC-diode array detection (DAD)-mass spectrometry (MS) analyses were performed using an Agilent 1100 Series LC/MSD system with a DAD coupled to a mass spectrometer (quadrupole analyzer) equipped with an ESI (electrospray ionization) interfaces (Agilent). Chromatographic separation was carried out using a 150 mm × 4.6 mm i.d., 5 μm SS Wakosil C18 with a 4 mm × 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 gradient was utilized as follows: 0−2 min, 10% B; 30 min, 20% B; 40 min, 30% B; 50 min, end. 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 the positive ion mode scanning from *m*/*z* 100 to 800 at a scan rate of 1.43 s/cycle.

Quantification of Anthocyanins. The wavelength used for quantification was 520 nm. For calibration, the external standard method was used and the standard curve was produced by the integration of absorption peaks generated from analysis of dilution series of cyanidin-3-glucoside.

RESULTS AND DISCUSSION

Anthocyanin Profile. The HPLC chromatogram of the purified anthocyanin extract from the skin of *R. aculeatus* berries is shown in **Figure 2**. As can be seen, six peaks are present in the chromatogram but the minor peaks (**3** and **4**) having an area percentage less than 3% and with a poor resolution were not considered. The four major anthocyanins, the structures of which are shown in **Figure 3**, represented about 97% of the total peak area and were identified by their HPLC retention times, elution order, spectroscopic characteristics, and fragmentation pattern (**Table 1**) from comparison with literature values (*18*−*20*) and our anthocyanin library. The major peaks identified by HPLC-MS were pelargonidin 3-*O*-glucoside, $M^+ = 433$ (**1**), pelargonidin 3-*O*-rutinoside, $M^+ = 579$ (**2**), and pelargonidin 3-*O*-*p*-coumarylglucoside, $M^+ = 579$ (**6**). The ESI-MS profiles of these compounds presented the molecular ions M^+ and the fragment resulting from the loss of the sugar molecule (271 amu) corresponding to the molecular ion of pelargonidin aglycone. The absorbance spectra of these compounds (**Figure 4**) confirmed the identity of the supposed anthocyanins. In fact, the UV−vis spectra of the three anthocyanins corresponding to peaks **1**, **2**, and **6** showed λ_{max} in the 504−510 nm region associated with a pronounced shoulder in the 400−450 nm region typical of the pelargonidin anthocyanidin type skeleton (*18*). The UV−vis spectrum of peak **6**, in particular, besides the characteristic visible maximum absorption at 510 nm, showed increased absorption in the 300−330 nm region (**Figure 4C**), which is indicative of a possible acylation of the glycoside with aromatic acids (*18*, *21*, *22*), confirmed by an increase of the retention time of the anthocyanin corresponding to peak **6**: acylation, in fact, usually increases the retention time of anthocyanins under reversed phase (RP) HPLC (*18*). On the basis of this evidence, peak **6** was assumed to be pelargonidin 3-*O*-*p*-coumarylglucoside. Peak **5** presented a molecular mass and fragmentation similar to peak **6**, but as shown in the chromatogram, it eluted 1 min earlier. Moreover, its UV−vis spectrum showing an absorption maximum at 508 nm was slightly different from that of peak **6** (**Figure 5**). It is known that in the case of cis and trans isomers, cis isomers elute first as compared to trans and are usually present in lower amounts. Also, slight differences in the absorbance spectra of cis and trans isomers have been reported (*23*). On the basis of these evidences, we have assumed this pattern of elution and assigned the cis and trans labels to peaks **5** and **6**, respectively.

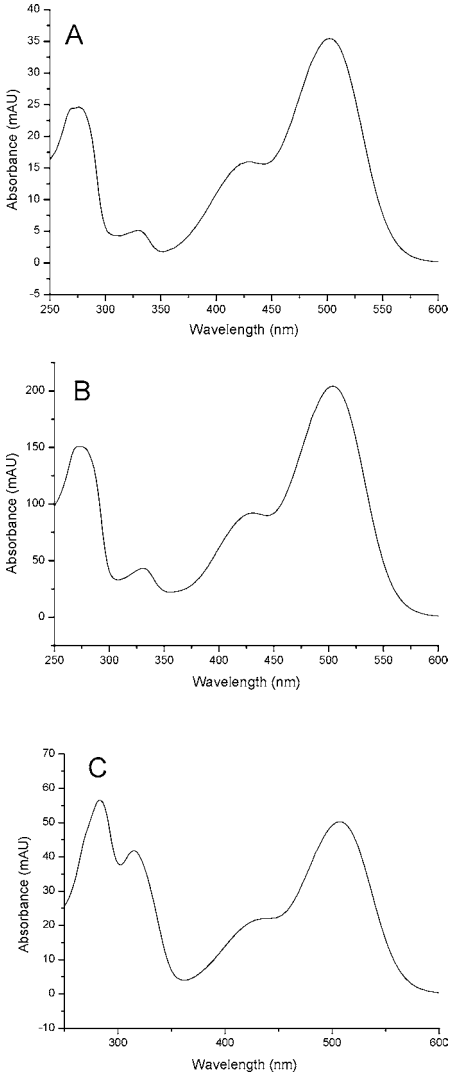

Figure 4. (**A**) UV–vis spectrum of pelargonidin 3-*O*-glucoside, recorded at 27.40 min. (**B**) UV–vis spectrum of pelargonidin 3-*O*-rutinoside, recorded at 28.34 min. (**C**) UV–vis spectrum of pelargonidin 3-*O*-*trans*-*p*-coumaryl-glucoside, recorded at 40.47 min.

According to the Harborne hypothesis (*24*), 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 that as compared to anthocyanins with glycosidic substitutions at position 5 or both positions 3 and 5. The $Abs_{440}/Abs_{\lambda max}$ ratios found for *Ruscus* anthocyanins confirmed their monoglucoside skeleton with glycosidic substitution in the C-3 position of the anthocyanidin (*19*).

As shown in the chromatogram of **Figure 6A**, acid hydrolysis of the *Ruscus* anthocyanins produced one major anthocyanidin corresponding to 60% of the total peak area (at 520 nm). The mass spectrum ($M^+ = 271$) of this anthocyanidin and its UV−vis spectrum with a λ_{max} at 514 nm and a pronounced shoulder at 424 nm indicated the presence of pelargonidin (**7**) as the only anthocyanidin present in *Ruscus* berries. The other two peaks corresponded to pelargonidin-3-glucoside, $M^+ = 433$ (**1**) and pelargonidin-3-*trans*-*p*-coumarylglucoside, $M^+ = 579$ (**6**),

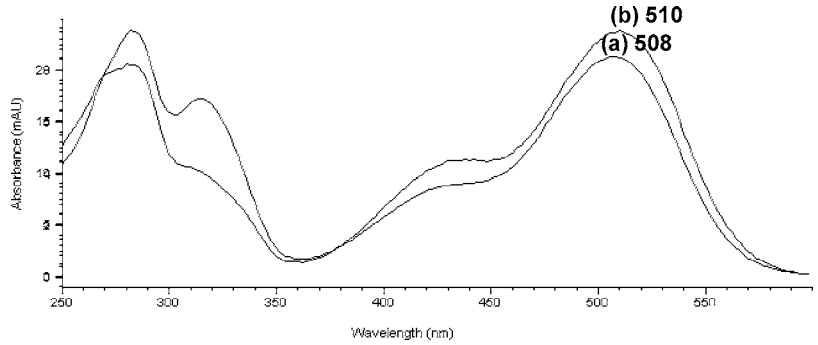

Figure 5. UV–vis spectra of pelargonidin 3-*O*-*cis*-*p*-coumarylglucoside (**A**) and pelargonidin 3-*O*-*trans*-*p*-coumarylglucoside (**B**).

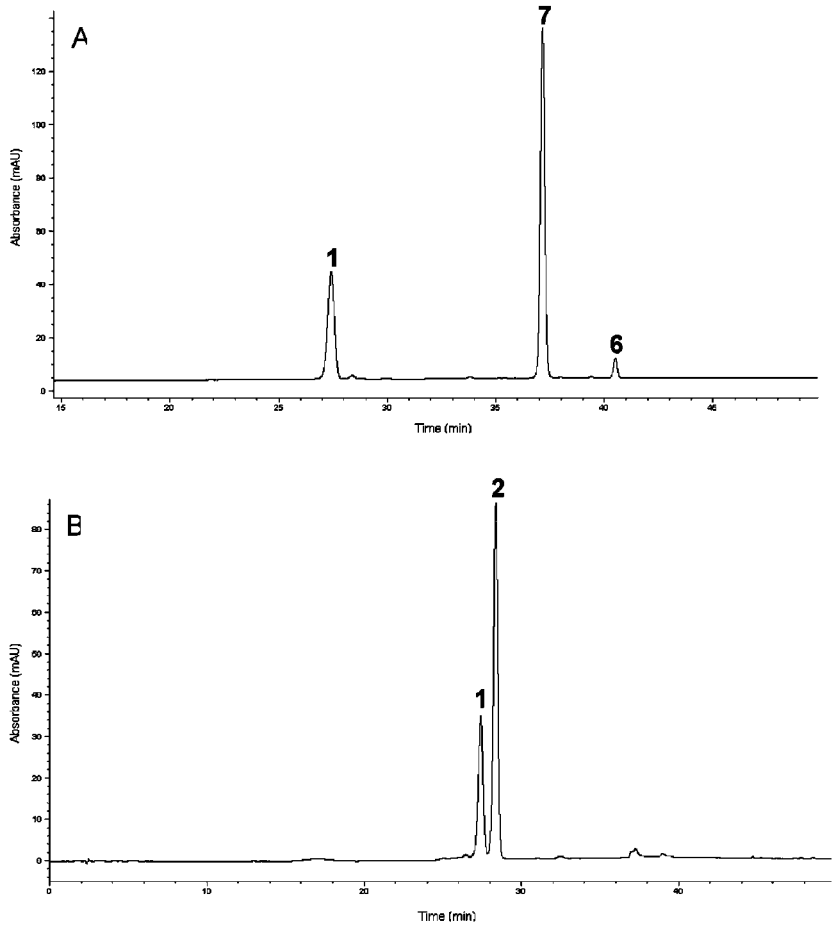

Figure 6. Chromatogram recorded at 520 nm corresponding to the purified extract of *R. aculeatus* berries after acid hydrolysis (**A**) and after alkaline hydrolysis (**B**).

respectively, which were not completely hydrolyzed in the acid hydrolysis conditions utilized. Alkaline hydrolysis of the anthocyanins produced the chromatographic profile shown in **Figure 6B**, similar to that of the purified anthocyanins (**Figure 2**) but with the absence of the peaks **5** and **6**. According to Hong and Wrolstad (*18*), this evidence could mean that these pigments are acylated or are sensitive to alkali. In our case, the disappearance of peaks **5** and **6** after alkaline hydrolysis suggested the presence of cinnamic acid as acylating groups and this hypothesis is confirmed by the fact that the anthocyanins corresponding to these peaks showed an increased absorption in the 300−330 nm region typical of the anthocyanin structures with acylating groups. In addition, according to Harborne (*24*), the $Abs_{310}/Abs_{\lambda max}$ ratios calculated for these compounds were indicative of a 1/1 molar ratio of the cinnamic acid to the anthocyanin. In conclusion, all of this information confirmed the identity of peaks **5** and **6** (**Figure 2**) corresponding to pelargonidin 3-*O*-*cis*-*p*-coumarylglucoside and pelargonidin 3-*O*-*trans*-*p*-coumarylglucoside, respectively.

Quantification of Anthocyanins. Because anthocyanins are unstable compounds, the availability of standards is poor and their price is high. For that reason, it is very difficult and expensive to obtain standards for all anthocyanins present in the sample. Therefore, many authors used to choose one glycoside as a reference for the calibration (*25*, *26*). This is possible because the chromatographic responses of these compounds at wavelengths around 520 nm are rather similar. Thus, we have used cyanidin 3-*O*-glucoside as the commercial standard to calculate the quantity of the anthocyanins contained in the *R. aculeatus* berries. In this way, we have found that the total amount of anthocyanins in the skin of *Ruscus* berries was 74.8 mg/g of skins and pelargonidin 3-*O*-rutinoside was the most

predominant anthocyanin (46 mg/g), followed by pelargonidin 3-*O*-glucoside (8.6 mg/g) and pelargonidin 3-*O*-*p*-coumaryl-glucoside trans isomer (6.0 mg/g).

To our knowledge, this is the first time that the anthocyanin composition of *R. aculeatus* berries has been described. Two of the anthocyanins found in these berries are acylated with hydroxycinnamic acid. Taking into account that the acylation of the anthocyanins protects them against some color-damaging factors, because the acyl groups allow the anthocyanins to adopt a folded conformation (*27*), we conclude that the berries of *R. aculeatus* should be a new and good source of colorants to be used in the food industry.

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 and Donato Cannoletta of the Engineering of Innovation Department, University of Lecce (Italy), for technical support.

LITERATURE CITED

(1) Cooper-Driver, G. A. Contributions of Jeffrey Harborne and co-workers to the study of anthocyanins. *Phytochemistry* **2001**, *56*, 229−236.

(2) Wang, H.; Nair, M.; Strasburg, G.; Chang, Y.; Booren, A.; Gray, J.; DeWitt, D. Antioxidant and antiinflammatory activities of anthocyanins and their aglycon, cyanidin, from tart cherries. *J. Nat. Prod.* **1999**, *62*, 294−296.

(3) Wang, S.; Jiao, H. Scavenging capacity of berry crops on superoxide radical, hydrogen peroxide, hydroxyl radical and singlet oxygen. *J. Agric. Food Chem.* **2000**, *75*, 5677−5684.

(4) Tamura, H.; Yamaganci, A. Antioxidative activity of monoacylated anthocyanins isolated from Muscat Bailey A grape. *J. Agric. Food Chem.* **1994**, *42*, 1612−1615.

(5) Pazmino-Duran, A.; Giusti, M.; Wrolstad, R.; Gloria, M. Anthocyanins from *Oxalis triangularis* as potential food colorants. *Food Chem.* **2001**, *75*, 211−216.

(6) Bridle, P.; Timberlake, C. F. Anthocyanins as natural food colours-selected aspects. *Food Chem.* **1997**, *58*, 103−107.

(7) Der Marderosian, A. *The Review of Natural Products*; Facts and Comparisons: St. Louis, 1999.

(8) Weiss, R. F. *Herbal Medicine*; Beaconsfield Publishers: Beaconsfield, England, 1988.

(9) Berg, D. Venous constriction by local administration of *Ruscus* extract. *Fortschr. Medizin* **1990**, *108*, 473−476.

(10) Bouskela, E.; Cyrino, F. Z.; Marcelon, G. Possible mechanisms for the inhibitory effect of *Ruscus* extract on increased microvascular permeability induced by histamine in hamster cheek pouch. *J. Cardiovasc. Pharmacol.* **1994**, *24*, 281−285.

(11) Facino, R. M.; Carini, M.; Stefani, R.; Aldini, G.; Saibene, L. Anti-elastase and anti-hyaluronidase activities of saponins and sapogenins from *Hedera helix*, *Aesculus hippocastanum*, and *Ruscus aculeatus*: Factors contributing to their efficacy in the treatment of venous insufficienty. *Arch. Pharm.* 1**995**, *328*, 720−724.

(12) Capra, C. Pharmacology and toxicology of some components of *Ruscus aculeatus*. *Fitoterapia* **1972**, *43*, 99−113.

(13) Cluzan, R. V.; Alliot, F.; Ghabboun, S.; Pacot, M. Treatment of secondary lymphedema of the upper limb with CYCLO 3 FORT. *Lymphology* **1996**, *29*, 29−35.

(14) Tarayre, J. P.; Lauressergues, H. Anti-edematous effect of an association of proteolytic enzymes, flavonoids, heterosides of *Ruscus aculeatus* and ascorbic acid. *Ann. Pharm. Fr.* **1979**, *37*, 191−198.

(15) Bombardelli, E.; Bonati, A.; Gabetta, B.; Mustich, G. Glycosides from rhizomes of *Ruscus aculeatus*. *Fitoterapia* **1971**, *42*, 127−136.

(16) Bombardelli, E.; Bonati, A.; Gabetta, B.; Mustich, G. Glycosides from rhizomes of *Ruscus aculeatus*. II. *Fitoterapia* **1972**, *43*, 3−10.

(17) Chaovanalikit, A.; Thompson, M. M.; Wrolstad, R. E. Characterization and quantification of anthocyanins and polyphenolics in blue honeysuckle (*Lonicera caerulea* L.). *J. Agric. Food Chem.* **2004**, *52*, 848−852.

(18) Hong, V.; Wrolstad, R. E. Use of HPLC separation/photodiode array detection for characterization of anthocyanins. *J. Agric. Food Chem.* **1990**, *38*, 708−715.

(19) Giusti, M. M.; Rodrìguez-Saona, L. E.; Wrolstad, R. E. Molar absorptivity and color characteristics of acylated and nonacylated pelargonidin-based anthocyanins. *J. Agric. Food Chem.* **1999**, *47*, 4631−4637.

(20) Mullen, W.; McGinn, J.; Lean, M. E. J.; McLean, M. R.; Gardner, P.; Duthie, G. G.; Yokota, T.; Crozier, A. Ellagitannins, flavonoids, and other phenolics in red raspberries and their contribution to antioxidant capacity and vasorelaxation properties. *J. Agric. Food Chem.* **2002**, *50*, 5191−5196.

(21) Woodall, G. S.; Stewart, G. R. Do anthocyanins play a role in UV protection of the red juvenile leaves of *Syzygium*? *J. Exp. Bot.* **1998**, *49*, 1447−1450.

(22) Mozetic, B.; Trebse, P.; Hribar, J. Determination and quantification of anthocyanins and hydroxycinnamic acids in different cultivars of sweet cherries (*Prunus avium* L.) from Nova Gorica Region (Slovenia). *Food Technol. Biotechnol.* **2002**, *40*, 207−212.

(23) Nùnez, V.; Monagas, M.; Gomez-Cordovès, M. C.; Bartolomè, B. *Vitis vinifera* L. cv. Graciano grapes characterized by its anthocyanin profile. *Postharvest Biol. Technol.* **2004**, *31*, 69−79.

(24) Harborne, J. B. *Comparative Biochemistry of the Flavonoids*; Academic Press: New York, 1976.

(25) Versari, A.; Barbanti, D.; Biesenbruch, S.; Farnell, P. J. Analysis of anthocyanins in red fruits by use of HPLC/spectral array detection. *Ital. J. Food. Sci.* **1997**, *9*, 141−148.

(26) Baj, A.; Bombardelli, E.; Gambetta, B.; Martinelli, E. M. Qualitative and quantitative evaluation of *Vaccinum myrtillus* anthocyanins by high-resolution gas chromatography and high-performance liquid chromatography. *J. Chromatogr.* **1983**, *279*, 365−372.

(27) Alcade-Eon, C.; Saavedra, G.; de Pascual-Teresa, S.; Rivas-Gonzalo, J. C. Identification of anthocyanins of pinta boca (*Solanum stenotomum*) tubers. *Food Chem.* **2004**, *86*, 441−448.

Received for review July 29, 2004. Revised manuscript received November 3, 2004. Accepted November 5, 2004. We thank MIUR, Rome (Italy), for financial support (progetto PON "Estrazione di Antocianine e Altri Principi Attivi Mediante CO_2 Supercritica da Substrati Vegetali").

JF0487250