Identification of Anthocyanins in Berries by Narrow-Bore High-Performance Liquid Chromatography with Electrospray Ionization Detection

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Qualitative determination of anthocyanins in extracts of red fruits by narrow-bore HPLC/ESI-MS was carried out. This method was used to investigate anthocyanin contents of black bilberry (*Vaccinium myrtillus* L.), blackberry (*Rubus* sp.), and mulberry (*Morus nigra*). An ultraviolet diode array and a mass spectrometer with ESI source were used for detection. Anthocyanin identifications were made by using retention time data and UV−vis and mass spectra and comparing them with those of commercially available standard compounds. The method allowed the identification of fourteen anthocyanins in black bilberry extract, six anthocyanins in blackberry extract, and five anthocyanins in mulberry extract.

Keywords: *Anthocyanin; bilberry; blackberry; mulberry; HPLC/ESI-MS; narrow-bore column*

INTRODUCTION

Anthocyanins are a group of naturally occurring phenolic compounds responsible for the color of many plants, flowers, and fruits (*1*). Anthocyanins are glycosylated polyhydroxy and polymethoxy derivatives of flavylium salts. The distribution of anthocyanins in the plant kingdom is so wide that, in many cases, information on the anthocyanin composition in a plant matrix is not available.

This group of compounds has great importance because of their demonstrated pharmacological activities (*2*−*5*). Berry extracts contain high amounts of anthocyanins, and their consumption in the diet or in some therapeutic applications is common (*6*−*8*). Anthocyanin compositions of different fruits are quite distinctive, and their analyses have been successfully used to detect adulteration of fruit jams (*9*), fruit juice products (*10*), and red wines (*11*) rich in anthocyanin pigments. In the last two decades, numerous papers have been published reporting the identification of anthocyanins in food (*12*), but frequently data obtained are not in accordance with those of other studies. In many studies the identification was carried out by comparing retention times and UV−vis spectra but frequently such information is not sufficient to distinguish similar structures.

Electrospray ionization mass spectrometry (ESI-MS) has been introduced as a highly sensitive and soft ionization technique for the mass spectrometric analysis of polar, nonvolatile, and thermolabile molecules (*13*). This technique has been used for anthocyanin characterization in different extracts (*14*, *15*), obtaining a useful fingerprint of the matrix examined. However, used in combination with HPLC, ESI-MS has proven to be a very powerful tool for anthocyanin characterization (*16*, *17*).

In this study a screening of anthocyanins content in some berry extracts (black bilberry, blackberry, and mulberry) by HPLC/ESI-MS in positive ion mode was carried out. Electrospray ionization is a soft technique, so that the mass spectra obtained at low voltage shows the molecular ion as the main peak of the spectrum. By applying higher voltage the anthocyanidin fragment $[M\text{-}Gly]^+$ appears due to loss of the glycosidic moiety, and at this voltage the aglycon fragment ion is the main peak of the spectrum. The six most common anthocyanidins show different molecular weights, so they can be easily distinguished. The mass spectra obtained with this technique were very simple and such information permitted a more reliable peak identification than the UV−vis spectra.

The use of a narrow-bore HPLC column permits the optimum flow rate into the ES source without splitting. Moreover, a better level of detection, due to the greatly reduced chromatographic dilution, is obtained because both UV and ESI-MS detectors are concentration-sensitive devices (*18*).

MATERIALS AND METHODS

Samples. Black bilberry, blackberry, and mulberry samples were obtained as commercial products. Ripe fruits were stored at −18 °C until analysis.

Standards. Cyanidin-3-glucoside, cyanidin-3-rutinoside, and pelargonidin-3-glucoside were purchased from Extrasynthese (Genay, France).

Sample Preparation. Fruit extracts were obtained by pounding in a mortar about 200 g of fruits. An aliquot of 5 mL of extract was centrifuged at 6000 rpm for 20 min. The supernatant so obtained was analyzed by HPLC/DAD and HPLC/ESI-MS.

HPLC/DAD Analysis. The HPLC/DAD analyses were performed on a Shimadzu HPLC system equipped with a SPD-

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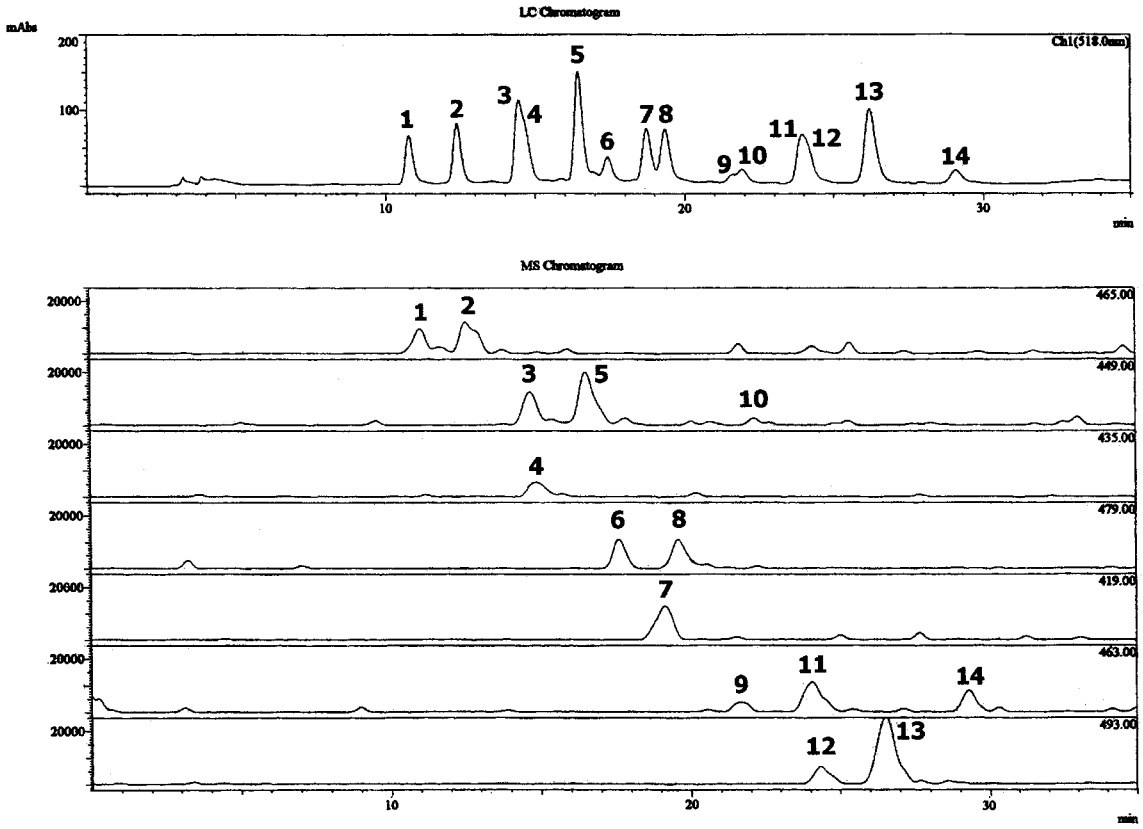


Figure 1. HPLC−UV chromatogram of a black bilberry extract and ion chromatograms (acquired at 45V) extracted at *m*/*z* corresponding to molecular weight of the identified anthocyanins. Peak identification: 1, delphinidin-3-galactoside; 2, delphinidin-3-glucoside; 3, cyanidin-3-galactoside; 4, delphinidin-3-arabinoside; 5, cyanidin-3-glucoside; 6, petunidin-3-galactoside; 7, cyanidin-3-arabinoside; 8, petunidin-3-glucoside; 9, peonidin-3-galactoside; 10, petunidin-3-arabinoside; 11, peonidin-3-glucoside; 12, malvidin-3-galactoside; 13, malvidin-3-glucoside; and 14, malvidin-3-arabinoside.

10AMvp photodiode array detector, and the data were collected using a Shimadzu Class VP-5.03 (Shimadzu, Milan, Italy) acquisition system. The column was a Restek Pinnacle ODS, 250 × 4.6 mm, 5-μm particle size (Superchrom, Milan, Italy); elution was with a binary high-pressure gradient at a flow rate of 1 mL/min. Injection volume was 20 μL of a solution of black bilberry, blackberry, or mulberry centrifuged extracts diluted 1:1 (v/v) in solvent A. Solvent A was H_2O/HCOOH, 9:1; solvent B was H_2O/HCOOH/CH_3CN, 4:1:5. The percentage of solvent B was increased linearly, after an initial hold of 1 min, from 12 to 30% in 25 min; then, to 100% in an additional 9 min. The column was reconditioned with the initial eluent for about 20 min. Acquisition range was set between 240 and 600 nm with a sampling period of 0.32 s and a time constant of 0.64 s. The chromatogram was monitored at 518 nm.

HPLC/ESI-MS Analysis. The HPLC/ESI-MS analyses were performed on a Shimadzu HPLC system equipped with UV−vis detector Shimadzu SPD-10Avp, and coupled on-line with a Shimadzu QP 8000 API mass spectrometer. UV and MS data were acquired and processed using Shimadzu Class 8000 software (Shimadzu, Milan, Italy). The column used was a Restek ODS Pinnacle C18, 250 × 2.1 mm, 5-μm particle size (Superchrom, Milan, Italy) with a 10 × 2.1 mm guard-cartridge packed with the same stationary phase as the column. UV−vis detection was by absorbance at 518 nm. The HPLC gradient used was the same as for HPLC/DAD analysis. The flow rate was 0.2 mL/min and injection volume was 2 μL. Black bilberry and blackberry centrifuged extracts were injected without dilution, but mulberry centrifuged extract was diluted 1:1 (v/v) in solvent A. Electrospray ionization (ESI) was performed in positive ion mode. MS conditions were nebulizer gas flow of 4.5 L/min; probe voltage of 4.5 kV; curved desolvation line (CDL) voltage of 130.0 V; CDL temperature of 230 °C; deflector voltage at 45 and 60 V; scan speed of 2000 amu/sec; and *m*/*z* acquisition from 100 to 800.

The sensitivity was evaluated with the commercially available standard cyanidin-3-glucoside. Solutions at different known concentrations were analyzed under the same experimental conditions. The molecular ion from a quantity of 50 ng could be detected with a signal-to-noise ratio of 3 under full scan conditions.

Reproducibility of retention times and of mass spectra was also tested, and the RSD% was always lower than 1.2%.

RESULTS AND DISCUSSION

Figures 1 and 2 show, under the HPLC−UV chromatogram obtained using the narrow-bore column, the extracted ion chromatograms at *m*/*z* values corresponding to the molecular weight of the anthocyanins found in the black bilberry extract (Figure 1) and the *m*/*z* values corresponding to the molecular weights of the six most common anthocyanidins (Figure 2). The MS conditions used for the analyses reported in Figures 1 and 2 used fragmentation voltages of 45V and 60V, respectively. At 45V a very strong $(M+H)^+$ ion is obtained, whereas at 60V an anthocyanidin fragment obtained from the loss of the sugar is the predominant ion.

Many studies have been carried out on the composition of the anthocyanins of *Vaccinium* species (*9*, *10*, *15*, *19*−*29*). In particular, the presence of 3-*O*-arabinosides, 3-*O*-glucosides, and 3-*O*-galactosides of cyanidin, delphinidin, peonidin, petunidin, and malvidin have been reported by many authors (*19*−*21*, *24*, *27*), but other authors reported only some of these compounds (*9*, *10*, *22*, *23*, *25*, *26*, *28*). Only Petri et al. (*29*) reported

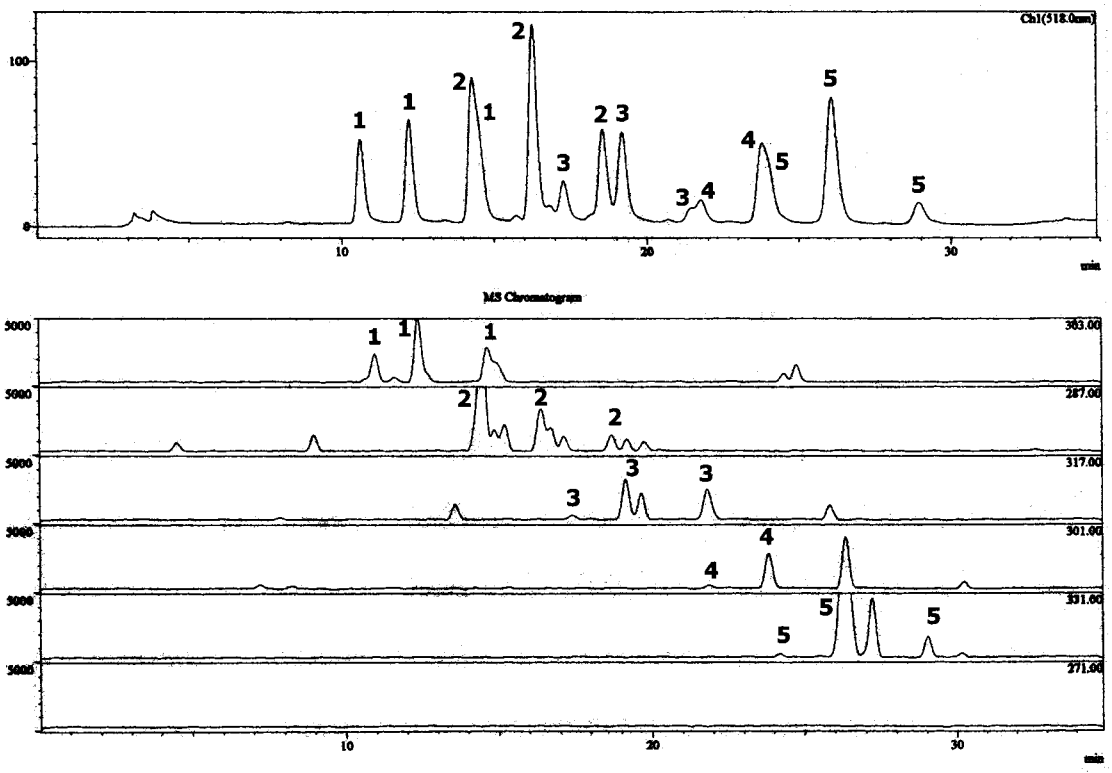


Figure 2. HPLC−UV chromatogram of a black bilberry extract and ion chromatograms (acquired at 60V) extracted at *m/z* corresponding to molecular weight of the six most common anthocyanidins. Peak identification: 1, delphinidin; 2, cyanidin; 3, petunidin; 4, peonidin; and 5, malvidin.

the presence of malvidin-3,5-diglucosides and petunidin and Baj et al. (*19*) reported the presence of petunidin and cyanidin in bilberry (*Vaccinium myrtillus* L.) extracts. Analytical methods have been mainly based on HPLC with UV−vis and photodiode array detection, but some data on GC−MS analysis of the anthocyanins treated with trimethylchlorosilane and hexamethyldisilazane have been reported (*19*). In this investigation, peak identification has been carried out using the molecular weight and structural information obtained from the HPLC−MS analysis with ES^+ ionization at different fragmentation voltages, in addition to the data of the UV−vis spectra. UV−vis spectra of anthocyanins are very similar, especially for those compounds that differ only for the glycosidic pattern, so the information obtained is not very characteristic.

The HPLC/ESI-MS analysis established the identification of 14 anthocyanins present in black bilberry. In particular, 3-*O*-arabinosides, 3-*O*-glucosides, and 3-*O*-galactosides of cyanidin, delphinidin, peonidin, petunidin, and malvidin have been found, with the exception of peonidin-3-arabinoside. Because black bilberry presents a large structural diversity of the anthocyanin fraction, and many of the identified components are not commercially available as standards, MS spectra of the identified components have been used to build a MS library, together with those commercially available. This library has been used for the identification of anthocyanins present in the other matrixes under investigation. The anthocyanin fractions of both blackberry and mulberry have been previously less extensively studied than that of bilberry, even though the compositions of their anthocyanin fractions are qualitatively less complex than that of bilberry.

Figure 3 shows the HPLC−UV chromatogram of a blackberry (*Rubus* sp.) extract obtained with the narrow-bore column and the extracted ion chromatograms at *m/z* values corresponding to the molecular weights of the anthocyanins present in the extract. As can be seen, seven components have been separated. Five of them have been identified on the basis of their MS spectra and retention times, and another component has been tentatively identified. Cyanidin-3-glucoside is the main component of the fraction, as previously reported in the literature (*9*, *10*, *27*, *28*, *30*−*33*). Cyanidin-3-*O*-galactoside, cyanidin-3-*O*-arabinoside, and malvidin-3-*O*-glucoside have been identified for the first time in blackberry extract. Pelargonidin-3-*O*-glucoside was also found; it was previously identified only by Torre and Barritt (*32*). An additional cyanidin with a pentose has been detected. The aglycon has been definitely identified as cyanidin because the *m/z* value of the ion obtained at 60V is 287. This compound has been tentatively identified as cyanidin-3-xyloside, in accordance with data previously reported by Sapers et al. (*31*).

Figure 4 shows the HPLC−UV chromatogram of a mulberry (*Morus nigra*) extract obtained with the narrow-bore column and the extracted ion chromatograms at *m/z* values corresponding to the molecular weights of the anthocyanins present in the extract. This fruit shows a very high content of total anthocyanins, and as can be seen from the chromatogram two are the main components of this fraction. On the basis of the UV and MS spectra and the retention time values, the main components have been identified as cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside. The second of these two compounds has been identified for the first time in mulberry. Moreover, cyanidin-3-*O*-sophoroside and pelargonidin-3-*O*-glucoside have been identified by comparison of their MS spectra with those of the library. A further pelargonidin has been identified on the basis of the *m/z* value obtained at 60V for this compound. On

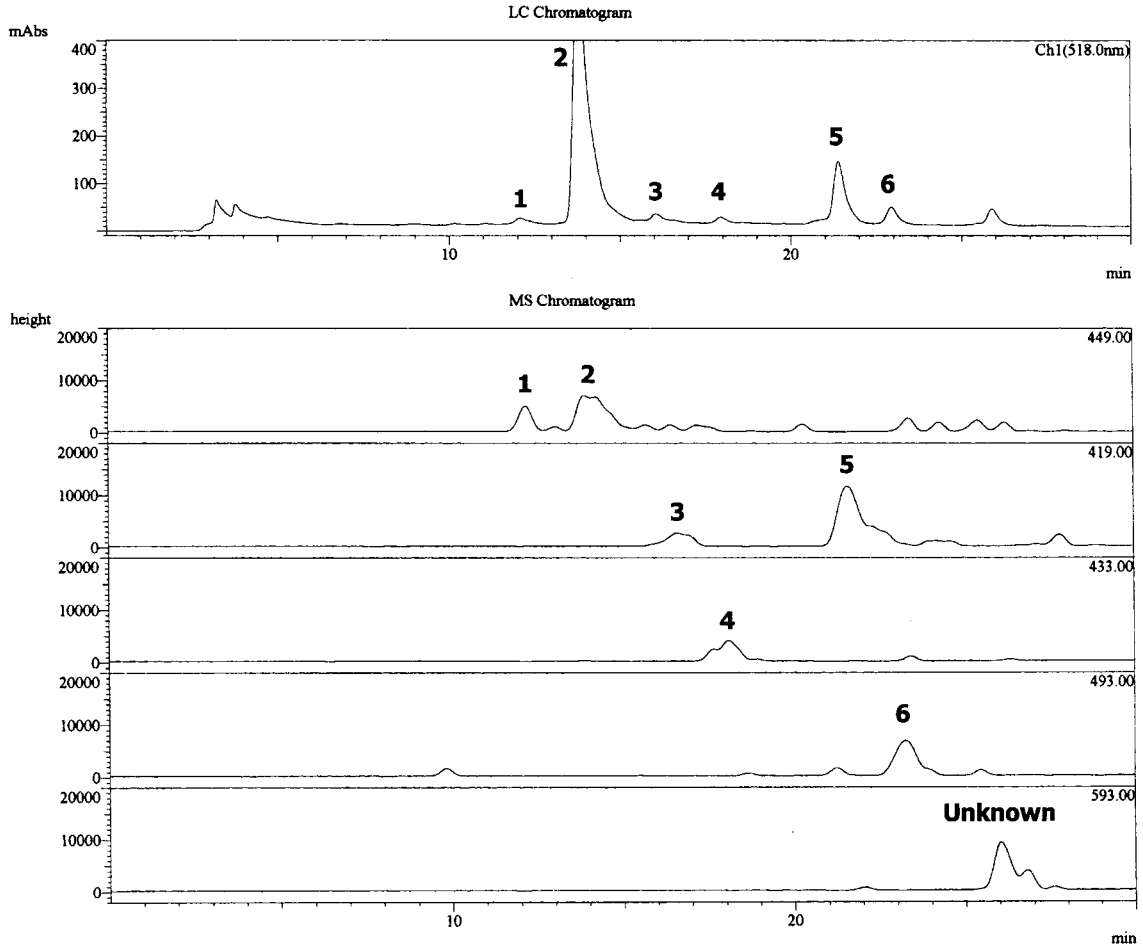


Figure 3. HPLC−UV chromatogram of a blackberry extract and ion chromatograms (acquired at 45V) extracted at *m*/*z* corresponding to molecular weight of the identified anthocyanins. Peak identification: 1, cyanidin-3-galactoside; 2, cyanidin-3-glucoside; 3, cyanidin-3-arabinoside; 4, pelargonidin-3-glucoside; 5, cyanidin-3-xyloside (tentative); and 6, malvidin-3-glucoside.

the basis of the *m*/*z* value obtained at 45V, the sugar may be a disaccharide, with a molecular weight corresponding to neohesperidose or rutinose. From literature data on the elution order of anthocyanins under reversed-phase liquid chromatography conditions (*27*, *34*) the peak can be identified as pelargonidin-3-*O*-rutinoside, because pelargonidin-3-*O*-neohesperidoside should be eluted at a shorter retention time than pelargonidin-3-*O*-rutinoside and pelargonidin-3-*O*-glucoside. There are few literature reports on the anthocyanin fraction of *M. nigra*. In 1934, Yamamoto (*35*) identified cyanidin-3-*O*-glucoside in the fruits of the mulberry; later, Maki and Inamoto (*36*) also detected pelargonidin-3-*O*-glucoside together with petunidin-3-*O*-rutinoside in a sample of *M. alba* and Toscano and Lamonica (*37*) detected, together with cyanidin-3-*O*-glucoside, an other unidentified pigment. The most recent data obtained by Gerasopoulos and Stavroulakis (*38*) were based on an attempted comparison with the anthocyanin pattern of red raspberry juice, which contains a well-defined anthocyanin pattern. These results agree with this work only for the identification of cyanidin-3-*O*-sophoroside.

Table 1 lists the identified anthocyanins and their retention times relative to that of cyanidin-3-*O*-glucoside, which is present in all three extracts. Structures of the anthocyanin aglycons are reported in Figure 5. HPLC with photodiode array detection, coupled to a mass detector with API interface, allowed the separation and identification of the anthocyanins present in black bilberry, blackberry, and mulberry extracts. The information obtained from the MS spectra acquired at different voltages permitted confirmation of the presence of 14 of the 15 anthocyanins most commonly identified in black bilberry extract; the presence of

Table 1. Anthocyanins Identified in Black Bilberry, Blackberry, and Mulberry Extracts

anthocyanin[a]	RRT[b]	black bilberry	blackberry	mulberry
dp-3-gal	0.65	X		
dp-3-glu	0.75	X		
cy-3-sop	0.83			X
cy-3-gal	0.87	X	X	
dp-3-ara	0.87	X		
cy-3-glu	1	X	X	X
cy-3-rut	1.11			X
cy-3-ara	1.15	X	X	
pt-3-glu	1.17	X		
pg-3-glu	1.28		X	X
pt-3-gal	1.29	X		
pn-3-gal	1.31	X		
pt-3-ara	1.33	X		
pg-3-rut	1.41			X
pn-3-glu	1.45	X		
mv-3-gal	1.45	X		
cy-3-xyl	1.55		X	
mv-3-glu	1.60	X	X	
mv-3-ara	1.76	X		

[a] Cy, cyanidin; pn, peonidin; dp, delphinidin; pt, petunidin; mv, malvidin; pg, pelargonidin; glu, glucoside; gal, galactoside; ara, arabinoside; rut, rutinoside; sop, sophoroside; xyl, xyloside. [b] RRT, relative retention time.

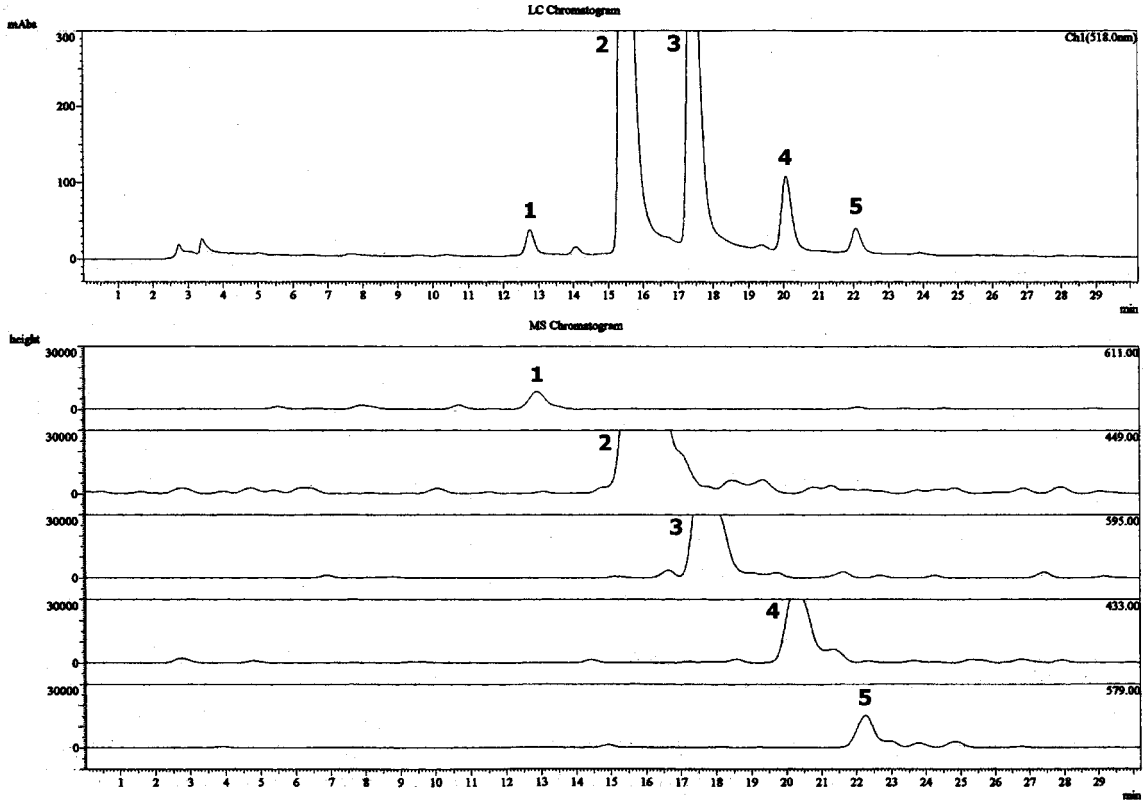
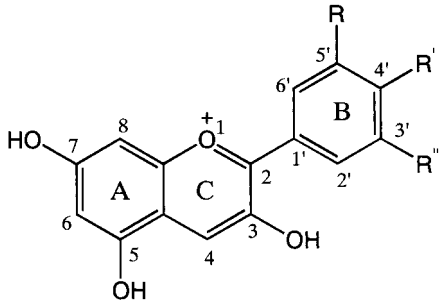


Figure 4. HPLC−UV chromatogram of a mulberry extract and ion chromatograms (acquired at 45V) extracted at *m/z* corresponding to molecular weight of the identified anthocyanins. Peak identification: 1, cyanidin-3-sophoroside; 2, cyanidin-3-glucoside; 3, cyanidin-3-rutinoside; 4, pelargonidin-3-glucoside; and 5, pelargonidin-3-rutinoside (tentative).



Anthocyanidin	R	R'	R''	MW
Delphinidin	OH	OH	OH	303
Cyanidin	OH	OH	H	287
Petunidin	OH	OH	OCH_3	317
Pelargonidin	H	OH	H	271
Peonidin	OCH_3	OH	H	301
Malvidin	OCH_3	OH	OCH_3	331

Figure 5. Structure of the 3,5,7,4′-tetrahydroxyflavilium ion and of the six anthocyaninidins found in the three extracts.

cyanidin-3-*O*-glucoside and pelargonidin-3-*O*-glucoside in blackberry extract; and, cyanidin-3-*O*-sophoroside, cyanidin-3-*O*-glucoside, and pelargonidin-3-*O*-glucoside in mulberry extract. Moreover, three anthocyanins have been identified for the first time in the blackberry extract (cyanidin-3-*O*-galactoside, cyanidin-3-*O*-arabinoside, and malvidin-3-*O*-glucoside), and one has been reported for the first time in the mulberry extract (cyanidin-3-*O*-rutinoside). Two compounds were tentatively identified: cyanidin-3-*O*-xyloside in blackberry extract and pelargonidin-3-*O*-rutinoside in mulberry extract.

This method is rapid and easy. The MS library developed may help in the characterization of other less-studied natural matrixes containing anthocyanins. The use of a narrow-bore HPLC column for separation improved mass detection sensitivity as the mobile-phase volume used was five times lower than that used in conventional HPLC. This improved sensitivity allowed a mass spectra to be obtained also for minor compounds present in the matrix.

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