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Characterization of the Anthocyanin Fraction of Sicilian Blood Orange Juice by Micro-HPLC-ESI/MS

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Anthocyanins of red orange juices were analyzed by micro-HPLC coupled on-line with an MS detector equipped with an ESI source. The use of microcolumn HPLC greatly enhanced detection performance, allowing direct identification of the anthocyanins present in the orange juices. The use of a soft ionization technique allows detection of the molecular ions of the aglycons. Eight components were identified, five of them for the first time in red orange juice. Three additional anthocyanins were detected, of which only the aglycon was identified.

KEYWORDS: Anthocyanins; HPLC-ESI/MS; red orange juice

INTRODUCTION

Anthocyanins are a group of naturally occurring phenolic compounds responsible for the color of many plants, flowers, and fruits (*1*). They are glycosylated polyhydroxy and polymethoxy derivatives of flavylium salts. Structures of the anthocyanin aglycons are reported in **Figure 1**. This group of compounds has great importance mainly related to their demonstrated antioxidant activitiy (*2*, *3*). Anthocyanin compositions of different fruits are quite distinctive, and their determination can be a parameter for the assessment of authenticity and quality of juices, jams, sorbets, liquors, and fruit wines rich in anthocyanin pigments (*4*, *5*).

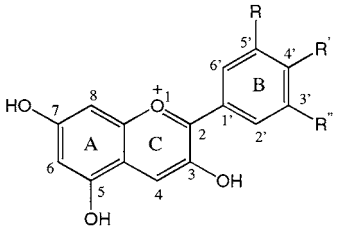
Many studies report the analysis of anthocyanins by HPLC with spectrophotometric detection, but recently ESI/MS has proven to be a very powerful tool for anthocyanin characterization (*6*, *7*).

Although many studies have been carried out on the investigation of the anthocyanin fraction of different matrices, only a few of them regard the composition of blood orange and its juice (*8*−*13*).

Blood oranges of cv. Moro, Tarocco, and Sanguinello are typical Italian products characteristic of eastern Sicily and represent a natural source of anthocyanins. Due to the increased interest in pharmacologically active components in food, the demand for pigmented orange juice is increasing, as is the number of soft drinks containing blood orange juice as an ingredient.

Previous studies carried out by HPLC with spectrophotometric detection have shown that cyanidin 3-glucoside is the main component of the fraction (*8*−*10*, *12*, *13*). The other major anthocyanin of the fraction, cyanidin 3-(6″-malonyl)glucoside, has been recently identified (*11*) and confirmed in further studies (*12*, *13*). The other minor pigments of the fraction are still unknown, due to the lack of standards and the difficulty in isolating sufficient amounts of them from the matrix. Attempts at identification were based on the comparison of retention times of standard anthocyanins or on the characteristics of the UV−vis spectrum and are not in good agreement (*8*−*10*).

In recent years, interest in microcolumn LC has increased considerably. This is mainly due to the ability to work with small sample sizes, small volumetric flow rates, and enhanced detection performance with the use of concentration sensitive



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

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detectors due to the reduced chromatographic dilution (*14*, *15*). Electrospray ionization (ESI) is the most widespread ionization technique in on-line LC-MS. It operates at atmospheric pressure and provides soft molecule ionization. The ESI interface can be operated at extremely low mobile flow rates, and for good efficiency of the ionization process, a splitter is commonly incorporated when conventional HPLC columns are used. The use of small-bore and capillary HPLC columns permits a decreasing or even elimination of the splitter.

In this study, a microcolumn for the HPLC-ESI/MS analysis of anthocyanins in blood orange juice produced in Sicily from different cultivars (Moro, Tarocco, and Sanguinello) has been used.

MATERIALS AND METHODS

Samples. Red orange fruits of three different cultivars (Moro, Tarocco, and Sanguinello) were obtained from a local producer. The fruits were immediately hand-squeezed and the centrifuged juices stored at −18 °C until analysis. For the analysis, the clarified juices were passed through a 0.45 μm filter and diluted with water.

HPLC-ESI/MS. The HPLC-ESI/MS analyses were performed on an HPLC system equipped with an SPD-10Avp UV−vis detector, coupled on-line with a QP 8000 API mass spectrometer (Shimadzu, Milan, Italy). UV and MS data were acquired and processed using Shimadzu Class 8000 software. To reduce the flow rate an Acurate flow splitter (LC Packings, Amsterdam, The Netherlands) was used between the pump and the injector. A U-Z view capillary flow cell (LC Packings) was fitted to the UV−vis detector. The column used was a 150 × 1 mm i.d., 3.5 μm Waters Symmetry C18. UV−vis detection was by absorbance at 518 nm. Elution was with a binary high-pressure gradient at a flow rate of 40 μL/min. Injection volume was 0.64 μL. Depending on the anthocyanin content, different dilutions of the extracts were injected: Moro juices were diluted 1:10 in H_2O and Tarocco juices 1:1 in H_2O, whereas Sanguinello juices were injected without dilution. 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 and then to 100% over an additional 9 min. Electrospay ionization was performed in positive ion mode. MS conditions were as follows: nebulizer gas (N_2) flow, 4.5 L/min; probe voltage, 4.5 kV; deflector voltage, 40 and 55 V; *m*/*z* acquisition from 100 to 800 amu.

RESULTS AND DISCUSSION

The anthocyanin fraction of red orange juice has been less extensively studied than other food matrices containing anthocyanins, and, with the exception of delphinidin 3-glucoside, only the two main components have been identified until now. For minor components of the fraction, data reported in the literature are not in accordance.

Figure 2A shows a micro-HPLC-UV−vis chromatogram acquired at 518 nm of the anthocyanin fraction of a blood orange juice. As can be seen, 13 components were detected. **Figure 2B** shows the micro-HPLC-ESI/MS chromatogram of the same sample acquired during the same analysis. In the total ion current (TIC) chromatogram obtained in full scan no peaks were detected due to the low sensitivity of ESI/MS for anthocyanins. Preliminary experiments carried out with standard cyanidin 3-glucoside showed that the ESI/MS conditions used for this analysis [curved desolvation line (CDL) voltage, −45 V; deflector voltages, 55 V] produced the fragmentation between the sugar moiety and the aglycons, allowing the detection of the ions corresponding to the anthocyanidins (aglycons). Extracting ion chromatograms at *m*/*z* values corresponding to molecular weight of the six most common anthocyanidins, signals appeared in correspondence to the peaks of the UV−vis chromatogram, as can be seen in the lower part of **Figure 2B**. In this way, it is possible to detect the nature of the anthocyanidins. As can be seen from the six ion chromatograms, peaks 2, 3, 6, 8, 9, 11, and 13 present *m*/*z* of 287 and can be identified as cyanidins. Peaks 1, 4, and 7 are delphinidins (*m*/*z* 303), peaks 5 and 12 are peonidins (*m*/*z* 301), and peak 10 was identified as petunidin (*m*/*z* 317). Ion chromatograms at *m*/*z* values of 271 and 331, corresponding, respectively, to pelargonidin and malvidin, did not present any significant signal.

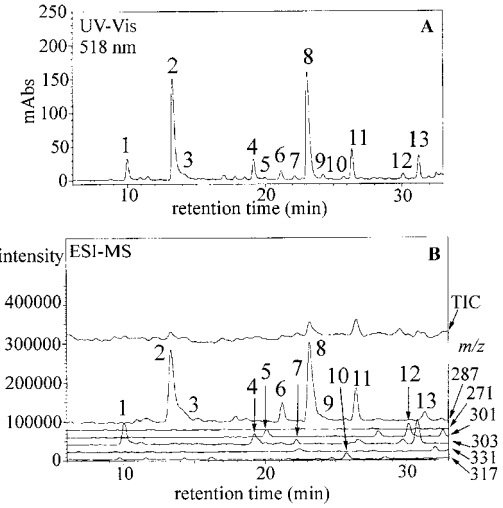

Figure 2. (A) Micro-HPLC-UV–vis and (B) micro-HPLC-ESI/MS chromatograms of the anthocyanins of a Moro orange juice. (B) TIC chromatogram and ion chromatograms extracted at *m*/*z* corresponding to the molecular weights of the six anthocyanidins. A fragmentation voltage of 55 V was used for ESI/MS. For peak identification see **Table 1**.

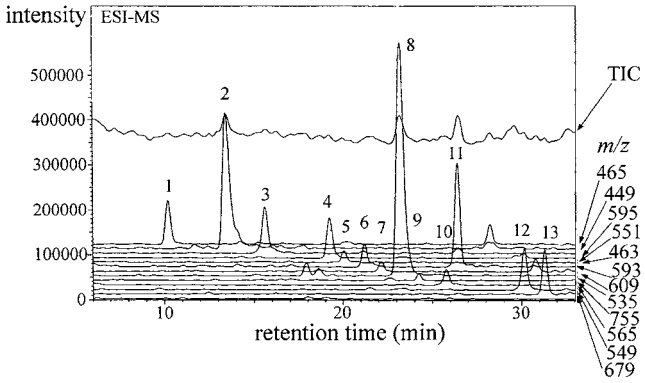

Figure 3. Micro-HPLC-ESI/MS chromatograms of the anthocyanins of a Moro orange juice: TIC chromatogram and ion chromatograms extracted at *m*/*z* corresponding to the molecular weights of the detected anthocyanins. A fragmentation voltage of 40 V was used for ESI/MS. For peak identification see **Table 1**.

Figure 3 shows the micro-HPLC-ESI/MS analysis of the same sample, but under MS conditions that allowed the detection of the molecular ion for each anthocyanin (CDL voltage, −45 V; deflector voltage, 40 V). Also in this case, the TIC chromatogram did not show significant signals. Ion chromatograms were extracted at values of retention times relative to cyanidin 3-glucoside, corresponding to those of the peaks of **Figure 2**. **Table 1** reports the *m*/*z* values obtained from the analysis reported in **Figures 2** and **3**. Because the six most common aglycons have different *m*/*z* values, it was easy to identify them from MS data. From the combination of *m*/*z* values obtained from the two analyses, identification of unknown components was possible. The three studied cultivars of blood orange differ only in the quantitative amount of anthocyanins,

Table 1. Anthocyanins Identified in Red Orange Juice Analyzed by Micro-HPLC-ESI/MS and Comparison with Literature Data

peak	name	MW	RRT	$[M]^+$ (40 V)	$[A]^+$ (55 V)	ref
1	delphinidin 3-glucoside	465	0.76	465	303	*8*, *9*
2	cyanidin 3-glucoside	449	1.00	449	287	*8–13*
3	cyanidin 3-rutinoside	595	1.13	595	287	
4	delphinidin 3-(6″-malonyl)glucoside	551	1.44	551	303	
5	peonidin 3-glu	463	1.49	463	301	
6	cyanidin ?		1.59	593	287	
7	delphinidin ?		1.66	609	303	
8	cyanidin 3-(6″-malonyl)glucoside	535	1.73	535	287	*10–13*
9	cyanidin ?		1.80	755	287	
10	petunidin 3-(6″-malonyl)glucoside	565	1.93	565	317	
10	cyanidin ?		1.93	?	287	
11	cyanidin ?		1.98	593	287	
12	peonidin 3-(6″-malonyl)glucoside	549	2.25	549	301	
13	cyanidin ?		2.34	679	287	
	cyanidin 3-ramnoside	433				*10*
	delphinidin 3,5-diglucoside	627				*8*, *9*
	cyanidin 3,5-diglucoside	611				*8*, *9*
	petunidin 3,5-diglucoside					*8*
	pelargonidin 3,5-diglucoside	595				*8*
	peonidin 3,5-diglucoside	625				*8*, *9*
	petunidin 3-glucoside	479				*8*
	pelargonidin 3-glucoside	433				*8*
	cyanidin 3-(acetyl)glucoside	491				*8*, *9*
	cyanidin 3-(ferulyl)glucoside	625				*9*
	cyanidin 3-(coumarylferulyl)glucoside	771				*9*
	cyanidin 3-(sinapyl)glucoside	655				*9*
	peonidin 3-(coumaryl)glucoside	609				*9*

but showed the same qualitative profile. **Table 1** also reports the anthocyanins previously found in blood orange juice (*8*−*13*).

As previously reported, cyanidin 3-glucoside and cyanidin 3-(6″-malonyl)glucoside are the main components of the fraction. As can be seen from **Figure 2** and **Table 1**, cyanidin is the predominant aglycon, followed by delphinidin, peonidin, and petunidin. Pelargonidin and malvidin aglycons were not detected. Delphinidin 3-glucoside was previously identified by comparison with standard, and this identification can be easily confirmed by ESI/MS data. Peonidin 3-glucoside has been identified for the first time, whereas pelargonidin 3-glucoside, previously reported, was not detected.

Delphinidin, peonidin, and petunidin acylated with malonic acid have been detected. These peaks were assigned to the isomer with malonic acid attached to the C-6 of the glucose, because this acyl group is generally bound to the C-6 of the sugar, and also by analogy with cyanidin 3-(6″-malonyl)-glucoside, for which the position of the acyl group has been previously determined by ^{1}H NMR (*11*). Obviously, this assignment is only tentative and needs to be confirmed by ^{1}H NMR analysis on the isolated components. Cyanidin 3-rutinoside has been detected for the first time in blood orange juice. Cyanidin 3-rutinoside has been confirmed by the injection of the standard and by the identification of this component in other matrices analyzed under the same analytical conditions, such as mulberry extract. Retention data of anthocyanins on C18 columns used together with ESI/MS data can be very useful for anthocyanin identification, because of the high number of different molecules with the same molecular weight. As an example, peak 10 has been identified as petunidin 3-(6″-malonyl)glucoside (MW 565). In the chromatogram of **Figure 2**, this peak presents an overlap of *m*/*z* values corresponding to cyanidin and petunidin aglycons. Considering the cyanidin aglycon, the *m*/*z* value of 565 may correspond to cyanidin 3-(6″-malyl)glucoside, a rare anthocyanin previously found only in *Dianthus caryophyllus* and *Dianthus deltoids* petals (*17*). Because retention behavior in RP-HPLC has shown that malylation lowers the retention time compared to malonylation, the *m*/*z* value of 565 cannot be due to the presence of a malyl derivative of cyanidin.

Other components with cyanidin or delphinidin aglycons have been detected. For these components, the *m*/*z* value of the molecular ion did not correspond to any anthocyanin reported in the literature (*18*). In particular, peaks 11 and 13, which have a cyanidin aglycon, are the most abundant among the minor components of the fraction. Lee (*13*), in a very recent paper on the characterization of red-fleshed Budd blood oranges from Florida, reported a tentative identification of these two peaks as malonated cyanidins with dimalonic substitution or probably as positional isomers. This identification was based on the elution order and UV−vis spectroscopic data, compared with anthocyanins from the red onion. From MS data here obtained, *m*/*z* values of the $[M]^+$ ions are not in agreement with the identification proposed (*13*). In this case, MS data are not enough for a complete identification, and further studies will be carried out for structure elucidation of these new anthocyanins. However, the *m*/*z* values of these not completely identified anthocyanins do not correspond to any of the other previously identified acylated anthocyanins, as can be seen from data reported in **Table 1**. The presence of signals at *m*/*z* values corresponding to the anthocyanin diglucosides and acylated glucosides previously reported in the literature was also tested, and none of those peaks was detected.

The micro-HPLC-ESI/MS analysis is rapid and easy. The use of microcolumns allows us to work with small sample sizes and small volumetric flow rates and to enhance detection performance due to reduced chromatographic dilution. The improved sensitivity allowed mass spectra to be obtained also for minor components present in the matrix. The use of the micro-HPLC-ESI/MS technique permitted the sure identification of six minor components of the anthocyanin fraction of blood orange juice, five of which were identified for the first time in this matrix. Analysis at higher deflector voltages permitted the identification of the aglycons present in the fraction: three

delphinidins, eight cyanidins, two peonidins, and one petunidin were detected, whereas pelargonidins and malvidins were not detected.

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