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Identification and Quantification of Phenolic Compounds in Grapes by HPLC-PDA-ESI-MS on a Semimicro Separation Scale

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Reversed phase high performance liquid chromatography (RP-HPLC) on a semimicro separation scale was employed to develop a straightforward method for the simultaneous separation, identification, and quantification of phenolic compounds occurring in whole berries of *Vitis vinifera*, which comprise phenolic acids, flavonols, catechins, stilbenes, and anthocyanins. A C-18 narrow bore column of 150 × 2.0 mm I.D. and a semimicro photodiode array detector (PDA) cell of 2.5 μL, in conjunction with a mass spectrometry detector equipped with an electrospray ionization source (ESI-MS) to confirm peak identification, were employed. The C-18 narrow bore column was eluted by a multisegment gradient of increasing concentration of acetonitrile in water−formic acid solution that was optimized on the basis of the results of a study carried out to evaluate the influence of mobile phase composition and gradient shape on separation performance and detection sensitivity by ESI-MS. The identification of individual phenolic compounds was performed on the basis of their retention times and both UV−visible and mass spectra, acquired by a mass spectrometer (MS) equipped with an electrospray ionization (ESI) source, employed in conjunction with the PDA detector. Libraries comprising retention times, UV−visible, and mass spectra for major phenolic compounds expected in grape berries were made by subjecting solutions of each phenolic standard to the optimized RP-HPLC method. Quantification of individual compounds was performed by the external standard method using a six point regression graph of the UV−visible absorption data collected at the wavelength of maximum absorbance of each analyte determined by the PDA spectra. The RP-HPLC method was validated in terms of linearity of calibration graphs, limits of detection, limits of quantification, repeatability, and accuracy, which was evaluated by a recovery study. The developed method was successfully applied to identify the phenolic compounds occurring in the whole berries of nine red and one white grape of different varieties of *Vitis vinifera*, comprising some autochthonous varieties of south Italy such as Aglianico, Malvasia Nera, Uva di Troia, Negroamaro, Primitivo, and Susumaniello. Large differences in the content of phenolic compounds was found in the investigated grape varieties. As expected, only glycosilated flavonols were quantified, and the total amount of these compounds was higher in the whole berries of red grapes than in the white Moscato, where the most abundant phenolic compound was quercetin 3-*O*-glucoside. In almost all samples, the most and least abundant anthocyanins were malvidin 3-*O*-glucoside and cyanidin 3-*O*-glucoside, respectively, with the exception of Uva di Troia where the least abundant anthocyanin was delphinidin 3-*O*-glucoside.

KEYWORDS: RP-HPLC; narrow bore columns; photodiode array detection; ESI-MS detection; phenolic compounds; grape berries

INTRODUCTION

Phenolic compounds are secondary metabolites, ubiquitous in the plant kingdom that are widely present in the human diet and have shown to exert beneficial influence on human health (*1*). Most secondary metabolites are not directly involved in development, growth, and reproduction of the plant but are synthesized for counteracting abiotic and biotic stresses, such as nutritional deficiency, drought, salinity, pollutants, adverse climatic conditions, pathogens, insects, and phytophagy (*2*). Phenolic compounds are potent antioxidants, and epidemiological studies have suggested a direct

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correlation between their high intake with diet and reduced risk of coronary heart disease mortality by suppressing the oxidation of low-density lipoprotein (*3*). Other studies have reported evidence that phenolic compounds may exhibit a great number of cell protective actions, such as modulation and induction of human cell receptors (*4*), enzymatic mechanisms of cell proliferation (*5*), and other beneficial effects against cancer (*6*), vasorelaxation (*7*), and allergy (*8*).

The phenolic compounds in *Vitis vinifera* include a plethora of compounds comprising phenolic acids, stilbenes, and flavonoids, which cover a large number of subclasses, such as flavonols, flavanols, and anthocyanins, and are expected to play an important role on the quality of grapes and wines (*9*). Anthocyanins are directly responsible for the color in grapes and young wines, whereas astringency and structure of wines seem to be mainly influenced by catechins and proanthocyanidins, which, as well as flavonols, are also believed to be responsible for their bitterness (*10*).

Occurrence and concentration of the various phenolic compounds in grape berries depend on the variety of grapevine and are influenced by viticultural and environmental factors (*11, 12*). Several studies have concluded that flavonols and anthocyanins may be ascribed as molecular markers for the classification and differentiation of grape cultivars and single cultivar wines (*13, 14*). According to these studies, either the flavonols or the anthocyanin profiles seem to be closely related to the genetic characteristic of the grape. However, other studies have evidenced variations in the occurrence of either anthocyanins or flavonols with seasonal conditions (*15*) or viticultural practice (*16*).

Reversed phase HPLC using analytical size columns (4.0−4.6 mm I.D.) and photodiode array detection has been widely reported for the identification and quantification of phenolic compounds in grape berries and wine. However, most of these methods target only a part of phenolic compounds, whereas their simultaneous determination is rather scarce (*17*). Recent reports describe the simultaneous determination of phenolic compounds of different classes in wine (*18*) and several phenolic families in fruit juices (*19*). In addition, the majority of the reported methods are devoted to studying the occurrence of phenolic compounds in the skin and seeds of grape berries or wines (*20*).

This article reports the results of a study carried out to develop a straightforward HPLC method for the simultaneous identification and quantification of the major phenolic compounds occurring in the whole berries of grape using a narrow bore reversed phase column (2.0 mm I.D.) and detection by in-sequence UV−visible photodiode array spectrophotometry, equipped with a semimicro detection cell, and electrospray ionization mass spectrometry. The selection of a narrow bore column, in combination with a microvolume (2.5 μL) detection cell, was motivated by the expected higher sensitivity of PDA detection, due to the minor dilution of samples during separation in comparison to using a conventional analytical size column (*21*). The method has been applied to investigate the occurrence of phenolic compounds in the whole berries of nine red and one white varieties of *Vitis vinifera*, comprising autochthonous varieties of the southern Italian region Apulia, such as Malvasia, Uva di Troia, Negroamaro, Primitivo, and Susumaniello.

MATERIALS AND METHODS

Chemicals and Standards. HPLC grade solvents and analytical-reagent grade formic acid were purchased from Carlo Erba (Milan, Italy). Deionized water was produced by a Milli-Q unit (Millipore, Bedford, MA, USA). Gallic acid, protocatechuic acid, catechin, epicatechin, quercetin 3-*O*-glucoside, and *trans*-resveratrol were purchased from Sigma (Milan, Italy); delphinidin 3-*O*-glucoside, cyanidin 3-*O*-glucoside, peonidin 3-*O*-glucoside, malvidin 3-*O*-glucoside, and kaempferol 3-*O*-glucoside were obtained from Extrasynthese (Genay, France); *trans*-piceid and petunidin 3-*O*-glucoside were supplied by Polyphenols Laboratories AS (Sandnes, Norway); and caftaric acid was purchased from Chromadex (CA, USA).

Table 1. Peak Capacity as a Function of the Concentration of Formic Acid in the Mobile Phase

concentration of HCOOH (%, v/v)	t_{ϖ} (min)	t_{α} (min)	average peak width (s)	peak capacity
0.5	49.66	7.84	40.2	62
1.0	59.87	4.64	43.6	76
5.0	59.82	4.47	38.2	87
10.0	49.91	3.11	65.7	43

Equipment. HPLC separation, identification, and quantification of phenolic compounds were performed on a Shimadzu LC-10A$_{VP}$ HPLC system consisting of an SCL-10A$_{VP}$ system controller; two LC-10AD$_{VP}$ μ solvent delivery units; a SPD-M10A spectrophotometric diode array detector, equipped with a semimicro flow cell of 2.5 μL; a CTO-10AS$_{VP}$ column oven; a DGU-14A online vacuum membrane degasser; and a Rheodyne (Cotati, CA, USA) Model 8125 semimicro injection valve with a 5-μL sample loop. Data were processed using the Shimadzu Class VP 5.6 HPLC Data System on a Pentium II 400 personal computer (Gigabyte, Milan, Italy). A second HPLC system was employed to confirm peak identification by electrospray ionization mass spectrometry (ESI-MS) detection in the single ion monitoring (SIM) mode in conjunction with photodiode array (PDA) detection. This instrument consisted of a Shimadzu High Performance Liquid Chromatograph/Mass spectrometer Model LCMS-2010 unit, comprising a SCL-10A*vp* system controller; two pump model LC-10AD*vp* μ Solvent Delivery Module; a SPD-M10Avp UV−visible Photodiode Array Detector, equipped with a semimicro flow cell of 2.5 μL; and a single quadrupole mass analyzer Model 2010, equipped with an electrospray (ESI) interface with nitrogen as the nebulizing and drying gas. The temperature of the column was controlled by a DBS (Vigonza, Padua, Italy) Model PCO 200 column oven. The MS acquisition was performed with the ESI interface in either the negative or positive ionization mode at the following conditions: nebulizing gas nitrogen at a flow rate of 4.5 L/min; temperature of block heater, 200 °C; temperature of the curved desolvation line (CDL), 225 °C; probe voltage (+), 4.5 V; probe voltage (−) −3.5 V; CDL voltage, 25 V; Q-array voltages (+), 50, 15, and 60 V; Q-array voltages (−), 0, −15, and −60 V; Q-array RF, 150. System control and data processing were carried out by the Shimadzu LCMS Solution software running on a Pentium IV personal computer (Gigabyte, Milan, Italy). Samples were introduced into the column by a Rheodyne Model 8125 semimicro injection valve with a 5-μL sample loop. The column effluent was first passed through the PDA detector before being directed to the quadrupole mass spectrometer with an ESI interface. The chromatographic separations were carried out on a reversed phase Polaris C-18A column (150 × 2.0 mm I.D, 5 μm; Varian Inc. Lake Forest, CA, USA) in conjunction with a C-18 (30 × 2 mm, 5 μm) guard cartridge column, both thermostatted at 30° ± 1 °C. The separations were performed by gradient elution of increasing concentration of acetonitrile in water acidified with either a volatile acid or a volatile buffer at a flow rate of 0.2 mL/min. Under optimized conditions, the starting eluent (A) and the gradient former (B) consisted of water and acetonitrile, respectively, both containing 5.0% (v/v) formic acid, and the elution was performed by a multisegment gradient, according to the following program: 3 min isocratic elution step with 5.0% B, followed by 12 min linear gradient from 5.0 to 9.0% B, 12 min linear gradient to 13.5% B, 5 min isocratic elution with 13.5% B, 10 min linear gradient to 18.5% B, 2 min isocratic elution with 18.5% B, 7 min linear gradient to 22.5% B, 4 min linear gradient to 30.0%, and 1 min linear gradient to 40.0% B. At the end of the gradient program, the eluent composition was brought to the initial condition in 1 min, and the column was equilibrated for 15 min before the next injection.

Grape Berries Extraction. Berry samples of wine *Vitis vinifera* of nine varieties were collected at enological maturity in 2006 from

Table 2. Interday and Intraday Precision

		repeatability									
		interday ($n = 5$)						intraday ($n = 15$ over 3 days)			
		retention time (min)			peak area[a]			retention time (min)		peak area[a]	
peak nr.	analyte	average	SD	RSD (%)	average	SD	RSD (%)	SD	RSD (%)	SD	RSD (%)
1	gallic acid	3.590	0.018	0.50	383905	3609	0.94	0.033	0.93	10980	2.86
2	protocatechuic acid	6.296	0.025	0.40	201959	1030	0.51	0.092	1.46	3918	1.94
3	caftaric acid	10.139	0.056	0.55	768697	5535	0.72	0.171	1.56	11992	1.56
4	catechin	12.529	0.066	0.53	509872	3008	0.59	0.189	1.54	11013	2.16
5	epicatechin	20.615	0.112	0.54	503498	2316	0.46	0.315	1.53	8811	1.75
6	delphinidin 3-*O*-glucoside	23.148	0.120	0.52	1277469	19417	1.52	0.292	1.26	32320	2.53
7	cyanidin 3-*O*-glucoside	26.761	0.121	0.45	411310	4360	1.06	0.254	0.95	8062	1.96
8	petunidin 3-*O*-glucoside	29.385	0.185	0.63	1158496	15524	1.34	0.376	1.28	32554	2.81
9	peonidin3-*O*-glucoside	33.088	0.064	0.19	288756	2800	0.97	0.205	0.62	7161	2.48
10	*trans*-piceid	34.465	0.146	0.42	2271895	26581	1.17	0.355	1.03	42939	1.89
11	malvidin 3-*O*-glucoside	35.903	0.060	0.17	1204781	23613	1.96	0.434	1.21	34939	2.90
12	rutin	42.340	0.299	0.71	1411637	19622	1.39	0.605	1.43	28374	2.01
13	quercetin 3-*O*-glucoside	42.986	0.359	0.84	196601	2221	1.13	0.709	1.65	4404	2.24
14	kaempferol 3-*O*-glucoside	49.164	0.117	0.24	166531	1732	1.04	0.629	1.28	3314	1.99
15	*trans*-resveratrol	50.496	0.170	0.34	747371	8221	1.10	0.495	0.98	18086	2.42

[a] Arbitrary units.

Table 3. Limit of Detection (LOD), Limit of Quantification (LOQ) and Results of Linear Regression Analysis of Calibration Graphs Based on Absorbance at the Reported Wavelength (λ)

analyte	λ	LOD (mg/L)	LOQ (mg/L)	linear range (mg/L)	equation[a]	correlation coefficient
gallic acid	280	0.05	0.39	0.40−25	$y = 1.85x + 0.65$	0.9989
protocatechuic acid	280	0.32	0.63	0.63−25	$y = 0.61x + 0.60$	0.9988
caftaric acid	320	0.10	0.32	0.32−250	$y = 3.67x - 0.35$	0.9999
catechin	280	0.47	1.25	1.25−300	$y = 0.86x - 0.74$	0.9998
epicatechin	280	0.63	1.25	1.25−100	$y = 0.79x + 0.71$	0.9998
delphinidin 3-*O*-glucoside	520	0.05	0.47	0.50−100	$y = 2.53x + 1.38$	0.9996
cyanidin 3-*O*-glucoside	520	0.16	0.52	0.52−62.5	$y = 5.62x - 5.40$	0.9995
peonidin3-*O*-glucoside	520	0.14	0.21	0.21−400	$y = 2.97x + 1.27$	0.9998
petunidin3-*O*-glucoside	520	0.18	0.43	0.50−400	$y = 3.42x + 1.12$	0.9995
trans-piceid	306	0.07	0.28	0.30−100	$y = 4.46x - 2.14$	0.9993
malvidin 3-*O*-glucoside	520	0.19	0.63	0.63−800	$y = 3.13x - 1.71$	0.9996
rutin	370	0.10	0.63	0.63−100	$y = 1.59x + 0.84$	0.9998
quercetin 3-*O*-glucoside	370	0.05	0.45	0.45−25	$y = 3.15x - 0.16$	0.9997
kaempferol-3-*O*-glucoside	370	0.31	0.63	0.63−25	$y = 0.68x - 0.07$	0.9992
trans-resveratrol	306	0.04	0.14	0.14−100	$y = 21.82x - 3.4$	0.9988

[a] y expresses the detection response (peak area in arbitrary units) and x the concentration for phenolic compounds (in mg/L).

commercial vineyards located in the Italian regions Apulia (Primitivo, Negro Amaro, Susumaniello, Uva di Troia, Aglianico, and Malvasia Nera) and Lazio (Cesanese and Merlot). The table grape Alphonse Lavallée from south Africa was purchased in a local market. With the exception of Aglianico, which originated in Campania and Basilicata, where it was brought by ancient Greeks, all varieties from Apulia are autochthonous wine grapes of this region. Also the Cesanese wine grape from Lazio is an autochthonous species of this region with ancient traditions.

Frozen grape berries were powdered in liquid nitrogen and lyophilized after withdrawal of seeds. The phenolic compounds were extracted using a previously reported method with minor modification as reported below (*22*). Weighted amounts of the lyophilized sample were quantitatively extracted with 8:2 (v/v) methanol−ethanol mixture at 25 °C for 2 h in a round-bottom flask. The extraction was repeated twice, and the collected supernatants were concentrated in a rotavapor with a water bath set at 35 °C. The residue was quantitatively recovered in 1 mL of 8:2 (v/v) methanol−water solution, which was employed for the identification and quantification of the phenolic compounds.

Identification and Quantification of Phenolic Compounds. The identification of individual phenolic compounds was performed on the basis of their retention times and both spectroscopic and mass spectrometric spectra. Libraries comprising retention times and UV−visible and mass spectra for major phenolic compounds expected in grape berry were made by subjecting solutions of each phenolic standard to RP-HPLC analysis with the optimized multisegment gradient and both PDA and ESI-MS detection. Using the Class VP software, a similarity index (SI) was calculated to evaluate how closer spectra of standard and corresponding phenolic compounds separated in the grape extracts resemble each other. According to the above software, SI closer to unity is indicative of higher similarity. In addition, the use of a purity index (PI), based on the comparison of all the spectra within a chromatographic peak to the spectrum at the peak apex, allowed one to exclude the presence of coeluting substances in the peaks of the phenolic compounds separated from the grape extracts. The ESI-MS detection was performed in negative ionization mode for phenolic acids, flavonols, catechins, and stilbenes, and in positive ionization mode for anthocyanins. Stock solutions of each standard compound were prepared by dissolving weighted amounts of each standard in 80% (v/v) methanol−water mixture and subsequently diluted with the 80% (v/v) methanol−water mixture to obtain six working solutions of each compound covering the linear concentration range with lower value close to its limit of quantification (LOQ). The limit of detection (LOD) was determined from the amount of analytes required to give a signal-to-noise ratio of 3, and the limit of quantification (LOQ), was defined as the lowest concentration giving a signal-to-noise ratio of 10. Quantification of individual compounds was performed by the external standard method using a six point regression graph of the UV−visible absorption data collected at the wavelength of maximum absorbance of each analyte reported in **Table 3**, which were determined by the PDA spectra acquired in the wavelength range 210−600 nm.

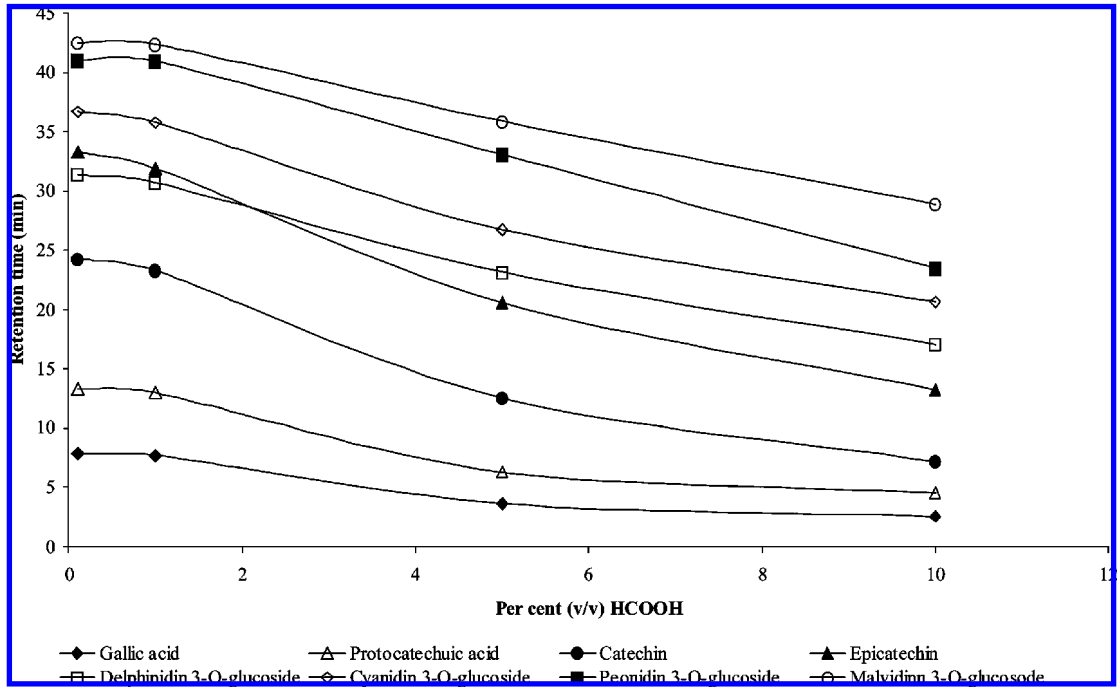

Figure 1. Dependence of retention time on the concentration of formic acid into the mobile phase consisting of the water−acetonitrile mixture. Detection by PDA at 520 nm for anthocyanins and at 280 nm for all other analytes; other conditions are as reported in Materials and Methods.

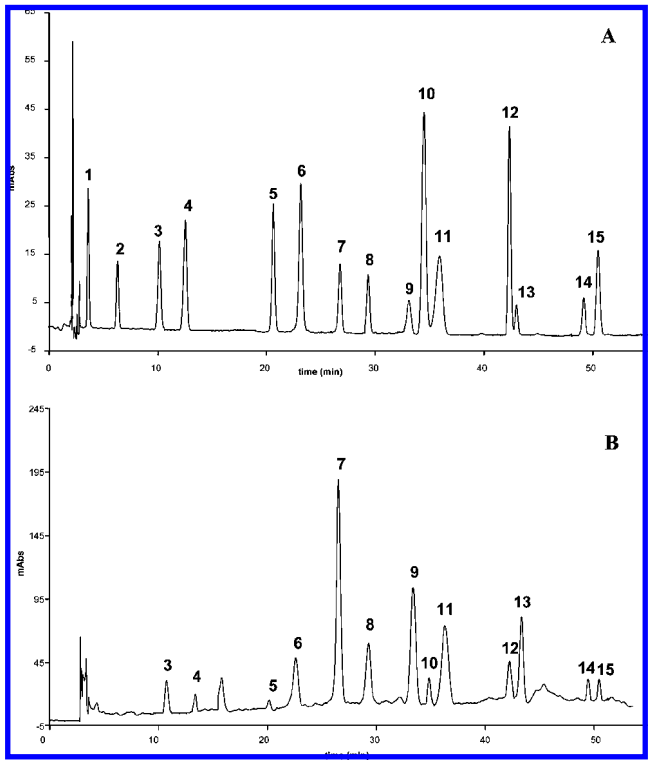

Figure 2. HPLC-PDA of a test mixture comprising phenolic acids, flavonols, catechins, stilbenes, and anthocyanins (**panel A**) and of the sample extracted from the berries of the red grape "Uva di Troia" (**panel B**). Detection by PDA at 280 nm and experimental conditions are as reported in Materials and Methods. Identification of peaks: **1**, gallic acid; **2**, protocatechuic acid; **3**, caftaric acid; **4**, catechin; **5**, epicatechin; **6**, delphinidin 3-*O*-glucoside; **7**, cyanidin 3-*O*-glucoside; **8**, petunidin 3-*O*-glucoside; **9**, peonidin 3-*O*-glucoside; **10**, *trans*-piceid; **11**, malvidin 3-*O*-glucoside; **12**, rutin; **13**, quercetin 3-*O*-glucoside; **14**, kaempferol 3-*O*-glucoside; **15**, *trans*-resveratrol.

Table 4. Recovery of Representative Phenolic Compounds in Samples of Alphonse Lavallée Grape Berries

analyte	content[a]	amount added[a]	found[a]	recovery (%)
caftaric acid	636.3	317.9[A]	939.1	95.2 ± 1.4
		635.9[B]	1304.1	105.0 ± 2.1
		953.8[C]	1558.0	96.6 ± 1.8
catechin	329.7	165.1[A]	501.7	104.2 ± 1.6
		330.0[B]	682.1	106.8 ± 1.3
		495.1[C]	776.3	90.2 ± 1.7
malvidin 3-*O*-glucoside	1838.2	919.1[A]	2742.3	98.4 ± 2.7
		1939.0[B]	3802.3	106.8 ± 2.4
		2757.3[C]	4675.5	102.9 ± 2.1
kaempferol 3-*O*-glucoside	4.2	2.0[A]	6.1	96.5 ± 1.8
		4.0[B]	8.1	96.8 ± 1.6
		6.0[C]	9.9	95.7 ± 1.5
trans-resveratrol	41.0	19.6[A]	60.1	97.6 ± 2.1
		39.2[B]	81.6	103.9 ± 1.8
		58.7[C]	102.1	104.0 ± 1.5

[a] Expressed in mg/kg dry weight. [A, B, C]Amounts corresponding to 50, 100, and 150%, respectively, of the values of the selected phenolic compounds determined in the non-spiked sample.

RESULTS AND DISCUSSION

Method Development. The influence of different factors influencing chromatographic performance and both photodiode array (PDA) and electrospray mass spectrometry (ESI-MS) detection of phenolic compounds was investigated with the purpose of optimizing the experimental conditions required to obtain the selective resolutions of phenolic compounds that accumulate in grapes, which include phenolic acids (*23*), catechins (*24*), flavonols (*13*), stilbenes (*22*), and anthocyanins (*25*). The experiments were conducted using a narrow bore C-18 column operated under gradient elution mode with the starting eluent and the gradient former consisting of water and an organic solvent, respectively, both containing a volatile acid to control the protonic equilibrium at acidic pH value, which was selected with the purpose to keep carboxyl and hydroxyl groups of the

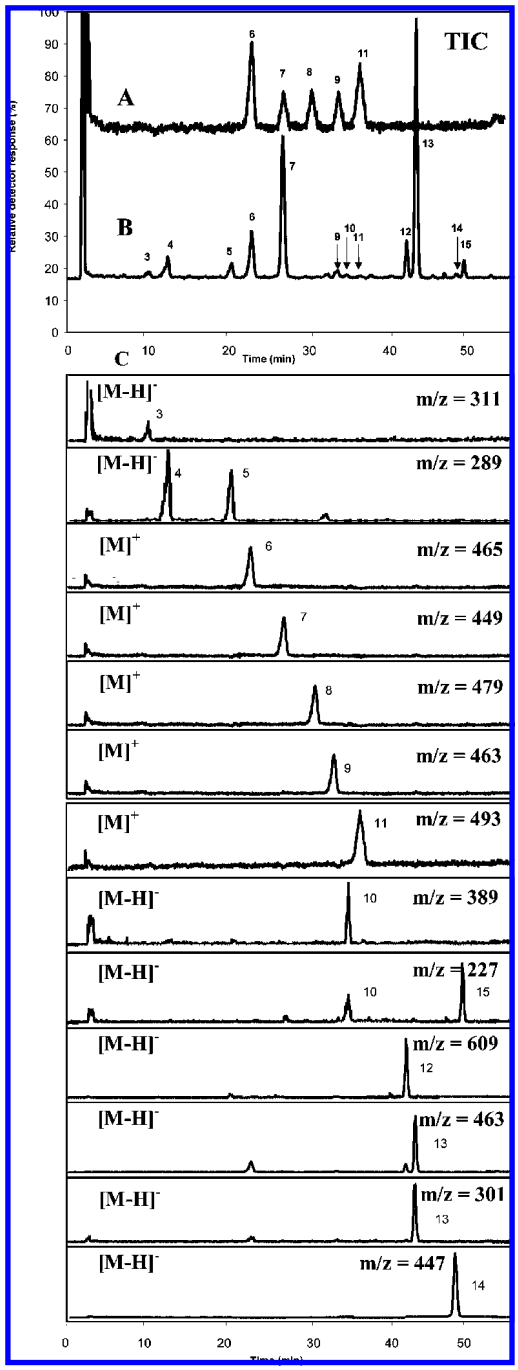

Figure 3. HPLC-ESI-MS of the sample extracted from the berries of the red grape "Uva di Troia": **Panel A**, total ion current chromatogram (TIC) acquired in positive full scan mode; **panel B**, total ion current chromatogram (TIC) acquired in negative full scan mode; **panel C**, ion chromatograms extracted at *m*/*z* values corresponding to either negative or positive ion or fragment ion of each identified phenolic compound. Peak identifications are as described in **Figure 2**.

analytes in their protonated form. This in order to avoid peak broadening caused by the simultaneous presence of protonated and ionized forms of the polyphenols and to improve their hydrophobic interactions with the C-18 stationary phase.

Acetonitrile was preferred over methanol because of its lower viscosity, which makes it more suitable than methanol in limiting the back pressure associated with the low permeability of the narrow bore HPLC column and of the capillary tube used in the electrospray interface. Formic acid was chosen to acidifying both components of the mobile phase because of its volatility

Table 5. Retention Times and Main Ions Observed in ESI-MS for Standard Phenolic Compounds

peak	analyte	retention time (min)	main observed ions[a]
1	gallic acid	3.590	169
2	protocatechuic acid	6.296	153
3	caftaric acid	10.139	311 (179)
4	catechin	12.529	289
5	epicatechin	20.615	289
6	delphinidin 3-*O*-glucoside	23.148	465
7	cyanidin 3-*O*-glucoside	26.761	449
8	petunidin 3-*O*-glucoside	29.385	479
9	peonidin 3-*O*-glucoside	33.088	463
10	*trans*-piceid	34.465	389 (227)
11	malvidin 3-*O*-glucoside	35.903	493
12	rutin	42.340	609
13	quercetin 3-*O*-glucoside	42.986	463 (301)
14	kaempferol 3-*O*-glucoside	49.164	447 (385)
15	*trans*-resveratrol	50.496	227

[a] Positive ion mode, anthocyanins; negative ion mode, all other analytes.

and ascertained compatibility with electrospray ionization of polyphenols (*26, 27*). In addition, preliminary experiments carried out with methanol as the organic modifier and with either acetic acid or ammonium acetate as the additive of either methanol or acetonitrile based mobile phases resulted in unsatisfactory separations of the selected polyphenols, representative of those potentially present in grape berries. Therefore, further experiments were carried out with the purpose of investigating the influence of the concentration of formic acid into the mobile phase on the separation performance and detection sensitivity by ESI-MS.

The influence of formic acid on the retention behavior of selected standard phenolic compounds, representative of the major phenolic compounds that accumulate in grapes, is depicted by plots of retention times as a function of the concentration of formic acid incorporated into both the aqueous and the organic component of the mobile phase, which are displayed in **Figure 1**. The retention time of almost all phenolic compounds decreased with increasing the concentration of formic acid. Besides lowering the pH and thus keeping carboxyl and hydroxyl groups of the analytes in their protonated form, formic acid is believed to interact with these functional groups via hydrogen-bond formation. Interaction with formic acid is expected to increase the virtual polarity of the polyphenols with the consequent reduction of hydrophobic interactions with the octadecyl stationary phase. As a result, the retention time of phenolic compounds decreases with increasing concentration of formic acid.

The effect of the concentration of formic acid on the performance of RP-HPLC separation was evaluated in terms of peak capacity (PC), which is defined as the upper limit of the number of peaks that can be placed within the chromatogram while retaining unit resolution for all adjacent peak pairs (*28*). After having verified that peak widths were approximately constant during the gradient time and that the contribution of dwell volume to the retention was neglected, we calculated PC using the following equation (*28*): $PC = t_\omega - t_\alpha/w_b$, where t_ω and t_α indicate the retention times of the last and the first peak, respectively, within the chromatogram, whereas w_b is the average peak width. Data reported in **Table 1** indicate that the highest PC was obtained with 5% (v/v) formic acid. Therefore, we selected this concentration of formic acid for performing further experiments even though better sensitivity in ESI-MS was obtained with mobile phases containing lower concentration of formic acid. This in accordance with the expectation that acidification suppresses deprotonation and, hence, the ionization

Table 6. Content[a] of Phenolic Compounds Determinated in Berries of Different Grape Varieties (mg/kg[b]±SD)

analyte	Primitivo	Negroamaro	Susumaniello	Uva diTroia	Malvasia Nera	Aglianico	Cesanese	Merlot	Moscato	Alphonse Lavallée
gallic acid		7.3 ± 0.2	45.0 ± 1.4		77.3 ± 0.7	151.9 ± 4.4		66.6 ± 0.7		
protocatechuic acid	13.4 ± 0.4	42.0 ± 1.1	8.5 ± 0.2		46.0 ± 0.2	37.4 ± 0.3	31.1 ± 1.0	328.7 ± 8.4		
caftaric acid	1.89 ± 0.01	8.5 ± 0.2	171.7 ± 1.5	93.3 ± 2.9	171.9 ± 4.5	320.4 ± 7.5	28.8 ± 0.7	746.3 ± 13.2	48.4 ± 0.7	645.0 ± 10.6
catechin	307.1 ± 1.5	118.2 ± 1.2	147.1 ± 0.7	127.6 ± 3.8	966.0 ± 27.0	3214.9 ± 71.2	178.8 ± 4.7	601.0 ± 2.9	21.5 ± 0.5	331.20 ± 9.8
epicatechin	49.8 ± 0.3	27.8 ± 0.7	73.5 ± 1.3	62.5 ± 1.5	734.7 ± 20.9	1890.1 ± 48.3	8.9 ± 0.3	980.7 ± 32.0	4.1 ± 0.1	32.4 ± 0.4
delphinidin 3-*O*-glucoside	44.7 ± 0.3	112.8 ± 1.0	764.0 ± 37.0	309.2 ± 8.7	586.6 ± 16.3	594.7 ± 16.1	1270.4 ± 31.3	974.5 ± 26.7		219.8 ± 3.1
cyanidin 3-*O*-glucoside	14.7 ± 0.4	50.6 ± 1.5	123.3 ± 3.1	443.2 ± 13.5	67.3 ± 2.0	52.1 ± 1.4	365.0 ± 5.6	25.93 ± 0.02		46.4 ± 0.7
petunidin-3-O-glucoside	168.4 ± 4.3	203.6 ± 4.5	881.5 ± 16.7	354.9 ± 8.9	625.3 ± 11.6	596.5 ± 11.0	1329.2 ± 30.5	534.4 ± 22.9		241.1 ± 6.2
peonidin 3-*O*-glucoside	207.7 ± 5.7	110.2 ± 1.5	873.0 ± 28.0	557.6 ± 14.9	827.5 ± 20.7	553.6 ± 12.9	1333.5 ± 38.1	580.2 ± 4.2		52.5 ± 16.2
trans-piceid	30.7 ± 0.9	4.14 ± 0.02	150.3 ± 4.6	15.3 ± 0.5	98.0 ± 2.0	75.7 ± 1.9	12.05 ± 0.02	26.3 ± 0.43		24.1 ± 0.5
malvidin 3-*O*-glucoside	1883.0 ± 58.0	662.6 ± 4.4	4131.0 ± 119.0	602.2 ± 15.9	3396.0 ± 47.6	3348.0 ± 96.0	2771.2 ± 83.9	12180.0 ± 159.7		1851.1 ± 24.4
rutin	6.2 ± 0.1	1.39 ± 0.03	10.6 ± 0.3	41.4 ± 1.2		6.1 ± −0.2	54.7 ± 1.7	30.2 ± 0.9	25.2 ± 0.3	
quercetin 3-*O*-glucoside	6.3 ± 0.2	1.03 ± 0.02	7.6 ± 0.2	15.0 ± 0.4	9.0 ± 0.3	6.7 ± 0.2	2.87 ± 0.09	45.0 ± 1.1	136.5 ± 2.2	4.1 ± 0.1
kaempferol 3-*O*-glucoside	33.9 ± 1.0	32.0 ± 0.7	22.0 ± 0.5	45.8 ± 1.4	31.2 ± 0.9	20.9 ± 0.5	40.7 ± 1.1	97.7 ± 24.6	101.0 ± 1.1	4.3 ± 0.10
trans-resveratrol	13.9 ± 0.4	3.6 ± 0.1	63.0 ± 0.9	4.60 ± 0.06	48.5 ± 1.0	61.1 ± 0.8	8.16 ± 0.02	9.2 ± 0.2	3.89 ± 0.01	40.0 ± 0.60

[a] Average value of triplicate analysis. [b] Dry weight.

efficiency of the electrospray ion source, with consequent reduction of the analyte signal (*29*).

The use of the water−acetonitrile mobile phase containing 5.0% (v/v) formic acid and proper selection of a multisegment gradient of increasing concentration of acetonitrile (see gradient in Materials and Methods) determined the concomitant resolution, in less than 55 min, of the phenolic compounds that were successively identified in the grape berries of the investigated samples. A representative separation of a standard mixture of these compounds comprising phenolic acids, flavonols, catechins, stilbenes, and anthocyanins, obtained by the developed method is shown in **Figure 2A**.

Identification of Polyphenols in Grape Berries and Method Validation. The optimized method was validated in terms of precision, linearity, and accuracy and subsequently employed for identification and quantification of phenolic compounds in grape berries of selected varieties of *Vitis vinifera*. The identification of the selected compounds in the samples extracted from grape berries of 10 varieties of *Vitis vinifera* was performed by the developed RP-HPLC method on the basis of their retention times, and UV−visible spectra acquired in the wavelength range comprised between 210 and 600 nm with the photodiode array (PDA) detector. The identification of each peak was confirmed by ESI-MS detection in the single ion monitoring mode, which resulted in the appearance of signals at *m*/*z* corresponding to the main ion of the searched compounds corresponding to the peaks of the PDA chromatogram. With experimental parameters of ESI-MS reported in Materials and Methods, almost all phenolic compounds produced mass spectra with the base peak corresponding to the so-called molecular ion.

The precision of the method was evaluated in terms of interday and intraday repeatability of both retention time and peak area for all phenolic compounds considered in the study, which were analyzed by the proposed method in quintuplicate during the same day and over three consecutive days. The results of this study are reported in **Table 2** as mean values, standard deviation, and relative standard deviation of retention times and peak area. It is shown that the interday and intraday repeatability of the retention times resulted in being better than 0.84 and 1.56%, respectively, whereas interday and intraday repeatability of peak area was better than 1.96 and 2.90%, respectively.

Linearity was evaluated on the basis of six point calibration graphs that were constructed by plotting the concentration of standard sample in mg/L as a function of peak area, for each of the six different concentrations, measured by the PDA detector at the wavelength corresponding to the maximum absorbance of each analyte. Linear least-squares regression analysis was employed to calculate slope, intercept, and correlation coefficient of the calibration graphs that are reported in **Table 3**, which also reports for each analyte the range of linearity, LOD, and LOQ. For all phenolic compounds, the correlation coefficient of the calibration graph was better then 0.9988, indicating good linearity.

The accuracy of the method was evaluated by a recovery study, which was carried out according to the following procedure. Known amounts of selected phenolic compounds, representative of the phenolic acids, flavonols, catechins, stilbenes, and anthocyanins that were identified in the sample of *Vitis vinifera* Alphonse Lavallée, were added to lyophilized berries of this grape variety, and the resulting spiked samples were subjected to the entire analytical method in parallel to a nonspiked sample of the same berries. Three different amounts of caftaric acid, catechin, kaempferol-3-*O*-glucoside, malvidin-3-*O*-glucoside, and *trans*-resveratrol were added to the samples, corresponding to 50, 100, and 150% of the values of the selected phenolic compounds determined in the nonspiked sample. All samples were injected three times, and an average of the response area was the basis for the found concentrations. The recoveries were calculated on the basis of the difference between the total concentration determined in the spiked samples and the concentration observed in the nonspiked samples. Results with the relative standard deviations are reported in **Table 4**. It can be seen that the recoveries were between 90.2 and 106.8%, indicating that the method has an adequate degree of accuracy.

Occurrence of Phenolic Compounds in Grape Berries. Using the developed method, up to 15 phenolic compounds comprising phenolic acids, flavonols, catechins, stilbenes, and anthocyanins were simultaneously separated and identified in the extracts of whole berries of 10 different varieties of *Vitis vinifera*. Peak identification was performed according to the method described in the Materials and Methods section. The separation of the components of the sample extracted from the berries of the red grape "Uva di Troia" obtained by HPLC with PDA detection is reported in **panel B** of **Figure 2**. The same separation detected by ESI-MS in negative and positive full scan mode is depicted by the total ion current (TIC) chromatograms reported in **panels A** and **B** of **Figure 3**. The identification of the peaks detected under full-scan conditions was obtained by analyzing the extracted-ion chromatograms of the ion current at *m*/*z* values corresponding to the ions of the individual

investigated compounds (see **Table 5**). Under the selected experimental conditions, fragmentation of the investigated phenolic compounds was very limited, and therefore, for almost all analytes, the main observed ion coincided with the so-called molecular ion. Exceptions to this general trend were observed for glycosilated flavonoids and stilbenes, which underwent fragmentation, giving rise to a product ion corresponding to their aglycone. **Panel C** of **Figure 3** clearly shows that acquiring the extracted-ion chromatogram of the ion current at *m/z* 389, corresponding to the $[M - H]^-$ ion, evidenced the presence of a peak at 34.46 min attributable to *trans*-piceid, the monoglycosylated form of resveratrol (MW 390). The identity of this peak was further investigated by acquiring the extracted-ion chromatogram of the ion current at *m/z* 227, corresponding to the $[M - H - 162]^-$ ion resulting from the loss of a 162 mass fragment, equivalent to the hexose group of the glycosilated *trans*-resveratrol. This signal appeared in correspondence to the peak acquired by SIM detection mode of the ion current at *m/z* 389, confirming its attribution to *trans*-piceid. Similarly, in the case of quercetin 3-*O*-glucoside, the SIM detection revealed two peaks at 42.98 min, corresponding to the retention time of this flavonol. One was detected acquiring the extracted-ion chromatogram at *m/z* 463, corresponding to the $[M - H]^-$ ion, the other at *m/z* 301, corresponding to the fragment ion $[M - H - 162]^-$ ion, due to the loss of glucose.

The identity of the anthocyanins delphinidin 3-*O*-glucoside, cyanidin 3-*O*-glucoside, petunidin 3-*O*-glucoside, peonidin 3-*O*-glucoside, and malvidin 3-*O*-glucoside was detected by ESI-MS in positive ionization mode.

The results of the quantification of the identified phenolic compounds in the whole berries of nine red and one white grape varieties are reported in **Table 6**. Considerable differences in the content of phenolic compounds were detected among varieties, including those cultivated in the same region. Similar findings are widely reported in the literature and have been related to the variety of grape, environmental and pedoclimatic factors, and in the different management of the vineyard (*30*−*32*). As expected, anthocyanins were determined only in red grape berries, and their content varied significantly with the cultivar. In accordance with previous findings (*33*), almost in all samples the most and least abundant anthocyanins were malvidin 3-*O*-glucoside and cyanidin 3-*O*-glucoside, respectively. This seems to be in accordance with the observation that cyanidin and malvidin are the precursor and terminal anthocyanidin in the biosynthesis of anthocyanins (*34*) and that the anthocyanin composition is closely linked to genetic heritance of the cultivar (*25*).

An exception to this general trend was observed in "Uva di Troia", where the least abundant anthocyanin was delphinidin 3-*O*-glucoside. It should be noted that in this grape the content of cyanidin 3-*O*-glucoside was relatively high, being 443.2 ± 13.5 mg/kg (dry weight), whereas the content of malvidin 3-*O*-glucoside was 602.2 ± 15.9 mg/kg (dry weight) and that of the other anthocyanins was comprised between this value and 309.2 ± 8.7 mg/kg (dry weight), which was the content of delfinidin-3-*O*-glucoside. As reported in the literature, such a relative high content of cyanidin 3-*O*-glucoside could be ascribed to a lower activity of the enzymes flavonoid-3,5-hydrolase that regulate the formation of the trihydroxylated anthocianins, with consequent increasing of the amount of cyanidine 3-*O*-glucoside and decrease of malvidin 3-*O*-glucoside (*25*).

Differences among varieties were also noted in the order of abundance of anthocyanins. The order of abundance malvidin 3-*O*-glucoside > peonidin 3-*O*-glucoside > petunidin 3-*O*-glucoside > delphinidin 3-*O*-glucoside > cyanidin 3-*O*-glucoside was monitored in Primitivo, Malvasia Nera, and Cesanese grapes, as was also observed in berry skins of the above varieties by Mattivi et al. in a recent study on the metabolite profiling of grapes (*35*). It should be noted that in another paper reporting the content of anthocyanins in the berry skins of red grapes grown in southern Italy, the order of abundance of anthocyanins in Primitivo was malvidin 3-*O*-glucoside > petunidin 3-*O*-glucoside > delphinidin 3-*O*-glucoside > peonidin 3-*O*-glucoside > cyanidin 3-*O*-glucoside (*31*), which is the order of abundance that we detected in Aglianico and Negroamaro, as was also monitored by Mattivi et al. in the berry skins of these grape varieties. However, the order of abundance of anthocyanins that we observed in the Merlot grape cultivated in the region Lazio overlapped that detected by Mattivi et al. for the same variety of grape, whereas it was different from those monitored by Ortega-Regules et al. (*25*) and by Kallithraka et al. (*36*) in Merlot grape cultivated in Spain and in Greece, respectively. Such observations are in agreement with the expectation that total content and relative abundance of anthocyanins may vary not only among varieties, but also with location of cultivation and growing seasons.

As expected, only glycosilated flavonols were quantified, and the total amount of these compounds in the whole berries of red grapes was higher than that detected in the white grape Moscato, where the most abundant phenolic compound was quercetin 3-*O*-glucoside, as observed by Rodriguez Moltelegre et al. in the skins of the white grape Gewürztraminer (*30*). Also expected is that in wine grapes the glycosilated stilbene *trans*-piceid was always found at higher concentration than its aglycone *trans*-resveratrol.

In conclusion, the results of our study confirm that RP-HPLC on a semimicro separation scale is an effective tool for the simultaneous separation, identification, and quantification of phenolic compounds, comprising phenolic acids, flavonols, catechins, stilbenes, and anthocyanins. The use of a narrow-bore column and of a semimicro detection cell in the PDA detector allow efficient separations, sensitive spectrophotometric detection, and the low values of the mobile phase flow rate requested by the ESI interface. Additional advantages of using a narrow bore reversed phase column lie in the reduced sample size and in the low consumption of eluent and, consequently, of organic solvents, with beneficial effects on the environment and on the costs of their purchase and disposal, which are drastically reduced.

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