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Direct determination of phenolic compounds in Sicilian wines by liquid chromatography with PDA and MS detection

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Abstract

Phenolic compounds in Sicilian wines were directly detected using an HPLC with a PDA detector coupled on-line with a MS system equipped with Electrospray Ionisation (ESI) source operated in the negative-ion mode and a quadrupole mass analyzer. In this work, MS spectra were recorded at different voltage, to obtain structural elucidations in addition to molecular mass informations. The different response of the compounds identified has been also evaluated. MS characteristics of *cis*- and *trans*-piceid were determined on the basis of the response obtained with the ESI interface.

The method allowed both the identification and determination of 24 phenolic compounds in 22 different commercial Sicilian red wines by direct injection, without any prior purification of the sample. The data on the levels of all the phenolic compounds in the red Sicilian commercial wines showed that the wine samples from *Merlot* grapes generally had the highest phenolic compounds content.
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1. Introduction

For a number of years, interest has been focused on the chemical composition of the wines because epidemiological studies have shown that coronary heart diseases are less prevalent in populations consuming moderate and regular amounts of wine (Friedman & Kimbal, 1986; Klautsky & Armstrong, 1993; St-Leger, Cochrane, & Moore, 1979). It has been hypothesized that the phenolic substances of wine might be responsible for these potential beneficial effects by their antioxidant and anti-inflammatory properties (Frankel, Kanner, German, Parks, & Kinsella, 1993; Teissedre, Frankel, Waterhouse, Peleg, & German, 1996). Moreover, wine phenolic compounds have been reported to have anticarcinogenic properties, delaying tumour onset in transgenic mice (Clifford et al., 1996).

The phenolic compounds are secondary plant metabolites that are contained within the skin, seed, and flesh of grapes and are extracted into wines (especially red) during the process of vinification. The types and concentrations of these compounds may depend on a number of factors: grape variety and ripening stage, soil and climatic conditions, vine cultivation and the treatment to which it is subjected (Viñas, LòpezErroz, Marin-Hernàndez, & Hernàndez-Cordoba, 2000).

Some recent studies have determined phenolic compounds in wine principally using liquid chromatographic techniques with UV or PDA detection (Golberg et al., 1996; Revilla & Ryan, 2000; Rodrıguez-Delgado,

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Malovanà, Pérez, Borges, & Garcìa-Montelongo, 2001; Viñas et al., 2000). However, UV–Vis spectra of flavan-3-ols, flavonoids, non-flavonoids and their derivatives are very similar, and often the possibility of unambiguous identification does not exist.

Recently the HPLC–MS technique has increased its popularity, mainly linked to the development of MS interface technology. Methods HPLC–MS for the analysis of phenolic compounds have been published (Ángeles Pozo-Bayòn, Hernàndez, Martın-Álvarez, & Polo, 2003; Vanhoenacker, De Villiers, Lazou, De Keukeleire, & Sandra, 2001). These methods utilized an enrichment procedure, based on extraction and concentration from the red wines, which does not furnish an extract content of all the phenolic compounds present in wine. For the purpose of estimate the correct phenolic compounds composition of the wine, we have developed an HPLC method with a photo-diode array (PDA) coupled online with a MS system equipped with Electrospray Ionisation (ESI) source operated in the negative-ion mode and a quadrupole mass analyser, for quantification of benzoic acids, cinnamic acids, flavonoids and stilbenes. We have included the MS detection in addition to PDA detection to exclude the possibility of interference and to be able to verify the glycosidic structure. In the present study, separation of 24 phenolic compounds was optimised; the method permitted both the identification and determination of the phenolic compounds in different type of commercial Sicilian red wine by direct injection, without any prior purification of the sample.

2. Materials and methods

2.1. Chemicals and materials

Acetonitrile and water were solvent HPLC grade, obtained from Carlo Erba.

Formic acid, (−)-epicatechin, (+)-catechin, gallic acid, 3,4-dihydroxybenzoic acid (protocatechuic acid), 4-hydroxy-3-methoxybenzoic acid (vanillic acid), 4-hydroxy-3,5-dimethoxybenzoic acid (syringic acid), 3,4-dihydroxycinnamic acid (caffeic acid), 4-hydroxy-3-methoxycinnamic acid (ferulic acid), 4-hydroxycinnamic acid (*p*-coumaric acid), tyrosol (2-(4-hydroxyphenil) ethylalcohol) and *trans*-resveratrol were purchased from Sigma–Aldrich.

The other phenolic compounds were procyanidin B1, procyanidin B2, ethylgallate, quercetin, isoquercitrin (quercetin-3-*O*-glucoside), kaempferol, kaempferol-3-*O*-glucoside, rhamnetin, isorhamnetin, isorhamnetin-3-*O*-glucoside, rutin (quercetin-3-*O*-rutinoside), and myricetin and were supplied by Extrasynthese.

The individual standards were dissolved in aqueous formic acid (pH 3)/methanol (90:10), and injected to determine individual retention times. The stock solutions of the individual standards were prepared by dissolving 100 mg standard into 100 ml aqueous formic acid (pH 3)/methanol (90:10). The stock solutions of the standards were diluted so to obtain five different diluted standards. They cover a range of concentration between: 1 and 50 mg/L for (+)-catechin and gallic acid; 1 and 30 mg/L for (−)-epicatechin, tyrosol, procyanidin B1, procyanidin B2, ethylgallate, quercetin, isoquercitrin, rutin and myricetin; 1 and 10 mg/L for vanillic acid, syringic acid, caffeic acid, ferulic acid, *p*-coumaric acid, *trans*-resveratrol, kaempferol, kaempferol-3-*O*-glucoside, rhamnetin, isorhamnetin, and isorhamnetin-3-*O*-glucoside. The five different diluted standards obtained from stock solutions were injected for linearity range and detection limit tests.

All the solutions were stored at −4 °C and protected from light.

Stock solution of *cis*-resveratrol was produced by UV irradiation of *trans*-resveratrol in methanol for 120 min at 366 nm (Trela & Waterhouse, 1996) since its commercial standard is not available. For the calibration of the *cis*-resveratrol, we have obtained a standard curve with five known amounts of the *trans*-resveratrol irradiated, as it is described by Romero-Pérez (Romero-Pérez, Lamuela-Raventòs, Waterhouse, & de la TorreBoronat, 1996).

All solutions were filtered through a 0.45-μm glassmicrofiber GMF Whatman chromatographic filter, before the HPLC analysis, and the mobile phase solvents were degassed before use.

2.2. Wine samples

A group of commercially available red wines were analysed. The wines selected were from the 2002 vintage and were produced according to standard procedures. All wines were stored in the dark at 4 °C, and each one was opened immediately before the analysis. Analyses were carried out without any prior purification of the sample; an aliquot (1 ml) was filtered through a 0.45-μm glassmicrofiber GMF Whatman chromatographic filter, before the HPLC analysis.

In all cases, analyses were performed in triplicate and the values were averaged. The standard deviation (SD) was also calculated. The statistical analysis was performed using SPSS 11.5 for Windows Software.

2.3. Instrumentation and condition

Analyses were performed on a Shimadzu system equipped with two pumps LC10-AD, a controller SCL-10A, and a photodiode array detector SPD-M10Avp equipped with a semimicro-cell and operating at wavelengths between 200 and 600 nm.

The system was coupled to a MS detector Shimadzu 2010 equipped with an ESI interface. UV and MS data were acquired and processed using operating system Windows NT 4.0.

Compounds were separated on a 150 mm × 2.1 mm, 5 μm particle size, Supelco Discovery C_{18} column; a Supelco guard column packed with the same stationary phase was also used.

The mobile phase was a gradient prepared from formic acid in water (pH 3, solvent A) and formic acid in acetonitrile (pH 3, solvent B): 0.01–20.00 min 5% B isocratic; 20.01–50.00 min, 5–40% B; 50.01–55.00 min, 40–95% B; 55.01–60.00 min 95% B isocratic. The gradient was reduced to initial condition in another 5 min; 10 min of equilibration was required before the next injection. The flow rate was 0.2 ml/min and the analyses were performed at 20 °C. Samples of 5 μm of wine were directly injected into the column using a Rheodyne (model 7725i) injection valve.

ESI source and negative ionisation mode was used with different fragment voltages. Nitrogen was used as the nebulizing and drying gas. The MS acquisition with the ESI interface was performed under the following condition: probe high voltage, 4 kV; nebulizing gas (N_2) flow rate, 4.5 L/min; curved desolvation line (CDL) voltage, 10 V; CDL temperature, 250 °C; acquisition mode, SCAN, 50–700 *m/z* and deflector voltage at −20 and −80 V.

The SIM (selected ion monitoring) mode was used when a search for some particular ions should be done.

3. Results and discussion

3.1. Optimisation of the phenolic compounds separation using photodiode array detection

In the present study, we have included analysis of the compounds listed in Table 1. The chromatographic conditions were first optimised by use of a standard mixture of phenolic compounds, to ensure that the compounds of interest were well resolved. Several experiments (various elution phase and corresponding chromatographic gradient) were carried out to achieve an appropriate resolution, as well as a better signal in the mass detector. This is because the composition of the phases in which the compounds of interest are eluted considerably affects the yield obtained in the transference of the analyte from the liquid phase to the mass spectrometer. The optimum conditions reached are those described in the preceding section.

To improve the peak shape, the retention behaviour of phenolic compounds was studied in presence of formic acid (Pèrez-Margariño, Revilla, Gonzàlez-SanJosé, & Beltràn, 1999). The formic acid concentration was optimised to improve the MS spectrometer sensitivity.

It was found that, when organic solvent such as methanol was used in preparation of stock solutions of the standards, the resolution of gallic, protocatechuic and vanillic acid were decreased. Moreover, it was observed that if methanol is about 10% v/V, the peak shape improved although the t_R values were slightly reduced.

Table 1
Retention time (t_R); absorbance maxima (λ); limits of detection (LOD; S/N = 3); calibration curve ($y = ax + b$); correlation coefficient (R^2); repeatability (RSD) of phenolic compounds

Compound (peak number)	$t_R \pm$ SD (min)	λ (nm)	LOD (mg/L)	*a*	*b*	R^2	RSD
Gallic acid (1)	6.60 ± 0.06	270	0.014	54,021	−5800.1	0.9996	1.3
Protocatechuic acid (2)	14.08 ± 0.14	258	0.018	87,788	−15,028	0.9995	1.7
Tyrosol (3)	27.90 ± 0.18	275	0.16	10,538	−419.28	0.9995	3.5
Vanillic acid (4)	31.95 ± 0.09	259	0.011	74,658	4807.2	0.9999	3.5
Procyanidin B1 (5)	32.27 ± 0.11	277	0.068	14,532	516.42	0.9994	1.8
(+)-Catechin (6)	33.60 ± 0.12	278	0.051	20,822	765.06	0.9987	0.9
Caffeic acid (7)	34.43 ± 0.16	323	0.006	133,408	−15,446	0.9996	3.0
Syringic acid (8)	34.83 ± 0.14	274	0.003	87,131	2492.6	0.9997	3.6
Procyanidin B2 (9)	35.43 ± 0.19	277	0.043	23,243	−607.91	0.9996	1.5
(−)-Epicatechin (10)	36.63 ± 0.12	278	0.055	13,128	466.80	0.9998	0.7
Ethylgallate (11)	37.89 ± 0.18	271	0.004	69,442	−6354.1	0.9990	1.1
Ferulic acid (12)	39.95 ± 0.12	323	0.004	166,249	44,074	0.9994	1.5
Rutin (13)	40.16 ± 0.11	354	0.003	37,056	−1088.1	0.9981	2.1
Isoquercitrin (14)	40.93 ± 0.11	354	0.003	44,461	10,063	0.9982	0.9
Kaempferol-3-*O*-glucoside (15)	42.64 ± 0.18	346	0.005	23,119	3389.5	0.9983	0.9
Isorhamnetin-3-*O*-glucoside (16)	42.87 ± 0.18	354	0.002	53,494	171,88	0.9970	1.0
p-Coumaric acid (17)	43.54 ± 0.18	276	0.003	223,020	−4189.6	0.9989	2.1
Myricetin (18)	44.49 ± 0.10	371	0.090	41,489	10,960	0.9891	0.8
trans-Resveratrol (19)	46.09 ± 0.10	305	0.001	289,989	130,93	0.9982	2.0
Quercetin (20)	48.60 ± 0.12	369	0.003	91,942	31,673	0.9955	1.4
cis-Resveratrol (21)	49.55 ± 0.13	285	0.004	258,953	12,006	0.9906	7.6
Kaempferol (22)	52.46 ± 0.13	365	0.002	280,334	−5801.2	0.9993	2.2
Isorhamnetin (23)	52.79 ± 0.11	369	0.002	250,036	−7106	0.9987	1.8
Rhamnetin (24)	55.57 ± 0.07	370	0.002	211,855	−15,774	0.9936	2.8

Considering that alcoholic content in commercially wines is between 8 and 14% v/V and that pH of this complex mixture of natural products is about 3.5, the stock solutions of the individual standards were prepared by dissolving standard into aqueous formic acid (pH 3)/methanol (90:10).

Calibration graphs were performed by plotting concentration (mg/L) against peak area. Table 1 shows the data obtained for the calibration graphs and the regression coefficients of 24 standard samples. The precision of the method was demonstrated by repetitive analyses, calculating the average relative standard deviation (RSD) for 5 replicate determinations of a solution containing each standard at the concentration of 1 mg/L. The limit of detection (LOD, S/N = 3) of individual compounds was calculated at the wavelength corresponding to their maximum absorbance. Values are also given in Table 1.

As shown in Fig. 1, the separation of a standard mixture of 24 phenolic compounds can be achieved in 60 min. Table 1 lists the retention times of individual phenolic compounds. Considering the UV spectra acquired at 278 nm (where all the phenolic compounds were contemporary detectable, Fig. 2), is evident that peak 12 (ferulic acid) was closed to peak 13 (rutin). Nevertheless, this was not a real problem because the identification and quantification of these two compounds was performed at different wavelength (maximum absorbance of each compound). However, some compounds such as kaempferol (peak 22, t_R = 51.80 min) and isorhamnetin (peak 23, t_R = 52.16 min), and their respective 3-*O*-glucosidic forms, are eluted with comparable retention time, and thus it is difficult to identify and quantify them by simply using retention time and UV spectrum.

In this work, HPLC–MS was used to obtain the spectrum of individual compounds, which was used for further confirm the peaks in samples.

3.2. Choice of the MS interface and optimisation of the conditions

Many class of compounds can be analysed by both APCI and ESI; however, APCI is suitable for medium-polar and lower molecular mass compounds, while

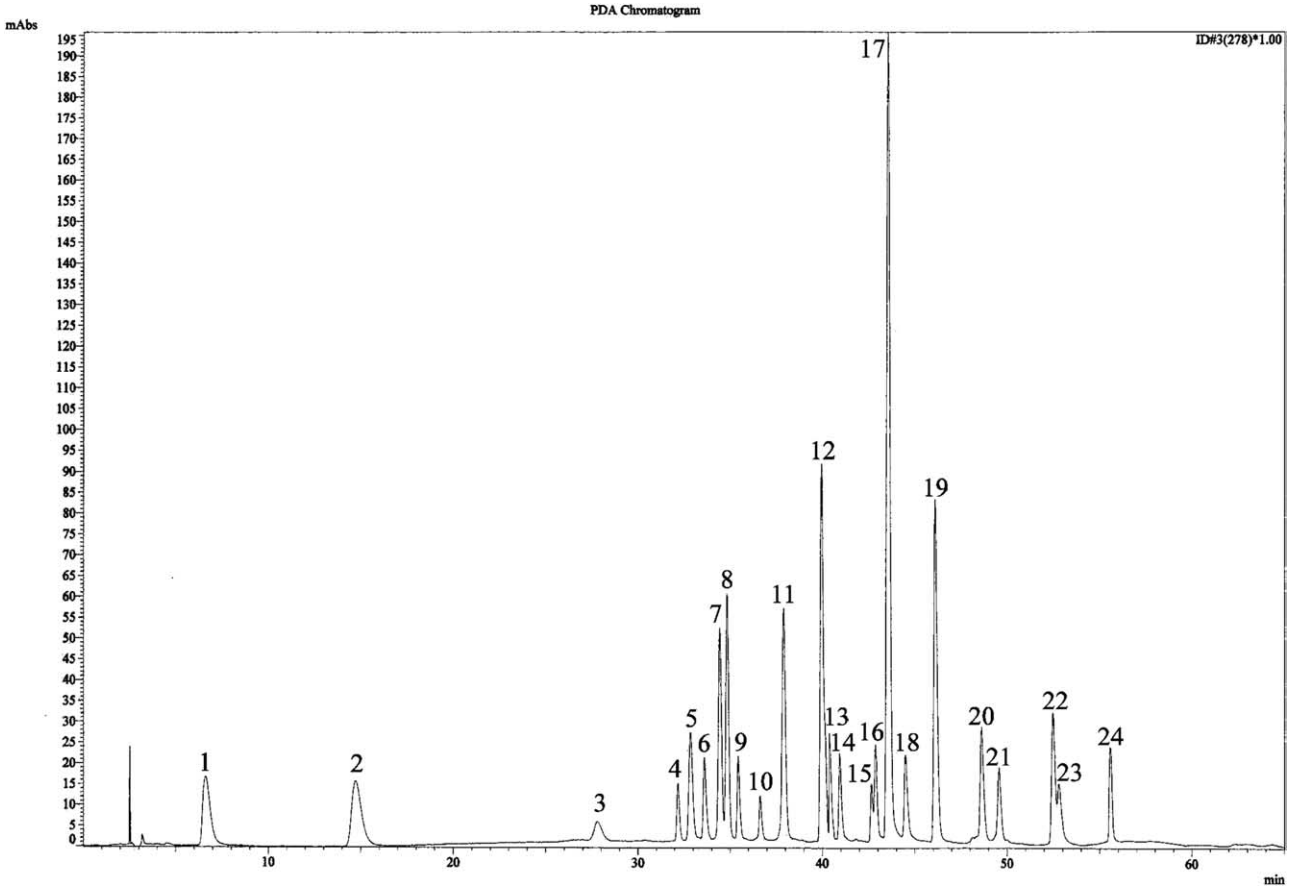

Fig. 1. HPLC–PDA of the mixture of standards. Detection at 278 nm. Peak identification: 1, gallic acid; 2, protocatechuic acid; 3, tyrosol; 4, vanillic acid; 5, procyanidin B1; 6, (+)-catechin; 7, caffeic acid; 8, syringic acid; 9, procyanidin B2; 10, (−)-epicatechin; 11, ethylgallate; 12, ferulic acid; 13, rutin; 14, isoquercitrin; 15, kaempferol-3-*O*-glucoside; 16, isorhamnetin-3-*O*-glucoside; 17, *p*-coumaric acid; 18, myricetin; 19, *trans*-resveratrol; 20, quercetin; 21, *cis*-resveratrol; 22, kaempferol; 23, isorhamnetin; 24, rhamnetin.

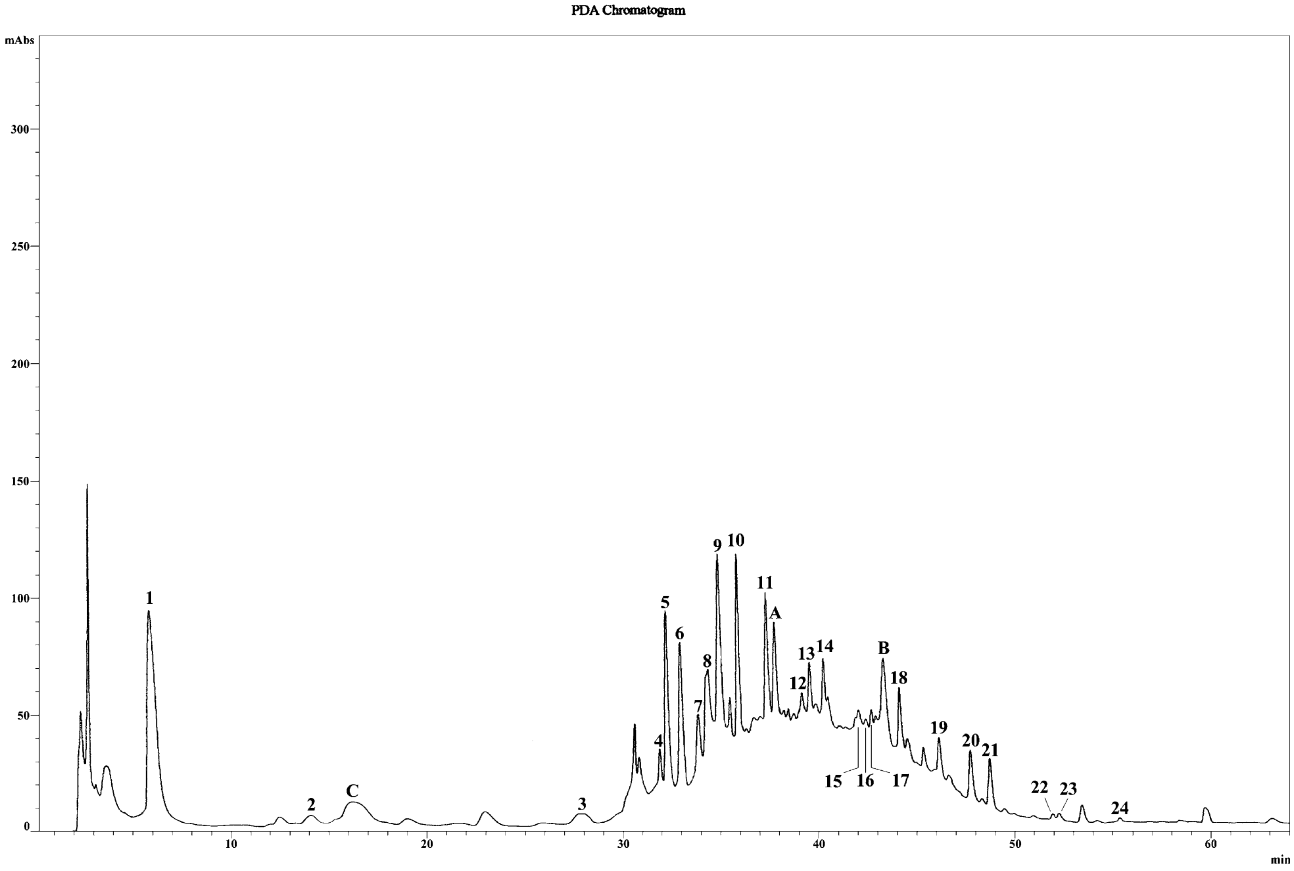

Fig. 2. HPLC–PDA chromatogram of a wine sample detected at 278 nm. Peak identification: 1, gallic acid; 2, protocatechuic acid; 3, tyrosol; 4, vanillic acid; 5, procyanidin B1; 6, (+)-catechin; 7, caffeic acid; 8, syringic acid; 9, procyanidin B2; 10, (−)-epicatechin; 11, ethylgallate; 12, ferulic acid; 13, rutin; 14, isoquercitrin; 15, kaempferol-3-*O*-glucoside; 16, isorhamnetin-3-*O*-glucoside; 17, *p*-coumaric acid; 18, myricetin; 19, *trans*-resveratrol; 20, quercetin; 21, *cis*-resveratrol; 22, kaempferol; 23, isorhamnetin; 24, rhamnetin; A, *trans*-piceid; B, *cis*-piceid; C, caftaric acid.

ESI coupled with HPLC provide to be the method of choice for the analysis of polar compounds and higher molecular mass than APCI.

To compare the two system of detection and to check whether one of them shows any systematic error, the value obtained by both system for a mixture of standards were analysed. Preliminary experiments showed that, in the case of low molecular mass phenolic compounds, APCI method exhibited a lower sensitivity, both with the positive- and negative-ion mode. At the same time, the results obtained in ESI technique were better and proved to be more sensitive and with less background noise than APCI, especially in negative-ion mode. Pèrez-Margariño and co-workers (Pèrez-Margariño et al., 1999) also observed this phenomenon. Furthermore, resveratrol, flavonoids, catechin, epicatechin, procyanidins and ethyl gallate were also studied using the two ion sources (APCI and ESI) and the two ionization modes (positive and negative). The results evidenced that these compound ionised in both positive and negative ion APCI and ESI. However, as in the case of low molecular mass phenolic compounds, ESI^- was a particularly effective technique for the analysis of these compounds. For this reason, since these types of compounds are easily deprotonated, further experiments were carried out using the ESI source in negative mode. Analyses were carried out at different deflector voltages to obtain different degrees of fragmentation, and, as a consequence, different structural information.

3.3. Identification of the phenolic compounds

Fig. 2 shows the chromatogram profile (at 278 nm) obtained for the determination of the phenolic compounds in a Sicilian wine by direct injection. As can be seen, 24 components were detected and each peak was above S/N. Obviously, at this wavelength, not all the compounds were perfectly separated; indeed, for the analytic determination we recorded the chromatograms at the wavelength corresponding to the maximum absorbance of each phenolic compound. To confirm identification of the peaks we carried out SIM detection; selecting ion chromatogram at *m*/*z* values corresponding to molecular mass of the phenolic compounds, signal appeared in correspondence to the peaks of the PDA chromatogram (Fig. 3(a) and (b)). To perform the

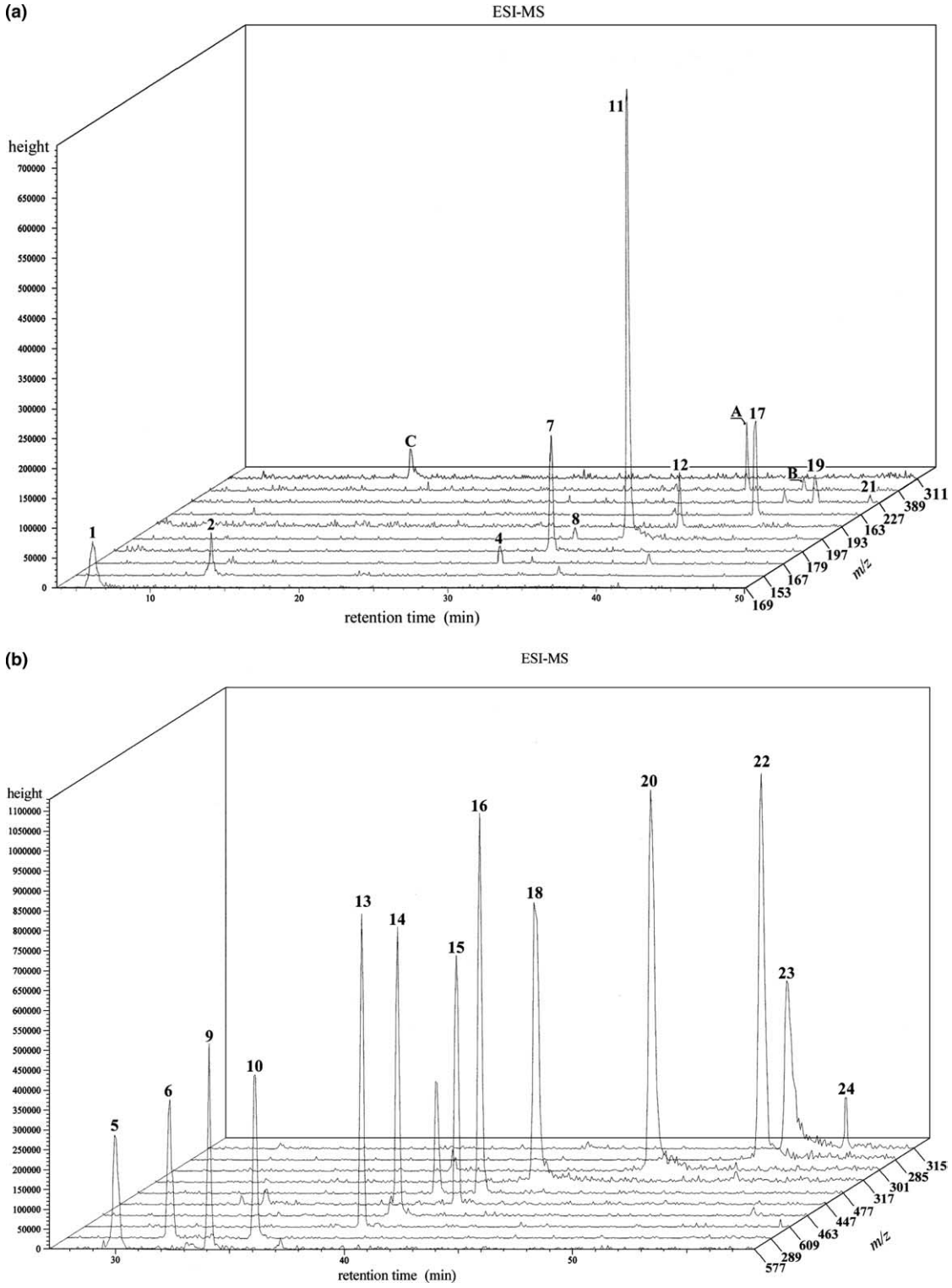

Fig. 3. (a) Ion chromatograms (acquired at −20 V) extracted at *m*/*z* corresponding to the molecular weight of the identified phenolic compounds. Peak identification: 1, gallic acid; 2, protocatechuic acid; 4, vanillic acid; 7, caffeic acid; 8, syringic acid; 11, ethylgallate; 12, ferulic acid; 17, *p*-coumaric acid; 19, *trans*-resveratrol; 21, *cis*-resveratrol; A, *trans*-piceid; B, *cis*-piceid; C, caftaric acid. (b) Ion chromatograms (acquired at −20 V) extracted at *m*/*z* corresponding to the molecular weight of the identified phenolic compounds. Peak identification: 5, procyanidin B1; 6, (+)-catechin; 9, procyanidin B2; 10, (−)-epicatechin; 13, rutin; 14, isoquercitrin; 15, kaempferol-3-*O*-glucoside; 16, isorhamnetin-3-*O*-glucoside; 18, myricetin; 20, quercetin; 22, kaempferol; 23, isorhamnetin; 24, rhamnetin.

HPLC–ESI/MS analysis under MS condition that allowed the detection of the molecular ion for each phenolic compound and to produce any fragmentation suitable for the detection of the corresponding ions, the ESI/MS analysis were performed in negative ionisation modes with two different fragment voltages [deflector voltage, −20 and −80 V].

All compounds were identified by means of HPLC–PDA and HPLC–MS analyses.

Table 2 reports the list of the major ions observed at −20 and −80 V for each compound. The obtained data provided evidence that all the identified phenolic compounds showed the molecular ion $[M - H]^-$ (deprotonated species) in the MS spectrum acquired at low deflector voltage (−20 V); increasing the fragment voltage (−80 V) in mass spectra were visible other ions useful for structure elucidation.

A typical fragment of benzoic acids (peaks 1, 2 and 8) and caffeic acid (peak 7) was due to the loss of a carboxylic group (ion $[M - 45]^-$), while ethyl gallate (peak 11), which have an ethoxy group, showed the ion at *m*/*z* 169 $[M - C_2H_5]^-$. In procyanidin B1 and procyanidin B2 mass spectra were observable ions at *m*/*z* 289, corresponding to loss of catechine and epicatechin, respectively. Moreover, (+)catechin (peak 6) and (−)-epicatechin (peak 10) showed a peak at *m*/*z* 245 (loss of a CH_2–CHOH– group) and the adduct formation at *m*/*z* 579 $[2M - H]^-$, probably due to the high self-polymerisation capacity of these compounds.

Fragmentation of flavonoids occurred only at a −80 V deflector votage. In particular, myricetin and quercetin mass spectral data are in agreement with the results of Vanhoenacker et al. (2001), however, unlike what previously observed (Vanhoenacker et al., 2001), when the fragment voltage was −80 V, kaempferol mass spectra showed some new fragments (*m*/*z* 269 and 151), though the molecular ion was still the most abundant one.

Increasing the deflector voltage, flavonoid glucosides lost glucose $[M - H - 162]^-$ [isoquercitrin (quercetin-3-*O*-glucoside), kaempferol-3-*O*-glucoside, isorhamnetin-3-*O*-glucoside,], rhamnose $[M - H - 146]^-$ and rutinose $[M - H - 146 - 164]^-$ [rutin (quercetin-3-*O*-rutinoside)].

The simultaneous use of HPLC with mass spectrometer also allowed the easy identification of rhamnetin (peak 24) and isorhamnetin (peak 23), two flavonoids isomers having the same molecular mass and UV spectra

Table 2
Ions observed in negative ESI/MS for compounds listed in Table 1

Compound (peak number)	MW	Main ion observed (*m*/*z*)	
		Deflector voltage −20 V	Deflector voltage −80 V
Tyrosol (3)	138	–	–
Carboxilic compounds			
Gallic acid (1)	170	169, 339	169, 125
Proto catechuic acid (2)	154	153, 307	153, 109
Vanillic acid (4)	168	167	167
Caffeic acid (7)	180	179	179, 135
Syringic acid (8)	198	197	197, 153
Ferulic acid (12)	194	193	193
p-Coumaric acid (17)	164	163	163
Ethylgallate (11)	198	197	197, 169
Flavan-3-ols			
(+)-Catechin (6)	290	289, 579, 245	289, 245, 149, 137
(−)-Epicatechin (10)	290	289, 579, 245	289, 245, 149, 137
Procyanidin B1 (5)	578	577, 451, 425, 289	577, 451, 425, 289
Procyanidin B2 (9)	578	577, 451, 425, 289	577, 451, 425, 289
Flavonoids			
Rutin (13)	610	609	609, 463, 301
Isoquercitrin (14)	464	463	463, 301, 179, 151
Kaempferol-3-*O*-glucoside (15)	448	447	447, 285
Isorhamnetin-3-*O*-glucoside (16)	478	477	477, 315
Myricetin (18)	318	317	317, 179, 151, 137
Quercetin (20)	302	301	301, 179, 151, 121
Kaempferol (22)	286	285	285, 269, 151
Isorhamnetin (23)	316	315	315, 300, 151
Rhamnetin (24)	316	315	315, 300, 165
Stilbenes			
trans-Resveratrol (19)	228	227	227
cis-Resveratrol (21)	228	227	227

(maximum at 369 nm). In the MS spectra of both compounds was identified an ion at *m/z* 300. The compound having a retention time of 52.79 min (isorhamnetin) had an ion at *m/z* 151, while for the compound having retention time of 55.47 min (rhamnetin) the other characteristic fragment ion was obtained at *m/z* 165. Using this different fragmentation spectrum and considering the retention time of an authentic sample, we identified the compound having t_R 52.79 min as isorhamnetin and the compound with t_R 55.47 min as rhamnetin.

PDA detection resulted more sensitive to identify tyrosol (peak 3). Indeed, to the concentration in which it was present in wine samples and under the condition used for MS detection (fragment voltage both −20 and −80 V), the ESI negative-ion mode did not produce clearly distinctive molecular ions.

Using these techniques, we probably recognized two other phenolic compounds without time-consuming pre-purification steps. In particular, we suppose that peak A (t_R 38.56 min) and peak B (t_R 42.21 min) are *trans* and *cis*-piceid. This identification was based on the elution order and UV–Vis and MS spectroscopic data. When fragment voltage was −20 V, only an ion at *m/z* 389 appeared (probably a quasi-molecular ion $[M - H]^-$). Increasing fragment voltage up to −80 V caused a reduction of the molecular ion signal and a new low-molecular-mass fragment was the base peak. This new peak at *m/z* 227 indicates a loss of a 162 mass unit and suggests a further loss of a hexose sugar. In this case, MS data are not enough for a complete identification because ESI/MS data obtained for these two compounds were very similar. However, while UV–Vis spectra of the compound having t_R 42.21 min showed a maximum at 281 nm, the compound with t_R 38.56 min exhibited further maximum at 301 nm. Considering that *trans*-resveratrol UV absorbance is maximum at 306 nm, we hypothesised that *trans*-piceid eluted first from the C_{18} HPLC column, with a retention time of 38.56 min, followed by *cis*-piceid at 42.21 min. Obviously, this assignment is only a tentative because we did not compare the results with authentic samples since they are not commercial products. Consequently, in order to perform the quantitation of these two glucosidic resveratrol derivatives with the least possibility of error, we operated an enzymatic hydrolysis with β-glucosidase (La Torre et al., 2004). A significant finding of our analysis was that after hydrolysis as *cis* and *trans*-resveratrol signal area increased, the hypothetical piceid signal area proportionally decreased.

The presence of 2-*O*-caffeoyltartaric acid (caftaric acid, peak C) was based on a tentative identification, because we could not confirm the results with those of an authentic sample, which is non-commercial. At a fragment voltage of −20 V, however, the fragment ion at *m/z* 311 was detected and mass spectra obtained by using higher fragment voltage (80 V) showed also a peak at *m/z* 179 and a lot of peaks with very low abundance. The *m/z* 311 value could be indicative of the molecular ion $[M - H]^-$ (deprotonated caftaric acid) and the ion at *m/z* 179 probably resulted from the loss of tartaric acid. Taking into account its relative position in the chromatogram and the UV–Vis data (absorbance maximum at 327 nm), the peak C was tentatively assigned to caftaric acid.

3.4. Determination of the phenolic compounds in wines

Once the analytic conditions for the separation and detection were optimised, the procedure was used to determine phenolic compounds in 22 commercial Sicilian red wines from different varieties.

The peaks were identified by:

- comparing the retention time obtained for the wine sample, the standards mixture and the wine spiked with the standards under identical conditions;
- using PDA detector to measure continuously the UV–Vis spectra of the eluted solute;
- comparing ESI-MS data for unambiguous detection and to eliminate misidentification of compounds with similar UV–Vis spectra.

Tables 3–5 shows the range and the median value of concentration of the phenolic compounds found in the Sicilian red wine samples analysed using the proposed HPLC–MS method. The data here presented were obtained as average values of triplicate analysis. The coefficient of variation (CV%) of the three analyses was always lower than 5%. The data on the levels of all the phenolic compounds in the analysed wines show that the content of these compounds are according to data in the literature (Frankel, Waterhouse, & Teissedre, 1995; Lòpez, Martínez, Del Valle, Orte, & Miró, 2001; Malovaná, García Montelongo, Pérez, & Rodrıguez-Delgado, 2001; Rodrıguez-Delgado, Gonzales-Hernández, Conde-González, & Pérez-Trujillo, 2002; Simonetti, Pietta, & Testolin, 1997).

It can be seen that the highest content in *trans* and *cis*-resveratrol was found in wines from *Merlot* grapes. Determination of *trans*- and *cis*-resveratrol levels in red wines showed mean values of 1.37 and 0.75 mg/L, respectively, for wines obtained form *Merlot* grapes; 0.35 and 0.11, respectively, for wines obtained form *Nero d'Avola* grapes; and 0.35 and 0.13, respectively, for wines obtained form allochthonous grapes. This result corroborates other studies showing that red wines from various countries, regions and cultivars have a low mean concentration of *trans* and *cis*-resveratrol (Goldberg et al., 1995; Lamuela-Raventòs, Romero-Pérez, Waterhouse, & deLaTorreBoronat, 1995). Concerning *trans* and *cis*-piceid, *cis*-piceid was typically found at lower concentration than *trans*-piceid in wine;

Table 3
Range (mg/L) and median value (mg/L) of phenolic compounds in 11 commercial Sicilian red wines of cv. *Nero d'Avola*

Compound	Range (mg/L)	Median values (mg/L)
Gallic acid	28.34–100.73	63.53
Protocatechuic acid	0.85–3.19	1.90
Tyrosol	4.01–64.79	30.01
Vanillic acid	6.06–11.19	7.52
Syringic acid	3.62–7.46	4.59
Caffeic acid	2.25–24.42	4.22
Ferulic acid	0.13–1.96	0.70
p-Coumaric acid	0.18–1.73	0.39
Procyanidin B1	16.88–61.31	42.61
Procyanidin B2	4.66–37.65	19.83
(+)-Catechin	17.72–41.87	24.68
(−)-Epicatechin	23.23–37.01	32.30
Ethylgallate	6.37–18.84	13.78
Rutin	1.86–27.25	13.25
Isoquercitrin	9.06–25.59	17.54
Isorhamnetin-3-*O*-glucoside	0.61–13.32	1.60
Kaempferol-3-*O*-glucoside	1.22–44.86	5.70
Myricetin	2.86–24.37	8.29
Quercetin	3.54–12.65	11.64
Kaempferol	0.14–0.57	0.37
Isorhamnetin	0.14–0.47	0.32
Rhamnetin	n.d.–0.08	n.d.
trans-Resveratrol	0.12–0.62	0.33
cis-Resveratrol	0.04–0.24	0.09
trans-Piceid	0.25–2.16	1.43
cis-Piceid	0.05–0.43	0.25

n.d., not detectable.

Table 4
Range (mg/L) and median value (mg/L) of phenolic compounds in 5 commercial Sicilian red wines of cv. *Merlot*

Compound	Range (mg/L)	Median values (mg/L)
Gallic acid	58.13–107.83	84.86
Protocatechuic acid	1.12–4.05	3.28
Tyrosol	23.33–43.77	33.03
Vanillic acid	4.60–9.46	6.57
Syringic acid	3.71–9.90	6.03
Caffeic acid	2.64–8.61	7.66
Ferulic acid	0.08–1.11	0.39
p-Coumaric acid	0.34–2.78	0.78
Procyanidin B1	39.54–127.5	69.80
Procyanidin B2	24.90–80.98	42.48
(+)-Catechin	14.24–78.67	62.14
(−)-Epicatechin	34.74–107.08	69.27
Ethylgallate	16.54–31.83	16.76
Rutin	4.78–29.28	8.50
Isoquercitrin	17.26–58.44	23.21
Isorhamnetin-3-*O*-glucoside	1.24–3.22	2.04
Kaempferol-3-*O*-glucoside	4.76–12.46	9.14
Myricetin	1.91–11.87	8.43
Quercetin	3.19–16.70	13.14
Kaempferol	0.17–0.54	0.44
Isorhamnetin	0.10–0.90	0.52
Rhamnetin	n.d.–0.22	n.d.
trans-Resveratrol	0.61–2.44	1.29
cis-Resveratrol	0.09–1.81	0.27
trans-Piceid	1.48–3.70	2.35
cis-Piceid	0.22–1.97	0.73

n.d., not detectable.

Table 5
Range (mg/L) and median value (mg/L) of phenolic compounds in 6 commercial Sicilian red wines of cv. alloctonous (cv.*Cabernet*, *Syrah* and *Petit-Verdot*)

Compound	Range (mg/L)	Median values (mg/L)
Gallic acid	39.07–106.66	68.50
Protocatechuic acid	1.22–2.57	1.77
Tyrosol	3.11–75.66	44.70
Vanillic acid	5.38–9.55	6.36
Syringic acid	3.76–7.35	5.02
Caffeic acid	4.26–19.65	6.99
Ferulic acid	0.04–1.34	1.03
p-Coumaric acid	0.29–2.75	1.59
Procyanidin B1	8.06–102.38	64.54
Procyanidin B2	17.54–58.61	36.51
(+)-Catechin	5.45–99.00	41.09
(−)-Epicatechin	31.65–136.01	62.46
Ethylgallate	5.45–22.25	17.06
Rutin	5.06–29.75	7.38
Isoquercitrin	14.68–34.62	22.82
Isorhamnetin-3-*O*-glucoside	2.05–21.89	5.14
Kaempferol-3-*O*-glucoside	0.87–37.72	10.87
Myricetin	2.84–30.92	6.99
Quercetin	4.63–16.63	11.76
Kaempferol	0.13–0.48	0.35
Isorhamnetin	0.15–0.78	0.53
Rhamnetin	n.d.–0.11	n.d.
trans-Resveratrol	0.10–0.88	0.25
cis-Resveratrol	0.05–0.28	0.11
trans-Piceid	0.36–3.74	3.14
cis-Piceid	0.09–0.54	0.19

n.d., not detectable.

their levels exceeded those of the free isomers and reached maxima concentration of 3.74 and 0.36 mg/L, respectively, in red wines from *Merlot* grapes.

In all our commercial wine samples, the predominant phenolic constituents were gallic acid (mean value 86.23 mg/L in *Merlot* wines; 62.90 mg/L in *Nero d'Avola* wines and 74.53 mg/L in wines from allochthonous grapes) and procyanidin B1 (mean value 76.87 mg/L in *Merlot* wines; 43.17 mg/L in *Nero d'Avola* wines and 60.03 mg/L in wines from allochthonous grapes). All the wine samples contained tyrosol (mean value 33.19 mg/L in *Merlot* wines; 28.42 mg/L in *Nero d'Avola* wines and 42.59 mg/L in wines from allochthonous grapes), (−)-epicatechin (mean value 71.99 mg/L in *Merlot* wines; 31.88 mg/L in *Nero d'Avola* wines and 74.80 mg/L in wines from allochthonous grapes), procyanidin B2 (mean value 44.43 mg/L in *Merlot* wines; 18.90 mg/L in *Nero d'Avola* wines and 38.00 mg/L in wines from allochthonous grapes) and (+)-catechin (mean value 52.44 mg/L in *Merlot* wines; 27.16 mg/L in *Nero d'Avola* wines and 45.88 mg/L in wines from allochthonous grapes). In particular, (+)-catechin, (−)-epicatechin, tyrosol and procyanidin B2 were the phenolic compounds present in higher concentration in wines from *Merlot* and other allochthonous grapes.

Among the cinnamic acids, appreciable quantities of vanillic acid were found in wines from *Nero d'Avola* grapes (mean value 7.89 mg/L), while good levels of caffeic acid were also found in wines from allochthonous grapes (mean value 8.73 mg/L).

Flavonols and flavonoid *O*-glucosides were detected in all the samples except for rhamnetin that was detected, at lower concentration, only in three *Nero d'Avola*, two *Merlot* and one allochthonous wine samples. At the same time, isoquercitrin was the most abundant *O*-glucoside compound in all the wine samples. On average, the highest concentration was found in *Merlot* wine samples.

4. Conclusion

In this paper, we developed a chromatographic method for the analysis of phenolic compounds in wines; the preliminary goal was to be able to routinely analyse 24 phenolic compounds in different type of wine by direct injection, without any prior purification of the sample. HPLC with PDA detection was used to separate and quantify the phenolic compounds, and MS used for unambiguous detection and to eliminate misidentification of compound with similar UV spectra. ESI-MS spectrometry resulted particularly effective for the analysis of flavonoid compound since it allows the formation of ions corresponding to the molecular ion of the compound and of its possible aglycon. Moreover, PDA detection can be performed under the same condition, thus providing UV–Vis spectra with confirmatory data. In this viewpoint, choosing of a double detection for each analyte furnish a more precise control of their identity.

The data on the levels of all the phenolic compounds in the red Sicilian commercial wines reported in this study showed that the Sicilian wine samples from *Merlot* grapes generally had the highest phenolic compounds content; so we can suppose that from these grapes is possible to produce in Sicily wines with phenolic compounds amount available to induce physiological effects.

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