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PSP
Angerstr. 12
85354 Freising - Germany
Phone: ++49 – (0) 8161-48420
Fax: ++49 - (0) 8161-484248
Email: parlar@psp-parlar.de
www.psp-parlar.de

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DETERMINATION OF PHENOLIC COMPOUNDS OF BIOLOGICAL INTEREST IN SOME ITALIAN RED WINES BY HPLC–DAD

C. Pavia[1], S. A. Bufo[1], A. Scopa[1], L. Scrano[1], A. Guerrieri[2] and T. R. I. Cataldi[2]

1 Dipartimento di Produzione Vegetale, Università degli Studi della Basilicata, Potenza, Italy
2 Dipartimento di Chimica, Università degli Studi della Basilicata, Potenza, Italy

SUMMARY

As there is a continuous interest to characterise wines of different origin with regard to their antioxidant activity, we have examined the phenolic content of some Aglianico red wines by gradient elution reversed-phase HPLC and UV diode array detection (DAD). The potential health benefits of red wine might partly be due to the presence of natural flavonoids, such as catechin, epicatechin, *cis*-resveratrol, *trans*-resveratrol, quercetin and myricetin. Catechin and epicatechin were quantified by direct sample injection, while the determination of free *cis*-resveratrol, *trans*-resveratrol, myricetin and quercetin was preceded by a concentration step on SPE C_{18} cartridges. The quantitative analysis was accomplished by standard addition using β-naphthol as internal standard. The phenolic content detected in this study in Aglianico red wines ranged from 49.7 to 68.6 mg/L. As expected the level of *cis*-resveratrol was lower than that of *trans*-resveratrol, with average values of 0.27 and 0.86 mg/L, respectively. The presence of hydroxytyrosol and myricetin in some Aglianico wines was ascertained by on-line spectral comparison.

KEYWORDS: HPLC analysis, phenolic compounds, red wines, Aglianico, *Vitis vinifera*

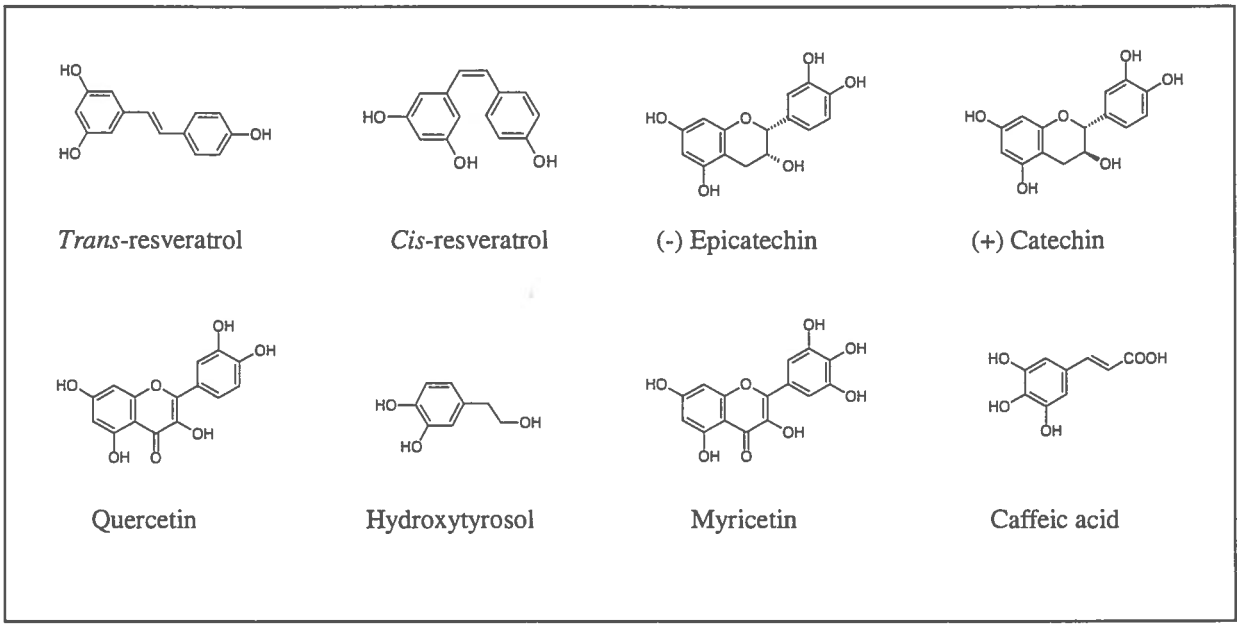
INTRODUCTION

There is currently a strong demand from the food and beverage industries for natural products with antioxidant activity, as news accounts have been full of warnings about unhealthful dietary habits. Flavonoids are natural antioxidants present in fruits, vegetable and beverages that are receiving considerable interest because of their nutritional and therapeutic effects [1]. Soleas *et al.* [2] and Sato *et al.* [3] reported that some of these compounds exhibit a potent activity against cancer due to their ability to scavenge free radicals breaking the peroxidative chain reaction. Unfortunately, most phenolic substances contained in vegetable foodstuffs are not soluble and readily bioavailable for their absorption in the gastrointestinal tract. Among moderate alcoholic beverages, red wine is characterised by a noticeable bioavailability of bioactive compounds (see Figure 1), and this makes it one of the main dietary sources of natural antioxidants [4].

Several epidemiological studies showed a relationship between wine consumption and reduced risks of cancer and cardiovascular diseases [5-7]. Apparently, these effects are related to the beneficial properties of several polyphenolic antioxidants contained in wine, such as catechin, epicatechin, quercetin, rutin and resveratrol [8]. The last compound is a phytoalexin found mainly in the skin of grapes. It exists in two isomeric forms, *cis* and *trans*, both of which show anticancer activity; they inhibit cellular events associated with tumour initiation, promotion and progression [9]. Moreover, it has been reported that *trans*-resveratrol inhibits platelet aggregation in vitro, alters lipoprotein metabolism and modulates eicosanoid synthesis toward a pattern likely to be protective against coronary heart disease [10-12]. Based on its beneficial properties, resveratrol has been the subject of intense research work, and numerous determinations in commercial wines were performed. Goldberg *et al.* [13, 14] employed a direct injection gas chromatographic-mass spectrometric method to assay *cis*- and *trans*-resveratrol contained in several red wines. High-performance liquid chromatography (HPLC) coupled with UV [15-18] or electrochemical detection [19] were also used. In this respect, HPLC has been very recently the subject of an extensive review [20]. Alternatively, Chu *et al.* [21] quantified the *trans*-resveratrol contained in wines by capillary electrophoresis.

In recent years, it is becoming even more evident that when certain phenolic substances are present in combination, they exhibit greater antioxidant activities than would be expected on the basis of their individual effect [22-24]. Therefore, the potential health benefits of wine

FIGURE 1 - Structures of studied phenolic compounds.



require knowledge not only of resveratrol concentration but also of other bioactive compounds such as catechin, epicatechin, rutin, and quercetin. Saucier *et al.* [25] observed that catechin exhibits a remarkable increase in antioxidant activity when is mixed with ascorbic acid and sulphur dioxide, which are commonly used in enology. Catechin and epicatechin protect low-density lipoproteins (LDL) against oxidation more effectively than α-tocopherol [26, 27], whereas quercetin and rutin show potential chemoprotective and cancer preventive effect [28].

Having well in mind the effects of these substances on human health, several authors described a variety of methods to analyse pools of phenolic compounds. A gas-chromatographic method, requiring a prior sample derivatization, was used to quantify hydroxylated stilbenes contained in American red wines [29]; the same analysis was proposed on Portuguese wines by liquid chromatography, thus avoiding the derivatization step [30]. Goldberg *et al.* [31] described a reversed-phase (RP) HPLC method with DAD detection to determine resveratrol and its glycoside along with catechin, epicatechin, rutin, and quercetin in wines. Finally, determination of resveratrol and other polyphenols in wines has been recently performed by capillary electrophoresis coupled with UV-DAD [32, 33].

The purpose of this study was to devise the most suitable chromatographic conditions by gradient elution RP-HPLC–DAD to identify and quantify the major free phenolic constituents of some Italian red wines of southern origin. We decided to focus on commercial wines produced with grapes of *Vitis vinifera*, Aglianico cultivar.

MATERIALS AND METHODS

Chemicals: Catechin hydrate 98%, epicatechin, rutin 95%, and *trans*-resveratrol 99% were purchased from Sigma Chemical Co. (Steinheim, G). Caffeic acid 97%, *p*-coumaric acid 98%, 2 M trimethylsilyldiazomethane solution in hexane, myricetin, lithium aluminium hydride 95%, and β-naphtol 98% were purchased from Aldrich Chemical Co. (St. Louis, MO). Fluka Chemie (Buchs, CH) supplied 3,4-dihydroxyphenylacetic acid 98%. Quercetin dihydrate 99% was purchased from Riedel-de Haën (Seelze, Germany). All these chemicals were used without further purification. Using a lamp at an intensity of 990 μW/cm^2 *cis*-resveratrol was obtained by exposure at 254 nm and 15 min of the *trans* isomer. The conversion yield was approximately 97%. Methanol, glacial acetic acid, ethanol and ethyl acetate (HPLC grade) were purchased from Panreac (Montcada i Reixac, Spain), while chloroform, diethyl ether, and tetrahydrofuran were supplied by Carlo Erba (Milan, Italy). Pure water from a Milli-Q system (Millipore, Bedford, MA) filtered through 0.45 μm cellulose nitrate filters (Aldrich) was used in all experiments. Stock solutions of polyphenols were prepared in a solvent mixture (v/v) of methanol 20%, glacial acetic acid 50% and water 30%. Just before use, standards to be injected were prepared from the stock solutions by dilution to the desired concentration.

Hydroxytyrosol synthesis: Bai *et al.* [34] synthesised hydroxytyrosol from 3,4-dihydroxyphenylacetic acid employing (trimethylsilyl) diazomethane as a rapid methylation agent, and reducing the reaction product by $NaBH_4$. Probably, because of the mild reducing power of $NaBH_4$.

we obtained an intermediate product with a single methylation. Yet, when lithium aluminium hydride was used in place of sodium borohydride, the yield of hydroxy-tyrosol was ca. 96%. Briefly, the reaction scheme is hereafter described. A stirred solution of 3,4-dihydroxy-phenylacetic acid (7.68 mmol), prepared in methanol (10 mL) and ether (35 mL), was treated with 5 mL of a 2 M trimethylsilyldiazomethane solution in hexane. The reaction mixture was stirred for 45 min at ambient temperature and concentrated to dryness; then, the residue was dissolved in 104 mL of anhydrous tetrahydrofuran and 0.68 g of $LiAlH_4$ were slowly added by stirring under nitrogen. The reaction was monitored by thin layer chromatography on silica gel eluted with a mixture chloroform-methanol, 7:1 (v/v), and stopped after 2 h by methanol; the solution was treated with brine and extracted with ethyl acetate. The organic phase was dried over Na_2SO_4, filtered and concentrated to dryness by evaporation; the crude reaction product was purified by chromatography on silica gel using chloroform-methanol (7:1) as eluting solvent. ^1H NMR spectroscopy and GC-MS analysis confirmed identity of hydroxytyrosol.

Chromatographic system and detection: All experiments were performed on a Hewlett Packard model 1090 with a ternary pump, a Rheodyne injection valve with a loop of 20 μL, and a diode array UV-visible detector coupled to an HP Chem-Station used for solvent delivery and detection. The column used was a Supelcosil™ LC-ABZ (250 mm x 4.6 mm i.d.), 5 μm particle size, with a guard column (40 mm x 4.6 mm i.d.) of the same material. Separations were obtained at constant flow rate of 1.0 mL/min with linear gradient elution using a mobile phase composed of water, methanol, and glacial acetic acid (see Table 1).

TABLE 1 - Gradient elution used for the separation of phenolic compounds occurred in Aglianico red wines.

Mobile phase component (% v/v)	Time	
	0.0 – 10.0 min	10.1 – 35.0 min
Water	82.0 → 60.0	60.0 → 32.0
Methanol	10.0 → 32.0	32.0 → 60.0
Glacial acetic acid	8.0	8.0

After each analysis, the column was washed with methanol (15 min) and re-equilibrated with the initial mobile phase for 10 min. Membrane-filtered (0.45 µm) and degassed Milli-Q water was used as solvent in the chromatographic gradient program. All separations were carried out at room temperature, 22±2 °C. The chromatograms were recorded at the sample wavelengths of 280 and 306 nm, using the reference signal at 550 nm. The bandwidths of the sample and reference wavelengths were ± 4 nm and ± 5 nm, respectively. The sample wavelength at 280 nm was used for the detection of catechin, epicatechin and hydroxytyrosol. Detection of *p*-coumaric acid, caffeic acid, myricetin, rutin, quercetin, *cis*- and *trans*-resveratrol, performed at 306 nm, was preceded by a concentration step on SPE C_{18} cartridges. The quantitative analysis was carried out by standard addition using β-naphthol as an internal standard; the results were expressed in milligrams of compound per litre of sample wine.

Wine samples and sample preparation: Four commercial Aglianico red wines from the Lucania region of the same 1997 vintage were kindly offered by local producers: D'Angelo snc, Martino srl, Paternoster srl, and Basilium srl, referred as Aglianico I, II, III, and IV (Portali), respectively. All wines were stored in the dark and each bottle was opened immediately prior to analysis. The samples were also protected from light to avoid light-induced isomerization of phenolic substances during treatment. All samples were adjusted to pH 3 with glacial acetic acid, spiked with 100 µM internal standard, then filtered through a 0.2 μm cellulose nitrate filters (Aldrich) and concentrated by solid-phase-extraction (SPE) on C_{18} cartridges (500 mg, 6 mL in volume) purchased from Superchrom (Milan, Italy). These cartridges were pretreated with 6 mL of ethyl acetate, 6 mL of 96% (v/v) ethanol and 12 mL of 10% (v/v) ethanol [12-14]. A sample volume of red wine, 2 or 5 mL, was slowly passed through the minicolumn. The cartridges were rinsed with 10 mL of water and the adsorbed fraction was eluted with 6 mL of ethyl acetate. The eluate was evaporated by rotary evaporation to a dry residue, which was dissolved with 1.0 mL of solvent mixture composed of glacial acetic acid, methanol and water in a ratio of 20:50:30 (v/v), and injected into the column. Recoveries were evaluated for each compound of interest by spiking the sample solutions with pure phenolics at the level of 50 and 100% of the measured content. Carrying out triplicate assays of the same wine sample the repeatability of recovery was assessed.

RESULTS AND DISCUSSION

Chromatographic analysis of red-wine phenolic compounds

For method development experiments, individual and pooled phenolic compounds were made up at the desired concentration in a solvent mixture composed of glacial acetic acid, methanol and water in a ratio of 20:50:30 (v/v). This solvent composition was the result of a compromise between the highest solubility of all compounds and the best chromatographic resolution, especially of catechins. Indeed, we observed that a concentration of acetic acid higher than 50% (v/v) further improved the separation of catechin and epicatechin but reduced the solubility of all other polyphenols. The use of an acidified mobile phase was already suggested by Dalluge *et al.* [35], as there is resolution enhancement of catechins and their peak tailing elimination.

Typical chromatograms of a standard mixture of phenolics recorded at two wavelengths, 280 nm, and 306 nm, are illustrated in Figure 2. Obtaining linear calibration curves with correlation coefficients (r) better than 0.999 validated the method, over the concentration range 0.1 mg/L – 1.2 g/L for catechin and epicatechin, and 0.02 – 50.0 mg/L for all other compounds.

RP-HPLC of phenolics in Aglianico red wines

It is conceivable that an accurate knowledge of the total phenolic contents in wines is crucial to assess the effects of these compounds on human health and disease. As Aglianico wines are derived from black "thick-skinned" grapes, it would be expected to contain a relatively high concentration of phenolic compounds and corresponding antioxidant activity [36]. In Figure 3 are shown two chromatograms of a sample of Aglianico I red wine recorded at 280 and 306 nm. Comparing retention times and UV spectra with those of standard solutions made possible the peak attributions. Catechin and epicatechin (peaks 2 – 3, curve a) were quantified by direct injection of a sample wine, while detection of *cis*-resveratrol, *trans*-resveratrol, myricetin and quercetin (peaks 4 – 7, curve b) was preceded by a concentration step accomplished by solid-phase extraction. Because of the low levels of resveratrol along with other phenolics, a concentration step was employed to ensure quantification by the present chromatographic method. β-naphthol turned out to be a good compound for internal standard (IS) quantification. The choice of such an IS is threefold: (1) it is absent from the wine samples, (2) its chromatographic peak does not overlap with other solutes and (3) it absorbs at both the wavelengths used in this study. The amount of phenolics retained by cellulose nitrate membranes was evaluated using standard solutions in different concentrations; no peak area differences were observed between filtered and authentic standard solutions. The most striking features, which arise from the chromatographic profile at 280 nm, is that all examined red wines exhibited a common phenolic pattern in which catechin and epicatechin were the major compounds.

In examining Figure 3, it is possible to distinguish (see inset of curve a) the presence of a small intensity peak (1) at a retention time of 5.31 min. On the basis of its spectral analysis, this peak was assigned as hydroxytyrosol; a standard solution was injected under the same experimental conditions (dashed curve, panel A of Figure 4). Hydroxytyrosol is a potent natural antioxidant, which has been found mainly in olive oil, but very recently its occurrence has been reported in Italian red wines [3]. Although the biological activity of this compound is still under investigation, epidemiological studies carried out in Mediterranean countries, where olive oil is the major dietary fat, have suggested that hydroxytyrosol might protect against coronary heart disease and atherosclerosis [37, 38]. Unfortunately, the poor chromatographic resolution and the low hydroxytyrosol concentration did not allow its quantification in Aglianico red wines. A further compound of interest for its antioxidant activity is myricetin, which along with quercetin was determined in its free form. In Figure 4B is illustrated the spectral analysis of such a flavonol in the sample wine (see peak 6 of Figure 3) and in a standard solution, solid and dashed lines, respectively. An extended survey of the free and conjugated myricetin and quercetin content in 65 red wines has been reported by McDonald *et al.* [39]. Although red wines may contain sizeable amounts of conjugated flavonols, free myricetin and quercetin are much more active than their conjugated derivates in preventing cardiovascular disease [40].

Quantification of phenolics

When considering the relationship between naturally abundant bioactive compounds and antioxidant activity, improved understanding is obtained using wine produced with the same grape cultivar. To this end, the method was tested on other red wines of the same geographical origin and variety (Aglianico). As the phenolic content depends not only on the grapes used but also on the wine aging, wines of the same vintage were analysed. Typical chromatograms at 306 nm of Aglianico red wines, upon the concentration step, are shown in Figure 5. Notably there is a marked difference in the chromatographic profile of these samples, which is characteristic of each wine. What is most important is, however, that *cis*-resveratrol, *trans*-resveratrol, myricetin and quercetin (peaks 1 - 4), were well resolved and generally free from interferences. Using solid phase extraction C_{18} cartridges, the recovery obtained with the concentration procedure was very satisfactory for *cis*-resveratrol, *trans*-resveratrol, myricetin, and quercetin, 97.5±1.3%, 98.0±1.4%, 97.0±1.4%, and 96.7±1.0%, respectively. Each value was obtained by averaging the recoveries of seven different red wines (i.e., using 1996, 1997, and 1998 vintages). Taking into account the concentration factor calculated for each sample wine, the amount of *cis*-resveratrol, *trans*-resveratrol, myricetin and quercetin was determined. The free phenolic content is summarised in Table 2. The values are in good agreement with data of red wines of different origin and cultivars [15, 32, 41]. Within the group of wines analysed, rutin, *p*-coumaric acid and caffeic acid were absent. While the mean level of quercetin was approximately 3.1 mg/L, myricetin was observed in only two of the analysed wines, Aglianico II and IV, 2.1 and 4.2 mg/L, respectively. Among the red wines examined, the present data show a greater amount of total phenolic content (68.6 mg/L) in Aglianico I wine. The relatively low content of phenolic compounds, which occurs in Aglianico II wine (49.7 mg/L), may reflect either the effects of a different method of vinification or different time at which grapes were picked, or both.

FIGURE 2
RP-HPLC-DAD of a mixture of phenolics recorded at two wavelengths, 280 nm and 306 nm. Peaks and concentrations: (1) hydroxytyrosol, 80 μM, (2) catechin, 200 μM, (3) epicatechin, 180 μM, (4) caffeic acid, 180 μM, (5) *p*-coumaric acid, 180 μM, (6) rutin, 60 μM, (7) *cis*-resveratrol, 15 μM, (8) *trans*-resveratrol, 30 μM, (9) myricetin, 60 μM, (IS) β-naphtol 100, μM, and (10) quercetin, 70 μM. Column, Supelcosil™ LC-ABZ plus guard with a flow rate of 1.0 mL/min.

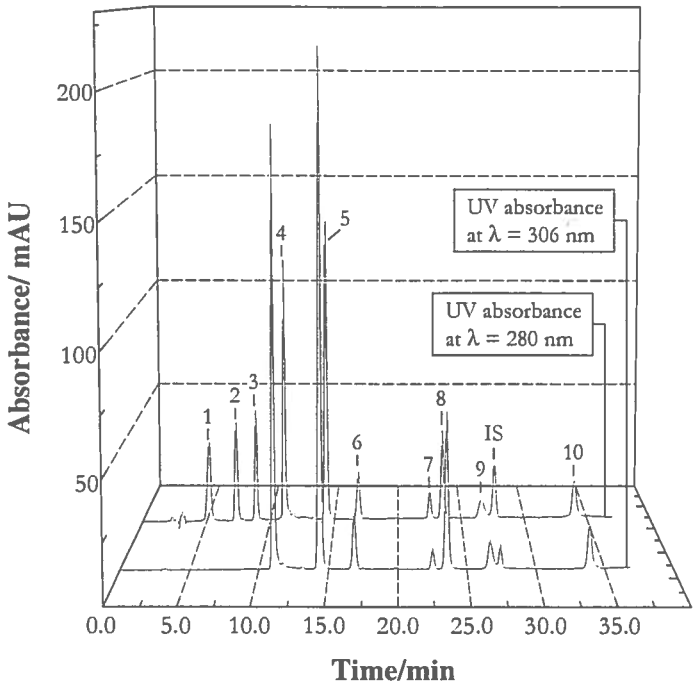


FIGURE 3
Chromatograms of a red wine Aglianico variety monitored at 280 nm (curve a) and 306 nm (curve b). Peaks identification: (1) hydroxytyrosol, (2) catechin, (3) epicatechin, (4) *cis*-resveratrol, (5) *trans*-resveratrol, (6) myricetin, (IS) β-naphtol 100 μM, (7) quercetin. The inset shows an expanded view of the hydroxytyrosol peak, retention time 5.31 min. Experimental conditions as in Figure 1.

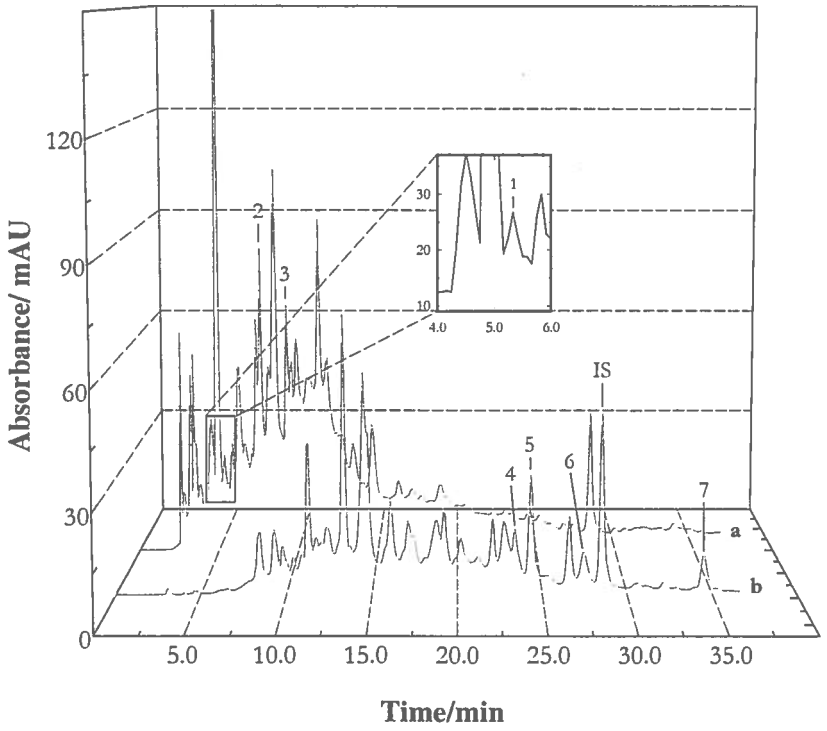


FIGURE 4
Spectral analysis of peaks eluting at the retention times of hydroxytyrosol and myricetin, (A) and (B), respectively. The solid lines are the spectra of a sample wine, and the dashed lines are the spectra of standard solutions containing hydroxytyrosol and myricetin. Match factors (MF) of hydroxytyrosol and myricetin were 994 and 986, respectively

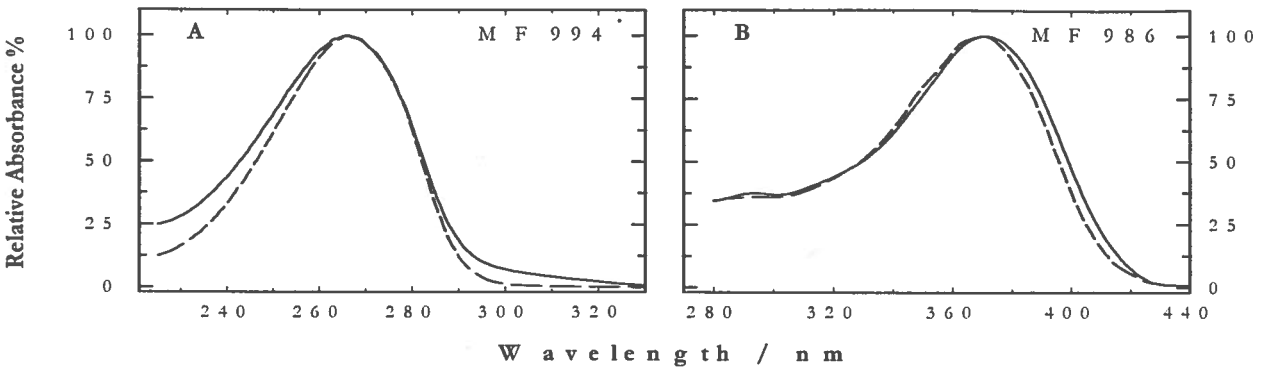


FIGURE 5
Chromatograms of three Aglianico red wines: D'Angelo (curve A), Portali (curve B), Paternoster (curve C) monitored at 306 nm. Peak identifications: (1) *cis*-resveratrol, (2) *trans*-resveratrol, (3) myricetin, (IS) *β*-naphtol, 100 μM, (4) quercetin. Experimental conditions as in Figure 1.

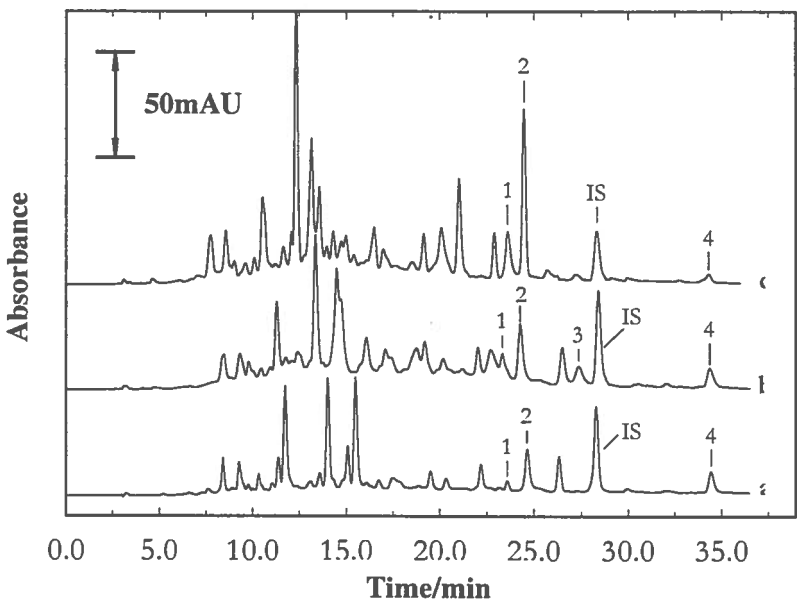


TABLE 2 - Free phenolic content of some Italian red wines (Aglianico, vintage 1997) determined by RP-HPLC-DAD.[a]

Wine labels		catechin		cis-resveratrol	trans-resveratrol	myricetin	quercetin	total amount
D'Angelo	I	32.2 ± 0.4	31.0 ± 0.5	0.31 ± 0.02	1.01 ± 0.04	nd	4.1 ± 0.1	68.6 ± 0.7
Martino	II	22.7 ± 0.4	20.9 ± 0.6	0.18 ± 0.05	0.66 ± 0.06	2.1±0.1	3.2 ± 0.1	49.7 ± 0.8
Paternoster	III	32.8 ± 0.5	21.4 ± 0.6	nq	1.03 ± 0.07	nd	2.1 ± 0.1	57.3 ± 0.8
Portali	IV	34.1 ± 0.4	21.0 ± 0.6	0.33 ± 0.07	0.72 ± 0.04	4.2±0.1	3.0 ± 0.1	63.4 ± 0.7

[a] Data are expressed as mg L^{-1} ± SE (n = 3); *nq* not quantified; *nd* not detected

As mainly derived from grape skins, the production of wines with high levels of phenolic compounds may be obtained upon a prolonged maceration time [42, 43]. Indeed, geographical or climatic reasons cannot be invoked as the main factors behind the relatively low content of total phenolics of Aglianico II wine, as all wines investigated here were produced using grapes of the Aglianico variety cultivated in the same area. We speculate that the present chromatographic method is also suitable for the determination of phenolic compounds in skins, seeds and pulp extracts.

Trans- and *cis*-resveratrol were quantified in all wines with the exception of Aglianico I wine in which the peak of the last compound was not completely resolved. Not surprisingly, in the other samples it was confirmed that *cis*-resveratrol levels are lower than *trans*-resveratrol, with mean values of 0.27 and 0.86 mg/L, respectively. These levels of resveratrol concentrations fell in the lower part of the range reported in the literature, which covers, for both the isomers, values lower than 4 mg/L [15, 32, 41]. Many studies have demonstrated that the concentration of resveratrol isomers and their glycosides in wines varies considerably as a function of multiple factors including climatic, fungal pressure, and maturation of the grapes along with grape variety and wine-processing techniques [44, 45]. As the same factors may affect the entire phenolic contents of wine, we intend to verify the influence of enological practices on the evolution of resveratrol and other phenolic compounds in grapevines, musts and wines of southern Italy origin.

CONCLUSIONS

The results of this study confirm the possibility of assessing the phenolic contents of red wines using an RP-HPLC method with UV-DAD detection. The experimental conditions allowed a satisfactory resolution of several biologically active antioxidants naturally occurring in red wines of Italian origin, as well as in skins, seeds and pulp extracts. Within the same vintage, variety and geographical region, large differences in the chromatographic profiles were observed. Apart from myricetin, which was observed in only two samples, catechin, epicatechin, *cis*-resveratrol, *trans*-resveratrol and quercetin were detected in all four red wines examined. The amounts of polyphenols detected are in good agreement with the values reported in literature for other wines of different grape variety.

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CORRESPONDING AUTHOR

S. A. Bufo
Università degli Studi della Basilicata
Dipartimento di Produzione Vegetale
Via N. Sauro, 85
I 85100 Potenza, Italy

Fax +39–0971–202269
email: bufo@unibas.it