Effect of Principal Polyphenolic Components in Relation to Antioxidant Characteristics of Aged Red Wines

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Aged red wines possess significantly different polyphenolic composition compared with young ones, mainly due not only to formation of polymeric compounds but also because of oxidation, hydrolysis, and other transformations that may occur in native grape phenolics during aging. Representative Greek, single-variety, aged red wines were examined for total phenol, total flavanol, and total anthocyanin content using spectrophotometry, and attempts were made to establish correlations with the antiradical, reducing, and hydroxyl free radical scavenging activity. In addition, HPLC analyses were carried out, to ascertain whether individual polyphenols are actually responsible for the antioxidant effects of aged red wines. It was found that total flavanols are the class of polyphenols that account for hydroxyl free radical scavenging efficacy and to a lesser extent for antiradical and reducing ability, whereas there was a less significant link between the antioxidant properties and the total phenolics and only a weak relationship to total anthocyanin content. The correlation of the antioxidant properties with the principal polyphenols showed that individual compounds are weakly associated with all the antioxidant parameters, suggesting that the expression of antioxidant activity in aged red wines is rather a consequence of synergism between various phenolics, and it is not simply attributed to specific constituents.

Keywords: *Aged red wines; anthocyanins; antiradical activity; benzoic acids; hydroxycinnamates; hydroxyl free radical scavenging activity; flavanols; flavonols; polyphenols; reducing power*

INTRODUCTION

In recent years much attention has been devoted to nutritional antioxidants and their association with suppressed rates of cardiovascular disorders and other health benefits. Many of these studies have focused on dietary phenolics and the epidemiological evidence that polyphenol-containing plant foods and products possess health-promoting effects have been based, to some extent, on their antioxidant properties. Red wine is an excellent source of various classes of polyphenols and may contain 1000−4000 mg L^{-1} of various phenols (*1*), which have different biological activities, including benzoic and cinnamic acid derivatives, flavanols, flavonols, and anthocyanins.

Much effort has been expended on the analysis of red wine polyphenols and the relationship between polyphenol content and antioxidant capacity. The antioxidant properties of red wines have been correlated with their content of flavanols (*2, 3*), anthocyanins (*4, 5*), and tannic acid (*6*), although it is believed that the antioxidant properties of red wines are linked with the total polyphenol concentration (*7, 8*), rather than individual polyphenols. It should be noted, however, that maturation of red wines is a process that involves extensive transformation of native grape polyphenols, and aged wines contain a particularly complex mixture of simple and polymeric polyphenols, the nature of the latter being largely unknown. Nevertheless, there have been very few studies concerning the polyphenolic composition of aged red wines, and thus there is a lack of knowledge concerning the composition of polyphenolic antioxidants in wines having undergone aging. This is of prime importance considering that only a small number of red wines are consumed young, whereas the vast majority are aged for at least 12 months in oak barrels and 12 months in the bottle. Thus wines are available for consumption after they have reached the appropriate level of maturity.

The present investigation was undertaken to determine the polyphenolic composition of selected, Greek aged wines and aimed at examining the relationship of principal polyphenolic groups and individual polyphenols to the in vitro antioxidant properties. For comparison reasons, wines were from the same vintage, and each was made from single, unblended grape variety.

MATERIALS AND METHODS

Wines. Ten representative, high-quality, single-variety red wines from the 1998 vintage were tested. All wines examined were produced according to standard procedures and with defined varietal composition (Table 1). Samples covered some of the most important viticultural areas in Greece, are available in the Greek market, and are widely consumed. All samples were stored at 10 °C in the dark and analyzed shortly after opening.

Chemicals. Water used for HPLC analyses was Nanopure. Acetonitrile (MeCN) was of HPLC grade. Luminol (3-aminophthalhydrazine), gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, syringic acid, catechin, *p*-dimethylaminocinnamaldehyde (DMACA), epicatechin, Folin-Ciocalteu reagent, rutin (quercetin 3-*O*-rutinoside), quercitrin (quercetin 3-*O*-rhamno-

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Table 1. Origin and Varietal Composition of the Wines Tested

appellation	cultivar(s)	location
Topikos Chalkidikis	Merlot	Macedonia (N)[a]
Naoussa	Xinomavro	Macedonia (N)
Nemea	Agiorgitiko	Peloponesse (S)
Sitia	Liatiko	Crete (S)
Macedonikos Topikos	Xinomavro	Macedonia (N)
Topikos Plagion Egialias	Cabernet Sauvignon	Peloponesse (S)
Topikos Imathias	Syrah	Macedonia (N)

[a] Letters N, S, and C assign Northern, Southern, and Central Greece, respectively.

side), caffeic acid, *p*-coumaric acid, ferulic acid, 2,2-diphenyl-β-picrylhydrazyl (DPPH$^\bullet$) radical, EDTA (disodium salt), and 2,4,6-tripyridyl-*s*-triazine (TPTZ) were from Sigma Chemical Co. (St. Louis, MO). Citric acid and Trolox were from Aldrich (Steinheim, Germany). Cobalt(II) chloride was from Merck (Germany). A standard mixture of wine anthocyanins containing 5 μM each of delphinidin, cyanidin, petunidin, paeonidin, and malvidin 3-*O*-glucosides was kindly provided by Dr. S. Kallithraka (Institute of Wine, Lycovrysi, Athens).

Determination of Total Phenols. Total polyphenol content of wines was determined with the Folin-Ciocalteu method (*9*), adapted to a microscale. In a 1.5-mL Eppendorf tube, 790 μL of distilled water, 10 μL of sample appropriately diluted, and 50 μL of Folin-Ciocalteu reagent were added and vortexed. After exactly 1 min, 150 μL of aqueous sodium carbonate 20% was added, and the mixture was vortexed and allowed to stand at room temperature in the dark, for 120 min. The absorbance was read at 750 nm, and the total polyphenol concentration was calculated from a calibration curve, using gallic acid as a standard. Results were expressed as mg L^{-1} gallic acid equivalents (GAE).

Determination of Total Anthocyanins. Measurements were performed using well-established spectrophotometric methodology (*10, 11*). The wine sample was placed in a 0.2-cm path-length quartz cuvette, 0.02 mL of a 20% aqueous sodium metabisulfite solution was added, and the sample was mixed well. After 1 min the absorbance was read at 520 nm (A_{520}^{SO2}). A 12% ethanolic solution was used as blank. Measurements were corrected to a 1.0-cm path length. In addition, wine (0.02 mL) was mixed with 0.98 mL of 1 N HCl solution (dilution 1:50) in a 1.5-mL Eppendorf tube, vortexed, and kept for 180 min at room temperature. The absorbance was read at 520 nm (A_{520}^{HCl}), using a 1.0-cm path-length cuvette. For the blank, 0.02 mL of a 12% ethanolic solution was used instead of wine. The concentration of total anthocyanins (mg L^{-1}) was calculated as follows:

$$20 \times [A_{520}{}^{HCl} - (5/3) \times A_{520}{}^{SO2}]$$

Determination of Total Flavanols. The total flavanol content was estimated using the *p*-dimethylaminocinnamaldehyde (DMACA) method (*12*−*14*). This method has a great advantage over the widely used vanillin method, since there is no interference by anthocyanins. Furthermore, it provides higher sensitivity and specificity (*13*). Wine (0.2 mL), diluted 1:100 with MeOH, was introduced into a 1.5-mL Eppendorf tube, and 1 mL of DMACA solution (0.1% in 1 N HCl in MeOH) was added. The mixture was vortexed and allowed to react at room temperature for 10 min. The absorbance at 640 nm was then read against a blank prepared similarly without DMACA. The concentration of total flavanols was estimated from a calibration curve, constructed by plotting known solutions of catechin (1−16 mg L^{-1}) against A_{640} ($r^2 = 0.9987$). Results were expressed as mg L^{-1} catechin equivalents.

HPLC Determination of Individual Phenolics. The concentration of individual polyphenols was determined by HPLC, employing a modified direct-injection method (*15*). Wines were filtered through 0.45-μm syringe filters prior to analysis. The equipment used was an HP 1090 liquid chromatograph, coupled with an HP 1090 diode array detector and

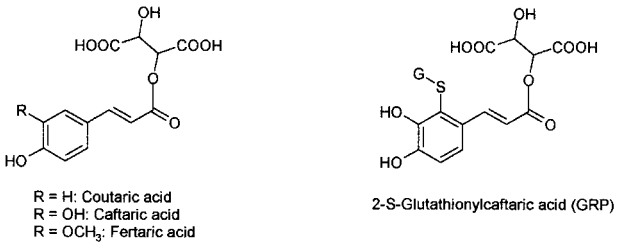

Figure 1. Chemical structures of hydroxycinnamates and caftaric acid adduct with glutathione, detected in the wines tested.

controlled by Agilent ChemStation software. The column was a LiChrospher RP-18, 5 μm, 250 × 4 mm (Merck), protected by a guard column packed with the same material. Both columns were maintained at 40 °C. Eluent (A) was 9 mM aqueous orthophosphoric acid (pH 2.5) and (B) MeCN:water (25:75), containing 9 mM orthophosphoric acid, and the flow rate was 1 mL/min. The elution program used was as follows: from 0 to 20 min, 100% A, then to 100% B in 100 min, and finally isocratic for another 20 min (total run time 140 min). Monitoring of the eluate was performed simultaneously at 280, 320, 360, and 520 nm. These wavelengths were used for benzoic acid and flavanol, hydroxycinnamate, flavonol, and anthocyanin quantification, respectively. Benzoic acids were quantified as gallic acid, flavanols as catechin, hydroxycinnamates as caffeic acid, and flavonols as rutin. Anthocyanins were quantified using the standard anthocyanin solution. All quantifications were carried out with external standard. Identification of individual compounds was based on retention times of original standards, where available, and spectral data. The identification of caftaric, coutaric, fertaric and 2-*S*-glutathionylcaftaric acid (GRP) (Figure 1) was based on UV−vis and LC-MS data published elsewhere (*15*).

Measurement of the Antiradical Activity (A_{AR}). All samples were diluted 1:10 with MeOH immediately before the analysis. An aliquot of 25 μL of diluted sample was added to 975 μL of DPPH$^\bullet$ solution (60 μM in MeOH) and vortexed, and the absorbance was read at $t = 0$ and $t = 30$ min. The antiradical activity (A_{AR}) was determined as follows

$$A_{AR} = 0.018 \times \%\Delta A_{515} + 0.017$$

as determined from linear regression, after plotting $\%\Delta A_{515}$ of known solutions of Trolox against concentration (0.08−1.28 mM, $r^2 = 0.9935$), where $\%\Delta A_{515} = [(A_{515(0)} - A_{515(30)})/A_{515(0)}] \times 100$. Results were expressed as mM Trolox equivalents.

Measurement of the Reducing Power (P_R). For the determination of the reducing ability of wines a protocol based on the ferric reducing/antioxidant power (FRAP) assay was developed, as described previously (*16, 17*), with modifications. Fifty microliters of wine, diluted 1:10 with distilled water, and 50 μL of ferric chloride (3 mM in 5 mM citric acid) were mixed well in a 1.5-mL Eppendorf tube and incubated for 30 min in a water bath at 37 °C. Following this, the mixture was added to 900 μL of 1 mM TPTZ solution in 0.05 M HCl and vortexed. After exactly 10 min the absorbance was read at 620 nm. The P_R was calculated from a calibration curve, established by plotting known amounts of ascorbic acid against A_{620}. Results were expressed as mM ascorbic acid equivalents using the following equation

$$P_R = (0.679 \times A_{620} - 0.008) \times F_D$$

where F_D is the dilution factor. For the blanks, distilled water was added instead of ferric chloride/citric acid. One blank was prepared for each wine tested. For all measurements, a computer-controlled HP 8452A diode-array spectrophotometer was used.

Measurement of Hydroxyl Free Radical Scavenging Activity (SA_{HFR}). A chemiluminescence method was used as described elsewhere (*18, 19*) with some minor modifications. One milliliter of 0.05 M borate buffer solution (pH 9), contain-

Table 2. Polyphenolic Composition and Antioxidant Properties of the Wines Examined[f]

wine code	total phenolics[a]	total flavanols[b]	total anthocyanins	A_{AR}[c]	SA_{HFR}[d]	P_R[e]
1	2117 ± 117	608.1 ± 3.5	101.8 ± 16.5	1.46 ± 0.07	54.7 ± 0.4	11.03 ± 0.15
2	2276 ± 323	606.5 ± 2.7	81.1 ± 2.5	1.48 ± 0.06	58.0 ± 1.1	10.27 ± 0.21
3	3098 ± 112	473.0 ± 6.6	212.6 ± 2.0	1.15 ± 0.07	47.8 ± 1.3	8.64 ± 0.05
4	3450 ± 171	568.5 ± 1.2	248.4 ± 2.1	1.32 ± 0.07	53.0 ± 0.9	9.06 ± 0.07
5	2898 ± 186	596.7 ± 0.7	363.1 ± 2.4	1.42 ± 0.08	60.0 ± 1.9	10.59 ± 0.05
6	1757 ± 145	347.0 ± 10.9	136.7 ± 5.0	1.10 ± 0.02	43.7 ± 1.5	5.86 ± 0.07
7	1217 ± 292	339.1 ± 6.1	109.7 ± 20.1	0.89 ± 0.06	42.2 ± 0.9	5.36 ± 0.04
8	1328 ± 235	424.4 ± 7.4	271.2 ± 18.5	0.88 ± 0.06	46.8 ± 0.6	5.49 ± 0.09
9	3772 ± 284	664.8 ± 9.5	360.1 ± 9.9	1.53 ± 0.07	61.4 ± 0.5	10.80 ± 0.18
10	3287 ± 250	643.6 ± 6.0	121.7 ± 3.8	1.39 ± 0.04	57.0 ± 1.0	8.35 ± 0.14
av	**2392**	**527.2**	**200.6**	**1.26**	**52.5**	**8.55**

[a] Gallic acid equivalents. [b] Catechin equivalents. [c] Antiradical activity (mM Trolox equivalents). [d] Hydroxyl free radical scavenging activity (%). [e] Reducing power (mM ascorbic acid equivalents). For the determination of A_{AR} and SA_{HFR} samples were diluted 1:10 and 1:200, respectively. 1, 2: Naoussa; 3, 4, 5: Nemea; 6: Sitia; 7: Macedonikos Topikos; 8: Topikos Chalkidikis; 9: Topikos Imathias; 10: Topikos Plagion Egialias. [f] Concentration is expressed as mg L^{-1}.

ing Co(II) (2 mg mL^{-1}) and EDTA (10 mg mL^{-1}) was mixed well with 0.1 mL of luminol solution (0.56 mM in borate buffer), 0.05 mL of H_2O_2 solution (50 mM), and 0.025 mL borate buffer, in a cuvette, and the chemiluminescence intensity was measured after exactly 5 min, where it reached a plateau. For hydroxyl free radical scavenging evaluation, 0.025 mL of sample was added instead of borate buffer. All samples were diluted 1:200 with borate buffer immediately before the analysis. The hydroxyl free radical scavenging activity (SA_{HFR}) was calculated as the percent decrease in chemiluminescence after 5 min. Chemiluminescence measurements were carried out using a JENWAY 6200 fluorimeter, keeping the lamp off and using only the photomultiplier of the apparatus.

Statistics. In all cases analyses were performed in triplicate, unless elsewhere specified, and the values were averaged. The standard deviation (SD) was also calculated. Correlation between A_{AR}, P_R, and SA_{HFR} with individual polyphenolic groups and individual phenolics was established using simple linear regression analysis.

RESULTS

Effect of Total Phenols, Total Flavanols, and Total Anthocyanins. The results for the determination of total phenolics, total flavanols, and total anthocyanins are presented in Table 2. Total phenolics content varied from 1217 to 3772 mg L^{-1} GAE (mean 2392 mg L^{-1} GAE), total flavanols from 347.0 to 664.8 mg L^{-1} catechin equivalents (527.2 mg L^{-1}), and total anthocyanins from 81.1 to 363.1 mg L^{-1} (200.6 mg L^{-1}). Plotting total phenol concentration against antiradical activity (A_{AR}), reducing power (P_R), and hydroxyl free radical scavenging activity (SA_{HFR}), the corresponding correlation coefficients (r^2) that obtained were 0.5226, 0.4652, and 0.5540, indicating that there is a rather moderate connection between the total phenol content and antioxidant properties of the wines. By contrast, the correlation of total flavanol concentration with antiradical activity, reducing power, and hydroxyl free radical scavenging activity gave r^2 of 0.8419, 0.7860, and 0.9242, respectively. This finding suggested that the total flavanol content may be strongly related to the antioxidant properties of the wines and highly associated with scavenging of hydroxyl free radicals. On the other hand, total anthocyanin content appeared to provide minor contribution, as the corresponding correlations gave r^2 of 0.0183, 0.0601, and 0.1510.

Determination of Individual Polyphenolic Compounds. Four benzoic acids including gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, and syringic acid could be separated and quantified by the HPLC method employed (Figure 2A). Gallic acid was by far the predominant benzoic acid, as it represented 85.3% of all benzoates (Table 3). Significant amounts of syringic acid were also found (mean 38.3 mg L^{-1}, 11.3%), but protocatechuic and *p*-hydroxybenzoic acids were minor constituents, their mean concentration being 4.4 (1.3%)

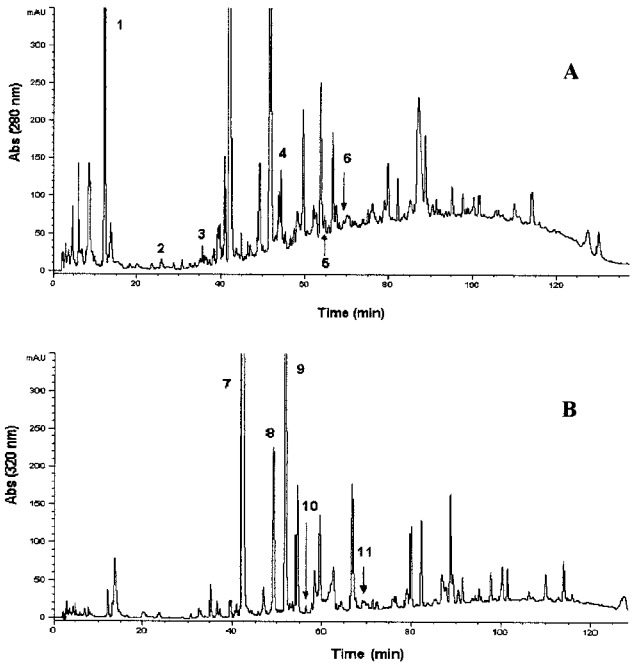

Figure 2. HPLC traces recorded at 280 (A) and 320 nm (B). Peak 1: gallic acid; 2: protocatechuic acid; 3: *p*-hydroxybenzoic acid; 4: catechin; 5: syringic acid; 6: epicatechin; 7: caftaric acid; 8: 2-*S*-glutathionylcaftaric acid; 9: coutaric acid; 10: caffeic acid; 11: coumaric acid.

Table 3. Analytical Phenolic Acid Composition of the Wines Tested[a]

wine code	gallic acid	protocatechuic acid	*p*-hydroxybenzoic acid	syringic acid	total
1	311.4 ± 17.6	3.2 ± 0.4	14.0 ± 1.1	28.3 ± 3.6	356.9
2	327.2 ± 2.7	nd	18.4 ± 0.7	55.7 ± 2.6	401.3
3	270.7 ± 6.0	4.7 ± 0.6	4.0 ± 0.6	28.9 ± 2.9	308.2
4	528.1 ± 1.5	2.9 ± 0.2	4.7 ± 0.0	65.7 ± 3.2	601.4
5	311.2 ± 4.7	2.6 ± 0.3	5.8 ± 0.0	35.1 ± 4.9	354.7
6	299.0 ± 13.4	7.5 ± 0.3	8.7 ± 0.8	55.1 ± 1.1	370.2
7	217.1 ± 2.1	9.9 ± 0.3	3.3 ± 0.5	8.2 ± 0.4	238.6
8	126.2 ± 6.6	7.5 ± 0.6	4.9 ± 0.2	18.4 ± 2.2	157.0
9	289.0 ± 6.4	3.2 ± 0.0	3.3 ± 0.1	57.1 ± 1.7	352.5
10	203.6 ± 10.4	2.1 ± 0.3	5.3 ± 0.5	30.9 ± 2.9	242.0
av	**288.4 (85.3)[b]**	**4.4 (1.3)[b]**	**7.2 (2.1)[b]**	**38.3 (11.3)[b]**	**338.3**

[a] Values represent means of triplicate determinations ($n = 3$) ± SD. Concentration is expressed as mg L^{-1}. [b] Values in parentheses are the percent of the total amount. Wine codes are as in Table 2.

Table 4. Analytical Hydroxycinnamate Composition of the Wines Tested[a]

wine code	caftaric acid	coutaric acid	caffeic acid	coumaric acid	GRP	total
1	561.7 ± 19.9	89.4 ± 2.5	1.4 ± 0.2	2.3 ± 0.3	36.4 ± 1.9	691.2
2	519.7 ± 17.9	55.2 ± 1.5	18.0 ± 1.1	1.5 ± 0.1	30.3 ± 3.8	653.3
3	199.5 ± 8.9	52.9 ± 2.0	2.2 ± 0.3	1.6 ± 0.2	40.5 ± 4.7	296.7
4	273.1 ± 2.7	95.5 ± 0.9	nd	1.4 ± 0.1	44.7 ± 1.3	412.9
5	479.5 ± 24.1	194.4 ± 15.5	1.4 ± 0.2	3.1 ± 0.3	52.4 ± 3.9	730.8
6	358.0 ± 9.1	53.6 ± 2.2	143.9 ± 4.4	105.9 ± 0.7	58.5 ± 2.1	719.9
7	269.0 ± 8.9	100.1 ± 3.4	1.0 ± 0.1	nd	35.1 ± 0.8	428.0
8	134.0 ± 9.4	25.8 ± 1.1	1.2 ± 0.2	2.6 ± 0.4	33.3 ± 1.9	197.0
9	330.8 ± 9.6	145.7 ± 3.0	1.1 ± 0.1	2.8 ± 0.7	33.2 ± 2.2	513.6
10	280.5 ± 10.5	103.6 ± 1.0	1.2 ± 0.0	2.4 ± 0.4	32.3 ± 1.3	419.9
av	**340.4 (67.9)**[b]	**91.6 (18.3)**[b]	**17.1 (3.4)**[b]	**12.7 (2.5)**[b]	**39.7 (7.9)**[b]	**501.6**

[a] Values represent means of triplicate determinations ($n = 3$) ± SD. Concentration is expressed as mg L^{-1}. [b] Values in parentheses are the percent of the total amount. GRP: 2-*S*-glutathionylcaftaric acid. Wine codes are as in Table 2.

Table 5. Analytical Flavanol and Flavonol Composition of the Wines Tested[a]

wine code	catechin	epicatechin	total	rutin	quercitrin	total
1	61.6 ± 2.1	110.5 ± 4.1	172.2	8.0 ± 0.1	25.4 ± 1.1	33.4
2	39.7 ± 2.1	32.2 ± 2.7	71.9	13.2 ± 0.9	12.2 ± 1.5	25.3
3	nd	nd		8.2 ± 0.4	26.0 ± 3.9	34.2
4	171.4 ± 7.7	172.0 ± 9.9	343.4	16.3 ± 2.1	18.5 ± 0.9	34.7
5	169.1 ± 4.6	51.2 ± 6.9	220.3	44.5 ± 1.8	96.2 ± 8.8	140.7
6	185.9 ± 5.8	13.1 ± 2.6	199.1	9.8 ± 0.3	81.3 ± 4.1	91.2
7	56.0 ± 4.1	95.7 ± 2.6	151.7	12.8 ± 0.3	14.9 ± 1.8	27.6
8	40.2 ± 1.8	45.7 ± 2.0	86.0	20.0 ± 0.9	25.8 ± 3.1	45.8
9	47.5 ± 3.5	82.8 ± 8.3	130.3	31.0 ± 2.9	122.6 ± 7.0	153.6
10	61.2 ± 4.8	22.9 ± 2.6	84.1	17.3 ± 1.5	16.2 ± 1.7	33.5
av	**83.3**	**62.6**	**145.9**	**18.1**	**43.9**	**62.0**

[a] Values represent means of triplicate determinations ($n = 3$) ± SD. Concentration is expressed as mg L^{-1}. Rutin (quercetin 3-*O*-rutinoside); quercitrin (quercetin 3-*O*-rhamnoside). Wine codes are as in Table 2.

and 7.2 (2.1%) mg L^{-1}, respectively. With respect to hydroxycinnamates, the average concentration was 501.6 mg L^{-1}, caftaric acid being the principal conjugate, as it accounted for 67.9% (Table 4). Coutaric acid also had an important contribution (mean content 91.6 mg L^{-1}, 18.3%), followed by 2-*S*-glutathionylcaftaric acid (39.7 mg L^{-1}, 7.9%). It is characteristic that neither fertaric nor free ferulic acid was detected, whereas caffeic and *p*-coumaric acids occurred in rather trivial amounts, their average content being 17.1 and 12.7 mg L^{-1}, respectively. These results are in agreement with previous findings (*20*).

Catechin and epicatechin were two of the major flavonoid compounds detected in wines (Figure 2A), having mean concentrations of 83.3 and 62.6 mg L^{-1}, respectively (Table 5). Two quercetin glycosides, rutin and quercitrin, were also found to occur in significant amounts (Figure 3A), and their corresponding mean values were 18.1 and 43.9 mg L^{-1} (Table 5). It should be noted, however, that in no case were free quercetin or myricetin detected, presumably because unconjugated flavonols occur in red wines at very low levels (*21*). With regard to anthocyanins, a typical anthocyanin profile is shown in Figure 3B (wine no. 5). It can be seen that, apart from the five standard anthocyanins, another two peaks were consistently detected and termed as A1 and A2 (peaks 19 and 20, respectively, Figure 3B). These compounds were quantified as malvidin 3-*O*-glucoside. According to previously published data (*22*−*27*), peaks A1 and A2 might correspond to malvidin 3-*O*-glucose acetate and *p*-coumarate, respectively. This assumption is supported by the fact that peak A2 also absorbed at 320 nm, indicating the existence of a hydroxycinnamate moiety (*23*), but this

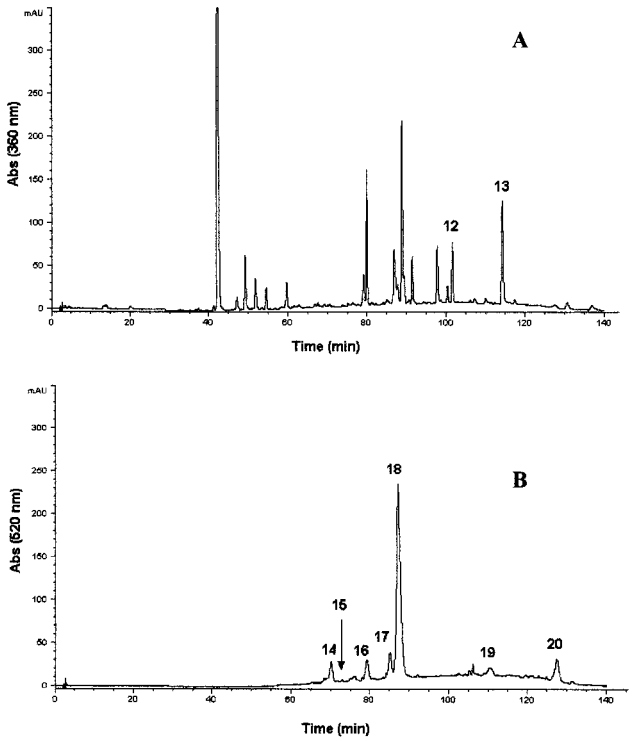

Figure 3. HPLC traces recorded at 360 (A) and 520 nm (B). Peak 12: rutin; 13: quercitrin; 14, 15, 16, 17, 18: glucosides of delphinidin, cyanidin, paeonidin, petunidin, and malvidin, respectively; 19 and 20: monomeric anthocyanins presumed to correspond to acetate and coumarate malvidin derivatives.

was not observed for A1. The quantitative determination showed that the total amount of pigments detected at 520 nm varied from 5.0 to 203.8 mg L^{-1}, the mean being 68.4 mg L^{-1} (Table 6). High total anthocyanin contents were found for wines nos. 4, 5, and 9, but wines nos. 2 and 7 were very poor in anthocyanins. In all cases the predominant anthocyanin was malvidin 3-*O*-glucoside (mean 42.2 mg L^{-1}), accounting for approximately 62% of all pigments. In contrast, cyanidin 3-*O*-glucoside had the lowest mean content (0.2 mg L^{-1}), its percentage to the overall concentration being only 0.4%. These findings are in accordance with previous studies on wines made from Cabernet Sauvignon and Merlot (*22*) and Tempranillo grapes (*28*).

Effect of Principal Polyphenols. The correlation of all three antioxidant parameters with the principal, in terms of quantity, phenolics showed that all compounds exhibited very weak correlations, and none of them appeared to influence any antioxidant characteristic to a significant extent. Moreover, it is

Table 6. Analytical Anthocyanin Composition of the Wines Tested[a]

wine code	Dp	Cy	Pt	Pn	Mv	A1	A2	total
1	4.0 ± 0.4	nd	4.3 ± 0.9	1.4 ± 0.1	26.0 ± 0.8	4.7 ± 0.8	6.9 ± 0.3	47.3
2	0.8 ± 0.00	nd	nd	nd	7.8 ± 0.9	1.4 ± 0.1	0.7 ± 0.0	10.8
3	7.3 ± 0.3	0.8 ± 0.00	9.2 ± 0.3	1.2 ± 0.1	23.4 ± 0.3	3.0 ± 0.5	2.7 ± 0.2	47.7
4	5.0 ± 0.5	nd	6.9 ± 0.5	3.9 ± 0.5	76.8 ± 1.6	9.5 ± 0.6	11.4 ± 1.1	113.6
5	14.9 ± 1.9	0.4 ± 0.1	14.4 ± 2.2	8.0 ± 1.0	139.6 ± 0.5	9.9 ± 1.2	16.7 ± 1.0	203.8
6	2.1 ± 0.1	nd	1.7 ± 0.1	0.8 ± 0.1	19.1 ± 1.4	1.5 ± 0.0	3.2 ± 0.4	28.3
7	nd	nd	nd	nd	2.0 ± 0.1	nd	3.0 ± 0.4	5.0
8	6.0 ± 0.5	nd	8.9 ± 0.9	3.8 ± 0.6	55.6 ± 0.5	15.3 ± 1.1	7.9 ± 0.4	97.6
9	9.6 ± 0.5	1.2 ± 0.4	13.8 ± 1.1	2.8 ± 0.2	57.3 ± 3.2	11.6 ± 0.8	7.8 ± 0.8	104.1
10	3.1 ± 0.5	nd	3.4 ± 0.3	nd	14.1 ± 1.3	2.8 ± 0.8	2.6 ± 0.2	25.9
av	**5.3 (7.7)**[b]	**0.2 (0.4)**[b]	**6.3 (9.1)**[b]	**2.2 (3.2)**[b]	**42.2 (61.7)**[b]	**6.0 (8.7)**[b]	**12.3 (17.9)**[b]	**68.4**

[a] Values represent means of triplicate determinations ($n = 3$) ± SD. Concentration is expressed as mg L^{-1}. Wine codes are as in Table 2. [b] Values in parentheses are the percent of the total amount. Dp, Cy, Pt, Pn, Mv: glucosides of delphinidin, cyanidin, petunidin, paeonidin, and malvidin, respectively. A1 and A2: unidentified monomeric anthocyanins.

worth mentioning that quercitrin was inversely correlated with all antiradical activity, hydroxyl free radical scavenging activity, and reducing power, a finding that might indicate that flavonols could act as prooxidants. Furthermore, it cannot be concluded that any of the phenolic classes (benzoates, hydroxycinnamates, flavanols, flavonols, anthocyanins) exerted strong antioxidant effects. Nevertheless, the relatively high correlation of total benzoic acids and total hydroxycinnamates with both antiradical activity and reducing power might suggest that these two classes could play a more important role with respect to the antioxidant characteristics of the wines, compared with other phenolics.

DISCUSSION

There have recently been a large number of investigations of the in vitro antioxidant capacity of red wines. It is irrefutable, however, that red wine is a dynamic system consisting of a plethora of polyphenols whose effect on the antioxidant properties of a given wine is extremely complicated and rather unpredictable. In addition, the polyphenolic composition of wines having undergone aging changes dramatically, as a consequence of reactions leading to condensation, polymerization, and oxidation. Because the nature of condensation and polymerization products which involve primarily anthocyanin/flavanol interactions is largely unknown, information on the constituents which are responsible for the antioxidant ability of red wines is incomplete.

In this study it was attempted to obtain not only an overall estimation of the total phenol concentration but also a determination of total flavanols and total anthocyanins, which represent the major flavonoid classes in red wines, and their correlation with certain representative antioxidant parameters. The results provided sound evidence that the total flavanol fraction, which consists of monomeric flavanols (catechin and epicatechin) and proanthocyanidins, possesses high antiradical, reducing, and hydroxyl free radical scavenging capacity. On the basis of data available in the literature, this finding is reasonable because flavanols such as catechin and proanthocyanidins have been shown to exhibit powerful antioxidant activities in different environments (*29*−*34*). Additionally, the antioxidant abilities of red grapes (*2*, *35*), juices (*36*), and wines (*2*, *3*, *7*, *37*, *38*) have always been correlated with the flavanol content. Further, the antioxidant potential of red wines might be influenced by the relative amounts of the individual flavanols. This hypothesis is supported by the fact that the antioxidant activity of proanthocyanidins is in part dictated by oligomer chain length. Thus flavanol monomers and dimers were found to inhibit LDL oxidation more efficiently than hexamers (*39*), and the inhibition of the $O_2^{\bullet -}$ tended to increase in the order of polymerization (*40*). Esterification in the 3-position with gallic acid was another important determinant with regard to scavenging hydroxyl free radical by grape seed proanthocyanidins (*29*).

In contrast to flavanols, the anthocyanin fraction was weakly correlated with all the antioxidant parameters. This could be attributed primarily to the significantly lower concentration of anthocyanins compared with flavanols. Nevertheless, despite the fact that anthocyanins have been proven very efficient antioxidants in a number of systems (*30*, *32*, *41*−*45*), and anthocyanin content correlated with antioxidant activities of red grape extracts (*35*), grape juices (*36*), and various red wines (*8*, *46*), other investigations indicated that anthocyanins may not be of high significance with respect to the antioxidant properties of red wines (*7*, *8*). It should also be noted the fact that the anthocyanin content determined with spectrophotometry does not provide an accurate estimation of the actual amount of monomeric anthocyanins (*10*), as differences in total anthocyanin content determined spectrophotometrically and by HPLC vary significantly with the age of a wine (*47*). Thus it would be reasonable to presume that the poor correlation of total anthocyanins with antiradical activity, reducing power, and hydroxyl free radical scavenging activity might be due to the fact that total anthocyanin values also represent polymeric and other types of pigments, which may not possess similar antioxidant characteristics with monomeric anthocyanins.

The HPLC analyses revealed that the prominent polyphenolic compounds are gallic acid, caftaric acid, and *p*-coutaric acid, although catechin, epicatechin, quercitrin, and malvidin were found to occur at levels that cannot be overlooked. Attempts to correlate these principal phenolics with the antioxidant parameters, however, did not meet with success. The correlation coefficients found were too low to support the assumption that these constituents directly influence the antioxidant behavior of wines. On the other hand, the higher coefficients calculated for total benzoic acids and total hydroxycinnamates indicate that the effect of these compounds are likely to be synergistic, and therefore the effect of minor constituents may be of importance.

Another point worth mentioning is the association between the different antioxidant parameters. Linear regression between antiradical activity and reducing power, antiradical activity, and hydroxyl free radical scavenging activity, and hydroxyl free radical scav-

enging activity and reducing power gave correlation coefficients of 0.8763, 0.8363, and 0.7832, respectively, suggesting that there is a close relationship among all three parameters. The assays are based on very different principles and represent different aspects of the antioxidant potential, so a connection among them would not normally be expected, as demonstrated by similar studies on human serum (*48*). However, the fact that there is such a correlation indicates that the total antioxidant activity of aged wines might be ascribed to particular class(es) of polyphenolic antioxidants, which are able to donate hydrogen atoms, scavenge hydroxyl radicals, and also participate in redox reactions. Since monomeric (catechin, epicatechin) and polymeric (proanthocyanidins) flavanols account for a great part of red wine polyphenols, it might be concluded that their content is a major factor affecting antioxidant properties.

The complexity of red wine composition does not permit the distinction of the class of compounds which define the antioxidant status of aged red wines, but from these results it is possible to highlight some significant details. First, it appears that monomeric flavanols along with proanthocyanidins are likely to govern to a great extent the antioxidant potential, but no clear conclusions could be drawn about the role of anthocyanins. Furthermore, the HPLC analyses provided the overall composition of wines and gave valuable information about the major polyphenols, and it was ascertained that it is unlikely that individual polyphenols could be able to directly influence the antioxidant behavior of wines. This indicates that the role of condensation and polymerization products, in which flavanols participate, should be taken into consideration, and further and more detailed studies are required to elucidate the mechanisms underlying the antioxidant functions of aged red wines.

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