Contribution of Proanthocyanidins to the Peroxy Radical Scavenging Capacity of Some Italian Red Wines

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Highly reactive radicals, ROO•, were generated from 2,2′-azobis[2-(2-imidazolin-2-yl)propane] and linoleic acid. The ROO• scavenging capacity of some Italian red wines was evaluated following the changes in oxygen consumption. Under the experimental conditions the time course of oxygen consumption shows two typical behaviors: trolox-like (class I) and gallic acid-like (class II). Usually the time course of wine was similar to that of gallic acid. The rate of oxygen consumption was found to decrease exponentially with the amount of wine or gallic acid added to the test solution. On this basis the capacity of red wines to scavenge peroxy radicals was expressed as content of gallic acid (S_{GA}). The S_{GA} values were found to be correlated to the amount of total proanthocyanidins and total polyphenols of some Italian red wines ($p < 0.01$). The proanthocyanidins extracted from seeds were shown to make a major contribution to the peroxy radical scavenging capacity of red wines, whereas, interestingly, the chemical class of the low molecular weight tannins reactive to vanillin did not correlate with the S_{GA} values.

Keywords: *Wine; antioxidant capacity; lipid peroxidation; radical scavenging; proanthocyanidins; phenolics; anthocyanins*

INTRODUCTION

The involvement of free radicals in many physiological and pathological processes such as aging, cancer, atherosclerosis, and rheumatoid arthritis is well recognized (Ames, 1989; Halliwell, 1994; Grisham, 1994). One of the primary sources of free radicals in man is lipid peroxidation, a chain reaction involving highly reactive ROO• radicals and leading to the formation of hydroperoxides (Miyazawa et al., 1990). Much attention has been focused on the use of antioxidants, because aspects of the antioxidant action include radical scavenging capacity, inhibition of lipid peroxidation, metal ion chelating ability, and reducing capacity (Bors et al., 1990). Flavonoids have been shown to have a strong antioxidant action (Bors et al., 1990, 1995; Rice-Evans et al., 1995, 1996) and to prevent the oxidation of human low-density lipoproteins (Frankel et al., 1995). On this basis the beneficial effects of red wine, which contains relatively large amounts of flavonoids (Goldberg et al., 1996), are becoming increasingly recognized (Renaud and de Lorgeril, 1992; Frankel et al., 1993).

Various methods have been developed to evaluate the antioxidant action of wine (Rice-Evans and Miller, 1994; Kanner et al., 1994; Vinson and Hontz, 1995; Simonetti et al., 1997; Fogliano et al., 1999). These methods are based on the reduction of relatively stable radicals such as those of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) and *N*,*N*-dimethyl-*p*-phenylenediamine. Alternatively, the inhibition of copper- or iron- catalyzed lipid peroxidation by the antioxidants present in wine was monitored. Usually the results obtained by these methods were compared with those obtained using as antioxidant 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), a synthetic and water soluble form of vitamin E (Simonetti et al., 1997).

The very good correlation between these methods and the total polyphenol content of wines measured according to the classical Folin−Ciocalteu method (Di Stefano et al., 1989b) is taken as proof that the antioxidant capacity of wine is due to polyphenols. However, this does not prove a direct relationship because these methods measure the reducing capacity of the wine and this capacity is compared with the results of the Folin−Ciocalteu method, which is based on the oxidative titration of phenolate anions by phosphotungstate and phosphomolybdate. Furthermore, the measurement of radical scavenging capacity of wines by the use of relatively unreactive radicals, such as the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) (Sanchez-Moreno, 1999), appears to be more a titration of reactive wine components than the measurement of the true wine scaveng-

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ing capacity of the very reactive secondary radicals generated in lipid peroxidation (Bors et al., 1990). Moreover, no paper has described a direct relationship between the peroxy radical scavenging capacity of wines and their content of specific antioxidants.

The present paper describes the application of a method (Niki, 1990; Pryor et al., 1993) that mimics the process of lipid peroxidation occurring in metabolic and pathological processes and reports the characterization of the peroxy radical scavenging capacity of some Italian wines. The correlations of these activities with the content of specific antioxidants in wines are also described.

MATERIALS AND METHODS

Reagents. The reagents, in particular, linoleic acid, sodium dodecyl sulfate, quercetin, catechin, 6-hydroxy-2,5,7,8-tetramethylchromane (trolox), sodium mono- and diphosphate, and ethanol, purchased from Fluka (Buchs, Switzerland), were of the highest available quality and were used without further purification. Ascorbic acid was from Aldrich and malvidin 3,5-diglucoside chloride (standard, chromatography grade) were from Carl Roth. Malvidin 3-monoglucoside chloride (standard, chromatography grade) and peonidin 3-monoglucoside chloride (technical grade) were from Extrasynthese. Deionized water, glass bidistilled, was used to prepare all of the standard and working solutions. The 2,2′-azobis[2-(2-imidazolin-2-yl)propane] (ABIP) was a kind gift of Wako Chemicals.

Measurement of the Radical Scavenging Capacity of Wine. The method is based on the inhibition of the rate of hydroperoxidation of linoleic acid in a model system consisting of micelles of sodium dodecyl sulfate in the presence of a constant source of free radicals (Niki, 1990; Pryor et al., 1993). In the present study the peroxidation process was monitored by measuring the rate of oxygen uptake by an oxygen electrode to avoid the optical interference of wine, which absorbs in the same wavelength range as linoleic acid.

A Yellow Springs oxygen electrode with a Metrohm 663 VA stand was used, inserted into a standard oxygraphic cell (volume = 3 mL), equipped with a magnetic stirrer. The current was recorded on a Y−t chart recorder (Lynseis, Italy). The working electrode was poised at −800 mV vs Ag/AgCl.

The reaction mixture was prepared by dissolving in 50 mM phosphate buffer, pH 7.4, 2.5 mM linoleic acid and 100 mM SDS. Three milliliters of this test solution was stirred continuously in the oxygraphic cell at 37 ± 0.1 °C. After 5 min, when thermal equilibrium was achieved, 50 μL of an aqueous solution of 0.5 M ABIP was added (8.2 mM final concentration) and incubated for an additional 10 min to establish the rate of oxygen consumption due to the uninhibited autoxidation of linoleic acid. The wine (0.5−50 μL) was then added to the test solution, and the rate of the inhibited reaction was recorded.

A similar procedure was used to measure the radical scavenging capacity of various antioxidants. In this case standard solutions of the various antioxidant compounds were prepared by dissolving 10 mg of the antioxidant in 10 mL of ethanol. The rate of oxygen consumption was calculated from the slope of the oxygraphic record, on the basis of the initial oxygen concentration. All of the assays were performed at 37 °C.

Wine and antioxidants caused a decrease of the rate of oxygen uptake, following an exponential dependence on their amount added to the test solution. The assay was calibrated using gallic acid, and the inhibitory effect of the tested wine was expressed as gallic acid equivalents (S_{GA}), that is, the concentration (millimolar) of gallic acid which induces the same inhibition as the tested wine on the rate of oxygen uptake.

The decomposition rate of ABIP was calculated by the measurement of the volume of nitrogen evolved in a homemade volumetric apparatus under controlled pressure and temperature conditions. This decomposition followed a first-order kinetic rate law and, under our experimental conditions, the kinetic rate constant, k_d, was found to be $(2.44 \pm 0.13) \times 10^{-4}$ min^{-1} at 37 °C.

Wines. Two sets of experimental wines were studied.

(A) Two lots of grapes of the variety Enantio and one of the variety Pinot noir were fermented at semi-industrial scale in the experimental winery of Istituto Agrario di S. Michele with three different techniques: without seeds, control (complete berries), and with double seeds, to obtain wines containing different amounts of catechins and proanthocyanidins extracted from seeds.

The seeds were selectively separated from 200 kg of crushed and destemmed berries (50 mg/L SO_2) with a mechanical screen, weighed, and added in appropriate amounts to the remaining fraction containing the must and the skins. Fermentation was obtained with selected wine strain yeasts (0.2 g/L). The contact of the must with the solid parts was prolonged for a total of 7 days for the Enantio and of 12 days for Pinot noir musts, and then the pomace was pressed (3.5 bar). The free-run and press fractions were pooled and subjected to natural malolactic fermentation, settled, filtered, and bottled (Nicolini et al., 1998).

(B) Nine samples of red wines—including the controls of the previous set—were produced in the experimental winery of Istituto Agrario di S. Michele from grape cultivars having different amounts and types of polyphenols (Mattivi and Nicolini, 1997). Four Pinot noir, three Teroldego, and two Enantio wines were obtained with the control protocol (complete berries) for the production of red wines through a traditional skin-contact technique.

Spectrophotometric Analysis of the Antioxidant Content. All analyses were done on wines aged at cellar temperature for 4−5 years in bottle. To remove the organic acids, residual sugars, free SO_2, amino acids, proteins, and other hydrophilic compounds that could cause interference, and to convert the phenols from the acqueous to the methanolic solvents before the assay, a preliminary cleanup of the phenols was performed with a Sep-Pak C-18 (Waters, Milford, MA, 0.5 g) previously conditioned with 2 mL of MeOH followed by 5 mL of 5 mM H_2SO_4. The elution and washing of the polar compounds were done at low pH to improve the recovery of acid phenols, such as gallic acid.

The relative standard deviation (RSD) under repeatability conditions [as defined (AA.VV., 1989)] was obtained experimentally with 12 analyses of the same sample of 1-year-old red wine, having the following average composition: total phenols, 1181 mg/L; index of vanillin, 475 mg/L; proanthocyanidins, 1167 mg/L; and total anthocyanins, 537 mg/L.

Total Phenols. The total phenols were assessed by the reduction of phosphotungstic and phosphomolybdic acids [Folin−Ciocalteu reagent (Folin and Denis, 1912)] to blue pigments by phenols in alkaline solution (Di Stefano and Guidoni, 1989a). The red wine was diluted (usually 10−20 times) with 0.5 M H_2SO_4, and the dilution factor was adjusted to obtain a final reading between 0.3 and 0.6 AU. One milliliter of diluted wine was then slowly loaded on the conditioned Sep-Pak, and the polar substances were removed with 2 mL of 5 mM H_2SO_4. The phenolic compounds were eluted into a 20 mL calibrated flask, with 2 mL of MeOH followed by 5 mL of distilled water. One milliliter of Folin−Ciocalteu reagent and, after ∼3−4 min, 4 mL of 10% Na_2CO_3 were added, and the solution was brought to 20 mL with distilled water. After 90 min at 20 °C, the absorbance of the sample (filtered at 0.45 μm) was read at 700 nm in a 10 mm cell, against a blank test prepared by using distilled water in place of the wine. Concentrations were determined by means of a calibration curve as gallic acid (mg/L). The RSD of the method was 2.45%.

Proanthocyanidins. The proanthocyanidins were evaluated by transformation into cyanidin (Di Stefano et al., 1989b). This method has been developed from the original acid butanol assay of Swain and Hillis (1959) and was optimized by adding adequate amounts of iron salts as catalyst to increase the reproducibility of yield of cyanidin and by replacing the toxic solvent *n*-butanol with the optimal percentage of ethanol, taking into account the suggestions of the main authors

(Ribereau-Gayon and Stonestreet, 1966; Pompei and Peri, 1971; Porter et al., 1986).

An aliquot of 2 mL of red wine diluted (10−20 times) with 0.05 M H_2SO_4 was loaded in a conditioned Sep-Pak. The column was washed with 2 mL of 5 mM H_2SO_4 and purged with air, and the proanthocyanidins were eluted with 3 mL of MeOH, collected into a 50 mL flask shielded from light (aluminum foil) and containing 9.5 mL absolute EtOH. An amount of 12.5 mL of $FeSO_4 \cdot 7H_2O$ (300 mg/L) in concentrated HCl was added, and the flask was then placed in a boiling water bath and refluxed for 50 min, after which time it was rapidly cooled by immersion in cold water (20 °C). The spectrum from 380 to 700 nm was recorded in a 10 mm cell after 10 min against a blank (water). The tangent from the minimum (∼450 nm) was drawn, and the absorbance between the maximum (∼550 nm) and the tangent was measured. To subtract natural anthocyanins present in the sample, the corresponding value of the wine prepared under the same conditions and put in ice instead of warming was subtracted to obtain the net value of absorbance. Under such conditions the average yield has been estimated from the author to be 20% (Di Stefano et al., 1989b) and the proanthocyanidins concentration (mg/L) can be conventionally expressed as 5 times the amount of cyanidin formed, by means of a calibration curve with cyanidin chloride [$\epsilon = 34700$ according to Di Stefano et al. (1989b)]. The RSD of the method was 2.74%.

Index of Vanillin. The catechins and proanthocyanidins reactive to vanillin were analyzed according to the optimized and controlled vanillin−HCl method of Broadhurst and Jones (1978), following the conditions described by Di Stefano et al. (1989b). The red wine was diluted (2−10 times, to obtain a final reading between 0.2 and 0.4 AU) with 0.5 M H_2SO_4, and 2 mL was loaded on a conditioned Sep-Pak. The column was washed with 2 mL of 5 mM H_2SO_4 and purged with air, and the flavanols were eluted with 5 mL of MeOH into a test tube. One milliliter of the methanolic solution containing the flavanols was placed in a test tube (shielded from light) together with 6 mL of vanillin (4% in MeOH) and immersed in a water bath at 20 °C. When cool, 3 mL of concentrated HCl was carefully added. After exactly 15 min, the absorbance of the pink complex was read at 500 nm in a 10 mm cell against a blank prepared in the same conditions, containing MeOH instead of vanillin. Concentrations were calculated as (+)-catechin (mg/L) by means of a calibration curve. The RSD of the method was 3.54%.

Total Anthocyanins. An aliquot of 5 mL of red wine diluted (5−20 times, to obtain a final reading in the range of 0.3−0.6 AU) with 0.5 M H_2SO_4 was loaded on a conditioned Sep-Pak. The column was washed with 2 mL of 5 mM H_2SO_4, and the red pigments were eluted with 3 mL of MeOH into a 20 mL calibrated flask. A volume of 0.1 mL of concentrated HCl was added, and the volume was brought to 20 mL with ethanol/water/HCl (70:30:1). The total anthocyanins were directly quantified on the basis of the maximal absorbance in the visible range (536−540 nm) against a blank (ethanol/water/HCl 70:30:1). The wine pigment content was calculated in milligrams per liter by assuming an average absorbance of the mixture of anthocyanins (average MW = 500) extracted from Cabernet Sauvignon grape to equal 18800 M^{-1} cm^{-1} (Glories, 1984; Di Stefano et al., 1989b). The conventional numerical value thus obtained can be easily converted in other common units by means of the appropriate absorbance of available standard compounds [experimental values in ethanol/water/HCl (70:30:1), malvidin 3,5-diglucoside chloride, $\epsilon = 32200$, 540 nm; malvidin 3-monoglucoside chloride, $\epsilon = 30100$, 542 nm; peonidin 3-monoglucoside chloride, $\epsilon = 22940$, 530 nm]. The RSD of the method was 1.39%.

Statistical Treatment. Analysis of variance and regression analysis were computed with Genstat 5 software (Lawes Agricultural Trust, IACR, Rothamsted, U.K.).

RESULTS AND DISCUSSION

Measurement of the Peroxy Radical Scavenging Capacity of Wine. The addition of a radical source (ABIP) providing a constant rate of radical production to a 50 mM phosphate solution, pH 7.4, equilibrated with air and containing 100 mM SDS, establishes a constant consumption of dissolved oxygen of ∼1.16 μM min^{-1}. No significant change of this rate was observed after the addition to this system of red wine up to 1% v/v. The same experiments performed in the presence of 2.5 mM linoleic acid showed different results. The ABIP addition starts the oxidation of linoleic acid without any induction period, and a constant rate of oxygen consumption (R_0) of 7.1 ± 0.3 μM min^{-1} was observed until the disappearance of oxygen (Figure 1, curve A). The addition of small amounts of red wine (≥0.1% v/v) to this test solution strongly decreased the rate of oxygen uptake (Figure 1, curves B−F). Conversely, no measurable effect was observed after the addition to the model system of ethanol (10% v/v), white wine (1% v/v), or sulfite (25 μM). The decrease in the rate of oxygen uptake is probably due to the large variety of antioxidants present in red wines.

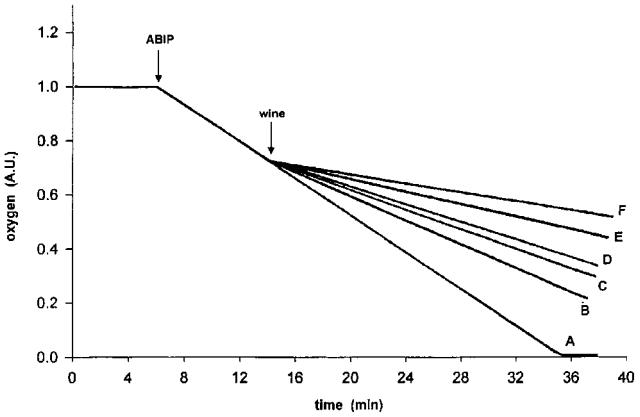

Figure 1. Inhibition by red wine of the oxygen uptake in the oxidation of linoleic acid: (curve A) time course in the absence of wine; (curves B, C, D, E, and F) initial rates after addition to the model system of 3, 5, 7, 10, and 20 μL of red wine (Enantio), respectively. The oxidation of 2.5 mM linoleic acid was carried out at 37 °C in 100 mM SDS in an aqueous dispersion and was initiated with 8.2 mM ABIP; 50 mM phosphate was used to keep the pH at 7.4.

In this regard, according to the method reported above, we tested various antioxidants, most of which are fundamental components of red wine. The time courses of oxygen uptake were found to depend on the type of antioxidant. Basically, two typical behaviors were observed under our experimental conditions: trolox-like (class I) behavior and gallic acid-like (class II) behavior.

In the case of class I (trolox, vitamin E, malvidin 3-monoglucoside, quercetin, and ascorbic acid), the addition of the antioxidant causes a strong decrease in the rate of oxygen uptake for a period of time (lag time), which appears to increase linearly with the concentration of the antioxidant (see Figure 2A, where the behavior of three antioxidants is reported). The actual decrease in the rate of oxygen uptake during the lag time was found to be only slightly sensitive to the concentration of the antioxidant. At the end of the lag time, the autoxidation proceeded at a rate similar to that observed before the antioxidant addition.

In the case of class II behavior (gallic acid, catechin, myricetin, caffeic acid, and *p*-coumaric acid), after the addition of the antioxidant, the rate of O_2 consumption decreases and appears to be almost constant until the

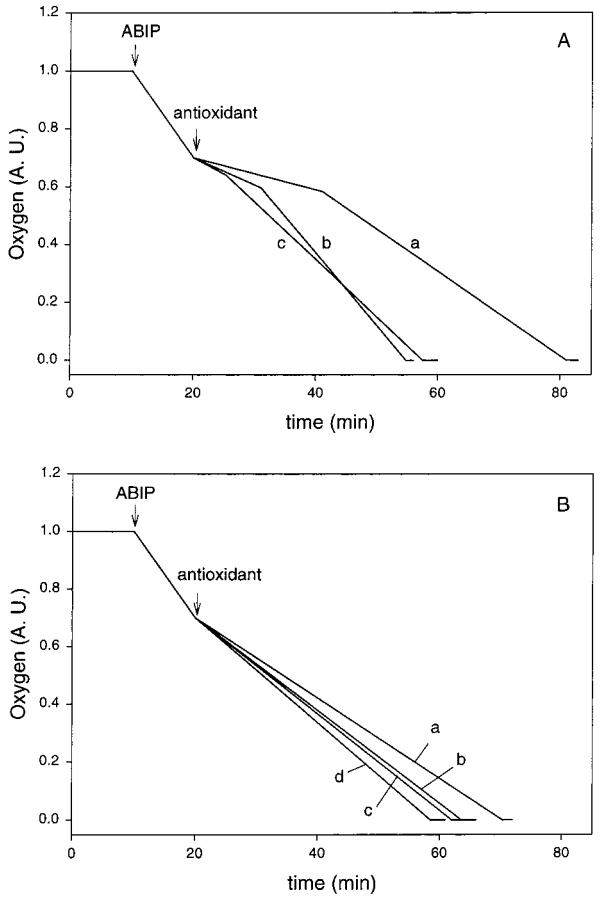

Figure 2. Representative traces of oxygen uptake of inhibited autoxidation of linoleic acid: (A) curves a, b, and c were obtained in the presence of 10 μM malvidin 3-monoglucoside, trolox, and quercetin, respectively; (B) curves a, b, c, and d were obtained in the presence of 10 μM caffeic acid, catechin, gallic acid, and myricetin, respectively. For experimental conditions, see Figure 1.

complete disappearance of O_2 (Figure 2B). In this second case the decrease in the rate of O_2 consumption appears to be more sensitive to the amount of the antioxidant added to the test solution. Differences of antioxidant inhibition patterns have already been suggested by Roginsky et al. (1996).

It has also been already reported that assessing antioxidant activity is problematic because the results are strongly dependent on the method used (Baderschneider et al., 1999). The different time courses of oxygen consumption of the compounds belonging to class I or class II demonstrate that accurate kinetic studies are strongly recommended to set up appropriate methods for the estimate of the radical scavenging capacity of wines. In particular, methods that are based on a one-point measurement can give rise to different results. The choice of appropriate standards for the calibration could be critical to obtain reliable results. In the case of our method, it is evident that the choice of the correct standard having a behavior similar to that of wine, such as gallic acid or catechin (Figure 2B) but not tocopherol or ascorbic acid (Figure 2A), is advisable.

Under the experimental conditions reported above, the red wines we have tested showed a gallic acid-like behavior. Only in a few cases, and particularly after the addition of a small volume of wine to the test solution, did the rate of O_2 consumption fail to be constant with time. In the case of Teroldego wine we observed a

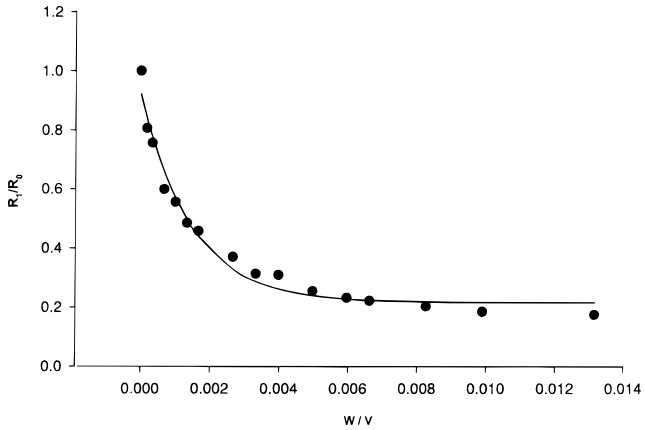

Figure 3. Initial rates of oxygen uptake in the presence of an increasing volume of red wine: (continuous curve) exponential fitting to the experimental data. For experimental conditions, see Figure 1; for composition of the wine, see the last line of Table 2, Teroldego).

behavior in some aspects similar to that of class I antioxidants, which could be due to the presence in this variety of high levels of malvidin 3-monoglucoside (Mattivi and Nicolini, 1997). In particular, the rate of oxygen uptake, which decreases following the addition of wine, shows a small recovery after a period of few minutes (<3−4 min) from the addition. This behavior was observed only in the presence of an amount of wine <0.1% v/v. With an increasing amount of wine, the rate of oxygen uptake further decreases and assumes a constant value throughout all of the kinetic run. This behavior could be explained by the presence in the wine of the two classes of antioxidants, the concentration of which control the time course of oxygen consumption. In Figure 3 we report the ratio R_1/R_0 versus the ratio w/v, where R_0 and R_1 are the rates of oxygen uptake measured before and after the addition of wine and w and v are the volumes of wine added to the test solution and the total volume of the test solution after the wine addition, respectively. The experimental data of Figure 3 provided an excellent fit ($r > 0.99$) to the equation

$$R_1/R_0 = K + (1 - K)\, e^{-(w/v)/(w_a/v)} \qquad (1)$$

where K is a constant corresponding to the relative rate of O_2 consumption extrapolated at a high concentration of wine, which value was found to be ~0.2 for all of the red wines we tested. The parameter w_a is the amount of wine required to decrease the ratio R_1/R_0 from 1 to $K + (1 - K)/e$, that is, from 1 to 0.494.

The value of w_a is highly variable and inversely correlated to the content of antioxidant of the wine. Therefore, on the basis of eq 1 we defined the "peroxy radical scavenging constant" of wine as $C_w = (w_a/v)^{-1}$ and therefore

$$R_1/R_0 = K + (1 - K)\, e^{-C_w(w/v)} \qquad (2)$$

The behavior reported in Figure 3 is characteristic for the red wines we tested, and the fitting of the experimental values to eq 1 was always satisfactory (r values > 0.97). The values of the radical scavenging constant of the various wines were calculated in duplicate (standard deviation = ±12%) and were in the range of 273−1011, corresponding to values of w_a/v in the range of 0.0037−0.00099 (Table 1).

Table 1. Content and Characteristics of Various Antioxidants in Red Wines Produced with Different Amounts of Seed during the Fermentation Process

variety	seeds	C_w[a]	S_{GA}[b]	total phenols[c]	proantho-cyanidins[d]	index of vanillin[e]	total antho-cyanins (mg/L)
Pinot noir	without	273	4.9	567	351	195	112
	control	458	8.3	1975	1314	1361	117
	double	824	14.9	2891	2623	2488	160
Enantio	without	366	6.6	1273	1083	434	195
	control	570	10.3	1963	1913	1168	294
	double	801	14.5	2771	2604	1795	307
Enantio	without	456	8.2	1300	1200	512	209
	control	666	12.1	2063	2027	1112	255
	double	1011	18.3	2931	2781	1788	330

[a] Peroxy radical scavenging constant = (v/w_a), see text. [b] Peroxy radical scavenging capacity, gallic acid equivalent (mM). [c] Gallic acid, mg/L. [d] Cyanidin chloride, mg/L. [e] (+)-Catechin, mg/L.

Table 2. Antioxidant Capacity and Content of Antioxidants of Red Wines from Three Grape Varieties (Enantio, Teroldego, and Pinot Noir)

variety	year	C_w[a]	S_{GA}[b]	total phenols[c]	proantho-cyanidins[d]	index of vanillin[e]	total antho-cyanins (mg/L)	polymerization index[f]
Pinot noir	1995	458	8.3	1975	1314	1361	117	1.04
Pinot noir	1994	355	6.4	1037	669	416	57	0.62
Pinot noir	1994	533	9.7	1503	1066	800	70	0.75
Pinot noir	1994	669	12.1	2529	2180	1741	65	0.80
Enantio	1995	570	10.3	1963	1913	1168	294	0.61
Enantio	1995	666	12.1	2063	2027	1112	255	0.55
Teroldego	1994	480	8.7	1663	1325	414	246	0.31
Teroldego	1994	633	11.5	2012	1992	676	317	0.34
Teroldego	1994	651	11.8	2352	2432	872	443	0.36

[a] Peroxy radical scavenging constant = (v/w_a), see text. [b] Peroxy radical scavenging capacity, gallic acid equivalent (mM). [c] Gallic acid, mg/L. [d] Cyanidin chloride, mg/L. [e] (+)-Catechin, mg/L. [f] Ratio vanillin assay/proanthocyanidins.

The plot of R_1/R_0 versus the concentration of the antioxidant in the test solution was fitted according to eq 2, for gallic acid (GA) and also catechin (CAT) which follow a class II behavior. The "radical scavenging constants" of the antioxidants, C_{GA} and C_{CAT}, respectively, were defined as the reciprocal of the concentration of these antioxidants in the text solution required to decrease the ratio R_1/R_0 from 1 to $K + (1 - K)/e$. Under our experimental conditions we found $C_{GA} = 55.2$ mM^{-1} and $C_{CAT} = 67.0$ mM^{-1}, respectively. In particular, in the case of gallic acid, a C_{GA} of 55.2 mM^{-1} corresponds to a concentration of 0.0181 mM gallic acid in the test solution. Furthermore, it should be noted that K values of ~0.2 were measured both for the wines we have tested (Figure 3) and for GA and CAT.

On the basis of the rate of the production of radicals from ABIP (kinetic decomposition constant, $k_d = 2.44 \times 10^{-4}$ min^{-1} at 37 °C) and of the lag times we have measured in the experiments using trolox as an inhibitor of linoleic acid peroxidation, an efficiency of 0.3 was calculated according to Pryor et al. (1993). This means that only 30% of the radicals generated in the decomposition of ABIP escape from the solvent cage (Frost and Pearson, 1961) and are involved in the peroxidation of linoleic acid. It therefore appears that, in the presence of linoleic acid and in the absence of an antioxidant, the radical chain length of peroxidation involves about six molecules of O_2 for each primary radical escaping from the cage. Furthermore, the strong effect of red wines on the rate of oxygen consumption that we have observed only in the presence of linoleic acid indicates the ability of the antioxidants present in the red wines to scavenge the peroxy radicals and not the radicals generated in the decomposition of ABIP.

The similar behaviors of gallic acid and red wine that we observed (see Figures 1 and 2) indicate that the lipid peroxidation-inhibiting potential of red wine is mainly due to antioxidants with class II behavior. The correspondence between C_{GA}^{-1} and C_w^{-1} can be conveniently used for the definition and the calculation of the "peroxy radical scavenging capacity of wine" (S_{GA}), expressed as the content of gallic acid or gallic acid equivalent: In fact, the amounts C_{GA}^{-1} and C_w^{-1} decrease the ratio R_1/R_0 from 1 to $K + (1 - K)/e$ and as a consequence the S_{GA} can be easily calculated according to the following equation:

$$S_{GA}(w_a/v) = 0.0181 \text{ mM}$$

that is

$$S_{GA}\,(\text{mM}) = 0.0181\,(v/w_a) = 0.0181\,C_w$$

The peroxy radical scavenging capacities calculated for the various wines we tested are reported in Tables 1 and 2.

Correlation between Peroxy Radical Scavenging Capacity and Chemical Composition of Wine. To investigate which classes of polyphenols were correlated with the peroxy radical scavenging capacity, different spectrophotometric methods were applied to the characterization of polyphenols of experimental red wines. The Folin−Ciocalteu index is the most widely used method to evaluate the global content of polyphenols. Catechins and proanthocyanidins in wine are of primary interest because they are the polyphenols present in higher concentrations in most red wines (Kennedy and Waterhouse, 2000) and because they all show the important feature of the presence in their structure of one or more catechol moieties, a key factor in determining the scavenging activity (Roginsky et al., 1996).

The proanthocyanidin assay is a very specific reaction based on conversion to anthocyanidins by means of autoxidation following acid-catalyzed cleavage of the interflavonoid bonds (Porter et al., 1986). The numerical value of this assay increases with the degree of polymerization of the proanthocyanidins, because only the "upper" unit may yield a carbocation, capable of producing cyanidin, and polymer chains are built up by the addition of further "upper" units.

The vanillin index provides an estimate of the number of free carbons C_6 and C_8 of both catechins and proanthocyanidins. This index decreases with the increase of the degree of polymerization because many of the C_6 and C_8 are involved in the polymerization step. Therefore, the result of the vanillin assay divided by the proanthocyanidin assay is a ratio that provides a rough estimate of the degree of polymerization of the flavanols mainly dependent upon the cultivar and the chemical age of wine (Ribereau-Gayon and Stonestreet, 1966). High values of this ratio are characteristic for the Pinot noir wines (Mattivi et al., 1991), a variety proved to have the largest amounts of monomeric tannins, (+)-catechin and (−)-epicatechin (Goldberg et al., 1998).

A first experiment was set up to study the effect of different amounts of seeds during fermentation (three different treatments) on the composition and S_{GA} of wines. The Pinot noir and Enantio wines fermented in the presence of different amounts of seeds had very different amounts of catechins and proanthocyanidins (Table 1). In agreement with the literature, the large majority of proanthocyanidins of Pinot noir are extracted from the seeds (Bourzeix et al., 1986; Nicolini et al., 1998). Our results, based on the increasing values of the ratio of vanillin assay divided by the proanthocyanidin assay when seeds are present (Table 1), indicated that grape seeds are also an important contributor of low molecular weight proanthocyanidins in wine. This is in very good agreement with recent literature (Labarbe et al., 1999).

The higher concentration of tannins extracted from seeds enhanced the concentration of red pigments formed by condensation between tannins and anthocyanins, which are more stable in time than free anthocyanins, thus increasing the final color of wines (Nicolini et al., 1998). Besides their participation to the formation of red pigments, the tannins extracted from seeds are expected to enhance the red color due to their copigment effect (Mazza and Miniati, 1993). A one-way analysis of variance (randomized block design) indicated that the presence of different amounts of seeds during the fermentation caused a very significant ($p < 0.001$) effect on the S_{GA}. The removal of seeds during the fermentation process reduced the S_{GA} by 32−41% with respect to control wines, whereas doubling the seeds increased the S_{GA} from 41 to 80% for the different trials (see Table 1). The effect was more evident in the case of Pinot noir, where the majority of tannins were extracted from seeds. It is likely that the proanthocyanidins extracted from seeds make a major contribution to the peroxy radical scavenging capacity of wines. As expected, the S_{GA} correlated very strictly with the value of the proanthocyanidins ($r = 0.968$). As a consequence of the particular wine-making technique, the two spectrophotometric indices used to quantify the wine tannins (the proanthocyanidins assay and the index of vanillin) were significantly self-correlated in the first ($r = 0.891$)—but not in the second ($r = 0.501$)—set of wines. The wines

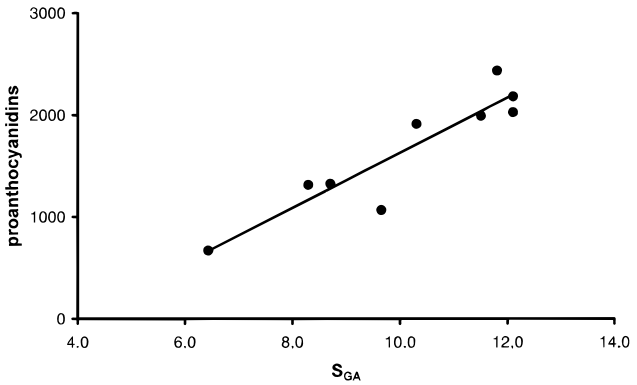

Figure 4. Correlation between the radical scavenging constant and the content of proanthocyanidins in various wines. For composition of the wines, see Table 2.

obtained with the standardized wine-making protocol (Table 2) were characterized by different amounts of total polyphenols and also by differences in the chemical classes of phenolics. In particular, more red pigments were present in the Teroldego and Enantio wines by comparison with Pinot noir wines, and the proportion of low molecular weight tannins reactive to vanillin was higher in the Pinot noir wines. The S_{GA} values were significantly correlated with the amounts of proanthocyanidins ($r = 0.921$, Figure 4) and total polyphenols ($r = 0.848$), but not with the total anthocyanins ($r = 0.508$) and, interestingly, not with the index of vanillin ($r = 0.495$). The linear regression with the proanthocyanidins accounted for 82.7% of the percentage variance of the S_{GA} values ($p < 0.001$) and was more predictive than the corresponding regression with the total polyphenols, which accounted for 67.9% of the variance.

The values of the proanthocyanidin assay and of the index of vanillin refer to the same class of compounds, all characterized essentially by the same flavonoid skeleton with 3′,4′-catechol moieties in ring B, three hydroxys at the 3-, 5-, and 7-positions, and a single bond at the 2,3-position. By similarity with the catechin, they are expected to be class II antioxidants. Because the different results of the two spectrophotometric methods are influenced by the degree of polymerization of the tannins, the fact that the chemical classes of the catechin and of the low molecular weight proanthocyanidins reactive to vanillin did not correlate with the S_{GA} values, whereas the proanthocyanidin assay was closely correlated, suggests an important increase of the peroxy radical scavenging capacity for polyphenol oligomers as observed by Arteel and Sies (1999) and deserves further studies.

By the method we set up it was possible to demonstrate that the capacity of red wine to scavenge $ROO^{\bullet}$ radicals is due mainly to the content of proanthocyanidins and total polyphenols. We compared the wine behavior to that of a well-characterized antioxidant (trolox), and we found a remarkable difference. In light of the different behaviors of red wine (absence of lag phase) and class II antioxidants, the use of trolox as a reference compound to quantify the radical scavenging capacity of wine (Ghiselli et al., 1998) could yield misleading results, in some cases. However, more detailed kinetic studies are advisable to explain these differences in terms of the antioxidant reaction mechanism.

Finally, the proposed method appears to be suitable for application to the study of the effect of the wine-making processes on the peroxy radical scavenging capacity of wines.

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