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Stable Free Radicals and Peroxyl Radical Trapping Capacity in Red Wines

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Thirty-two experimental red wines, obtained from eight cultivars and aged in bottles for 2 and 7 years, were examined for the presence of stable free radicals (SFR), for the peroxyl radical trapping capacity (PRTC), and for the concentrations of some important polyphenol families. Aging significantly increases SFR, polyphenol polymers with $n \geq 5$ (HMWP), and PRTC and is accompanied by a strong decrease of free anthocyanins. Multivariate regression analyses show that HMWP and SFR are independently associated with PRTC while HMWP and anthocyanins are independently associated with the formation of SFR. These results indicate that polymeric polyphenols generated from anthocyanins and proanthocyanidins during wine aging are able to convert highly reactive free radicals into nonreactive radicals through electron delocalization. The strict correlation between SFR and antioxidant activity that we found suggests that these characteristics are related to the functional properties of food.

KEYWORDS: Free radical; antioxidant activity; polyphenol polymers; lipid peroxidation; proanthocyanidins; anthocyanins

INTRODUCTION

The superior antioxidant properties of red wine are well-known (*1*−*3*). These properties are mainly due to the presence of a large amount of polyphenols, with anthocyanins and flavanols the major families in red wine (*4*). Among the flavanols are (+)-catechin, (−)-epicatechin, and flavan-3,4-diols. The latter compounds are not found as such in grape or wine but undergo condensation reactions, giving rise to oligomeric proanthocyanidins (PROC) and to their polymers. PROC have been shown to have a high free radical scavenging potential (*5*, *6*), which is supposed to be correlated to antioxidant capacities (*7*). Epicatechin oligomers were demonstrated to be particularly efficient at protecting against oxidation reactions (*8*). The free radical scavenging potential was also associated with oligomerization following nucleophilic addition of o-quinones formed by initial semiquinone disproportionation (*9*). Furthermore, it has been demonstrated that at the low pH of wines, anthocyanins and PROC react with the formation of adducts, which can be oxidized to large conjugated structures such as xanthylium salts (*10*).

It has been observed that red wines, but not white wines, show a stable electron spin resonance (ESR) signal characterized by a single resonance assignable to a free radical (*11*, *12*). This resonance becomes more intense with wine aging, and we suggested that it is due to stable semiquinones (*11*). The presence of this ESR signal supports the hypothesis that relatively stable free radical species are involved in the aging of red wine and can play a role in its antioxidant properties. Moreover, a relatively high concentration of stable free radicals (SFR) was also found in beverages with strong antioxidant properties such as coffee (*13*) or fresh orange juice (unpublished results). The relationships between the presence of SFR and the antioxidant properties of these beverages are still lacking, although the stability of phenoxyl radicals generated by polyphenols is considered one of the most important parameters governing their antioxidant activity (*14*).

In this paper, the content of some families of polyphenols present in red wines was measured and compared to the concentration of SFR and to peroxyl radical trapping capacity (PRTC) with the aim of obtaining information on correlations between PRTC and SFR and between these properties and the compositional variables.

MATERIALS AND METHODS

Reagents. The reagents were purchased from Fluka (Buchs, Swiss) and were of the highest available quality. The 2,2′-azobis[2-(2-imidazolin-2-yl)propane] (ABIP) was a kind gift of Wako Chemicals (Germany).

Aqueous solutions were prepared with doubly distilled water and when necessary were passed throughout a column of Chelex-100

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(Biorad, Richmond, CA), to minimize the concentration of heavy metal ions. Malvidin-3,5-diglucoside chloride (standard, chromatography grade) was purchased from Carl Roth (Germany).

Wines. Red wines from grape cultivars having different amounts of polyphenols were produced in the experimental winery of IASMA (Trento, Italy) through a traditional skin−contact technique. The wines were then subjected to natural malolactic fermentation, settled, filtered, bottled under a nitrogen atmosphere (5 months after vinification), capped with airtight screwcaps, and stored at cellar temperature until the time of the analysis. The wines were produced from each of the following eight cultivars: Teroldego (Ter), Enantio (En), Lagrein (Lg), Cabernet Sauvignon (Cs), Marzemino (Mz), Merlot (Mt), Pinot noir (Pn), and Schiava (Sc). The experimental measurements were performed on 16 wines (two wines for each cultivar) bottled in 1993 and on 16 wines bottled in 1998, after 7 and 2 years of aging, respectively.

PRTC Test. The PRTC of wines was measured according to the method of Wayner et al. (*15*) with some modifications (*6*). This method reproduces in vitro the free radical-induced lipid peroxidation process, which is monitored by electrochemical measurement of oxygen consumption. The standard assay solution contains 2.5 mM linoleic acid and 50 mM sodium dodecyl sulfate in 20 mM phosphate at pH 7.4, equilibrated with atmospheric oxygen. A constant free radical flux is induced by thermal decomposition of 8 mM ABIP, which at the temperature of the test (37 °C), occurs with a kinetic rate constant of 2.44×10^{-4} min^{-1} (*6*). A typical kinetic run shows that the oxygen consumption rate in the assay solution is inhibited by red wine to a much lower rate. This inhibition lasts for a short time (lag time, LT) after which the oxygen consumption rate increases again. From LT values and from the rate of generation of free radicals (ROO$^{\bullet}$), which effectively trigger lipid peroxidation, and taking into account the wine dilution in the assay solution (usually 0.001 v/v), we calculated the amount of peroxyl radical trapped by wine.

Chemical Analysis of the Antioxidants Present in the Red Wines. *Spectrophotometric Methods.* Under the optimized conditions described by Rigo et al. (*6*), total phenols (TP) were directly measured by the Folin−Ciocalteu reagent while PROC were indirectly determined by their transformation into cyanidin. Total anthocyanins (TA) were determined on the basis of their maximum absorbance in the visible range (520 nm), in ethanol−water−HCl 70:30:1, and quantified on the basis of a calibration curve with malvidin-3,5-diglucoside chloride.

Normal Phase High-Performance Liquid Chromatography (HPLC) Method. The high molecular weight phenolics of wine were characterized by means of the normal phase HPLC method of Kennedy and Waterhouse (*16*), under the conditions suggested by Vrhovsek et al. (*17*). By integrating two areas of the chromatogram at 280 nm separately, the values corresponding to PROC from 2 to 4 units of low molecular weight polymers (LMWP) and to PROC formed by 5 or higher units of high molecular weight polymers (HMWP) were obtained. The latter amount was defined as the polyphenol polymer content of wines. A cacao bean proanthocyanidin extract was injected at the beginning and at the end of each sequence (of about eight wine samples) in order to calibrate the retention times of different oligomers. Quantitative data were calculated on the basis of a calibration curve, with the external standard method, and expressed as epicatechin, mg/L.

Reversed Phase HPLC Method. An aliquot (5 to 20 mL) of wine sample, diluted four times with water, was applied to a C18-SPE cartridge (1 g, Waters), previously activated with methanol (5 mL) and water (10 mL). The cartridge was washed with 6 mL of 0.3% $HClO_4$ in distilled water and then eluted with 10 mL of methanol into a 100 mL pear-shaped flask. The eluate was evaporated under reduced pressure at 35 °C and reconstituted in 1 mL of diluted methanol (27% in water and 0.3% $HClO_4$). The final samples were filtered through 0.22 μm, 13 mm PTFE syringe tip filters (Millipore, Bedford, MA) into LC vials and immediately injected into a 1090 HPLC equipped with a diode array detector (Agilent, Waldbronn, Germany). Separation of the 15 main free anthocyanins (FA) was obtained at 40 °C on a Purospher RP18 column, 250 mm × 4 mm, 5 μm, with a precolumn (Merck, Germany). The flow was 0.45 mL/min, eluent A was 0.3% $HClO_4$ in distilled water, and B was methanol. The following binary gradient was applied, from 27 to 43% of B in 32 min, then to 68.5% of B in 13 min, to 100% B in 2 min, and isocratic 100% B for 3 min; total analysis time, 50 min. Delphinidin 3-glucoside, cyanidin 3-glucoside, petunidin 3-glucoside, peonidin 3-glucoside, malvidin 3-glucoside, and their relevant acetic acid and *p*-coumaric acid esters were identified according to Castia et al. (*18*) and quantified at 520 nm with a calibration curve with malvidin-3,5-diglucoside chloride. The sum of these 15 compounds gives the total amount of FA.

Table 1. Polyphenol Content, SFR, and PRTC of Some Typical Italian Red Wines

	years of aging	PRTC[a]	SFR[b]	TP[c]	PROC[d]	LMWP[e]	HMWP[e]	TA[f]	FA[f]
Ter	7	13.5	45	2839[g]	2892[g]	544[g]	2406	308	36
Ter	2	7.7	12.5	1467[g]	1133[g]	492[g]	1069[g]	378	280
En	7	13.4	38.5	2678	2627[g]	544[g]	2303	168	19
En	2	13.7	16.0	2412	2201[g]	647[g]	1889[g]	319	230
Lg	7	8.0	34.0	1768	1738	507	1384[g]	203	26
Lg	2	8.2	9.5	1757	1347	536	1308	372	305[g]
Cs	7	11.4	32.0	2163	2715	466	2034	209	25
Cs	2	8.1	10.0	1803	1711	502[g]	1228	234	190
Mz	7	5.6	23.0	1365	1145	429[g]	1295	162	22
Mz	2	6.3	9.0	1584	1322	589	1184	321	219
Mt	7	10.3	17.5	1601	1380	475	1540	167	24
Mt	2	7.1	10.0	1481	1179	535	1179	300	255
Pn	7	7.3	13.0	2040	1546	695	1650	66[g]	5
Pn	2	5.8[g]	5.5	1830[g]	1225[g]	614[g]	1269[g]	109	83
Sc	7	nm	8.0	1238	923	269[g]	1150	47	5
Sc	2	nm	0.0	919	621	350	561	70	65

[a] mM. [b] nM. [c] Catechin, mg/L. [d] Cyanidin, mg/L. [e] Epicatechin, mg/L. [f] Malvidin 3,5-diglucoside chloride, mg/L. [g] The average value reported differs between 20–25% from the two wines values.

ESR Method. Samples of the red wines were transferred directly from the bottle into the ESR cell by a glass transfer line, under a positive argon atmosphere, to avoid contact with atmospheric oxygen. The concentration of SFR was calculated by normalizing the spectra to Mn^{2+} as an internal standard. In particular, a small sealed vial containing MnO-doped CaO was fixed on the exterior surface of the quartz flat cell. The absolute concentration of radicals was calculated by calibration of Mn^{2+} signal using 2,2,6,6-tetramethylpiperidinoxyl (*11*).

Statistics. Univariate relations were assessed by the Pearson linear correlation method (*19*). Multiple linear regression analyses were used to identify significant independent predictors for PRTC and for SFR concentration, with the selection made on the basis of a stepwise regression procedure. The results were considered significant at $P < 0.05$. In order for linear regressions to be applied, an analysis of residuals was previously performed to verify normal distribution of the data. All statistical analyses were performed on IBM compatible Personal Computer using Statistica 5.1 software (Statsoft, Tulsa, OK).

RESULTS

PRTC, SFR Concentration, and Polyphenol Content in Red Wines. Thirty-two red wines, 16 aged for 2 years and 16 aged for 7 years, obtained from eight grape cultivars, were considered. For each cultivar and vintage year, two wines produced by grapes from different vineyards were analyzed for PRTC, SFR, and the content of some classes of red wine polyphenols. The results obtained are presented in Table 1 as the average of the values obtained analyzing the two wines from different vineyards. In general, the influence of vineyard was usually small. In fact, only in a few cases, denoted by footnote *g*, the differences between the wines were between 20 and 25% of the average value reported in Table 1. These differences appeared related to TP content, which depends on the characteristics of the vineyard (sun exposure, microclimate, soil, etc.) and of the vintage. The wines chosen for the trial, made from grape varieties ranging from low to high polyphenols content (*20*) and including both young and aged wines, showed a wide

Table 2. Summary Statistics of Studied Red Wines after 2 or 7 Years of Aging[a]

	aging		
variable	2 years[b]	7 years[b]	*P*
TP[c]	1656 ± 492	1957 ± 646	0.1133
PROC[c]	1348 ± 550	1871 ± 820	0.0321
TA[c]	262 ± 118	166 ± 81	0.0001
FA[c]	202 ± 89	20 ± 11	<0.0001
HMWP[c]	1211 ± 423	1720 ± 504	0.0018
LMWP[c]	533 ± 112	491 ± 131	0.2398
SFR[d]	9.1 ± 5.0	26.4 ± 13.2	<0.0001
PRTC[e]	7.11 ± 3.9	8.70 ± 4.42	0.0481

[a] Differences were considered significant at $P < 0.05$. [b] Mean values ± standard deviation. [c] mg L^{-1}. [d] nM. [e] mM.

range of values for the compositional variables. This allowed us to study the correlations between biochemical and composition parameters of wines.

When the various cultivars and the aging process are considered, a very strong variability of SFR and of FA concentrations (about 1 order of magnitude) and a large variability of all of the other characteristics (by a factor ranging from 2 to 5) were found; see Table 1. As regards SFR, it must be remarked that in all of the tested wines an ESR signal, assignable to a free radical centered at $g = 2.0037 \pm 0.0002$, was found. The spectral characteristics of this signal (resonance field and line shape) were as described in a previous paper by Rossetto et al. (*11*), where the ubiquitous presence of a stable free radical, both in commercial and in experimental red wines, was reported. As found in commercial samples of red wines (*11*), the lowest ESR signals were observed in the Sc and Pn wines, that is, in varieties having the lowest concentration of red pigments. Moreover, because of the low inhibition efficiency of lipid peroxidation of the Sc cultivar, it was not possible to calculate a PRTC value for this wine.

From the data of Table 1, it appears that PROC are the largest class of polyphenols present in the studied red wines, while the second largest class is represented by anthocyanins, which range from a few percent to 25% of TP. Furthermore, the data reported in Table 1 clearly show that 70−95% of TP are present as polymeric aggregates (HMWP).

Dependence of Wine Characteristics on Aging. To evaluate the dependence of wine characteristics on aging, average values, standard deviations, range, and significance of PRTC, SFR, and some polyphenol families of the studied wines are reported in Table 2, according to the aging period.

The aging process appears to be significantly related ($P < 0.05$) with an increase of PRTC (on average 22%), of PROC (on average 39%), of HMWP (on average 42%), and of SFR (about three times) content and with a decrease of both FA (about 1 order of magnitude) and TA (to 63%). No significant variations of TP and of LMWP concentration appear to occur during wine aging.

Linear Regressions between PRTC or SFR and Composition Variables. On the basis of the above-discussed dependence of wine characteristics on the aging process, the studied red wines were classified into three groups: the pooled wines (2 and 7 years of aging) and the wines characterized by 2 and 7 years of aging, respectively.

For these three groups of wines, univariate relationships between PRTC or SFR and polyphenol composition were tested; see Tables 3 and 4, respectively.

In all three groups of wines, PRTC was correlated with TA ($P < 0.004$) and in particular with TP, PROC, HMWP, and SFR ($P < 0.0002$) concentrations. In particular, for all three groups of wines, high correlation coefficients ($r^2 \geq 0.64$) were found between PRTC and TP, between PRTC and PROC, between PRTC and HMWP, and, in the case of the wines aged for 2 and 7 years, between PRTC and SFR. In the pooled wines, PRTC was not related to FA while it was related to LMWP.

Table 3. Univariate Linear Regression Analyses between PRTC and Composition Variables in Red Wines

	aging								
	2 and 7 years			2 years			7 years		
variable	R^2	*t*	*P*	R^2	*t*	*P*	R^2	*t*	*P*
TP	0.64	7.34	<0.0001	0.74	6.38	<0.0001	0.56	4.24	0.0008
PROC	0.66	7.57	<0.0001	0.74	6.25	<0.0001	0.63	4.85	0.0003
TA	0.26	3.27	0.0027	0.46	3.43	0.0040	0.53	3.95	0.0014
FA	0.01	0.52	0.6091	0.36	2.83	0.0134	0.49	3.65	0.0026
HMWP	0.65	7.48	<0.0001	0.79	7.20	<0.0001	0.63	4.92	0.0002
LMWP	0.29	3.49	0.0015	0.48	3.61	0.0028	0.26	2.23	0.0423
SFR	0.47	5.17	<0.0001	0.78	7.06	<0.0001	0.63	4.90	0.0002

Table 4. Univariate Linear Regression Analyses between SFR and Composition Variables in Red Wines

	aging								
	2 and 7 years			2 years			7 years		
variable	R^2	*t*	*P*	R^2	*t*	*P*	R^2	*t*	*P*
TP	0.42	4.66	0.0001	0.50	3.89	0.0016	0.46	3.46	0.0038
PROC	0.56	6.17	< 0.0001	0.61	4.72	0.0003	0.55	4.14	0.0010
TA	0.02	0.79	0.4381	0.55	4.10	0.0011	0.69	5.41	0.0001
FA	0.18	2.54	0.0167	0.42	3.22	0.0061	0.62	4.97	0.0002
HMWP	0.55	6.05	<0.0001	0.62	4.74	0.0003	0.41	3.11	0.0076
LMWP	0.01	0.63	0.5339	0.35	2.71	0.0168	0.06	0.98	0.3422

The SFR concentration in the cases of wines aged for 2 and 7 years was strongly correlated to TP, PROC, HMWP, TA, and FA. However, when all of the wines were considered together, the SFR concentration appears to still be related to the above-reported variables with the exclusion of TA. This behavior should be due to the strong dependence of anthocyanin concentration on aging. Considering the two vintages separately, the correlation of SFR with TA was positive and highest in the wines aged for 7 years, where the large majority of TA are combined in condensed pigments. SFR and LMWP appear not well-correlated.

Considering univariate relations among composition variables in the whole wine sample, high and significant correlation coefficients were found between the following pairs of variables: TP and PROC ($r^2 = 0.83$), TP and HMWP ($r^2 = 0.77$), and PROC and HMWP ($r^2 = 0.79$). In the case of wines aged for 2 and 7 years, a good correlation was also found between TA and FA ($r^2 = 0.83$). These high correlation coefficients indicate a strong interdependence between the above considered variables.

Multiple Regression Analyses. Multiple regression analyses were performed to assess the independent association of PRTC and of SFR in all three groups of wines with potential determinants including TP, TA, FA, and HMWP; see Tables 5 and 6, respectively.

From Table 5, it appears that HMWP and SFR are independently associated with PRTC when the wines aged for 2 and 7 years are considered separately, while when these wines are considered as a single group, HMWP and TA concentrations are independently associated with PRTC.

As regards SFR concentration, from Table 6, it results that HMWP and TA are independently associated with SFR when

Table 5. Multiple Linear Regression Analyses with PRTC as the Dependent Variable in Red Wines, Considering TP, TA, FA, SFR, and HMWP as Potential Determinants

years of aging	independent predictor	*t*	*P*	R^2	*F*	SE	*P*
2				0.88	47.00	1.45	<0.001
	HMWP	3.23	0.0065				
	SFR	3.12	0.0081				
7				0.77	21.92	2.27	<0.0001
	HMWP	2.82	0.0144				
	SFR	2.80	0.0150				
2 and 7				0.79	55.22	1.97	<0.0001
	HMWP	8.59	<0.0001				
	TA	4.43	0.0001				

Table 6. Multiple Linear Regression Analyses with SFR Concentration as the Dependent Variable in Red Wines, Considering TP, TA, FA, and HMWP as Potential Determinants

years of aging	independent predictor	*t*	*P*	R^2	*F*	SE	*P*
2				0.79	24.94	2.43	<0.0001
	HMWP	3.95	0.0017				
	TA	3.33	0.0054				
7				0.68	29.26	7.76	0.0001
	TA	5.41	0.0001				
2 and 7				0.80	37.73	6.17	<0.0001
	HMWP	2.32	0.0279				
	TA	5.97	<0.0001				
	FA	5.32	<0.0001				

the wines aged for 2 and 7 years are considered separately. Furthermore, in the pooled wines, FA must also be considered as an independent predictor. It should be noted that when LMWP was added to the potential determinants (TP, TA, FA, and HMWP) to assess independent association of PRTC and of SFR, no change with respect to the associations reported in Tables 5 and 6 occurred, except in the case of the dependence of SFR in the pooled wines. In this case, HMWP (with a positive coefficient) and LMWP (with a negative coefficient) result as independent predictors of SFR.

DISCUSSION

Among the results obtained in this study, we note the large amount of polyphenol polymers generated in wine aging, up to 95% of TP in some wines after 7 years of aging, and the high increase of SFR with aging. This behavior is accompanied by a decrease of FAs by about 1 order of magnitude and is in agreement with literature reports indicating that during aging a progressive polymerization of polyphenols occurs involving flavanols and anthocyanins (*21*, *22*). This polymerization process causes the disappearance of FA from wine, while extensive modification of anthocyanins during the polymerization process leads to a decrease of TA since the flavilium form of the anthocyanins is partially lost in some of the condensation products.

From this study, it appears also that there are (i) strong and positive correlations of PRTC with HMWP and with SFR, in addition to the expected correlations of PRTC with the concentrations of TP, PROC, and TA, see Table 3, and (ii) strong and positive correlations of SFR with HMWP, PROC, and TA, see Table 4.

Some of these results agree with literature data (*23*, *24*), which show that antiradical activity during aging is strongly related to an increase in proanthocyanidin concentration. Moreover, the TA content appears to contribute to antiradical activity even if the concentration of these compounds is relatively low (*24*, *25*).

A clearer response was obtained by multiple regression analysis with SFR as a dependent variable. This analysis clearly indicates that TA is a good independent predictor in all three classes of wines; see Table 6. In other words, a high concentration of TA in wine is independently associated with the presence of SFR. This behavior strongly indicates that the SFR found in red wine are produced during the aging modifications occurring in wine, involving extensive polymerization of the anthocyanins with PROC. This polymerization process leading to the formation of SFR is supported by the fact that HMWP is an independent predictor of SFR in the case of the wines aged for 2 years and in the case of the pooled wines; see Table 6. Furthermore, when in the latter case LMWP was considered among the potential determinants, the concentration of SFR appears significantly related to the increase of HMWP and to the decrease of LMWP.

In the case of PRTC analyzed as a dependent variable, multiple regression analysis (see Table 5) indicates polymer concentration as an independent predictor. Furthermore, when the wines aged for 2 and 7 years are taken into consideration, SFR appears also as an independent predictor of PRTC. These data are in accord with the findings that during aging a progressive polymerization of polyphenols (in particular of anthocyanins and flavanols) occurs, leading to the formation of polymeric aggregates (*26*), which show unusual antioxidant properties (*5*). These species contain large conjugate structures, such as xanthylium salts (*21*, *22*), which may stabilize unpaired electrons throughout their delocalization over aromatic rings; therefore, they may contribute to the antiradical activity of this beverage (*27*).

The strong relationship between PRTC and SFR (see Table 3) is not a trivial consequence of similar dependence of these two parameters on wine characteristics (in particular on the polyphenol content of wines) since the multivariate analysis indicates SFR formation as an independent predictor of PRTC. To our knowledge, this is the first time that a strict correlation between the presence of SFR and the antioxidant activity in food has been assessed. This correlation must also have thermodynamic bases: the energetic of the one-electron capture of highly reactive free radicals by polyphenols becomes more favorable with the increase of stability of the generated polyphenol free radicals. This increase of stability should also correspond to a higher concentration of these species.

In conclusion, the analyses of the correlations found between the parameters associated with free radical scavenging properties of red wines, that is, between PRTC and SFR and between these properties and the content of the most important classes of antioxidants, confirm the superior role of PROC and of anthocyanins as precursors of polymeric species containing large unsaturated structures, leading to stabilization of free radicals through delocalization of unpaired electrons across these structures.

ABBREVIATIONS

SFR, stable free radicals; PRTC, peroxyl radical trapping capacity; Ter, Teroldego; En, Enantio; Lg, Lagrein; Cs, Cabernet Sauvignon; Mz, Marzemino; Mt, Merlot; Pn, Pinot noir; Sc, Schiava; FA, free anthocyanins; TA, total anthocyanins; TP, total phenols; PROC, proanthocyanidins; LMWP, low molecular weight polymers; HMWP, high molecular weight polymers.

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