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Radical-scavenging capacity of several Italian red wines

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Abstract

The main object of the present study was to investigate the correlation between the radical-scavenging powers (measured by evaluating the quenching of the stable 2,2-diphenyl-1-picrylhydrazyl radical) of seven red Italian wines from different geographical origins, and their contents of total phenols and of anthocyanins and proanthocyanins, as well as of other antioxidant components (ethanol, ascorbic acid, sulphur dioxide and glutathione).

All red wines tested in our study showed evident radical-scavenging properties. In particular the radical-scavenger efficiencies of these wines may be attributed, in a significant number at least, to their content of total phenols, and are affected by alcohol only in a marginal way. Furthermore, the antioxidant efficiency of red wines tested appears to be largely influenced by the proanthocyanidin level, with anthocyanins playing a minor role. Finally, ascorbic acid, thiol groups and SO_2 (antioxidant compounds present in red wines especially as consequence of the wine-making processes) play only a minor role in the radical-scavenging power of the red wines under investigation. In conclusion, the present findings support the results from several experimental and epidemiological studies, suggesting that the supply of antioxidant phenols through a moderate daily consumption of red wines may provide additional protection against *in vivo* oxidation of cellular biomolecules.

Keywords: Red wines; Proanthocyanidins; Anthocyanins; Phenols; Radical-scavenging

1. Introduction

Oxidative stress has been proposed to contribute to several human disease conditions, such as cancer, cardiovascular disease, aging and neurodegenerative diseases. Many natural and synthetic antioxidants have been demonstrated to induce beneficial effects on human health and disease prevention.

The moderate consumption of wine, especially red wine, is recently receiving more prominence due to a possible link with reduction in mortality from cardiovascular diseases, the so-called French paradox (Ferrieres, 2004; Goldfinger, 2003; Mukamal et al., 2003; Zilkens & Puddey, 2003). This beneficial effect of wine has been attributed to its content of phenolic compounds, which are well-known antioxidant compounds (Burns et al., 2000; Cuevas et al., 2000; Iijima, Yoshizumi, & Ouchi, 2002).

Wine polyphenols, which contribute to wine colour and to other sensorial characteristics of wines such as bitterness and astringency, comprise both flavonoids and non-flavonoids (Pérez-Magariño & González-San José, 2004; Thorngate, 1997). Flavonoids include flavonols (such as quercetin), flavan-3-ols (such as catechin), anthocyanins (such as malvidin-3-glucoside) and proanthocyanidins (which are oligomers or polymers of polyhydroxy flavan-3-ol units). Hydroxycinnamates and their conjugates represent the greatest part of the non-flavonoid phenolics found in wines, together with phenolic acids, phenolic alcohols and stilbenes.

Not all phenolic compounds possess the same biological activity, and phenolic composition in wines can be strongly affected, not only quantitatively, but also qualitatively, by grape cultivars, maturity degree of the grapes used,

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environmental factors, wine-making techniques and technological treatments (Jeandet, Bessis, Sbaghi, Meunier, & Trollat, 1995; Manzocco, Anese, & Nicoli, 1998; Pellegrini et al., 2000).

Furthermore wine contains alcohol and its protective effects against cardiovascular diseases seem also to be the related to the content of ethanol (Giugliano, 2000; Scott et al., 2000). The observed beneficial effect of alcohol cannot be explained by a uniform biochemical mechanism; in particular its role in oxidation processes, especially *in vivo*, is unclear (Belleville, 2002; Rodrigo & Rivera, 2002).

Besides being influenced by content of native antioxidants, the resistance to oxidation of wines is also function of the concentration of added antioxidants (Fogliano, Verde, Randazzo, & Ritieni, 1999; Manzocco, Mastrocola, & Nicoli, 1998; Oliveira, Silva Ferreira, Guedes de Pinho, & Hogg, 2002; Vaimakis & Roussis, 1996). In fact, additional antioxidants are used in wine-making, namely ascorbic acid, sulphur dioxide and glutathione. The antioxidant power of these compounds is currently a source of some controversy. The antioxidant power of sulphur oxide is frequently contested, and ascorbic acid can be an oxidation promoter at high concentrations; glutathione is a significant factor in must oxidation, since it traps caftaric acid quinones and acts as a general reducing agent. Furthermore, S-glutathione conjugates have been revealed as natural compounds of white and red wines (Peyrot Des Gachons, Tominaga, & Dubourdieu, 2002).

However, data concerning the relationship between the antioxidant activity of red wines and their content of specific and individual antioxidants are relatively limited. The main object of the present study was to investigate the correlation between the antioxidant activities of several red Italian wines, and their contents of total phenols and of specific antioxidant components, in particular anthocyanins, proanthocyanidins, ethanol, ascorbic acid, sulphur dioxide and glutathione.

2. Materials and methods

2.1. Wines

Seven Italian red wines from different geographical origins, were obtained from a local market. The wineries, countries, grape varieties (*Vitis vinifera* L.) and vintage of all wines are listed in Table 1.

To separate the non-alcoholic wine fraction, wine samples were dealcoholized in a rotary evaporator at room temperature for 4 h and then diluted to the original volume with distilled water.

2.2. Chemicals

Folin-Ciocalteau phenol reagent, ferrous sulfate heptahydrate, vanillin and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\cdot}$) were purchased from Sigma-Aldrich (Milan, Italy). Sulfuric acid, hydrochloric acid, methanol and ethanol were from Carlo Erba Reagenti (Milan, Italy). Sep-Pak Cartridge C18 were purchased from Millipore Corporation (Milford, Mass, USA). Malvidin 3,5-diglucoside chloride, gallic acid and (+)-catechin were from Extrasynthése (Genay, France).

2.3. Determination of total phenols

The amount of total phenols in the whole wines were determined according to the Folin-Ciocalteu colorimetric method (Di Stefano & Guidoni, 1989; Rapisarda et al., 1999; Spagna et al., 2002); total phenols were expressed as gallic acid equivalents (g/l). Each determination was performed in triplicate and repeated at least three times; results are expressed as means ± S.D.

2.4. Determination of total anthocyanins

In brief, an aliquot (5 ml) of wine was diluted with 0.5 M H_2SO_4 to obtain a final reading in the range of 0.3–0.6 AU and loaded on to 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; then 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 absorbance measured at 540 nm on a Shimadzu UV-1601 UV–visible spectrophotometer against a blank (ethanol/water/HCl 70:30:1) and expressed as malvidin 3,5-diglucoside chloride (mg/l) by means of its molar absorptivity (experimental value in ethanol/water/HCl

Table 1
Italian red wines

Wine	Country	Winery	Variety	Vintage
Badiola	Poggio alla Badiola (Tuscany)	Mazzei in Fonterutoli	Sangiovese	1998
Barbera D'Alba	Barolo (Piemont)	Marchesi di Barolo	Barbera	1998
Cabernet	Gambellara (Veneto)	Zonin S.p.A.	Cabernet Franc Cabernet Sauvignon	1998
Chianti Classico	Castellina in Chianti (Tuscany)	Cecchi & figli S.r.l.	Sangiovese Canaiolo nero Colorino Trebbiano toscano	1998
Cirò	Cirò Marina (Calabria)	Librandi A. & N. S.n.c.	Gaglioppo	1998
Donnafugata	Contessa Entellina (Sicily)	Donnafugata S.r.l.	Nero d'Avola	1998
Terre di Franciacorta	Capriolo (Lombardy)	Gualberto Ricci & figli	Cabernet Franc Cabernet Sauvignon Barbera Merlot Nebbiolo	1998

$\epsilon = 30{,}000$ at 540 nm). Each determination was performed in triplicate; results are expressed as means ± S.D.

2.5. Determination of proanthocyanidins

The method for determining proanthocyanidins is based on their transformation into anthocyanidins, leading to a shift of colour towards the red zone in a warm and acid environment (Margheri & Falcieri, 1972; Spagna et al., 1996; Spagna et al., 2002).

An aliquot of 2 ml of red wine diluted (10–20 times) with 0.05 M H_2SO_4 was loaded onto 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 (aluminium foil) and containing 9.5 ml absolute EtOH. An amount of 12.5 ml of $FeSO_4 \cdot 7\ H_2O$ (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 that time it was rapidly cooled by immersion in cold water (20 °C). After 10 min; the absorbance at 550 nm was registered on a Shimadzu UV-1601 UV–visible spectrophotometer. To subtract natural anthocyanins present in the sample, the corresponding absorbance value of the wine sample prepared under the same conditions, but cooled in ice instead of warmed, was subtracted to obtain the net value of absorbance. Under such conditions, the average yield was 20% and the proanthocyanidin concentration can be conventionally expressed as 5 times the amount of cyanidin formed, by means of a calibration curve with cyanidin chloride [$\epsilon = 34{,}700$ according to Di Stefano, Cravero, and Gentilini (1989)]. Each determination was performed in triplicate; results are expressed as means ± S.D.

2.6. Determination of vanillin index

The concentration of polyphenols reactive to vanillin in a highly acid environment was determined according to the method described by Margheri and Falcieri (1972) and Spagna et al. (2002). Vanillin is an aldehyde relatively stable at high concentrations of H_2SO_4; it reacts with free carbons C_6 and C_8 of flavanols, leading to the formation of a red complex with maximum absorbance at 500 nm.

The 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 were loaded onto a conditioned Sep-Pak. The column was washed with 2 ml of 5 mM H_2SO_4 and purged with air and eluted with 5 ml of MeOH into a test tube. One millilitre of the methanolic solution 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 were carefully added. After exactly 15 min, the absorbance of the pigment was read on a Shimadzu UV-1601 UV–visible spectrophotometer at 500 nm against a blank prepared under the same conditions, containing MeOH instead of vanillin. Concentration, expressed as (+)-catechin equivalents (mg/l), was determined by means of a calibration curve ranging between 0.001 and 0.080 g/l and submitted to the same procedure described above.

The curve obtained provided an excellent fit ($r = 0.9991$) to the following equation:

$$A = (7.788 \times C) + 0.0143$$

where A is the absorbance at 500 nm and C is the catechin concentration.

Each determination was performed in triplicate; results are expressed as means ± S.D.

2.7. Determination of ethanol, ascorbic acid, total and free sulphur oxide and sulphydrylic groups

The ethanol, ascorbic acid and sulphur oxide (SO_2) contents of red wines were determined with enzymatic test kits from Boehringer Mannheim (Mannheim, Germany). The total sulphur oxide content was determined in the whole wine; the free sulphur oxide was calculated by subtraction of the SO_2 content (bound SO_2) in the dealcoholized wine from the total SO_2 content determined in the respective whole wine.

The content of sulphydrylic (SH) groups was determined using Ellmann's reagent in accordance with the method reported by Rice-Evans, Diplock, and Symons (1991) and slight modified. Briefly, 2.4 ml of phosphate buffer (5 mM, pH 8) were added to 0.6 ml of wine and the solution mixed. The background absorbance at 412 nm was then measured. Then, 0.3 ml of a solution (1 mM in 5 mM phosphate buffer, pH 8) of the thiol reagent, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) was added, and the solution mixed and incubated for 1 h at 37 °C. For each wine sample, the absorbance at 412 nm was read on a Shimadzu UV-1601 UV–visible spectrophotometer against a blank prepared under the same conditions, containing phosphate buffer instead of DTNB. The concentration of SH groups, expressed as glutathione (mg/l), was determined by means of a calibration curve ranging between 2.0 and 20 mg/l and submitted to the same procedure as described above, without reading the absorbance at 412 nm before the colour development. Each determination was performed in triplicate; results are expressed as means ± S.D.

2.8. Quenching of 2,2-diphenyl-2-picrylhydrazyl radical (DPPH test)

The antiradical activities of the (both whole and dealcoholized) wines investigated were determined using the stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\cdot}$) and the procedure described by Saija et al. (1998) and Rapisarda et al. (1999). In its radical form, DPPH$^{\cdot}$ has an absorption band at 515 nm which disappears upon reduction by an antiradical compound. Different amounts (2–20 μl) of each wine were added to 1.5 ml of daily prepared DPPH$^{\cdot}$ solution (0.025 g/l in methanol). The same

volumes (2–20 μl) of the vehicle alone were added to control tubes. Absorbance at 515 nm was measured on a Shimadzu UV-1601 UV–visible spectrophotometer 20 min after starting the reaction. The DPPH· concentration in the reaction medium was calculated from a calibration curve analyzed by linear regression. The percentage of remaining DPPH· (%$DPPH^{\cdot}_{REM}$) was calculated as follows:

$$\%DPPH^{\cdot}_{REM} = [DPPH^{\cdot}]_T/[\ DPPH^{\cdot}]_0 \times 100$$

Each determination was carried out in triplicate and repeated at least three times. Results were expressed as percentage decrease with respect to control values; the mean scavenging concentration (SC_{50}) and 95% confidence limits were calculated by using the Litchfiels and Wilcoxon test.

3. Results and discussion

Red wine represents a rich source of polyphenols, e.g. anthocyanins, catechins, proanthocyanidins, flavonols, stilbenes and other phenolics, all potent antioxidants possessing biological properties that may protect against cardiovascular disease (Dell'Agli, Buscala, & Bosisio, 2004). However many of these studies show conflicting results and the dietary components responsible for the lower cardiovascular risk still remain to be identified. During the past few years the proanthocyanidins have been suggested as candidates to explain the superior effect of red wines white wines and other alcoholic beverages, since the proanthocyanidins are specifically extracted from the grape seeds and skin during the mash fermentation of red wine (Rasmussen, Frederiksen, Struntze Krogholm, & Poulsen, 2005)

All red wines tested in our study showed an evident antioxidant effect (Table 2). In particular the wines with higher total phenol levels were better radical-scavengers than were those with lower phenol contents (Table 2). In fact, a direct correlation between the antioxidant effectiveness of red wines and their total phenol content was demonstrated by linear regression analysis, both in whole wines ($R^2 = 0.943$) and in dealcoholized wines ($R^2 = 0.710$). The only slightly lower correlation degree calculated for dealcoholized wines, together with the really low R^2 value (0.014) calculated for the correlation between SC_{50} values of whole red wines and their ethanol content, may be attributed, in a significant number at least, to their content of total phenols, and it is influenced by alcohol only in a marginal way. Furthermore, as one can see in Table 3, also ascorbic acid, thiol groups and SO_2 (antioxidant compounds present in red wines especially as consequence of the wine-making processes) play only a minor role in the

Table 2
Total phenol content and DPPH radical-scavenging activity of seven red wines

Wine	Whole wines SC_{50}(95% C.L.[a])		Dealcoholized wines SC_{50}(95% C.L.[a])		Total phenols[b] g/l[c]
	μl of wine	μg as phenols	μl of wine	μg as phenols	
Badiola	0.90 (0.71–1.14)	5.27 (4.16–6.68)	1.13 (0.90–1.43)	6.39 (5.09–8.09)	5.86 ± 0.53
Chianti	1.71 (1.44–2.04)	8.36 (7.04–9.97)	1.90 (1.46–2.47)	9.04 (6.94–11.75)	4.89 ± 0.39
Donnafugata	1.72 (1.41–2.12)	8.43 (6.91–10.39)	1.24 (0.93–1.63)	6.13 (4.60–8.06)	4.90 ± 0.35
Cirò	1.77 (1.46–2.14)	8.23 (6.79–9.95)	1.83 (1.44–2.33)	8.27 (6.50–10.53)	4.65 ± 0.42
Cabernet	1.92 (1.56–2.37)	8.23 (6.69–10.16)	2.06 (1.57–2.69)	8.79 (6.70–11.48)	4.29 ± 0.32
Franciacorta	2.29 (1.84–2.87)	8.88 (7.14–11.13)	3.04 (2.35–3.92)	12.00 (9.28–15.48)	3.88 ± 0.45
Barbera	2.61 (2.12–3.21)	9.84 (7.99–12.10)	2.25 (1.65–3.05)	8.59 (6.30–11.65)	3.77 ± 0.38
R^{2d}	0.943		0.710		

Mean scavenging concentrations (SC_{50}) are expressed as μl of wine or as μg of total phenols.
[a] Confidence limits.
[b] Values represent means ± S.D. of three independent analyses carried out in whole wines.
[c] As gallic acid equivalents.
[d] Squared correlation coefficient between total phenols and scavenging activity.

Table 3
Detection of ethanol, ascorbic acid, sulphydryl groups, total and free sulphur oxide in seven red wines

Wine	Ethanol (%)	Ascorbic acid (mg/l)	SH groups (mg/l)	Total SO_2 (mg/l)	Free SO_2 (mg/l)
Badiola	13.0 ± 1.12	5.54 ± 0.32	10.0 ± 0.87	97.2 ± 8.53	48.4 ± 4.17
Chianti	12.3 ± 0.92	2.11 ± 0.19	5.51 ± 0.49	50.8 ± 4.98	19.9 ± 1.62
Donnafugata	12.1 ± 1.11	7.96 ± 0.47	7.81 ± 0.69	48.0 ± 4.59	14.6 ± 1.18
Cirò	12.8 ± 1.09	0	7.44 ± 0.62	42.3 ± 3.87	5.6 ± 0.15
Cabernet	12.5 ± 0.96	1.83 ± 0.15	7.29 ± 0.71	37.6 ± 3.24	12.0 ± 1.18
Franciacorta	12.2 ± 1.06	0	3.04 ± 0.29	27.6 ± 2.24	9.24 ± 0.74
Barbera	13.4 ± 1.25	0.92 ± 0.08	6.60 ± 0.61	40.1 ± 3.57	15.2 ± 1.27
R^{2a}	0.014	0.343	0.485	0.742	0.558

Results represent means ± S.D. of three independent analyses carried out in the whole wines.
[a] Squared correlation coefficient between SC_{50} values and relative parameters of whole wines.

radical-scavenging power of the red wines tested, as shown by the R^2 values (0.343, 0.485 and 0.742, respectively) calculated for the whole wines.

In our study we have employed the Folin-Ciocalteu index, the most widely used method to evaluate the global content of polyphenols, based on the oxidative titration of phenolate anions by phosphotungstate and phosphomolybdate. Thus we have correlated the scavenging activity of the wines tested also with their contents of anthocyanins and proanthocyanidins.

Grape proanthocyanidins have been reported to possess a broad spectrum of biological, pharmacological and therapeutic activities against free radicals and oxidative stress, as shown by *in vitro* and *in vivo* studies (Bagchi et al., 2000; Cos et al., 2004; Dell'Agli et al., 2004; Natella, Belelli, Gentili, Urini, & Scaccini, 2002). In particular, proanthocyanidins in wines are of primary interest, not only because they are the phenols present in highest concentrations in most red wines, but also 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.

According to the method employed in our study, the numerical value of the proanthocyanidin assay increases with the degree of polymerization of the proanthocyanidins because only the upper units 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 C_6 and C_8 of both catechins and proanthocyanidins; proanthocyanidins are slightly less reactive than the catechins only when at least one of the two sites, 6 and 8, is free. This index decreases with the increase in polymerization degree because many of the C6 and C8 positions are involved in the polymerization step.

As shown in Table 4, the SC_{50} values calculated for the whole wines tested are not well correlated with the total content of anthocyanins ($R^2 = 0.013$) but, interestingly were significantly correlated ($R^2 = 0.868$) with the amounts of proanthocyanidins and with the index of vanillin ($R^2 = 0.913$).

Thus the antioxidant efficiency of red wines tested appears to be largely influenced by the proanthocyanidin level, with anthocyanins playing a minor role. In accordance with our findings, several authors have shown that proanthocyanidins are more effective radical-scavenging compounds than are the monomeric flavanols and flavones, and that these substances, ratherthan the other flavonoids, could represent the oxidative principles of red wines (Das et al., 1999; Sánchez-Moreno, Cao, Ou, & Prior, 2003). Furthermore, Rigo et al. (2000) demonstrated that the peroxy radical-scavenging capacities of some Italian red wines was related to the amount of total proanthocyanidins. However, anthocyanins also are endowed with strong antioxidant activity and may contribute to the antioxidant power of red wines. For example, Ghiselli, Nardini, Baldi, and Scaccini (1998) have demonstrated that anthocyanin is the main component in the antioxidant activity of red wines.

The polymerization index is the result of the vanillin assay divided by the proanthocyanidin assay; this ratio provides a rough estimate of the degree of polymerization of the flavonols, and is mainly dependent upon the cultivar and the chemical age of the wine. High values of this ratio are characteristic of wines having largest amounts of monomeric tannins, (+)-catechin and (−)-epicatechin. Similar values of polymerization index, ranging from 0.356 to 0.470, were calculated for all wines tested in our study, indicating similar degrees of polymerization for proanthocyanidins contained in these wines. According to this result, no statistical correlation ($R^2 = 0.000$) appears to exist between scavenging activity and polymerization index of the red wines under investigation (Table 4).

Taken together, our findings also show that the radical-scavenger activity of red wines is not a property of single phytochemical compounds but is widely distributed among the phenolic constituents. Similarly, other authors have demonstrated that phenols are responsible for the antioxidant activity of wines acting synergistically (Arnous, Makris, & Kefalas, 2001; Lopez-Velez, Martinez-Martinez, & Del Valle-Ribes, 2003) in a mechanism in which the easily oxidized phenols are regenerated by less active phenols.

In conclusion, the present findings support the results from several experimental and epidemiological studies, suggesting that the supply of antioxidant phenols, through a moderate daily consumption of red wines, may provide additional protection against *in vivo* oxidation of cellular biomolecules.

Table 4
Detection of anthocyanins, proanthocyanidins, vanillin index and polymerization index in seven red wines

Wine	Anthocyanins (mg/l)	Proanthocyanidins (mg/l)	Vanillin index (mg/l)	Polymerization index
Badiola	217 ± 19.4	4249 ± 383	1612 ± 158	0.379
Chianti	255 ± 21.4	3054 ± 274	1089 ± 114	0.356
Donnafugata	233 ± 22.6	3269 ± 301	1146 ± 103	0.350
Cirò	102 ± 9.8	3161 ± 253	1322 ± 123	0.418
Cabernet	405 ± 35.6	2269 ± 190	1067 ± 106	0.470
Franciacorta	207 ± 21.9	2645 ± 223	943 ± 85	0.356
Barbera	247 ± 23.1	1877 ± 191	712 ± 59	0.379
R^{2a}	0.013	0.868	0.913	0.000

Results represent means ± D. of three independent analyses carried out in the whole wines.
[a] Squared correlation coefficient between SC_{50} values and relative parameters measured in whole wines.

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