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The antioxidant capacity of red wine in relationship with its polyphenolic constituents

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1. Introduction

Many epidemiological studies have shown a correlation between a non-well-balanced diet and coronary heart diseases, a few types of cancer and diabetes [\(Fernàndez-Pachòs, Villano, Gar](#page-4-0)[cia-Parrilla, & Troncoso, 2004; Landrault, Poucheret, Ravel, Gasc,](#page-4-0) [Cros & Teissedre, 2001](#page-4-0)). Epidemiologists have observed that a diet rich in polyphenolic compounds may provide a positive effect due to their antioxidant properties ([Frankel, Waterhouse, & Teissedre,](#page-4-0) [1995; Hertog, Feskens, Hollman, Katan, & Kromhout, 1993\)](#page-4-0). Wine is an important component in Mediterranean dietary tradition because it is very rich in antioxidant compounds. The polyphenolic contents of wine consist in two classes of components (flavonoids and non-flavonoids) and depend on the grape variety, vineyard location, cultivation system, climate, soil type, vine cultivation practices, harvesting time, production process and ageing [\(Shahidi](#page-4-0) [& Naczk, 1995](#page-4-0)). The polyphenolic molecules have a functional role, in that they behave as antioxidants against the free radicals and show a physiologic role as well; in fact, they increase the antioxidant capacity in the human body after red wine consumption ([Serafini, Maiani, & Ferro-Luzzi, 1998\)](#page-4-0). The latter is due to the reduction-oxidation properties of the phenolic hydroxyl groups and the potential for delocalization of Π electrons within the individual ring.

In the literature, there are few studies on the antioxidant capacity of red wine and its correlation with each of its phenolic

ABSTRACT

The ''antioxidant power" of a food is an expression of its capability both to defend the human organism from the action of the free radicals and to prevent degenerative disorders deriving from persistent oxidative stress. Purpose of this study is to analyse the antioxidant capacity (measured by means of the crocin bleaching method) of several samples of Sicilian red wines and to evaluate their dependency on the vintage and on the grape variety. Finally, the correlation between the single flavonoids compounds and the antioxidant capacity has been investigated. The analyses show that the antioxidant properties of red wine appear to be unequally influenced by the vintages for the different cultivars and that the correlation between antioxidant capacity and the total phenolic contents is weak. The latter can be explained by the fact that the wine's antioxidant properties are influenced differently by each polyphenolic molecule. - 2008 Elsevier Ltd. All rights reserved.

> compounds. The purpose of this study was to evaluate the correlation between antioxidant capacity and total polyphenolic contents, the concentration of each flavonoid compound and its contribution to the antioxidant activity in different Sicilian red wines; after that, the relationship between the antioxidant capacity and the grape variety; finally, the influence of the vintage both on the antioxidant capacity and on the polyphenolic concentration. The crocin bleaching method has been adopted to evaluate the antioxidant capacity of wine.

2. Materials and methods

Twenty-three samples of red wine selected from different grape varieties grown in Sicily and with different vintages were analysed. The samples derived either from pure grape varieties (Cabernet-Sauvignon, Nero D'Avola, Merlot, Sangiovese, Syrah) or from blends (Sangiovese-Merlot, Nero D'Avola–Cabernet-Sauvignon). All the samples shared the same vineyard location, cultivation system, climate, soil types, vine cultivation practices, harvesting time, production process and barrel aging. Some of these samples, such as Cabernet-Sauvignon 2004 (pair 1a and 1b in [Table 1\)](#page-1-0) or Nero D'Avola 2003 (pair 10a and 10b in [Table 1](#page-1-0)) and others, derive from the same grape variety and vintage but they belong to different production lots. Therefore, they have been produced with the same process (pressing, winemaking method, yeast, etc.) but they have been bottled at different times. The vintages 2002, 2003, and 2004 have been taken into consideration. The samples were fractioned into aliquots of 50 mL; deprived of oxygen, then put under nitrogen gas and stored at 4 ± 1 °C in amber bottles; finally, they

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Table 1

Each value represents mean value \pm standard deviation of three replicates. (r) represents the relative linearity of the regression curves obtained the plotting the relative rates $(\Delta V_0/\Delta V_a)$ against the (red wine samples/crocin) ratios, for each sample of wine.

were tested shortly after opening of the bottles. For each sample both the total polyphenolic contents and the antioxidant capacity were studied; furthermore, for the samples of vintages 2002 and 2003 the phenolic profile has been analysed, in order to evaluate its influence on the antioxidant capacity.

2.1. Total polyphenolic contents determination by the Folin-Ciocalteu method

The total phenolic concentration was determined spectrophotometrically according to the Folin-Ciocalteu colorimetric method. Samples of wine (1 mL) were diluted with distilled water (4 mL). Then, an aliquot (0.2 mL) of red diluted wine and 1 mL of Folin-Ciocalteau reagent, purchased from Sigma Chemicals Co (St. Louis, MO, USA), were mixed into a 20 mL calibrated flask. Exactly after 1 min, 4 mL of Sodium Carbonate (20% v/v) was added and the volume was made to 20 mL with distilled water; finally, the mixture was allowed to stand at room temperature in obscurity for 30 min and the absorbance of the solution at 750 nm was measured with a Beckman 640 UV/Vis spectrophotometer (single beam). The total polyphenolic concentration was calculated from a calibration curve using gallic acid as a standard (50–500 mg/L). Gallic acid was provided by Sigma Chemicals Co (St. Louis, MO, USA). Data were expressed as mg of gallic acid equivalents (GAE)/L, averaged from three measurements.

2.2. Analysis of red wines antioxidant capacity by the crocin bleaching assay (CBA)

The "crocin bleaching assay" (CBA) has been applied for the evaluation of the antioxidant capacity of individual compounds, plant extracts, and plasma [\(Chatterjee, Poduval, Tilek, & Devasaga](#page-4-0)[yam, 2005; Di Majo, Giammanco, La Guardia, Tripoli, Giammanco &](#page-4-0) [Finotti, 2005; Manzocco, Calligaris, & Nicoli, 2002; Peretti, Finotti,](#page-4-0) [Adamuccio, Della Sera, & Montanari, 2004; Ordoudi & Tsimidou,](#page-4-0) [2006\)](#page-4-0). This protocol is proposed by [Bors, Michel, & Saran, 1984](#page-4-0) and adapted by [Tubaro, Micossi, & Ursini, 1996](#page-4-0). A critic investigation of method performance was recently accomplished by [Ordou](#page-4-0)[di and Tsimidou \(2006\)](#page-4-0). This method is interesting because it can be used both in the lipophilic and in the hydrophilic environment ([Finotti & Di Majo, 2003](#page-4-0)). It has an advantage over the others, in that it is able to detect either the antioxidant or the pro-oxidant action of the compound or mixture under analysis.

This method is based on the crocin bleaching as a result of its oxidation by a source of radicals, AAPH [2,2'-azo-bis(2-aminopropane)dihydrochloride]. Peroxyl radical scavenging was evaluated according to the protocol of [Tubaro et al., 1996](#page-4-0) with some modifications. The basic requirement of lag-time-based test is that the rate constant of the reaction of antioxidant with peroxyl radicals must be much greater than that of the molecules to be protected. In the case of red wine, the rate constant for the reaction with peroxyl radicals is much greater than that of the crocin (Fig. 1).

Crocin was extracted twice from authentic commercial saffron (origin grade) according to the validated protocol ([Ordoudi & Tsim](#page-4-0)[idou, 2006](#page-4-0)) and the estimation of its concentration to \sim 3 μ M was based on an extinction coefficient reported in the literature, $\varepsilon^{\text{MeOH}}$ = 1.33 \times 10⁵ mol⁻¹ cm⁻¹ ([Finotti & Di Majo, 2003](#page-4-0)). Crocin working solutions were daily prepared in methanol (Merk) so that after adjustment the A_{443} value was \sim 3.9. All the treatments were carried out away from direct exposure to light. A certain volume of

Fig. 1. Crocin bleaching reactions for the crocin alone (a), crocin with red wine (b), and crocin with Trolox (c). The added antioxidants (red wine or trolox) have the same concentration as crocin.

crocin working solution was diluted with methanol to 25 mL (total volume) so that the A $_{443}$ value was ${\sim}1.$ Then an AAPH, purchased from Wako Chemicals, stock solution (12.5 mM) in distilled water was prepared. Red wine samples were diluted 1:25 (v:v) with distilled water. The reaction mixture, in presence of red wine samples, contains 0.15 mL of AAPH, 0.15 mL of crocin diluted, increasing amount (0.15–0.02 mL) of diluted red wine and distilled water to a final volume of 1 mL; on the contrary, in absence of antioxidants, the mixture is composed of 0.15 mL of AAPH, 0.15 mL of crocin and distilled water to a final volume of 1 mL. The reactions start with the introduction of the source of radicals and they were carried out at 40° C. The bleaching rate of crocin becomes linear approximately 2 min after the addition of the diazocompound and the correspondent decrease of absorbance at 443 nm was monitored for 10 min, using a Beckman 640 UV/visible spectrophotometer, against a blank. A ''blank" is the solution composed by the same reagents without crocin. It was run to rule out spectral interferences between the compounds and the crocin. Each kinetic analysis was compared to a kinetic crocin bleaching.

2.3. Expression of results on the antioxidant capacity

According to the competition kinetics [\(Bors et al., 1984\)](#page-4-0), the crocin bleaching rate by a peroxyl radical (V_0) decreases in absorbance at 443 nm when either an antioxidant or a pseudo-antioxidant compound is added because they compete with crocin for the peroxy radicals. As a consequence, the new crocin bleaching rate is reduced to V_a . The loss in absorbance values within 10 min of reaction both in the absence (ΔV_0) and in the presence of increasing amounts of diluted red wine (ΔV_a) was calculated. The bleaching reactions for crocin both in absence and in presence of red wine are shown in [Fig. 1](#page-1-0). Various levels of red wine (0.15– 0.02 mL) were also tested so that linear regression curves of relative rates $(\Delta V_0/\Delta V_a)$ against the (red wine samples/crocin) ratios could be built. The five-point linear regression slopes representing the relative rate constants ($K_{\text{rel}} = K_{\text{a}}/K_{\text{c}}$) were calculated according to the following equation:

 $V_0/V_a = 1 + K_a/K_c * [A]/[C],$

where K_a is the rate constant for the reaction between antioxidant and peroxyl radicals; K_c is the rate constant for the reaction between crocin and peroxyl radicals; [A] is the concentration of red wine samples and [C] is the concentration of the crocin. All the concentrations are expressed as %v/v (volume-to-volume percentage).

The value of the ratio K_a/K_c indicates the relative capacity (antioxidant capacity) of red wine to interact with peroxyl radicals.

 V_0 is the rate of the reaction of the crocin with the peroxyl radical and can be calculated with the following formula:

$V_0 = \Delta$ [Absorbance₄₄₃ ($t = 2$ min) – Absorbance₄₄₃($t = 12$ min)] for crocin in the absence of red wine:

 V_a is the rate of the reaction of the crocin with the peroxyl radical in the presence of different levels of red wine (0.15–0.02 mL) and it was determined with the following formula:

 $V_a = \Delta$ [Absorbance₄₄₃ $(t = 2 \text{ min})$ – Absorbance₄₄₃ $(t = 12 \text{ min})$] for each concentration $(\%v/v)$ of red wine analysed.

By dividing the $(K_{rel} = K_a/K_c)$ of a wine sample by the K_{rel} value of a millimolar concentration of Trolox, we obtain a ratio between the rate constants; this value represents the antioxidant capacity expressed as Trolox equivalents (TRE). [Fig. 1](#page-1-0) compares the bleaching of the crocin in a millimolar Trolox solution vs. an equally concentrated Trolox-less solution.

The construction of a curve for each wine sample requires three replications, as suggested by the analytical guidelines ([Eurachem](#page-4-0) [working group, 1998; Eurachem working group, 2000](#page-4-0)) and in [Table](#page-1-0) [1](#page-1-0), the relative linearity of the regression curves (r) obtained plotting the relative rates ($\Delta V_0/\Delta V_a$) against the (red wine samples/crocin) ratios for each sample of wine is shown.

2.4. Analysis of the phenolic compounds by HPLC

The analysis of phenolic compounds in wine was carried out using a WATERS liquid chromatography equipped with two pumps (Model 626), an automated gradient controller (Model 600 S) and a WATERS 717 plus autosampler; the detection was carried out with a Photodiode Array UV Detector (Model 996). The system was connected to a Data Station (Millenium³²) for collection and data analysis. The analytical column was a VARIAN C_{18} 250 \times 4.6 mm I.D. with 5 μ m particle diameter. Aliquots of 50 μ L were injected into HPLC system under the following conditions: the column was initially balanced with a mixture of water/ formic acid (95:5, v/v) as solvent A for 15 min and the system was thermostated at 25 \degree C. The phenolic compounds were eluted with a four stage linear gradient: from 5% to 8% of A in 5 min, from 8% to 27% of A in 45 min, from 27% to 50% A in 25 min, from 50% to 100% A in 10 min, 100% A in 5 min, back to 5% A in 2 min, 5% A in 8 min. The methanol was used as solvent B. The flow rate was 1 mL/min. A wavelength of λ = 280 nm was used for the detection of gallic acid and flavan-3ols (catechin and epicatechin), while a λ = 520 nm was used for anthocyanins and anthocyanidins. The identification of each compound was established by comparing the retention time and UV– Vis spectra of the peaks in wine with those previously obtained by the injection of standards. Each compound was quantified as mg/L by means of calibration with an external standard using the equation obtained from the linear regression between surface and different concentrations of standard.

2.5. Statistical analysis

The analysis on the same sample was made in three replications and the results were expressed as mean value ± standard error. The direction and the magnitude of the correlation between the variables were calculated using analysis of variance (ANOVA test) and Kruskal–Wallis test. The criterion for statistical significance was $p \leq 0.05$.

3. Results and discussion

Phenol-rich beverages like wine contain a variety of low-mass molecules and many of these have been ascribed a primary antioxidant role. In the literature, contrastant and confused data exist about the correlation between the antioxidant capacity and the polyphenolic contents of wine. In agreement with a few authors ([Ghiselli, Nardini, Baldi, & Scaccini, 1998](#page-4-0)), a linear correlation exists between antioxidant capacity and total polyphenols contents; others claim that the statistic correlation is relevant between total polyphenols and just the flavonoids fraction, while it is not when considering the non-flavonoids fraction (Katalinić, Milos, Modun, [Music´, & Boban, 2004\)](#page-4-0); finally, [Arnous, Makris, & Kefalas, 2002](#page-4-0) maintain that the anti-radicalic activity is due to the flavan-3-ols fraction and not due to the anthocyanins. The data in [Table 1](#page-1-0) shows that there is a low linear correlation between the phenolic components and the antioxidant activity of Sicilian red wine $(p = 0.3383$ ANOVA test; $p = 0.352$ Kruskal–Wallis test). In fact, wines like Syrah or Nero D'Avola of 2003, for example, have a lower antioxidant power and a higher polyphenols amount. Others, like the Merlot and the Cabernet-Sauvignon of 2003, have proved to be richer in polyphenols and to have a high antiradical activity; on the contrary, the Syrah of 2002 and the Merlot of 2004, for

example, contain a lower polyphenols amount and show a higher antioxidant capacity. The possible explanations of the above-mentioned results could be

- (i) The influence of the different flavonoid and non-flavonoid subgroups on the antioxidant capacity. According to [Finotti](#page-4-0) [and Di Majo \(2003\),](#page-4-0) various phenolic compounds have different responses based on the number of OH and OCH3 groups and their position on the ring.
- (ii) The degree of polymerization and the ratio between monomeric and polymeric forms; in fact [Saint-Criq de Gaulejac,](#page-4-0) [Provost, and Vivasournal, 1999](#page-4-0) maintain that the inhibition of the radicals tend to increase along with the order of polymerization. [Arnous et al. \(2002\)](#page-4-0) are convinced that the polymeric and other types of pigments may not have similar antioxidant characteristics in comparison with monomeric anthocyanins.
- (iii) The possible synergy or antagonism among the different classes of polyphenols.
- (iv) The radicalic molecules contained in wine.

As reported in [Table 1](#page-1-0), in 2002 it is the Syrah (5.83 ± 0.23) mmol/L TRE), the variety showing the highest antioxidant power; in 2003 it is the Cabernet-Souvignon $(5.54 \pm 0.50 \text{ mmol/L} \text{ TRE})$, and in 2004 it is the Merlot $(2.90 \pm 0.08 \text{ mmol/L} \text{ TRE})$. For the blends of all the three vintages, [Table 1](#page-1-0), the reported antioxidant capacity values ranged from 1.43 to 3.93 mmol/L (TRE), average 2.52 mmol/L (TRE).

The analysis of the individual phenolic constituents (Table 2) and their correlation with the total antioxidant capacity ([Table 3\)](#page-4-0) confirm what was stated above. Differences in the concentration of certain phenolic compounds (Table 2) are related with differences in the antioxidant capacity of the wines [\(Table 1\)](#page-1-0). In fact, wines such as Merlot 2003 or Cabernet-Souvignon 2003 have high concentrations of catechine (Table 2) and higher antioxidant capacity values [\(Table 1](#page-1-0)); on the contrary, others like Sangiovese 2003 or Sangiovese-Merlot 2002, have the lower values of catechin and consequently of antioxidant capacity. For the quercetin the influence on the antioxidant capacity was marginal; for example, wines like Nero D'Avola 2002 have shown to be richer in quercetin but these have lower antioxidant capacity values. The best r^2 values were found for catechin, myricetin, gallic acid and peonidin-3-O-glucoside, the other compounds gave the lowest correlations.

In agreement with the literature, wine phenolic composition is influenced by different factors: grape variety, vineyard location, cultivation system, climate, soil type, vine cultivation practices, harvesting time, production process and ageing ([Shahidi & Naczk,](#page-4-0) [1995\)](#page-4-0). This study shows that the vintage does not influence the antioxidant capacity ($p = 0.7193$ ANOVA test; $p = 0.9636$ Kruskal– Wallis test) of a wine equally across all the cultivars; in fact, according to the data presented in [Fig. 2](#page-4-0) and [Table 1](#page-1-0) there is not any better vintage than the others in which all the grape varieties show the higher values of antioxidant capacity. Comparing the data outcoming from the analysis of the antioxidant capacity of four cultivars (Cabernet-Sauvignon, Nero D'Avola, Merlot, Syrah) in the vintages 2002, 2003, 2004 ([Fig. 2\)](#page-4-0), it can be observed that some varieties of grapes as Nero D'Avola in 2004 show a greater antioxidant power $(AC = 2.30 \pm 0.26 \text{ mM}$ Trolox) than those in 2003 (AC = 1.36 ± 0.09 mM Trolox) and in 2002 (AC = 0.65 ± 0.09) 0.54 mM Trolox). On the contrary, the Syrah has shown the opposite behaviour, in fact in 2004 it has a lower antioxidant capacity $(AC = 1.34 \pm 0.20 \text{ mM}$ Trolox) than in 2003 $(AC = 1.69 \pm 0.09 \text{ mM})$ Trolox) and in 2002 ($AC = 5.78 \pm 0.07$ mM Trolox). The other two, Merlot versus Cabernet-Sauvignon, have had the same response in 2003 and opposite behaviour in 2002 and 2004. The statistical analysis has shown that for all the three years the data correlation

Table 3

The correlation between single phenolic compounds and antioxidant capacity values of red wines

 (r^2) represents the correlation coefficient between antioxidant capacity values and the content of single phenolic compounds (Microsoft Office Excel 2003).

Fig. 2. Antioxidant capacity ($p = 0.7193$ ANOVA test; $p = 0.9636$ Kruskal–Wallis test) of wines produced from different cultivars (Cabernet-Sauvignon, Nero D'Avola, Merlot, Syrah) in relationship with the vintage (years 2002, 2003, 2004).

is meaningful with values: ANOVA $p = 0.0001$; Kruskal–Wallis $p = 0.02$ in 2002; ANOVA $p = 0.0000$; Kruskal–Wallis $p = 0.0680$ in 2003; ANOVA $p = 0.0213$ Kruskal–Wallis $p = 0.0794$ in 2004. In conclusion, the antioxidant properties of red wine, at the same conditions of production process, vineyard location, cultivation system, climate, soil type, harvesting time and ageing, appear to be unequally influenced by the vintage for the different cultivars. In fact, in different years each of the four different cultivars has produced four different trends of responses. The low linear correlation between the phenolic component contents and the antioxidant activity of the analysed Sicilian red wines indicates that the type and presumably the polymerization grade have a high impact on the overall antioxidant status. Furthermore, the antioxidant capacity of red wines tested appears to be largely influenced by cathechin, myricetin, gallic acid and peonidin-3-O-glucoside levels, while the other compounds gave lowers correlations. The antioxidant capacity of wine identifies its ability to protect itself from the free radicals which might be generated while being stored.

Thus, we think that a study on the antioxidant capacity of foods or beverages should always take the following into account:

- (i) Structure-activity relationships of the antioxidant components (Di Majo et al., 2005).
- (ii) The contribution of specific polyphenolic fractions to the total antioxidant capacity (Arnous et al., 2002).

(iii) The polymerization grade of the phenolic compounds. According to Rice-Evans, Miller and Bolwell (1995), the polymerized polyphenolic compounds account for presumably 75% of the antioxidant capacity of a wine.

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