Method for Measuring Antioxidant Activity and Its Application to Monitoring the Antioxidant Capacity of Wines

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A novel method for measuring the antioxidant activity using *N*,*N*-dimethyl-*p*-phenylenediamine (DMPD) was developed. The radical cation of this compound gives a stable colored solution and a linear inhibition of color formation can be observed in the presence of 0.2−11 μg of TROLOX. The experimental protocol, which is rapid and inexpensive, ensures sensitivity and reproducibility in the measure of antioxidant activity of hydrophilic compounds. The effectiveness of the DMPD method on real foods was verified by evaluating the antioxidant ability of wine samples coming from different areas of Campania, Italy. Antioxidant capacity of wines is strictly related to the amount of phenolic compounds. The results obtained by the DMPD method are very similar to those obtained on the same samples when the radical cation of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Miller et al., 1996) was used.

Keywords: *Antioxidant activity; DMPD; radical cation; ABTS; wine*

INTRODUCTION

The intake of antioxidant compounds present in food is an important health-protecting factor. It is well-known that Mediterranean diet, which is rich in natural antioxidants, leads to a limited incidence of cardio- and cerebrovascular diseases (Hertog et al., 1993). It is known that compounds belonging to several classes of phytochemical components such as phenols, flavonoids, and carotenoids are able to scavenge free radical such as $O_2^{\bullet}$, $OH^{\bullet}$, or lipid peroxyl radical $LOO^{\bullet}$ in plasma (Frei et al., 1988). The effective intake of single food antioxidants and their fate in the human body have been defined only for a few compounds (Ramarathnam et al., 1995; Manach et al., 1998). It is reasonable that the higher the antioxidant content in foods is, the higher the intake by the human body will be.

A rapid test able to measure total antioxidative power of food commodities and in particular of fresh vegetables, which are the main source of natural antioxidants, is required. This tool would allow one to perform large screening of different vegetable species, to determine variety, ripening time, and agronomic conditions which result in a high content of natural antioxidants.

Several assays have been introduced (Frankel, 1993) for the measurement of the antioxidant ability of single compounds and/or a complex mixture. It is accepted that the effects exerted by different compounds in various environments require different methods to be evaluated. In fact, the assessment of antioxidant activity may be considered from the standpoint of at least three usual environmental conditions: bulk food lipids, dispersed food lipids, and watery fluids.

Free radical oxidation of the lipid components in foods is a major problem for food manufacturers (Frankel, 1980), thus the early attempts to measure antioxidative activity were mainly focused on lipid protection. Several methods based on the measure of the secondary products of lipid oxidation have been described in the literature. The tiobarbituric acid method (Ottolenghi, 1959) and the tiocianate method (Mitsuda et al., 1981) have been widely used to evaluate the effectiveness of potentially antioxidative compounds to prevent lipid oxidation. The main drawback of these methods is that only an advanced oxidation status can be detected. A different approach is to measure the induction period before the rapid oxidation phase, which occurs in a lipid matrix exposed to conditions of accelerated oxidation (Frankel, 1993). More recently two methods based on a kinetic measure of linoleic acid peroxidation in micelles of detergents have been proposed: the method of Pryor (Pryor et al., 1993; Foti et al., 1996) and the crocine method (Tubaro et al., 1996). Cao and co-workers (1995) set up a method able to measure directly the oxygen radical absorbance capacity (ORAC) to assess the antioxidant capacity of fruits and vegetables. These methods are more representative of what happen in a dispersed food lipid matrix such as sauces or salad dressing. They are very sensitive and allow evaluating the initial steps of the oxidation process; on the other hand, they are expensive and time-consuming.

A different approach based on the chromatic properties of stable radical cation was first envisaged by Blois (1958) who used the radical of α,α-diphenyl-β-pycryl-hydrazyl (DPPH) to measure the antioxidative ability of several natural compounds. More recently, Miller et al. (1993), using the colored solution of the ABTS radical cation, were able to set up a very simple and efficient method to measure antioxidant status of human plasma. The same authors (Miller et al., 1996; Rice-Evans et al., 1997; Miller and Rice-Evans, 1997) use ABTS also to assess the efficiency of antioxidant compounds from different vegetables developing a decolorization assay.

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A similar approach was followed by us using *N*,*N*-dimethyl-*p*-phenylenediamine (DMPD). In the presence of a suitable oxidant solution a colored DMPD radical cation is formed ($DMPD^{\bullet+}$). Antioxidant compounds, which are able to transfer a hydrogen atom to $DMPD^{\bullet+}$, cause a decoloration of the solution.

In this paper the experimental protocol of the DMPD method and its application to wine samples collected in Campania, Italy, is reported. Several experimental works regarding the measure of antioxidative efficiency of wine were carried out by using different method (Frankel et al., 1995; Rice-Evans et al., 1996; Sato et al., 1996; Simonetti et al., 1997). Thus, results obtained by the DMPD method can be compared to those obtained by other available methods.

MATERIALS AND METHODS

N,*N*-Dimethyl-*p*-phenylenediamine dihydrochloride (DMPD) and gallic acid were from Fluka (Switzerland). L-Ascorbic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (TROLOX), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), butylated hydroxytoluene (BHT) and α-tocopherol were purchased from Aldrich, Germany. ABAP, 2,2′-azobis(2-amidinopropane) dihydrochloride, was from Waco. All solvents (HPLC grade) were from Carlo Erba (Italy). Seven red (1993−1996) and four white (1996) wine samples originating from different areas of Campania, Italy, were purchased from local markets.

Spectrometric measurements were recorded by using an UV−vis Shimadzu 2100 (Japan) apparatus. The instrument was equipped with Peltier electronics temperature control and magnetic stirring.

Measurement of Antioxidative Ability by the DMPD Method. DMPD, 100 mM, was prepared by dissolving 209 mg of DMPD in 10 mL of deionized water; 1 mL of this solution was added to 100 mL of 0.1 M acetate buffer, pH 5.25, and the colored radical cation ($DMPD^{\bullet+}$) was obtained by adding 0.2 mL of a solution of 0.05 M ferric chloride (final concentration 0.1 mM). One milliliter of this solution was directly placed in a 1-mL plastic cuvette and its absorbance at 505 nm was measured. An optical density of 0.900 ± 0.100 unit of absorbance was obtained and it represents the uninhibited signal. The optical density of this solution, which is freshly prepared daily, is constant up to 12 h at room temperature.

Standard solutions of the different antioxidant compounds were prepared as follows: 1 mg/mL of ascorbic acid was prepared by dissolving 0.1 g of ascorbic acid in 100 mL of deionized water; 1 mg/mL of TROLOX was prepared by dissolving 0.1 g of TROLOX in 100 mL of methanol. Fifty microliters of standard antioxidants or of wine samples (diluted in water 1:20 for the red wines, undiluted for the white wines) were added in the spectrometric cuvette and after 10 min at 25 °C under continuous stirring the absorbance at 505 nm was measured. The buffered solution was placed in the reference cuvette.

A dose−response curve was derived for TROLOX, ascorbic acid, and SO_2 by plotting the absorbance at 505 nm as percentage of the absorbance of the uninhibited radical cation solution (blank) according to the equation

$$\text{inhibition of } A_{505}\ (\%) = (1 - A_f/A_0) \times 100 \qquad (1)$$

where A_0 is the absorbance of uninhibited radical cation and A_f is the absorbance measured 10 min after the addition of antioxidant samples.

Antioxidant ability of wine samples was expressed as TEAC (TROLOX equivalent antioxidant capacity) according to Miller et al. (1993), using the calibration curve plotted with different amounts of TROLOX (see Figure 4). Each result is the mean (CV < 5%) of eight determinations of different amounts of wine within the range of the TROLOX dose−response curve.

Scheme 1

$$DMPD_{(uncolored)} + \text{oxidant } (Fe^{3+}) + H^+ \rightarrow DMPD^{\bullet+}_{(purple)}$$

$$DMPD^{\bullet+}_{(purple)} + AOH \rightarrow DMPD^{+}_{(uncolored)} + AO$$

Measure of Antioxidative Ability by the ABTS Method. Measure of the antioxidant ability of wine samples was carried out as described by Miller et al. (1996) and by Miller and Rice-Evans (1997). ABTS radical cation ($ABTS^{\bullet+}$) solution was diluted in PBS 5 mM to obtain an optical density at 734 nm of about 0.800 unit of absorbance. The solution was placed in a plastic cuvette, 50 μL of the antioxidant solutions were added, and the absorbance was read after exactly 1 min. A dose−response curve was plotted for TROLOX and antioxidant ability was expressed as TEAC as already described for DMPD.

Total Phenolic Content of Wine Samples. The phenolic content of the different wines was determined by Folin−Ciocalteu reagent (Singleton and Rossi, 1965). Each sample (0.1 mL at proper dilution) was added to 4.2 mL of deionized water and 0.5 mL of Folin−Ciocalteu reagent (Sigma). After 1 min of mixing, 1 mL of an 80% solution of sodium carbonate and 4.2 mL of deionized water were added. The mixture was left 2 h at room temperature in the dark and the absorbance at 760 nm was measured. The concentration of the total phenolic content was determined by a comparison with the values obtained with a standard solution of gallic acid.

Ascorbic Acid Determination. Ascorbic acid content of wine samples was determined by the titrimetic method using 2,6-dichloroindophenol (Fluka) (AOAC, 1990).

Sulfur Dioxide Determination. Total SO_2 content of wine samples was determined by the alkalimetric method (Rankine and Pocock, 1970) using sodium bisulfite as standard.

RESULTS AND DISCUSSION

Standardization of the DMPD Assay. The principle of the assay is that at an acidic pH and in the presence of a suitable oxidant solution DMPD can form a stable and colored radical cation ($DMPD^{\bullet+}$) (Scheme 1, step 1). The UV−visible spectrum of $DMPD^{\bullet+}$, reported in Figure 1, shows a maximum of absorbance at 505 nm. Antioxidant compounds (AO) which are able to transfer a hydrogen atom to $DMPD^{\bullet+}$ quench the color and produce a decoloration of the solution which is proportional to their amount (Scheme 1, step 2). This reaction is rapid (less than 10 min) and the end point, which is stable, is taken as a measure of the antioxidative efficiency. Therefore, this assay reflects the ability of radical hydrogen-donor to scavenge the single electron from $DMPD^{\bullet+}$.

Preliminary experiments show that the choice of oxidant solution and the ratio between the concentration

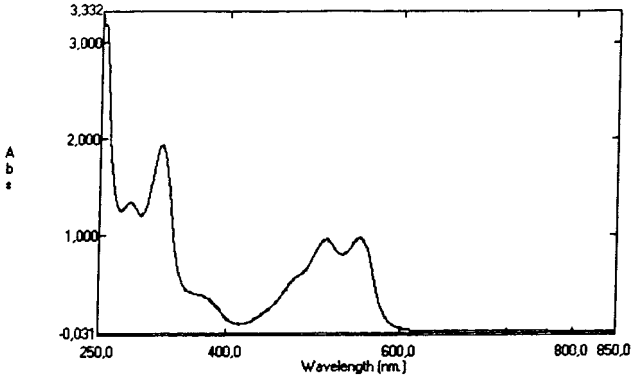

Figure 1. UV−visible spectrum of the DMPD radical cation ($DMPD^{\bullet+}$).

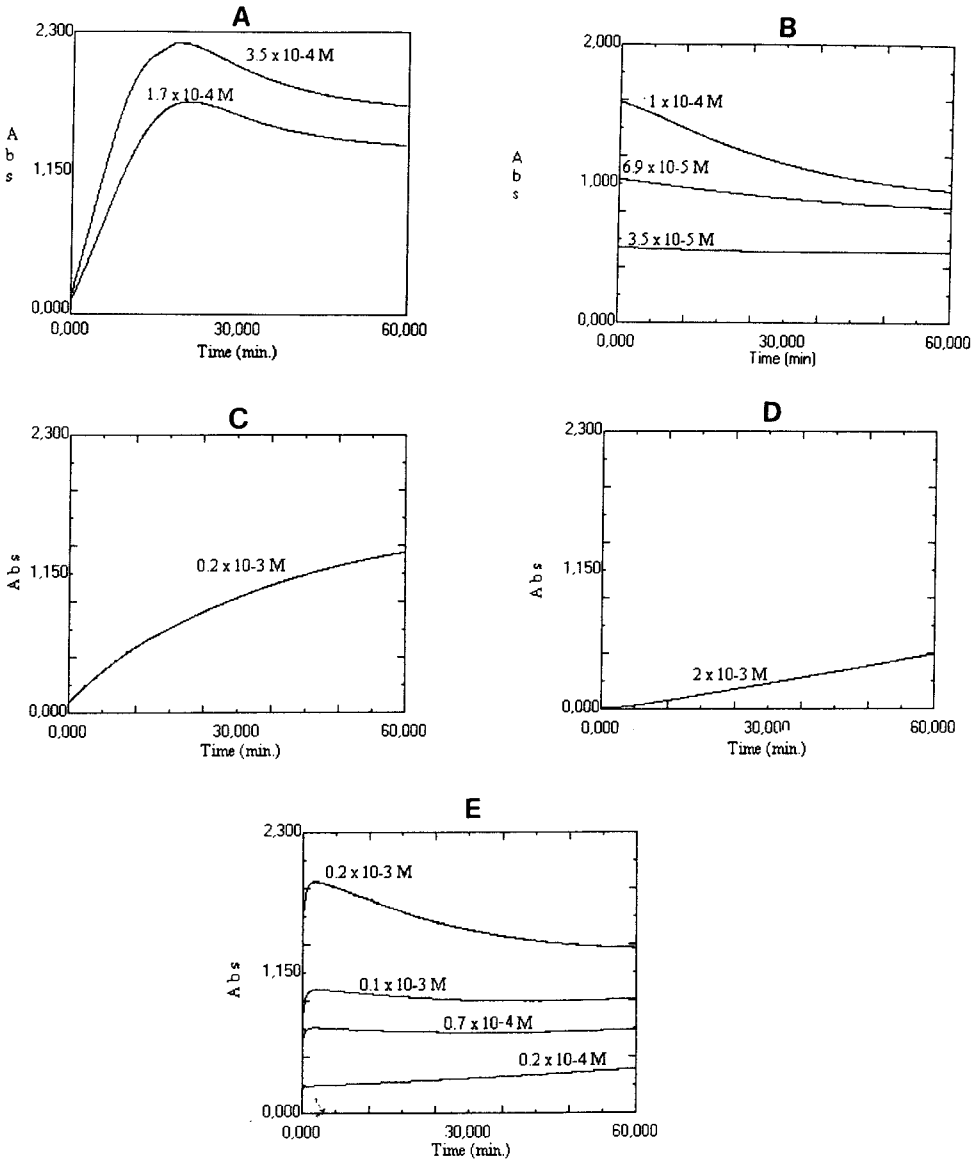

Figure 2. Time course of the $DMPD^{\bullet+}$ formation using different oxidants. The final concentration of the oxidant compounds is reported above each curve. DMPD concentration was 0.1 mM in all experiments. Panel A, hydrogen peroxide; panel B, sodium ipochlorite; panel C, copper chloride; panel D, ABAP; panel E, ferric chloride.

of DMPD and the concentration of the oxidative compound are crucial for the effectiveness of the method. The time course of $DMPD^{\bullet+}$ formation obtained with different oxidant solutions is reported in the panels of Figure 2. The use of hydrogen peroxide (panel A) produced a colored solution which is not very stable. The use of sodium ipochlorite (panel B) allows one to overcome this problem and the formation of the colored radical cation is immediate. However, this compound was also ruled out because of the instability of the commercial solution which causes changes in concentration and a consequent low reproducibility of the measures. The use of Cu^{2+} (panel C) and ABAP (panel D) is not adequate; in fact, formation of radical cation (also at a higher temperature using ABAP) is very slow and it resulted in a continuous increase of the absorbance. The best results were obtained with $FeCl_3$ (panel E), which gives, up to a final concentration of 0.1 mM, a stable colored solution; moreover, this method ensures low cost and highly reproducible analysis.

It should be outlined that to have both high sensitivity of the measurements and a sufficient inhibition range, a starting point between 0.80 and 1.00 of absorbance at 505 nm is necessary. This value can be reached with a chromophore concentration of 1 mM (Figure 3). A further increase of DMPD concentration does not produce an increase of color and results in a more difficult color inhibition. As shown in Figure 2, panel E, different amounts of $FeCl_3$ have been tested by using a DMPD concentration of 1 mM: a final concentration of 0.1 mM allows a sufficient color formation avoiding the residual presence of ferric ion in the colored solution. In conclusion the best results were obtained with a DMPD:Fe^{3+} molar ratio of 10:1.

To evaluate the sensitivity of the method, the system was tested by using different concentrations of ascorbic acid and TROLOX, the α-tocopherol analogue with enhanced water solubility. Standard solutions of these two antioxidants are stable for 1 week when stored at 4 °C and at least 6 months at −20 °C.

The results of the dose−response curves obtained by using TROLOX from eight different sets of experiments are shown in Figure 4. The standard deviation is very low and the dose−response curve is highly reproducible.

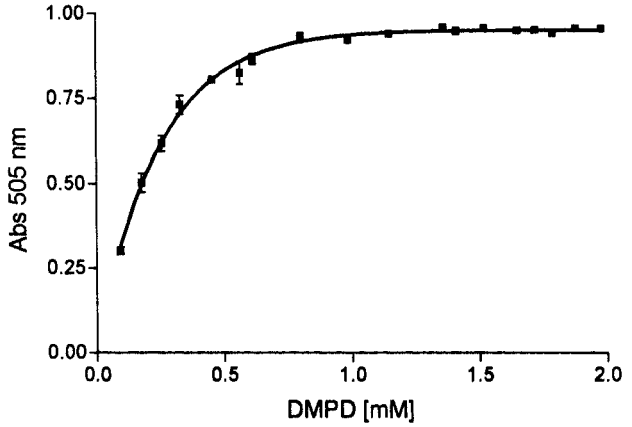

Figure 3. Dependence of color formation on DMPD concentration. Absorbance at 505 nm was recorded 10 min after ferric chloride addition. The value of optical density remains stable for several hours.

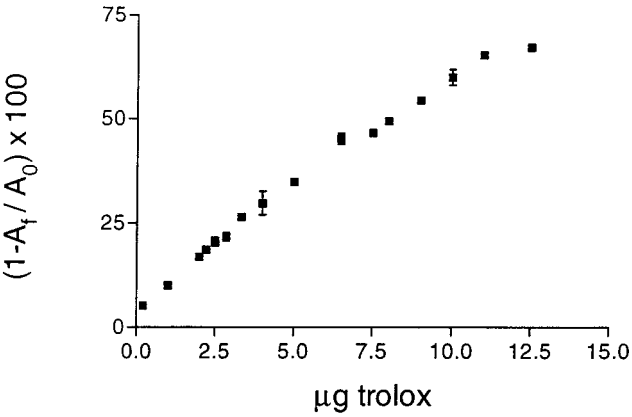

Figure 4. Degree of inhibition of the absorbance at 505 nm as a function of the TROLOX concentration Values are means ± SD (n = 8).

Inhibition of the absorbance at 505 nm is linear between 0.2 and 11 μg of TROLOX. The relationship calculated within this range for the standard compound is

$$A_{505}(\text{inhibition}) = 5.3\ (\mu\text{g of TROLOX}) + 7.0 \quad (2)$$

$$r^2 = 0.987$$

TROLOX and ascorbic acid have different kinetics in DMPD scavenging: in fact, while the absorbance inhibition induced by ascorbic acid is immediate, TROLOX requires a few minutes to exert its antioxidative action. On the other hand the end point of the curves is stable for both standard or unknown compounds; hence a lag-time of 10 min was always adopted between addition of antioxidant solution and measure of the color inhibition.

This assay is based on the extent of radical cation reduction at a fixed time point and not on the rate of reduction. This feature ruled out the complications due to the monitoring of the time course of color inhibition which are present in other methods (Pryor et al., 1993; Tubaro et al., 1996) and allows the simultaneous analysis of many samples. Compared to the widely used method based on the reduction of ABTS radical cation (Miller and Rice-Evans, 1997), the procedure described above has a comparable range of linearity and sensibility (0.2 and 11 μg of TROLOX for DMPD, 1−25 μg for ABTS). On the other hand, in contrast to the ABTS procedure the DMPD method guarantees a very stable end point of the measure. In this way we get free of the variable "time of the measure" which is crucial for the ABTS decolorization assay. This is particularly important when a large-scale screening is required.

Table 1

type of wine	amt of phenols, ppm of gallic acid	total SO_2, ppm	free SO_2, ppm	ascorbic acid[a] ppm
Aglianico, 89, red	1300	42	15	nd
Aglianico, 93, red	2300	64	20	nd
Aglianico, 96, red	2200	83	27	nd
Guardiolo, 96, red	1400	79	12	nd
Solopaca, 96, red	1200	59	10	nd
Gragnano, 95, red	900	77	18	nd
Lacrima christi, red, 95	700	65	12	nd
Coda di volpe, 96,white	120	137	5	30
Solopaca, 96, white	150	80	nd	20
Falanghina, 96, white	140	70	nd	17
Lacrima christi, 96, white	110	140	10	38

[a] nd not detected.

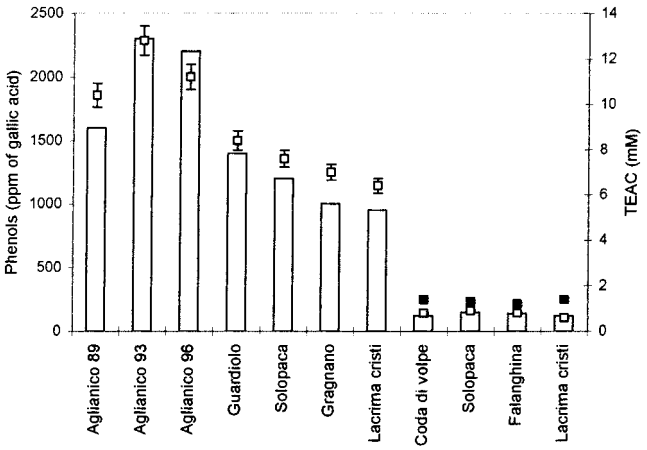

Figure 5. Amounts of phenols (bars) and values of TEAC (white squares) of the tested wines. Black squares indicate the TEAC values before subtraction of the ascorbic acid contribution (present only in white wines).

The main drawback of the DMPD method is that its sensitivity and reproducibility dramatically decreased when hydrophobic antioxidants such as α-tocopherol or BHT were used. The same behavior is observed when methanol is used as DMPD solvent.

Antioxidant Power of Wines. Wine was widely studied for its antioxidative properties due to the well-known health importance of its phenolic component (National Research Council, 1989). Several papers report on the evaluation of wine antioxidant power (Frankel et al., 1995; Rice-Evans et al., 1996; Sato et al., 1996; Simonetti et al., 1997). Wine samples were selected to test the effectiveness of the DMPD method on complex matrixes. Antioxidant compounds in wine are mainly hydrophilic and their antioxidant activity could be well evaluated by the DMPD method.

The 11 wine samples tested for their antioxidant ability are indicated in Table 1 together with the content of phenols measured by using the Folin−Ciocolteu method.

The antioxidant ability of wine samples was measured by using the DMPD method as described under Materials and Methods. Sample dilution was selected to reduce the measurement within the appropriate part of the Trolox standard curve. Results are reported in Figure 5. The antioxidative efficiency was expressed in TEAC (TROLOX equivalent antioxidant activity) according to

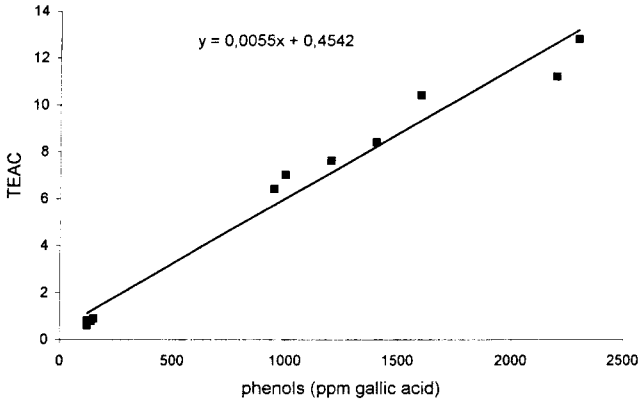

Figure 6. Correlation between TEAC and amount of phenols for the 11 wine samples tested.

Table 2

type wine	TEAC (DMPD)	TEAC (ABTS)
Aglianico, 89, red	12.8	11.6
Aglianico, 93, red	11.2	10.4
Aglianico, 96, red	10.4	7.8
Guardiolo, 96, red	8.4	9.4
Solopaca, 96, red	7.6	8.5
Gragnano, 95, red	7.0	7.3
Lacrima christi, red, 95	6.4	6.1
Coda di volpe, 96,white	0.9	1.9
Solopaca, 96, white	0.8	1.6
Falanghina, 96, white	0.8	1.4
Lacrima christi, 96, white	0.6	1.5

Miller et al. (1996), using the curve of standard TROLOX reported in Figure 4. There is a correlation between the content of phenols and the TEAC of each red wine and a clear difference between the value of TEAC of red wine samples and the white ones. The total phenol content of the white wines is too low to account for their TEAC values (see the black squares of Figure 5). This finding could be related to the addition of antioxidants such as sulfur dioxide and ascorbic acid, which are widely used as preservatives, in white wines.

In all samples total and free SO_2 as well as ascorbic acid were determined (Table 1). The curves of dose−response of DMPD for SO_2 and ascorbic acid were plotted. Whereas the TEAC due to free SO_2 is negligible (no antioxidative effect is detectable below 1 μg of SO_2 in the sample) the TEAC due to ascorbic acid is very important. In fact its amount ranges between 17 and 38 μg/mL. This means that 40−60% of the total TEAC measured for the white wine samples is due to ascorbic acid.

As shown in Figure 5 (white squares), the TEAC values obtained after subtraction of the ascorbic acid contribution are better related to the phenol content also for white wines.

In Figure 6 the TEAC value of each wine versus the respective amount of phenols is plotted. The correlation between these two parameters is more than 0.96. Therefore, using the line equation showed in Figure 6, it is possible to calculate the TEAC of a wine from its phenol content. This relationship could be useful especially for red wines.

To verify the results obtained by the DMPD method, the TEAC value of all wine samples was also measured by the ABTS method according to Miller et al. (1996); results are reported in Table 2 after subtraction of the ascorbic acid contribution. The results are comparable to those obtained with DMPD: in fact the absolute values are very similar. Moreover, ranking the 11 wine samples according to their TEAC value, only for the wine Aglianico 96 a significant shift is visible, while the other positions remain unchanged.

CONCLUSIONS

In this paper a new method to measure antioxidant power based on the DMPD colored radical cation is reported. The assay is particularly suitable for a large-scale screening of watery food extracts. It is cheaper and less laborious compared to all methods involving the addition of an antioxidant to a radical generating system (Pryor et al., 1993; Tubaro et al., 1996).

The results obtained by DMPD were compared to those obtained by using the ABTS decolorization assay proposed by Miller and Rice-Evans (1997). Sensitivity and reproducibility are similar to those obtained with ABTS both on standard antioxidants and on real samples such as wine. It is worth noting that the high stability of the point fixed for the measure makes the variable time negligible (which is a crucial feature of the ABTS assay), allowing a high interassay reproducibility. The radical cation of DMPD is less sensitive than ABTS to the hydrophobic antioxidants. The procedure here described proved to be suitable for hydrophilic antioxidants, while further studies are in progress to exploit the effectiveness of the DMPD method in organic solvents.

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