PAPER

EFFECT OF SULFITES ON THE *IN VITRO* ANTIOXIDANT ACTIVITY OF WINES

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ABSTRACT

The objective of this study was to assess the contribution of SO_2 to the overall antioxidant activity of wines. In this study, white, red, and model wines, with increasing sulfite content, were used. The radical scavenging activity of the wines was evaluated by ABTS and DPPH assays, while the reducing capacity of the wines was assessed by the FRAP assay. SO_2 positively affected the antioxidant properties of the wines and, in some cases, its contribution to the overall antioxidant activity of wines was higher than that of naturally occurring antioxidants. Depending on the assay, SO_2 showed both synergistic and antagonistic effects with the antioxidants naturally present in wines.

- Keywords: antioxidant activity, ABTS, DPPH, FRAP, wine, sulfites -

INTRODUCTION

Wine is one of the most important dietary sources of antioxidants with both *in vitro* and *in vivo* antioxidant activity (MANZOCCO *et al.*, 1998; FRANKEL *et al.*, 1995; VINSON and HONTZ, 1995; SERAFINI *et al.*, 1998; TSANG *et al.*, 2005). The *in vitro* antioxidant activity of wine, which has been studied in depth for decades, is highly correlated to its phenolic content (SIMONETTI *et al.*, 1997; BURNS *et al.*, 2000; ALONSO *et al.*, 2002; FERNÁNDEZ-PACHÓN *et al.*, 2004; YILDIRIM *et al.*, 2005). *In vivo* studies have shown that the consumption of wine modulates the serum non-enzymatic antioxidant capacity in humans. However, the direct antioxidant effect of polyphenols *in vivo* is still under debate (SERAFINI *et al.*, 2011; HOLLMAN *et al.*, 2011).

Even though the antioxidant activity of wines has been mostly attributed to the presence of phenolic compounds (MANZOCCO *et al.*, 1998; YILDIRIM *et al.*, 2005; BURNS *et al.*, 2001; VILLAÑO *et al.*, 2006), exogenous antioxidants, such as sulfites, are added during the wine-making process. Sulfur dioxide is one of the most commonly used additives in the food and beverage industries (WHO, 1998) due to its antioxidant, antiseptic, and preservative properties (BRANEN *et al.*, 2002). In wines, sulfur dioxide has positive effects by inhibiting oxidation and microbial growth, increasing pigment extraction, and reducing color loss and phenolic polymerization (RIBÉREAU-GAYON *et al.*, 2000). However, the use of sulfites in certain food products has either been banned (FDA, 1986) or strictly limited (EEC, 1995) and is currently under regulation due to its allergenic effects in hypersensitive individuals (EFSA, 2004).

Even though sulfites have reducing and antioxidant properties, there are contradictory findings on the contribution of sufur dioxide to the overall antioxidant capacity of wines. Some studies have reported that sulfur dioxide reacts with DPPH radicals and improves the radical scavenging activity of wines (ABRAMOVIC *et al.* 2015). Other studies have found that sulfur dioxide plays a minor role in the antioxidant capacity of wines (MANZOCCO *et al.*, 1998; CIMINO *et al.*, 2007). Additionally, as reported by KILMARTIN *et al.* (2001), the contribution of sulfur-containing antioxidants is lost when their reducing properties are determined by cyclic voltammetry methods equipped with glassy carbon electrodes.

On the other hand, authors have reported that sulfur dioxide might play a significant role in the antioxidant activity of beverages and sauces (LONG *et al.*, 2000; LACHMAN *et al.*, 2009; MITSUHASHI *et al.*, 2001), especially of white wines, which contain high sulfite levels and low natural antioxidant levels. Additionally, the *in vivo* effect of sulfites on the antioxidant activity of foods is still unknown (CAMPANELLA *et al.*, 2004; LAGGNER *et al.*, 2005).

The objective of this study was to evaluate the contribution of sulfur dioxide to the overall *in vitro* antioxidant capacity of wines using the ABTS, DPPH, and Ferric Reducing Antioxidant Power (FRAP) assays. These assays differ in several properties including mechanism of action (radical or redox reaction) and environmental conditions (solvent polarity and pH). In this study, three wine types (white wine, red wine, and a model wine) were used. This experimental approach was used to assess possible matrix effects which, to the best of the authors' knowledge, have not been evaluated.

MATERIALS AND METHODS

Materials

Three types of wines were analyzed: white wine (Trebbiano d'Abruzzo Pietrosa, 2003 vintage, winery Dora Sarchese), red wine (Montepulciano d'Abruzzo, 2004 vintage, Miglianico social winery), and a model wine made from distilled water, ethanol (12% v/v), and tartaric acid (0.033 M, pH 3.6). The content of alcohol, total polyphenols, and sufur dioxide, and the pH value of the three wines are shown in Table 1. Different levels of sodium metabisulphite ($K_2S_2O_5$) were added to the wines. All reagents used in this study were of analytical grade.

Chemical and chemico-physical analyses

Alcohol content, total and free sulfur dioxide content, and pH values were determined by official EU methods (EEC, 1990). Total polyphe-

Table 1 - Total and free sulphur dioxide content, pH, alcohol amount, total polyphenol index and total dry extracts of the white and red wines under investigations.

Samples (mg l^{-1})	$SO_{2\,TOT}$	$SO_{2\,FREE}$ (mg l^{-1})	pH	Alcohol (%v/v)	TPI (mg GAE l^{-1})	Total dry extract (g l^{-1})
white wine	77	65	3.24	12.90	1052	22.80
red wine	70	54	3.27	13.05	1837	23.55

Data coefficient of variation <2%.

nol content was determined by the method reported by SINGLETON and ROSSI (1965).

ABTS assay

The radical-scavenging activity of the samples was determined by the ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical decolorization assay (RE *et al.*, 1999). The bleaching rate of the ABTS radical in the presence of sample was monitored at 734 nm. ABTS radical solution (2.97 mL; Abs = 0.70±0.02) was mixed with 30 μL of diluted wine samples (1:2, 1:5, 1:10, and 1:20) using the model wine as diluent. ABTS radical bleaching was monitored at 25°C for 60 min; the decoloration degree after 5 min was used as an indicator of antioxidant activity. In the dilution range considered, the ABTS radical bleaching was proportional to the concentration of sample added to the medium; a dose-response curve was fitted to a linear model. Antioxidant activity, which was calculated as the ratio between the regression coefficient of the dose-response curve of the sample and the regression coefficient of the dose-response curve of Trolox (hydrophilic homologue of tocopherol), was expressed as μmoles of Trolox equivalents per mL of sample ($TEAC_{ABTS}$: Trolox Equivalent Antioxidant Capacity).

FRAP assay

The reducing activity of the samples was determined according to the method described by BENZIE and STRAIN (1996), with slight modifications. Sample (0.1 mL) was mixed with FRAP reagent (2.9 mL) obtained by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl-*s*-triazine) solubilized in 40 mM HCl, and 20 mM $FeCl_3$ in a 10:1:1 ratio. Absorbance was measured at 593 nm for 6 min. A calibration plot was generated based on $FeSO_4$ $7H_2O$; the results were expressed as mM Fe^{2+}.

DPPH• assay

The antiradical activity of the samples was measured by the DPPH (2,2-diphenyl-1-picrylhydrazyl) decolorization assay as reported by BRAND-WILLIAMS *et al.* (1995), with slight modifications in data computation. A dose-response curve was generated by adding 0.1 mL of sample at different dilutions (1:2, 1:5, 1:10, and 1:20) to 2.9 mL of a $6.1 \cdot 10^{-5}$ M DPPH-methanol solution. Radical bleaching was monitored at 25°C for 60 min. Dilutions were performed with the model wine as diluent. The $TEAC_{DPPH}$ value was calculated as the ratio between the regression coefficient of the dose-response curve of the sample and the regression coefficient of the dose-response curve of Trolox and expressed as μmoles of Trolox equivalents per ml of sample.

Statistical analyses

Three aliquots were sampled from each wine; each aliquot had different levels of sodium metabisulfite. All analytical determinations were carried out in triplicate. Data were reported as mean ± standard deviations. Linear regression was applied to assess the relationship between sulfite content and antioxidant activity; the goodness of fit was evaluated by the coefficient of determination (R^2). The antioxidant activity of wines in the absence of sulfites was obtained by extrapolation of the intercept value; the accuracy of the predicted values was assessed from the standard deviation. All statistical analyses were performed with Statistica® for Windows (Statsoft, Tulsa, OK).

RESULTS AND DISCUSSION

The proximate composition and sulfite content of the wines are shown in Table 1. The polyphenol content of the white wine was quite high because the wine was processed by cryo-maceration, while that of red wine was relatively low because it was a 'cerasuolo-type' red wine. These two types of wine were selected for this study because they had similar alcohol and total dry extract contents.

The sulfite content of the three wines increased with increasing sodium metabisulfite addition. The total sulfur dioxide content, which was assessed by titration, was 50, 100, 150, and 200 mg L^{-1} in the model wine; 77, 113, 125, 153, 185, and 209 mg L^{-1} in the white wine; and 70, 100, 125, 150, 175, and 200 mg L^{-1} in the red wine. The amount of sodium metabisulfite added to the wines was calculated using data in Table 1. The free sulfur dioxide content was measured immediately and 2 h after sodium metabisulfite addition; no significant changes in bound sulfilte levels were obtained between these two time points. This time lapse is usually required for antioxidant activity determinations.

Antioxidant activity was determined by ABTS (TEAC method), DPPH, and FRAP assays. The ABTS and DPPH assays have similar mechanism of action towards AR-OH, because they can be neutralized either by direct reduction via electron transfer or by radical quenching via H atom transfer (PRIOR *et al*, 2005), even though in the case of DPPH radical, the hydrogen atom removal from AR-OH could be considered as a marginal reaction because it occurs very slowly in strong hydrogen bond-accepting solvents such as methanol (HUANG *et al.*, 2005). The environmental conditions of the two radical scavenging assays are quite different because the ABTS assay is performed in aqueous media versus pure methanol in the DPPH assay.

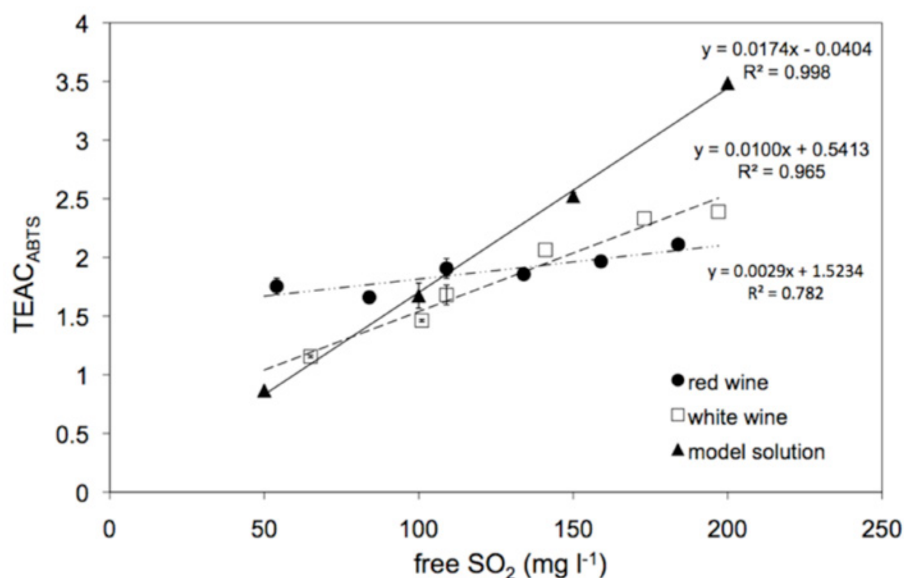

Fig. 1 - Antiradical activity as evaluated by the ABTS radical decolorization assay of the model wine solutions and of the white and red wines as a function of free SO_2 concentration.

Radical scavenging activity as determined by the ABTS radical decolorization assay

The antiradical activity of the wines, as determined by the ABTS decolorization assay, is shown in Fig. 1. Antiradical activity improved with increasing sulfur dioxide concentration. The model wine containing 50 ppm SO_2, an amount that is likely to occur in real wines, was characterized by a $TEAC_{ABTS}$ value of 0.85 μmoles Trolox equivalents per ml of sample, while the 200 ppm model wine had a $TEAC_{ABTS}$ value of 3.48 μmoles Trolox equivalents per ml of sample.

Taking into account the fact that the antioxidant activity of the white wine measured by the ABTS assay may vary between 0.8 and 4.24 μmoles Trolox equivalents per mL (ALONSO *et al.*, 2002; DE BEER *et al.*, 2003; VILLAÑO *et al.*, 2004), these results suggest that sulfites may play a more significant role in the antiradical properties of wines than polyphenols. However, all wine samples had SO_2 added in its free form; commercial wines are likely to have SO_2 bound to different compounds.

To evaluate the effect of sulfur dioxide on the antioxidant capacity in a real wine, the antiradical activity determinations were carried out in white wine with different contents of total sulfur dioxide. The results revealed that the white wine, with a total SO_2 content of 77 mg L^{-1}, was characterized by a $TEAC_{ABTS}$ value of 1.16 μmoles of Trolox equivalents per ml. Taking into account that 77 mg L^{-1} of sulfur dioxide in the model wine exerted a TEAC value of 1.30, it can be hypothesized that most of the antioxidant capacity of white wine is attributed to its sulfur dioxide content. By extrapolating the antioxidant activity of white wine without sulfites from the regression curve (Fig. 1), the wine had a $TEAC_{ABTS}$ value of 0.40 μmoles of Trolox equivalents per ml of sample. Therefore, sulfur dioxide contributed to the antioxidant activity of white wine to such an extent that an amount of 50 mg L^{-1} sulfur dioxide can double the $TEAC_{ABTS}$ value. These results were in agreement with those obtained by LONG *et al.* (2000), who reported that small quantities of sulfites can affect the total antioxidant activity of the product.

In white wine, an increase in sulfur dioxide concentration from 77 to 200 mg L^{-1} doubled its antioxidant activity. This result is quite significant because most wine research studies have not evaluated sulfite interference or sulfite contribution to the overall wine antioxidant activity (VILLAÑO *et al.*, 2006; LACHMAN *et al.*, 2009; 24, VILLAÑO *et al.*, 2004; ARNAO, 2000), even when the fractionation of polyphenolic compounds could not explain the overall antioxidant activity of the samples (FERNÁNDEZ-PACHÓN *et al.*, 2004). The regression coefficient of the dose-response curve of the white wine was lower than that of the model wine (Fig. 1), which could be attributed to matrix effects.

To further investigate the matrix effect on the antioxidant capacity of sulfur dioxide, the antiradical activity was also determined in red wine. Fig. 1 shows that the red wine, with a sulfur dioxide content of 70 mg L^{-1}, had a $TEAC_{ABTS}$ value of 1.70 μmoles of Trolox equivalents per ml. Considering that the model wine with similar sulfur dioxide content had a $TEAC_{ABTS}$ value of 1.18, it could be hypothesized that a considerable percentage of the antiradical activity of red wine is attributed to its sulfur dioxide content. However, when the antioxidant activity of the red wine with no sulfites was extrapolated in the regression curve (Fig. 1), the $TEAC_{ABTS}$ value was 1.52. Taking into account the regression equa-

tion, the sulfur dioxide contribution to the overall antiradical capacity was 10–38% in the tested concentration range, which was lower than that of white wine.

In decreasing order of regression coefficient magnitude, the wines were model wine > white wine > red wine. This result confirmed the presence of a matrix effect on the determination of antioxidant activity; this matrix effect was higher in the red wine than in the white wine. It has been extensively reported that sulfites in wine can bind to several compounds such as acetaldehyde and polyphenols. Polyphenol content is usually much higher in red wines than in white wines (ALONSO *et al.*, 2002; DE BEER *et al.*, 2003). Additionally, red wines contain a high amount of anthocyanins, which bind to sulfur dioxide (ANTONELLI and ARFELLI, 1993; TIMBERLAKE and BRIDLE, 1967). However, in this study, the free sulfite content was taken into consideration (Fig. 1); therefore, in our experimental conditions it could be assumed that natural antioxidants and sulfites interfered with the antioxidant activity assays. In fact, both synergistic and antagonistic effects among antioxidants were observed in different *in vitro* antioxidant activity assays.

Reducing activity as determined by the FRAP method

The antioxidant properties of the wines were evaluated with the FRAP assay (BENZIE and STRAIN, 1996). In contrast with the previously described methods, this method is based on the reducing capacity of a compound rather than its antiradical activity.

The FRAP values of the model, white, and red wines are shown in Fig. 2. The model wine had low reducing activity; however, the addition of sulfites (70–200 ppm) resulted in a threefold increase relative to the initial value. The addition of sodium metabisulfite to the white and red wines increased their reducing power. The higher the sulfite content, the higher the reducing properties, likely due to the protective role of sulfur dioxide against polyphenol oxidation. An increase in sulfur dioxide from 71 to 200 ppm contributed to a 73% and 158% increase in the reducing capacity of the red and white wines, respectively.

In the red and model wines, it was possible to extrapolate the FRAP value without sulfite addition. Based on the results, sulfur dioxide is responsible for most of the reducing power of the wine samples. However, with respect to the white wine without sulfite addition, the experimental data did not allow an accurate estimation of the FRAP value because of a non-linear response (Fig. 2). This result could be attributed to synergistic effects between natural antioxidants and sulfur dioxide. The synergistic effects between natural antioxidants and sulfur dioxide could account for a non-linear response between the FRAP assay and the SO_2 dose, which was evident in the red and white wines (Fig. 2). If the individual effect of an antioxidant on FRAP is linear within a certain concentration range, the synergistic effect of two antioxidants could show an increase or decrease in the response due to variations in their molar ratios (HIDALGO *et al.*, 2010).

In order of decreasing regression coefficient magnitude, the wines were red wine >white wine > model wine. There were no negative matrix effects in the red and white wines. Contrary to the results obtained from the ABTS assay, sulfites

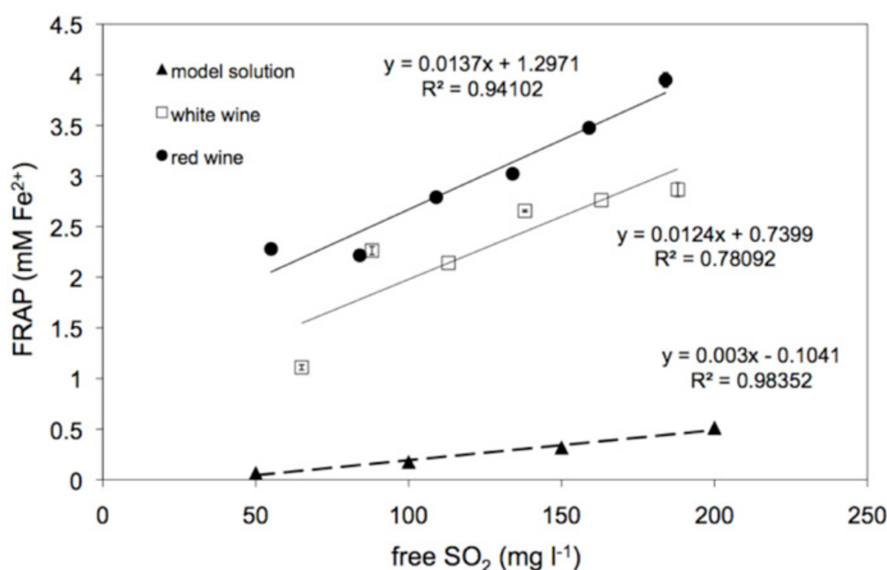

Fig. 2 - Reducing capacity as evaluated by the FRAP method of the model wine solutions and of the white and red wines as a function of free SO_2 concentration.

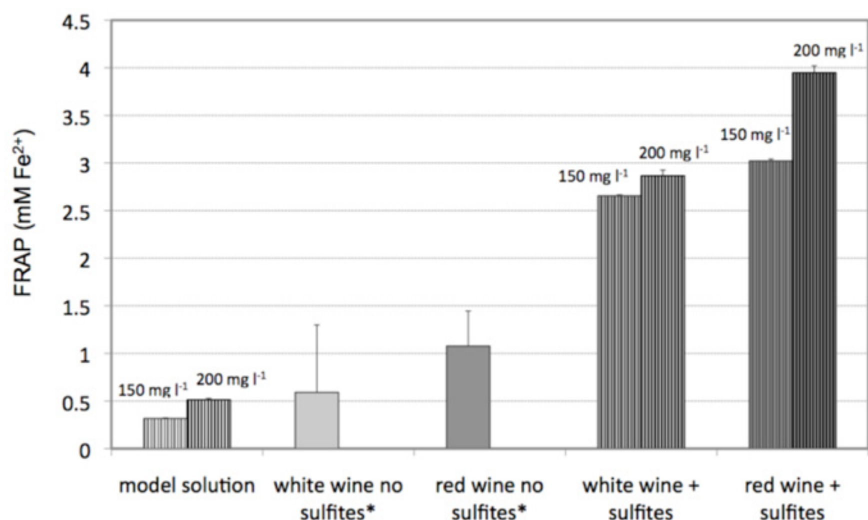

Fig. 3 - Reducing capacity of the samples with no sulfites (*obtained by extrapolation of data linear regression) and in the presence of 150 and 200 mg l^{-1} total sulphur dioxide content.

and naturally occurring antioxidants (i.e., polyphenols) had a synergistic effect on the reducing power of wines (Fig. 3). This result may be attributed to several factors: (i) in the experimental conditions of the FRAP assay (pH= 3), there is a lower amount of bound SO_2 (RIBÉREAU-GAYON *et al.*, 2000) than in the ABTS assay (pH= 7); (ii) free SO_2 could scavenge hydrogen peroxides produced via the Fenton reaction from catechols; and (iii) polyphenols could prevent the prooxidant action of peroxomonosulfate radicals resulting from Fe(III)-initiated bisulfite oxidation (DANILEWICZ, 2007; DANILEWICZ *et al.*, 2008). The synergistic effect between sulfites and polyphenols support the facts that SO_2 and catechols are not individual antioxidants, and that the antioxidant activity of wine is a result of multiple antioxidants (DANILEWICZ *et al.*, 2008).

Radical scavenging activity as determined by the DPPH• radical cation decolorization assay

The antiradical activity of sulfur dioxide was evaluated by the DPPH decolorization assay (BRAND-WILLIAMS *et al.*, 1995), which relies on a methanol-soluble stable radical in an amphiphilic environment. Dose response curves were generated with different dilutions of hydroalcoholic solutions containing increasing amounts of total sulfur dioxide. Fig. 4 shows the antioxidant activities of the model wine, expressed as $TEAC_{DPPH}$, plotted against the total sulfur dioxide content (25–200 mg L^{-1}).

The model wine containing sulfites had limited antiradical activity in the amphiphilic environment (MANZOCCO *et al.*, 1998). Water-metha-

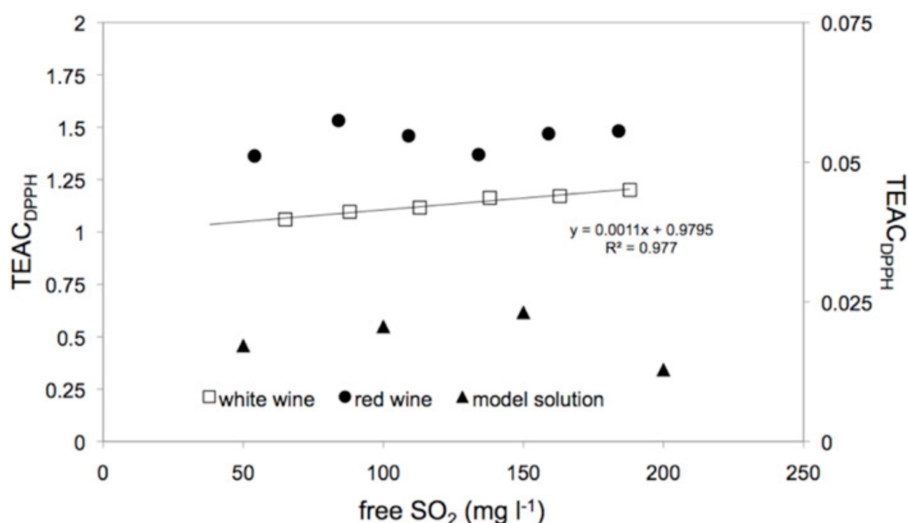

Fig. 4 - Antiradical activity as evaluated by the DPPH radical decolorization assay of the model wine solutions (secondary y axe) and of the white and red wines (primary y axe) as a function of free SO_2 concentration.

nol mixtures are not ideal solutions; in such mixtures, small volumes of water in methanol result in the stabilization of methanol clusters as a result of hydrophobic and hydrogen bond interactions (TAKAMUKU *et al.*, 2000; WAKISAKA *et al.*, 1998; OKASAKI *et al.*, 1984), resulting in a phase separation that could limit the contact between the water soluble antioxidant (sulfites) and the methanol-soluble radicals with negative effects on the estimation of the antioxidant capacity.

Fig. 4 shows the dose response curves of the white and red wines. In the white wine, increasing sulfur dioxide content from 77 to 200 mg L^{-1} caused a slight increase in the antiradical activity (+13%). In the red wine, the increase in sulfur dioxide contributed to increased but fluctuating antiradical activity values. A reduction in antioxidant activity due to sulfite addition (>150 mg L^{-1}) was observed in the model wine (Fig. 4).

Based on the results obtained from the DPPH• assay, there were no negative matrix effects on the antiradical activity of sulfur dioxide. White and red wines, as opposed to the model wine of this study, contain phenolic compounds, which exhibit a surface activity that affects their radical scavenging efficiency in multiphasic systems (DI MATTIA *et al.*, 2009; 2010). A possible explanation for this result is that amphiphilic compounds like polyphenols could have acted as surfactants allowing sulfur dioxide to exert its antiradical activity with positive effects on the $TEAC_{DPPH}$ value. In this case, the interfacial effect of polyphenols may justify the positive combined effects between the two antioxidants. Another possible explanation is the synergistic effects between polyphenols and SO_2 on reducing activity.

The synergistic effects between natural antioxidants and sulfur dioxide could account for a non-linear response between the DPPH assay results and the SO_2 dose, which was evident in the red wine (Fig. 4). In the case of the FRAP assay, a non-linear response was observed in the white wine, which contains less polyphenols (Fig. 2), while in the case of the DPPH• assay, a non-linear response was evident in the red wine, which contains more polyphenols (Fig. 4). This result could be due to the different solvents used in the two assays: hydrophilic solvents in the FRAP assay and amphiphilic solvents in the DPPH• assay. Additionally, the different activities of phenolic antioxidants in the two assays is pH dependent (JOVANOVICH *et al.*, 1994).

CONCLUSIONS

Sulfur dioxide had antioxidant activity in the white, red, and model wines. In some cases, the contribution of sulfur dioxide to the overall antioxidant capacity of the wines was higher than that of naturally occurring antioxidants. Moreover, in wines, sulfur dioxide had both antagonistic and synergistic effects with naturally occurring antioxidants on the total antioxidant activity of wines.

The role of sulfur dioxide on the total antioxidant capacity of wines is of utmost importance to assess the technological and potentially health-promoting properties of different products. Future studies should evaluate the *in vivo* antioxidant effects of sulfur dioxide in wines.

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