Determination of reactions between free radicals and selected Chilean wines and transition metals by ESR and UV–vis technique

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Keywords:
Wine
Polyphenols
Transition metals
ESR
Antioxidant
Free radical scavenging properties

A B S T R A C T

Four different types of Chilean wines (Cabernet Sauvignon, Merlot, Carmenere and Syrah) were selected and examined in their free radical scavenging capacities by electron spin resonance (ESR) and spectrophotometric methods. The free radical scavenging properties were evaluated against 2,2-diphenyl-1-picrylhydrazyl (DPPH•) radical, 2,6-di-*tert*-butyl-alpha-(3,5-di-*tert*-butyl-4-oxo-2,5-cyclohexadien-1-ylidene)-*p*-tolyloxy (Galvinoxyl) radical and hydroxyl radical (HO•). The possible effect on these scavenging properties of added transition metals to these wines was evaluated. Among the wines evaluated, Cabernet Sauvignon was the one with the highest activity against all radicals tested. The presence of added copper or iron to wines resulted in a reduced free radical scavenging capacity for all type of wines studied. The formation of redox inactive complexes between polyphenols of wine and transition metals is the possible cause of this reduction in antioxidant activity.

1. Introduction

Growing evidence has been collected suggesting that high consumption of phenolic compounds-rich foods and their derivatives may reduce the risk of cardiovascular diseases, cancer and other chronic diseases [1]. Vegetables, fruits and their seeds are rich sources of vitamins C, E, and β-carotene that prevent the organism against cancer. Several plants have been reported to contain compounds including bioflavonoids and proanthocyanidins, which exhibit chemopreventive and or anticancer properties [2–4]. As a derivative of grapes, the wine is a beverage widely consumed around the world, is a product with high phenolic content. The composition of wine is highly complex. Most of the components of wine come from the grape and the fermentation process. The concentration and variety of flavonoids in grape, and therefore in wine, depend on a large number of factors such as grape variety, climate and land, time of harvest, grape pressing procedure, time of fermentation and contact with skin, grapes, etc. [5]. The strong antioxidant properties of wine have been widely studied [6]. These properties are mainly due to the presence of a large amount of polyphenols with anthocyanins and flavanols, the major families in red wine [7–9].

The propensity of a flavonoid to inhibit free radical-mediated events is governed by its chemical structure. The chelating properties of flavonoids and tannins contribute to their antioxidant activity [10,11]. Chelation of a divalent cation may result in changes to a different extent of free radical scavenging activity [10,12,13].

Many methods (spectrophotometric methods, TRAP, FRAP, among others) have been used to study antioxidant activity of wines [5,6]. More recently, electron spin resonance spectroscopic (ESR) methods have been applied to evaluate the antioxidant activity of different foodstuffs [14,15]. Since the flavonoid composition in wines is highly variable, the net effect of divalent transition metal cations in the free radical scavenging activity remains uncertain.

The present study was conducted to determine the radical scavenging activities of four different types of red wines. Spectrophotometric methods an ESR were used to determine and evaluate the free radical scavenging activities of these wines. We evaluated the change in radical scavenging activities when bivalent copper or iron compounds were added to these four types of wines. We also studied the effect of added complexing agent to the wine–metals mixture.

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2. Materials and methods

2.1. Reagents

Disodiumethylenediaminetetraacetate (EDTA), 2,2-diphenyl-1-picrylhydrazyl (DPPH•) radical, 2,6-di-*tert*-butyl-alpha-(3,5-di-*tert*-butyl-4-oxo-2,5-cyclohexadien-1-ylidene)-*p*-tolyloxy (Galvinoxyl) radical, 5,5-dimethyl-*N*-oxide pyrroline (DMPO), gallic acid, Folin Ciocalteau reactive A and reactive B were purchased from Sigma–Aldrich. All other chemicals and solvents were of the highest commercial grade and used without further purification.

2.2. Samples

Four different types of Chilean red wines were used as samples for the determination of antioxidant activity: Cabernet Sauvignon 2003 vintage, Cabernet Sauvignon 2004 vintage, Merlot 2004 vintage and Shiraz 2004 vintage.

The native concentrations of iron and copper were determined by atomic absorption measurements. The wines were then fortified with copper and iron solutions, in order to increase the native concentration of copper and iron. Two additions were prepared for both copper and iron. The former was increased in 0.6 mg/L and 1.2 mg/L, the latter in 3 mg/L and 6 mg/L. The resulting concentrations after the addition of both metals were still in the permitted range for wines.

2.3. Determination of the antioxidant activity with the UV–vis spectrophotometric 2,2-diphenyl-1-picrylhydrazyl radical scavenging method

The antioxidant activity of wines was measured by UV–vis spectrophotometric methods, as described previously [16]. An approximately 50 μM ethanolic solution of DPPH• was prepared. A total reaction volume of 1.4 mL was prepared by the addition of the ethanolic DPPH• solution and 10% ethanol diluted wine. Absorbance measurements commenced immediately. The decrease in absorbance at 517 nm was determined continuously with data capturing at 0.2 s intervals with an UV2-100 (ATI Unicam, England) UV–vis spectrophotometer until absorbance stabilized (100 s). The decrease at different volumes of 10% diluted wine in the absorbance at 517 nm was registered. Kinetic parameters were calculated assuming a second-order exponential decay model in the absorbance. Consumption and initial rate for each point were calculated as $(A1 + A2)$ and $(A1/t1 + A2/t2)$, respectively, according to the parameters obtained. Both parameters were finally expressed as the slope of the obtained curve of consumption or initial rate vs. wine volume.

2.4. Determination of the antioxidant activity with the UV–vis spectrophotometric 2,6-di-tert-butyl-alpha-(3,5-di-tert-butyl-4-oxo-2,5-cyclohexadien-1-ylidene)-p-tolyloxy (Galvinoxyl) radical scavenging method

Identical conditions as the DPPH radical experiment were applied. In this case, a 10-μM Galvinoxyl radical ethanolic solution was used. Calculations were the same described in Section 2.3.

2.5. Determination of the antioxidant activity with the electron spin resonance 2,2-diphenyl-1-picrylhydrazyl radical scavenging method

DPPH radical scavenging capacities of individual wines were determined by electron spin resonance spectrometry method. ESR analyses were conducted using a Bruker ECS 106 X-Band ESR spectrometer at room temperature. Each wine was mixed with 10 mM DDPH• stock solution to initiate the antioxidant-radical reaction. The final concentration was 2 mM for DDPH• in all reaction mixtures. Different volumes of 50% diluted wines were tested. Both DPPH• and wines were prepared in water. ESR signals were recorded at 1 min intervals following the start of the reaction with 50 KHz field modulation. The scavenging activity of each wine was estimated by comparison with DPPH• signal in the control reaction and was expressed as % DPPH• remaining.

In order to test potential effects of transition metals (copper, iron) on the antioxidant activity, aqueous solutions of either copper or iron were added to the wines. Final concentrations of both metals added ranged from equal to the native concentration found in wine to twice the maximum permitted concentration. Results obtained for wines with added metal were also compared with that of control reaction mixtures.

Additionally, the effect of added disodiumethylenediaminetetraacetate to the antioxidant activity was evaluated under the same conditions.

2.6. Determination of antioxidant activity with the electron spin resonance, 6-di-tert-butyl-alpha-(3,5-di-tert-butyl-4-oxo-2,5-cyclohexadien-1-ylidene)-p-tolyloxy (Galvinoxyl) radical scavenging method

Similar conditions were applied for measuring the antioxidant activity of wines by ESR with the Galvinoxyl radical. Briefly, different volumes of 50% diluted wines were mixed with a 10-mM Galvinoxyl aqueous stock solution. Final concentration of Galvinoxyl in the reaction mixture was 2 mM. ESR signals were recorded at 1 min after the start of the reaction. As in DPPH• experiments, the effect of metal addition was evaluated.

2.7. Determination of total content of polyphenols in wines

Total polyphenols in wines were determined using the Folin Ciocalteau spectrophotometric method. A modification of the method described by Singleton and Rossi was applied [17]. The reaction mixture contained 250 μL Folin Ciocalteau reactive, 50 μL of wine sample, 750 μL of 20% sodium carbonate and distilled water. The final reaction volume was 5 mL. The reaction mixture was incubated for 30 min at 37 °C, and finally, the absorbance at 765 nm was determined. A calibration curve was prepared under these conditions, using gallic acid as standard. The polyphenols content in the wine samples was expressed as gallic acid equivalents.

3. Results and discussion

3.1. Determination of the antioxidant activity with the UV–vis spectrophotometric 2,2-diphenyl-1-picrylhydrazyl radical scavenging method

The antioxidant activity of four different types of wines was determined by means of spectrophotometric methods. Each of the four wines assessed were tested against 2,2-diphenyl-1-picrylhydrazyl radical, and their antioxidant activities were expressed as the consumption and initial rate.

When calculating consumption in these four wines, it was observed that Cabernet Sauvignon 2003 vintage was the one with highest consumption of DPPH radical. As shown in Table 1, both Cabernet Sauvignon wines had stronger scavenging activity than Merlot and Shiraz against DPPH radical. The scavenging activity of Merlot and Shiraz was comparable.

When calculating the initial rate of consumption of DPPH radical, a similar result was obtained. Again, Cabernet Sauvignon 2003

Table 1
Consumption and initial rate of radical consumption in selected red wines

Wine	DPPH		Galvinoxyl	
	Consumption	Initial rate of consumption ($\times 10^{-5}$)	Consumption	Initial rate of consumption ($\times 10^{-5}$)
Cabernet Sauvingon 2003	0.0088	7.22	0.015	3.83
Cabernet Sauvingon 2004	0.0078	3.16	0.0131	2.90
Merlot 2004	0.0051	2.78	0.0073	1.80
Shiraz 2004	0.0048	2.82	0.0087	2.46

vintage was, by far, the most potent one among selected wines against DPPH radical, when comparing their initial rate of DPPH consumption. The initial rate of DPPH consumption determined for Cabernet Sauvignon 2003 was twice larger than the initial rate calculated for the other three wines. Table 1 shows the results obtained for initial rate of DPPH consumption of these four wines.

Fortified wines were then subjected to the same assay. When comparing the consumption and initial rate of DPPH consumption, all four wines showed the same result. Both parameters decreased when performing the assay with fortified wines either with copper or iron addition. The decrease in consumption was approximately 50% in all cases. There was no significant difference between the lowest and the highest additions of metals. Results were similar to those obtained with Galvinoxyl radical, shown in Table 2.

3.2. Determination of the antioxidant activity with the UV–vis spectrophotometric 2,6-di-tert-butyl-alpha-(3,5-di-tert-butyl-4-oxo-2,5-cyclohexadien-1-ylidene)-p-tolyloxy (Galvinoxyl) radical scavenging method

The scavenging activity against Galvinoxyl radical was determined by means of consumption and initial rate of radical consumption. As previously done for DPPH radical, the four selected wines were assayed and their scavenging parameters were calculated. Similarly, a difference in consumption and initial rate was observed among assessed wines. Cabernet Sauvignon 2003 was again the wine with higher consumption and initial rate. Cabernet Sauvignon 2004 had slightly lower consumption of Galvinoxyl radical, while both Shiraz and Merlot had significant lower consumption of this radical (Table 1).

When determining initial rate of consumption of the radical, a similar result was obtained (Table 1).

When analyzing the effect of added copper or iron to red wines, the kinetic parameters of Galvinoxyl consumption showed the same effect that was observed with DPPH radical, that is, a decrease in consumption and initial rate. Table 2 shows the effect of added copper and iron to the red wines. Results obtained for both consumption and initial rate were in all cases similar when analyzing the effect of copper or iron addition, despite of the wine analyzed.

Table 2
Consumption and initial rate of Galvinoxyl consumption in metal fortifie selected red wines

Wine	Galvinoxyl	
	Consumption	Initial rate of consumption ($\times 10^{-5}$)
Wine, no addition[a]	0.0087	–
Wine, first iron addition[a]	0.0071	–
Wine, second iron addition[a]	0.0060	–
Wine, no addition[b]	–	1.86
Wine, first copper addition[b]	–	1.30
Wine, second copper addition[b]	–	1.39
Wine, no addition[c]	–	6.61
Wine, EDTA addition[c]	–	7.25
Wine, copper addition[c]	–	3.05
Wine, copper plus EDTA addition[c]	–	5.00

[a] Cabernet Sauvignon 2004 wine. First iron addition, 3 mg/L; second iron addition, 6 mg/L.
[b] Shiraz 2004 wine. First copper addition, 0.6 mg/L; second iron addition, 1.2 mg/L.
[c] Merlot 2004 wine. Copper addition 0.6 mg/L; EDTA addition, 10 mM.

After the effect of copper and iron addition on kinetic parameters was demonstrated, the effect of a strong chelating agent was evaluated. EDTA was added to wine (previously added with copper or iron), and the effect on consumption and initial rate of Galvinoxyl consumption in selected red wines was observed. The addition of this chelating agent produced a recovery in both consumption and initial rate. This effect was observed in all wines, both with added copper or iron. Table 2 shows the recovery in initial rate of Galvinoxyl consumption.

Table 2 shows that adding EDTA to wine with copper addition results in a recovery of approximately 50% of the antioxidant activity, when expressing the latter as the initial rate of consumption of Galvinoxyl radical. An equal effect was observed in all the four wines evaluated.

3.3. Determination of the antioxidant activity with the electron spin resonance

The activity of wines against stable radicals Galvinoxyl and DPPH was assessed by electron spin resonance. The activity was evaluated by measuring the peak area of the corresponding signal obtained under the instrumental conditions previously described. The higher antioxidant activity was observed as the lower peak area of the radical. As mentioned in Section 2, the activity of all four selected red wines was measured in the presence of two added concentrations of copper or iron.

3.4. Determination of the antioxidant activity with the electron spin resonance 2,2-diphenyl-1-picrylhydrazyl radical scavenging method

When measuring the activity of red wines by ESR, results clearly showed their antioxidant activity against this radical. The DPPH peak area was strongly reduced in the presence of different amounts of wine in the reaction. Only 100 μL of diluted wine was enough to completely scavenge DPPH radical. In the presence of this volume of wine in the reaction, the signal of DPPH completely disappeared.

When evaluating the effect of added copper or iron, results agreed with those observed by UV–vis spectrophotometric experiments. A reduction of the antioxidant activity in wine was produced by the addition of increasing concentrations of copper or iron. It was observed as an increase in DPPH peak area, as shown in Fig. 1.

As observed in Fig. 1, the higher concentration of copper or iron added to wine, the lower antioxidant activity. In this wine, the addition of 0.6 mg/L and 1.2 mg/L of copper resulted in 22% and 46% peak area increase with respect to native wine, respectively. In the same way, the addition of 3 mg/L and 6 mg/L of iron produced 46% and 69% peak area increase with respect to native wine, respectively.

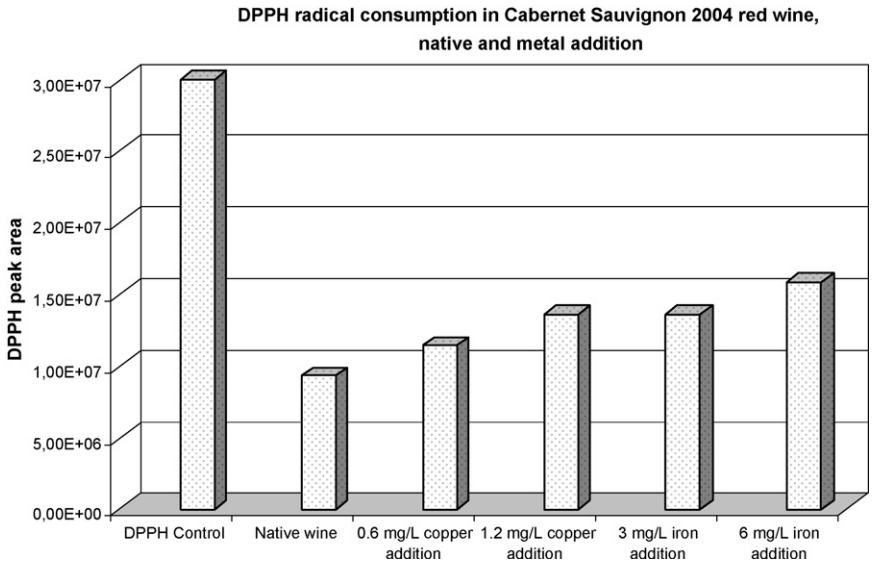

Fig. 1. DPPH radical consumption in Cabernet Sauvingnon 2004 red wine. Bars represent DPPH area obtained when the reaction was done with the corresponding wine: DPPH control, no added wine; native wine, Cabernet Sauvignon red wine with no metal addition.

3.5. Determination of antioxidant activity with the electron spin resonance (Galvinoxyl) radical scavenging method

When measuring the antioxidant activity against Galvinoxyl radical, results were similar to those obtained with DPPH radical. The effect of metal addition was observed as a decrease of Galvinoxyl radical scavenging, that is, an increase in the radical peak area. The higher the addition of metal (either copper or iron), the lower the scavenging activity against Galvinoxyl radical. A similar trend was observed in all four wines analyzed. Fig. 2 shows the effect of metal addition to Cabernet Sauvignon 2004 red wine.

The reduction in Galvinoxyl radical scavenging activity of wine when adding different concentrations of copper or iron is observed in Fig. 2. When expressing this reduction as percentage of variation in Galvinoxyl peak area, the following results was obtained: a 10% increase with 0.6 mg/L copper addition, 116% increase with 1.2 mg/L copper addition, 14% increase with 3 mg/L iron addition and 15% increase in the presence of 6 mg/L iron addition.

Results obtained by these two techniques clearly show an interaction between added transition metals and antioxidant compounds in wine. This interaction requires a stabilization time between addition and measurement of activity. The addition of metal to wine without stabilization time produces erratic and contradictory results.

3.6. Determination of total polyphenols (TPP) content in wines

The total polyphenols content in red wines was measured by the Folin Ciocalteau spectrophotometric method. This method measures the total available hydroxyl groups. At reaction pH, phenols in sample are as phenolate ion, which can reduce the Folin Ciocalteau reactive. For this reason, results from this method can be interpreted as polyphenol content, but also, it can be interpreted as reducing capacity of the sample, and so, as a way of measuring its antioxidant activity.

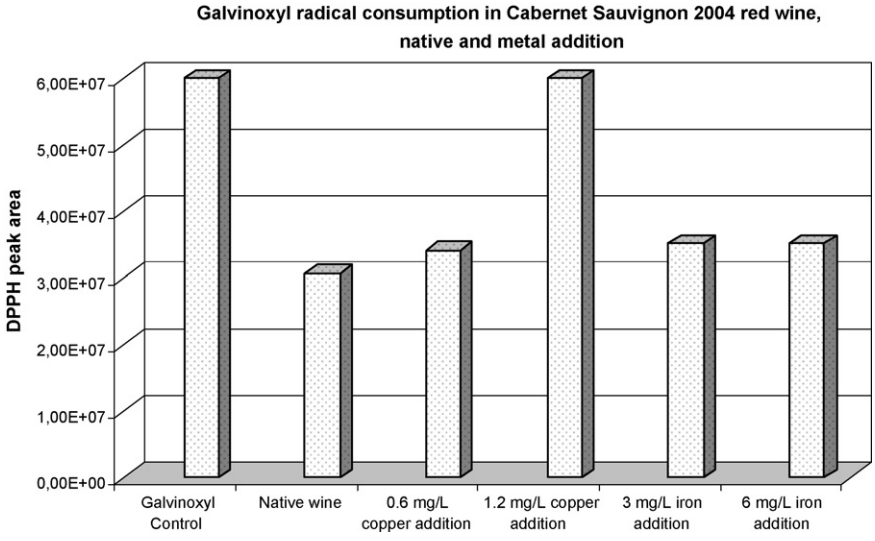

Fig. 2. Galvinoxyl radical consumption in Cabernet Sauvingnon 2004 red wine. Bars represent Galvinoxyl area obtained when the reaction was done with the corresponding wine: DPPH control, no added wine; native wine, Cabernet Sauvignon red wine with no metal addition.

Table 3
Total polyphenols in selected red wines

Sample	TPP (mg equivalent galic acid/g)
Cabernet Sauvignon 2003	2.41
Cabernet Sauvignon 2004	2.46
Merlot 2004	2.59
Shiraz 2004	2.37

Total polyphenols in selected wines were measured. Results are shown in Table 3.

TPP was also measured in wines with added metal. In all cases, all four analyzed wines showed the same trend. In the presence of added copper or iron, TPP dropped with respect to the corresponding native wine. This reduction was dependent of the metal and concentration added. Fig. 3 shows the effect observed in Cabernet Sauvignon 2003.

The antioxidant activity of wines was studied based on results obtained by different techniques. In all cases, the term "antioxidant activity" was used to express the radical scavenging capacity of the samples. In the case of TPP measurements, the reducing capacity of wines was associated to antioxidant activity.

All techniques used in this work showed the same trend in results. Results obtained by using different techniques were consistent with each other. In all cases, an effect of metal addition on antioxidant activity of wines was observed (Table 2, Figs. 1–3). An inverse relation between metal content and antioxidant activity was detected. The addition of Cu^{2+} or Fe^{2+} to wines produced a reduction in antioxidant capacity.

As previously mentioned, the addition of metals to wine requires a stabilization time in order to obtain logic results. The addition of metals without this stabilization time (data not shown) produced erratic and inconsistent results in all cases (mainly in ESR studies, were a signal is obtained with copper and iron which they are themselves paramagnetic). This clearly indicates that the interaction between metals and antioxidant compounds of wine requires a certain time to be established. This type of behavior is frequently observed in the formation of complexes. The literature often raises the hypothesis of a ligand–chelate type interaction between these two [18]. Results obtained in this work agree with this hypothesis.

Measurements done in UV–vis spectrophotometry using Galvinoxyl radical included the use of EDTA as strong chelating agent. These experiments showed that in the presence of this chelating agent, a reversion of the metal effect on antioxidant activity was obtained, when measuring the Galvinoxyl scavenging capacity. Table 2 clearly shows that the addition of EDTA produces an increase in initial rate of Galvinoxyl consumption, with respect to the corresponding copper added wine. This increment corresponds to approximately 50% recovery of antioxidant activity. This result demonstrates that EDTA sequestrates metal ions, preventing the formation of the polyphenol–metal complex by competing with polyphenols for metal ion. This supports the hypothesis of a complex type of interaction between polyphenols and metals.

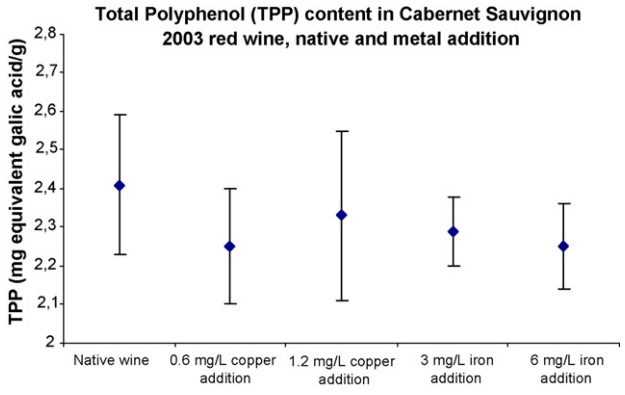

Fig. 3. Total polyphenol content (TPP) in red wine. Bars represent the one standard deviation of results.

The total polyphenols measurement shows a result that is concordant with those obtained by UV–vis and ESR experiments. Again, as observed, the addition of metals produces a reduction of antioxidant activity of wine. In this case, this antioxidant activity is related to available hydroxyl groups in phenolic compounds in wine, which give the reducing (antioxidant) capacity to the matrix. Again, this result demonstrates the effect of copper and iron on the reducing capacity of wine, and also that this reduction occurs due to an interaction between metal ions and available hydroxyl groups in phenolic compounds in wine.

When considering the result of Folin Ciocalteau measurements as a reflection of antioxidant "amount", we decided to use this parameter to normalize and compare the antioxidant activity obtained and measured with other techniques. Basically, the overall antioxidant activity of a sample is a result of (1) the amount of antioxidant compounds contained in the sample and (2) the reactivity of the antioxidant compounds contained in it. By normalizing the data, it is possible to observe whether the difference between wines is due to amount or reactivity of antioxidant compounds.

Data obtained by UV–vis experiments were normalized by simply dividing the result (consumption and initial rate) by TPP. This allowed to isolate the amount of polyphenols from the reactivity of them, and to see which wine sample presented the highest reactivity. When calculating the kinetic parameters consumption and initial rate, it was possible to construct a "ranking" of antioxidant activity of wines. This showed that Cabernet Sauvignon wines were those with higher consumption and initial rate of radical consumption. After normalizing data, it was observed that the overall antioxidant activity "ranking" was maintained, but the difference between wines was larger than without normalization, showing that the difference previously observed between wine types was due mainly to reactivity of antioxidant compounds contained in the matrix.

Finally, it should be noted that ESR technique was successfully applied to wine samples, opening the possibility of a more detailed studies in this matrix by means of this powerful technique.

4. Conclusions

The techniques applied in this work were adequate to the evaluation of antioxidant activity of wine samples. The results obtained in all three techniques applied were consistent with each other, showing an overall effect of metals on the antioxidant activity of wines. It was clearly observed in all experiments that the increase of cooper or iron concentration in wines produces a reduction in antioxidant activity of wine. The interaction between metals and antioxidant (phenolic) compounds of wine is possibly via the formation of metal–antioxidant complex. The formation of this complex would require a certain time, and would cause a reduction in available hydroxyl groups, thus reducing the antioxidant activity of the wine, as observed in Folin Ciocalteau total polyphenol determination.

Acknowledgment

We thank the CEPEDEQ (Centro de Estudios para el Desarrollo de la Química, Center of Studies for Chemistry Development) for the use of its infrastructure and equipments.

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