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Antioxidant Profile of Red Wines Evaluated by Total Antioxidant Capacity, Scavenger Activity, and Biomarkers of Oxidative Stress Methodologies

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The study of the antioxidant capacity of foodstuffs requires the use of diverse determination methods to gain a wider picture of their multiple effects. The aim of this work was to evaluate the "antioxidant profile" of red wines applying TAC (total antioxidant capacity) methods: 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl, *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride, oxygen radical absorbance capacity, ferric reducing/antioxidant power, hydroxyl and superoxide radical scavenger activities, and biomarkers of oxidative stress methods such as lipid peroxidation inhibition and inhibition of damage to DNA. Furthermore, levels of total polyphenols (TPP) of wines were also evaluated. Three bottles of 107 different Spanish red wines (total samples 321), made from different grape varieties, aging processes, and vintages, were analyzed. The validation of TAC methods, the first step in this work, provided a good linearity, proportionality, and low detection limits. Among these methods, the ABTS was the most satisfactory for its rapidity, cost, and precision. All wines showed an important capacity to scavenge hydroxyl radicals and were capable of blocking superoxide radicals but with 10 times lower intensity. Wines also showed important protective action on biomarkers of oxidative stress; they were much more active to inhibit lipid peroxidation than DNA oxidation. Few statistically significant correlations among levels of TPP and antioxidant properties of wines were detected. Furthermore, values of these correlations were very low.

KEYWORDS: Red wine; ABTS; DPPH; DMPD; ORAC; FRAP; scavenger activity; biomarkers of oxidative stress; total polyphenols

INTRODUCTION

Moderate consumption of wine seems to reduce the risk of cardiovascular diseases and cancer (*1*, *2*). In fact, numerous studies have demonstrated that the phenolic compounds found in wine have an antioxidant capacity and free radical scavenging activity (*3*−*7*).

A great many studies have developed and applied methods to measure the total antioxidant capacity (TAC) of foodstuffs and other substrates (for examples, see *8* and *9*). All of these studies have shown that no single assay can provide all of the information needed to evaluate antioxidant capacity. Multiple assays are therefore required to build an antioxidant profile of particular foodstuffs.

The most commonly used methods in the measurement of TAC differ with regard to their reaction mechanisms. Some of them such as oxygen radical absorbance capacity assay (ORAC), telomeric repeat amplification protocol, *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride (DMPD), and low-density lipoprotein oxidation use a hydrogen atom transfer (HAT) mechanism, and others such as ferric reducing/antioxidant power (FRAP), 2,2′-azinobis 3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^{\bullet+}$), and 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) use a single electron transfer (SET) mechanism (*8*). Very briefly put, the HAT assay quantifies the capacity to donate hydrogen atoms and the SET assay measures the reductive capacity of antioxidants. From the point of view of the total polyphenols (TPP) assay, which has been applied for many years as a measure of the total phenolic content in natural products, it is based on an oxidation−reduction reaction; this method can be considered another TAC method (*9*). The TPP assay is also a SET method.

Nowadays, consumer demand and public pressure in general for information on the health benefits of foods call for methods that present relevant biological aspects, that is, methods that are able to reflect the action of a particular antioxidant in vivo. Among the range of biologically relevant methods are those that evaluate scavenger capacity toward reactive oxygen species (ROS) generated in the organism itself due to aerobic metabolism. An example of this method includes evaluation of scavenging capacity in the presence of hydroxyl and superoxide

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radicals. Furthermore, and considering the fact that oxidative stress in biological systems arises from an imbalance between oxygen species and antioxidants, the study of biomarkers of oxidative stress is also a biologically relevant method (*10*). The measurement of lipid peroxidation inhibition and the study of DNA damage enable an assessment of the protective effect of foods on oxidative stress, and they are two of the most applied methods (*7*, *10*, *11*).

The last two groups of methodologies are in vitro biological assays. It is well-known that these methodologies allow an approximation of the healthy effects of the diet, in this case, with the moderate consumption of wines. It is evident that they are limited by bioavailability aspects; however, it has been recognized that the in vitro nature of these assays should not compromise their value. On the contrary, a valid in vitro assay is an invaluable tool for clinical studies if it is combined with bioavailability data (*9*).

During the last years, papers about the TAC of food have been increasing continuously, and in the case of wines, the last 2−3 years have been the most productive (*5*, *6*, *11*−*18*). However, previous work about antioxidant properties of wines usually showed data from small group of wines: The largest number has been 54 wines (*19*), followed by 42 (*5*, *12*); it is very usual to analyze less than 20 wines. Furthermore, these papers showed data from a reduced number of methods: The maximum number was four methods (*5*) and, more frequently, three methods (*14*−*18*). Under these conditions, the results shown sometimes seem to be contradictory, inducing erroneous conclusions and creating some confusion about the actual antioxidant value of the wines. For that reason, it is necessary to carry out new studies using large numbers of samples, and different methodologies could give more precise information about the global "antioxidant profile" of these products, which will be useful in clarifying the confusing situation.

For this reason, the principal aim of this work was to evaluate the antioxidant profile of red wines, using different assays or methodologies, which allow measurement of the TAC, including SET and HAT methods, the scavenging activity, and the protection of biomolecules through biomarkers of the oxidative stress. Furthermore, among methodologies of the same group, it will be carried out in a comparative study to identify what methods might be of most interest, in view of the time and cost of analysis. In other words, the study seeks to identify the best methods for routine analysis with a high number of samples. Finally, the possible correspondence among antioxidant properties (TAC/scavenger/biomarkers) of red wines was analyzed.

The methods used to measure TAC were ABTS, DPPH, DMPD, ORAC, FRAP, and TPP assays. The scavenging activities of hydroxyl and superoxide radicals and two assays of biomarkers of oxidative stress, lipid peroxidation and DNA damage inhibition, were also evaluated.

MATERIALS AND METHODS

Reactives. ′ABTS, DPPH•, DMPD, β-phycoerythrin (PE), 2,2′-diazobis(2-aminodinopropane)dihydrochloride (ABAP), 6-hydroxyl-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox), 2,4,6-tris(2-pyridyl)-*S*-triazine (TPTZ), 2-deoxy-D-ribose, phenazin methosulfate (PMS), and calf thymus DNA were from Sigma-Aldrich Co. (St. Louis, MO). Potassium persulphate ($K_2O_8S_2$), ferric(III) chloride acid ($FeCl_3$), ferrous(II) sulfate ($FeSO_4$), Cu(II) sulfate ($FeSO_4$), hydrogen peroxide (H_2O_2), l-ascorbic acid, and trichloroacetic acid (TCA) were obtained from Panreac (Barcelona, Spain). Methanol (high-performance liquid chromatography grade) came from Lab-Scan (Dublin, Ireland). Thiobarbituric acid (TBA) was purchased from Merck (Darmstadt, Germany). Bovine serum albumin, NADH disodium salt, and 4-nitroblue tetrazolium chloride (NBT) were from Roche (IN). Tris, ethidium bromide, and ethylenediaminetetraacetic acid (EDTA) tretrasodium salt were from Amresco (OH).

Samples. Because of the particular heterogeneity of wines, to reduce the intrinsic variability factors, only red wines were studied. However, to extrapolate results to other red wines, variability due to factors such as grape variety, vintage, or age were considered. One hundred seven Spanish red wines from different grape varieties (Tinta de Toro, Mencía, and Tempranillo), from different vintages (2000−2004), and from different aging processes (young, less than 3 months old; 1, 2, and 3 years old, with wood or bottle aging and with both of them) were studied. Three different bottles of each wine were analyzed. So, interbottle variability was also considered.

ABTS•+ Method. This assay is based on decolorization that occurs when the radical cation ABTS•+ is reduced to ABTS′ (*20*). The radical was generated by reaction of a 7 mM solution of ABTS in water with 2.45 mM $K_2O_8S_2$ (1:1). The mixture was held in darkness at room temperature for 16 h, as this is the time needed to obtain stable absorbance values at 734 nm. Once prepared, the reactive mixture may be used over 4 days. The assay was made up with 980 μL of ABTS•+ and 20 μL of the sample (at a dilution of 1:50 in water). Optimization studies on the absorbance stability of the mixture indicate that the measurements must be made after 15 min of reaction time. The results were expressed in millimolar Trolox, using the relevant calibration curve.

DPPH• Method. This method is based on the reduction of the free radical DPPH• (*21*), which leads to its decolorization. The presence of the antioxidant leads to a loss of color in the reactive in methanol at a concentration of 60 μM. At this concentration, the solution immediately reaches an absorbance value of around 0.7 at 517 nm. According to our own studies in this field, the reaction mixture remains stable for a period of 4 days when kept in darkness at room temperature. The reaction takes place when 980 μL of DPPH• (60 μM) was mixed with 20 μL of the sample (at a dilution of 1:50 in water). A reaction time of 2 h is calculated at room temperature, which is the time needed to arrive at a stable reading of the reactive with the sample. Results were expressed in millimolar Trolox using the dose−response curve described by this substance.

DMPD•+ Method. This method as described by (*22*) was applied with slight modifications. The pink DMPD•+ radical, due to antioxidant action involving the transfer of a hydrogen atom, undergoes decoloration as it transforms into the reduced, colorless form of DMPD. The radical was obtained by mixing 1 mL of DMPD solution (200 mM), 0.4 mL of ferric chloride(III) (0.05 M), and 100 mL of sodium acetate buffer solution at 0.1 M, modifying the pH to 5.25. According to the studies carried out, it reached stable absorbance values at 505 nm following a period of between 18 and 21 h after its preparation at values of around 0.9. It was observed that the reactive mixture had to be kept in darkness, under refrigeration, and at a low temperature (4−5 °C) (*23*).

The reaction takes place when 50 μL of the sample (a dilution of 1:10 in water) is added to 950 μL of the DMPD•+ solution. Absorbance was measured after 10 min of continuous stirring, which is the time taken to reach constant decolorization values. The results were quantified in millimolar Trolox on the relevant calibration curve.

ORAC. This method is based on the fluorescent qualities of PE, which is a pigment extracted from algae (*24*). The presence of an oxidant (ABAP) leads to the formation of peroxyl radicals that directly attack the PE protein molecule and reduce its fluorescence. The action of an antioxidant halts the decay of the molecule, and its fluorescence is therefore not completely reduced. The reaction was prompted by mixing 553 μL of PE (16.7 nM) with 20 μL of the sample (diluted at 1:10 with water) and 1177 μL of 75 mM potassium phosphate buffer solution (pH 7.4). After 15 min of incubation at 37 °C, 750 μL of ABAP was added (32 mM final concentration) and fluorescence was measured. The results were calculated by measuring the difference of net areas under the curve (AUC_{net}) taken from the spectrofluorimeter between the white and the sample colors, expressed as millimolar Trolox on an appropriate calibration curve. The fluorescence decay curve was measured for 80 min, using emission and excitation wavelengths of 584 and 544 nm, respectively.

Table 1. Calibration Model for Measurement of Antioxidant Capacity

method	standard	range	model	*R*
DPPH	Trolox	0.08–1 mM	mM Trolox = 1.6903 × Dif *A* – 0.0056[a]	0.9986
ABTS	Trolox	0.16–1.8 mM	mM Trolox = 2.0968 × Dif *A* + 0.0139[a]	0.9975
FRAP	Fe(II)	0.2–1.6 mM	mM Fe(II) = 1.535 × Dif *A* – 0.0137[a]	0.9983
DMPD	Trolox	0.016–7.9 mM	mM Trolox = (5.2164 × Dif *A* + 0.054)^{2}[b]	0.9900
			mM Trolox = –1.1281 + 15.25 × Dif *A*[a]	0.9609
ORAC	Trolox	0.5–6 μM	μM Trolox = 1/(0.0141 + 3.2665/AUC_{net})[c]	0.9993
			μM Trolox = 0.083 + 0.2861 × AUC_{net}[a]	0.9970
TPP	gallic acid	10–500 mg/L	mg/L gallic acid = 473.21 × $A^{1.09}$[d]	0.9993
			mg/L gallic acid = 485.4 × *A* – 13.22[a]	0.9958

[a] Linear model. [b] Square root-Y model. [c] Double-reciprocal model. [d] Multiplicative model.

FRAP. This method is used to measure the reductive power of a sample (*25*). It is based on increased absorbance at 593 nm due to formation of tripyridyl-*S*-triazine complexes with ferric(II) [TPTZ−Fe(II)] in the presence of a reductive agent. The reactive mixture is prepared by mixing 25 mL of 0.3 M sodium acetate buffer solution at pH 3.6, 2.5 mL of TPTZ (10 mM), 2.5 mL of $FeCl_3$ (20 mM), and 3 mL of water. Thirty microliters of the sample (diluted in water at 1:50) was added to 970 μL of the latter reactive mixture and was incubated at 37 °C for 30 min. The results were expressed as millimoles of Fe(II), using linear calibration obtained with different concentrations of $FeSO_4$.

TPP. The total phenolic contents of the wine samples were determined with the Folin−Ciocalteau reagent, using gallic acid as standard. The Singleton and Rossi improved method was applied (*26*). The results were expressed as gallic acid equivalents (mg/L).

Hydroxyl Radical Scavenging Activity (HRSA). Desoxyribose (2-desoxy-D-ribose) decays when exposed to hydroxyl radicals generated by the Fenton reaction (*27*). The hydroxyl radicals ($HO^{\bullet}$) were generated through the following system: 10 μL of $FeCl_3$ (0.1 mM), 10 μL of ascorbic acid (0.1 mM), 10 μL of H_2O_2 (1 mM), and 10 μL of EDTA (0.1 mM). Fifteen microliters of the sample (diluted at 1:50 in water) were incubated at 37 °C for 1 h, with 20 μL of desoxyribose (1 mM final concentration) in the presence of $FeCl_3$, ascorbic acid, H_2O_2, and EDTA. A 1.5 mL amount of TCA (28% w/v) and 1 mL of TBA (1% w/v, 0.05 M NaOH) were added to 1 mL of the sample under incubation and held for 15 min at 100 °C after which it was left to cool to room temperature. The MDA (malondialdehyde) formed from the decay of the desoxyribose was evaluated in reaction with TBA and measured at 532 nm. The result was expressed as an inhibition % in relation to a control test (without the sample).

Superoxide Radical Scavenging Activity (SRSA). The superoxide radical reacts with NBT (*28*) to generate a colored compound with absorbance to 560 nm. The antioxidant scavenging superoxide radical is correlated with leading of the coloration. The reactive was made up with 50 μL of NADH (77 μM), 50 μL of NBT (50 μM), and 5 μL of PMS (3.3 μM final concentration) in a medium of 16 mM Tris-HCl, pH 8, and 10 μL of the sample (diluted at 1:5). The result was expressed as an inhibition % in relation to a control test (without the sample).

Lipid Peroxidation Inhibition. The microsomes from the liver of a 250−300 g male Wistar rat were extracted in line with the method of Kessler (*29*). The total microsomal protein content was determined using the Bradford method (*30*). The microsomal fraction (1 mg/mL of protein) was incubated in a solution of ABAP (10 mM), prepared in a Tris-HCl buffer solution immediately prior to use, in the presence of 50 μL of the sample (diluted at 1:50). The incubation temperature was set at 37 °C for a period of 90 min. Lipid peroxidation was evaluated with the TBA assay to quantify MDA. Absorbance measured at 532 nm was proportional to the quantity of peroxyl radicals generated, and the results were expressed as an inhibition % in relation to a control test (without the sample).

Damage to DNA. *Incubation of DNA Calf Thymus.* The DNA calf thymus (200 μg) in the absence and the presence of 200 μL of undiluted wine was exposed to the action of hydroxyl radicals generated by the mixture of 100 μL of ascorbic acid (1 mM final concentration) and 10 μL of copper sulfate(II) (100 μM final concentration). The mixture was incubated at 37 °C for 1 h after which the fragments were separated by electrophoresis.

Agarose Gel Electrophoresis. Electrophoresis was carried out with 1% agarose at room temperature using a Bio-Rad power-Pac 1000 (Hercules, CA) electrophoresis system. Subsequently, the gel containing 15 μL of ethidium bromide (10 mg/mL) was observed under ultraviolet light, using a transilluminator, and photographed. The results were expressed as DNA bp fragments, calculated using the standard molecular weight markers such as the 100 bp DNA ladder of 100−1500 bp and the λ DNA/Hind III of 125 bp to 23.1 Kb, both from Invitrogen (CA).

Statistical Analysis. The data sets were subjected to comparative analysis using analysis of variance (ANOVA) and the least significant differences (LSD) test calculated to a significance level of $\alpha = 0.05$. Simple regression models were applied using the Statgraphics Plus software program (*31*). The validation process was carried out applying the computer program developed by Cela (*32*).

RESULTS AND DISCUSSION

Nowadays, there is a great interest in evaluating the protective activity of natural antioxidants, and the health benefits associated with antioxidants in foodstuffs are commonly studied. Many studies have demonstrated the antioxidant abilities of foodstuffs using various methods based on the transference of electrons, hydrogen atoms, or the detection of lipid and protein oxidation activities. It is of immense interest to establish or distinguish between the most satisfactory methods to quantify and describe the antioxidant profile of drinks, as wines, which are related to possible health implications.

The use of optimal analytical methods in the determination of antioxidant capacity constitutes one of the necessary requirements for the satisfactory interpretation of results and their comparison. Hence, the first part of this work concerns with the validation of the methods.

Validation of Methods. The first step is to determine the way in which to express the results. Various forms of quantification appear in the bibliography, the most frequently used being external references and IC_{50} (50% inhibitory concentration) (*5*, *6*, *21*−*25*). In our experience, both methods are valid and useful in the development of comparative studies. However, the method of calculating IC_{50} is more tedious and implies longer analysis times, which is hardly recommendable when there are a high number of samples to be analyzed. For this reason, the quantification was based on external references.

The most appropriate regression models for each method (mM of Trolox vs difference in absorbances), using standard solutions of Trolox concentrations, were calculated. Linear models gave better fits for the ABTS, DPPH, and FRAP methods; a square root (Y) plot proved best for DMPD, and a double-reciprocal model was best for ORAC; a multiplicative model was the best for TPP, although in this case linear model was also satisfactory

Table 2. Linearity Test for the Measurement of Antioxidant Capacity[a]

method	CV F_r (%)	CV b (%)	ANOVA F_{exp}
DPPH	4.54	0.94	11298
ABTS	4.57	1.15	7566
FRAP	3.49	1.25	6373
TPP	4.18	0.85	13770

[a] Acceptance criteria: CV F_r < 5%; CV b < 2%; ANOVA $F_{exp} > F_{tab}$; F_{tab} (1, $n - 2$, 0.05) = 4.17.

Table 3. Proportionality Test for the Measurement of Antioxidant Capacity[a]

method	$a \pm t_{tab}Sa$	t_{cal} (a)	t_{cal} (b)
DPPH	−0.0056 ± 0.0104	−0.91	106.29
ABTS	0.0139 ± 0.0213	1.11	86.98
FRAP	−0.0137 ± 0.0219	−1.07	79.83
TPP	−13.22 ± 14.16	−4.95	117.34

[a] Acceptance criteria: interval should include 0; t_{cal} (a) < t_{tab}; t_{cal} (b) > t_{tab}; t_{tab} ($n - 2$, 0.05) = 1.70.

Table 4. Detection Limits for the Measurement of Antioxidant Capacity, Calculated for a False Positive Probability of 0.05 and False Negative of 0.5

method	detection limit (mM)
DPPH	0.029
ABTS	0.072
FRAP	0.052
DMPD	0.020
ORAC	0.010[a]
TPP	0.025[b]

[a] Values expressed as μM. [b] Values expressed as mg/L.

(**Table 1**). These models presented coefficient correlation values above or equal to 0.990 and random distributions of the residues.

The linear models were checked with the linearity test considering the coefficient of variation of the response factor (CV F_r), the coefficient of variation of the slope (CV b), and the analysis of global variance (ANOVA) (**Table 2**). They all showed good values, with a low CV b and a good fit between experimental and calculated data.

In addition, a proportionality test that indicates whether the mathematical model could be used for predictive purposes (*33*) was performed. The confidence interval of the ordinate at origin a was calculated, which in this case should ideally be 0 and which is defined as $a \pm t_{tab}Sa$, where t_{tab} is the distribution value of Student's t for $n - 2$ degrees of freedom with a probability of 0.05. In addition, the Student's t values for the slope and the ordinate at the origin were also calculated, which should, respectively, be lesser and greater than t_{tab} (**Table 3**). The condition of proportionality was corroborated in all cases.

Having studied the acceptability of the linear models, the next step was to determine the detection limits (*34*), which is to say the minimum quantity of compound with which it is feasible to carry out the analysis (**Table 4**). The detection limits were determined using the Detarchi program (*35*). Very low limits were observed to allow determination of sufficiently small quantities of antioxidants.

The direct evaluation of the antioxidant capacity of the wines is not usually possible, because operating in this way, the limit of saturation (maxima concentration that is possible to assay) is very often exceeded. Then, it was also necessary to study this fact, looking forward for the appropriate dilution of the wines. This study was carried out using six different wines. The appropriate dilution and quantity of the sample for each method were understood as that which gave values within the range of linearity, in each case, and which approached the IC_{50}, which is to say values situated around the inhibition of 50%. It was found that for the ABTS, DPPH, FRAP, and HRSA and for the inhibition of lipid peroxidation methods, the sample had to be diluted 50 times; for DMPD, ORAC, and TPP methods, it had to be diluted 10 times; for SRSA, it was diluted five times, whereas inhibition of DNA damage was studied directly with the undiluted wines. All dilutions were made up using bidistilled water.

Table 5. Evaluation of Method Precision

method	CV (%)[a] repeatability	CV (%)[b] reproducibility
DPPH	1.60	4.38
ABTS	2.57	4.89
FRAP	1.90	8.45
DMPD	1.33	7.99
ORAC	2.63	8.76
HRSA	1.45	6.99
SRSA	3.02	7.68
peroxidation	2.83	6.20
DNA damage	1.66	7.58
TPP	1.32	4.87

[a] Acceptance criteria: CV (%) < 3%. [b] Acceptance criteria: CV (%) < 10%.

The following phase was to evaluate the precision of the methods, defined as the distribution of analytical values around their mean, evaluated in terms of the repeatability and reproducibility of the method. The former was evaluated from the analytical results obtained from the same person analyzing the same sample 10 times simultaneously. It said, using the same apparatus and under the same conditions. The CV was the statistical parameter considered to evaluate the precision of each method (**Table 5**). In all cases, the CV showed values below 3%, which is very satisfactory.

The reproducibility was calculated by three analysts, which analyzed by triplicate the same sample at three different days, thereby obtaining nine data sets. Considering the rapid evolution of the wines after opening the bottles, the wine bottles used for the reproducibility test were opened and rapidly rebottled in other smaller bottles. These were analyzed at the corresponding date and by the corresponding analyst. All analyses were completed over 1 week. Results showed, in all cases, CVs below 10%, thereby demonstrating that the precision of the methods was acceptable. For lipid peroxidation and DNA damage methods, repeatability and reproducibility were evaluated from five and six replicate data sets, respectively.

TAC. The wines under analysis showed significant differences of TAC values, so a large range of variation and high values of the CV were detected (**Table 6**). These results were expected in view of the heterogeneity of the studied wines. The strong influence of factors such as the grape variety, the winemaking process, the ages, and the vintages on the final composition of the wines is a well-known fact, which is especially important on some particular phenolic compounds as anthocyanins and tannins (*36*−*38*), which are the main compounds responsible for TAC in wines and other drinks (*3*, *7*, *13*, *39*−*41*).

To better understand the quantitative differences detected among methods, it must be considered that each method works with a different mechanism and the sensibility of each ones. In

Table 6. Antioxidant Capacity Measured as ABTS, DPPH, DMPD, ORAC, FRAP, and TPP[a]

method	antioxidant capacity	CV %	range
ABTS (mM Trolox)	22.09 ± 8.30 c	37.6	4.28–41.02
DPPH (mM Trolox)	14.34 ± 4.62 b	32.2	1.19–25.49
DMPD (mM Trolox)	13.60 ± 12.51 b	92	0.05–50.02
ORAC (mM Trolox)	0.039 ± 0.019 a	48.7	0.008–0.091
FRAP (mM Fe(II)	35.08 ± 15.66	44.6	9.41–101.53
TPP (mg gallic acid/L)	2422 ± 332	13.7	1775–3014

[a] Results are the mean ± SD of 321 samples. Values with different letters are significantly different (LSD test, $p < 0.05$).

this sense, it is important to remember that DPPH has been described as more specific for lipophilic antioxidants, DMPD and FRAP for hydrophilic antioxidants, and ABTS and ORAC for both classes (*9*).

Therefore, because ABTS is a good method to evaluate lipophilic as well as hydrophilic antioxidants, it seems logical that this method gave higher values, registering the activity of both types of antioxidant present in the wine. The low values registered by ORAC might initially appear surprising for the same reason; nevertheless, similar results have previously been published (*5*, *15*). Furthermore, some works referring to ORAC do not measure TAC, measuring only the scavenging activity toward peroxyl radicals (*42*). ANOVA results indicated that average values of DPPH and DMPD were similar. However, the range of variation and the CV of their values among wines were markedly different. In fact, the DMPD method showed the highest CV among wines. Usually, wines showed lower values for DPPH than for DMPD.

The reductive capacity of many phenolic compounds present in wines is a well-known fact. Furthermore, oenologists know well the problems associated with the capacity of phenolic compounds to react with metals and especially with iron. Accordingly, the high FRAP values are not surprising, especially considering that the values were taken from red wines, the richest wines in phenolic compounds.

As it was expected, the three methods that work with SET mechanisms showed significant and positive correlations among them, being much closer between ABTS and DPPH or FRAP than between the latter two (**Table 7**). This fact seems to be correlated with the particular antioxidant specification of each method: DPPH for lipophilic antioxidants, FRAP for hydrophilic antioxidants, and ABTS for both types.

The DMPD method, based on a HAT mechanism, showed significant, positive, and close correlations with ABTS as well as with DPPH and somewhat less so with FRAP. ORAC also shows significant positive correlations with the SET methods although they are not as close, which might be due to the low values obtained by this method.

These results seem to indicate that these five methods, despite their differences, give similar information or at least much correlated information about TAC of the wines. For this reason, and to simplify the effort until large routine analysis to evaluate the TAC of the red wines, it could be recommended that one use mainly the ABTS method.

The TPP method also showed an important range of variation but a relatively low value of CV (**Table 6**). This parameter, in spite of evaluating antioxidant capacity or the reduction power of the wines, seems not to be widely correlated with TAC methods (**Table 7**); an even negative correlation was detected, indicating that wines with the highest levels of TPP did not always show the highest values of TAC. These results are contrary to some others published previously (*5*, *6*, *12*, *19*, among others), which described significant and positive correlations between TPP levels of wines and their antioxidant capacities evaluated by ABTS and DPPH, among others methods.

One possible explanation for the obtained results is the fact that the antioxidant capacity of the phenols depends more on their structure and conformation than their concentration (*39*); it is more important the kind of phenolic present in the wines than their global content. It is well-established that the antioxidant efficiency of red wines is significantly correlated with flavonoids, especially with flavanol and anthocyanin fractions (*39*−*41*). Flavanols and anthocyanins are probably the groups of flavonoids that show the most important variability among wines from different varieties, ages, or making process, including aging (*3*, *13*, *17*−*19*, *36*−*38*). In fact, varietal and vintage factors, together with aging processes, are some of the most influential factors on the antioxidant capacities of wines (*19*). Therefore, the heterogeneity of studied wines produced a high intrinsic variability (**Table 6**, CV) and strong dispersion of the data (**Figure 2**), which makes the detection of significant linear correlation difficult. This hypothesis is also supported by the fact that significant linear correlations among total levels of polyphenols and TAC values were found by grouping the wines. Therefore, by grouping wines by similar age, vintage, or variety, coefficients of correlations with statistical significance were found, which showed high values. For example, grouping the wines by age, the coefficients of linear correlation between TPP and ABTS showed values ranging from 0.3171 to 0.7444; between TPP and DPPH showed values ranging from 0.2958 to 0.7622, and values ranging from −0.7975 to 0.5995 were found between TPP and FRAP values. All of these results seem to indicate that the relation between the amount of total phenols and the protective effect of wines is not so evident, and the establishment of general conclusions must be done out with prudence.

Scavenging Activity of ROS. All wines showed a significant capacity to scavenger hydroxyl and superoxide radicals (**Table 8**), being more active with hydroxyl. These results must be correlated with the components of the wines capable of scavenging these radicals, and the differences may be explained by the different mechanisms involved in the capture of each one. Therefore, Jovanovic (*43*) suggested that superoxide radical activity was solely mediated by the donation of one unpaired electron from the radical to the flavonoid. However, various mechanisms such as the transference of hydrogen atoms, the stability acquired by the phenoxyl radicals generated in that transference, and the donation of electrons appear to be involved in HRSA, hence, the higher activity values. Furthermore, Roussis (*14*) described flavanols and anthocyanins as the active compounds involved in superoxide radical activity. Furthermore, this author indicated that flavonols, together with anthocyanins and flavanols, are the compounds that exhibited the highest activity in the presence of hydroxyl radicals. These facts explain that both methods showed significant and positive linear correlation ($r = 0.4981$; $p = 0.0000$). These data noted that the "scavenger" methods give complementary information, and it is recommendable to evaluate both activities to obtain a complete antioxidant profile of the wines.

Biomarkers of Oxidative Stress. It has been widely demonstrated that red wine possesses a protective effect against oxidative stress (*1*, *2*, *4*). Similarly, biomarkers of oxidative stress have been used to monitor the involvement of oxidative damage in the pathogenesis of certain diseases or in the toxicity

Table 7. Lineal Correlation Coefficients among the Different Methods for Quantify the Antioxidant Capacities[a]

	ABTS	DMPD	DPPH	FRAP	TPP
DMPD	0.7577 (0.0000)				−0.1733 (0.0284)
DPPH	0.7156 (0.0000)	0.7379 (0.0000)			
FRAP	0.7412 (0.0000)	0.5801 (0.0000)	0.5882 (0.0000)		0.1729 (0.0456)
ORAC	0.5415 (0.0000)*	0.6353 (0.0000)	0.4754 (0.0000)	0.4113 (0.0000)	−0.3003 (0.0001)

[a] The correlation was evaluated by linear regression using the Statgraphics Plus software. Only linear coefficients with significance lower than 0.05 are shown; (*p* values)* ≤ 0.05 (*n* = 321).

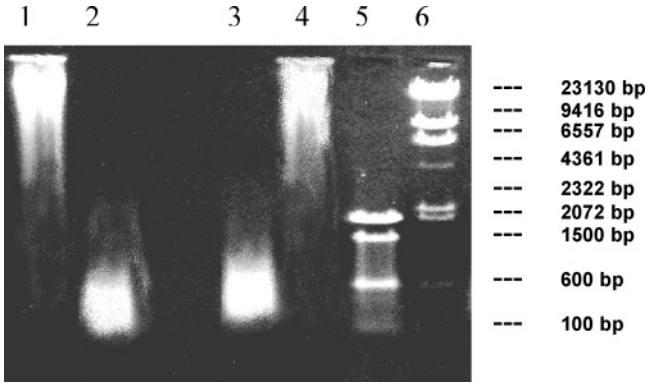

Figure 1. Agarose gel electrophoresis separation of damaged DNA induced by Cu(II)–ascorbic acid and the effect of red wine. The numbered lanes represent DNA alone (1), DNA exposed to Cu(II) (100 μM) and ascorbic acid (1 mM) (2), DNA plus Cu(II)–ascorbic acid plus 100 μL of red wine (3), DNA plus Cu(II)–ascorbic acid plus 200 μL of red wine (4), 100–2000 bp DNA ladder molecular weight standard (5), and 125 bp 23,1 Kb λ DNA/Hind III Fragments molecular weight standard (6).

of xenobiotics (*10*). Hence, it might well be a good strategy to look carefully at the possible health benefits or protective effects against oxidative stress and to measure various biomarkers simultaneously that are representative of damage to different cellular components, such as membrane, DNA, or proteins.

Thus, it was decided to evaluate the protective effect of wines on the inhibition of microsomal peroxidation and of damage to DNA. The results of the inhibition of lipid peroxidation, as in the previous cases, showed a great variability among wines: range, 0−89; average value of 36.1%; deviation of ±23.1; and percentage variation of CV, 64%. It was necessary to dilute the sample 50 times for this evaluation, which is to say, all of the wines showed important protective capacity against lipid peroxidation that was similar to their scavenging capacity toward hydroxyl radicals.

The assay of damage to DNA also showed significant protective action by the wines under study that, in general, was clearly observable with a dose of 200 μL. **Figure 1** shows an example of the obtained results. This methodology is valid if we accept that greater mobility-distance from the well to the zone of greatest density is directly proportional to the damage to DNA and inversely proportional to the size of the fragments that are obtained. So, the fragments ranged from 3594 pb up to 13302 pb with a mean average value of 8076 pb ± 2351, which implies a coefficient of variation of 31.34%. These results are considered positive although a total protective effect was not achieved. In general, the DNA was partially protected, as demonstrated by the presence of fragments with larger sizes than those obtained when the wine was not in the incubation medium. Furthermore, in some cases, the wines offered a great deal of protection (fragments of 13302 bp). Moreover, no pro-oxidant effects were detected.

Both biomarker methods showed significant and positive correlation between them ($r = 0.5043$; $p = 0.0000$), which

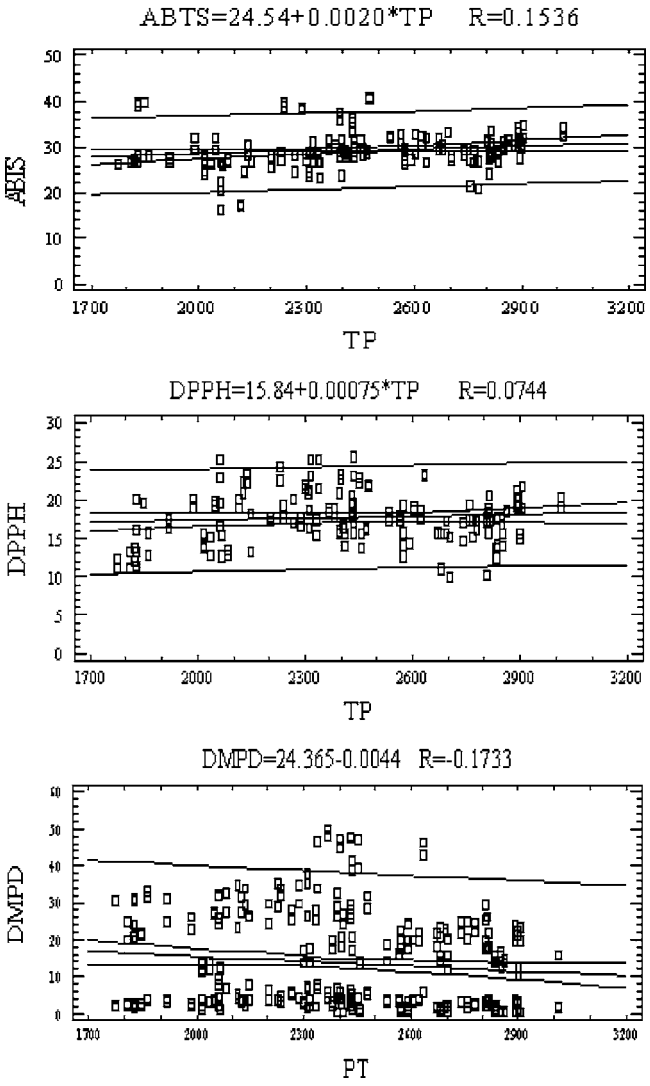

Figure 2. Distribution of studied wines on the described spaces: TTP vs ABTS, TPP vs DPPH, and TPP vs DMPD. Simple linear corresponding models are shown. External lines indicated the confiance interval for α = 0.05.

Table 8. Scavenger Activity Measured as HRSA and SRSA[a]

method	% of inhibition	CV %	range
HRSA (1:50)[b]	37.8 ± 11.6	31	5.55–80.42
SRSA (1:5)[b]	59.6 ± 28.3	47	0–98.03

[a] Results are the mean ± SD of 321 samples. [b] Dilution of samples.

means that in general the wines that showed greater protective effects against peroxidation were also the most effective against damage to DNA. This result seems to indicate that in general the protective effect of wines against oxidative stress can occur at various levels, all of which are significant and correlate

Table 9. Linear Correlation Coefficients among Antioxidant Capacities, Scavenging Activities, and Biomarkers of Oxidative Stress[a]

	HRSA	SRSA	DNA damage	inhibition lipid peroxidation
ABTS		0.1291 (0.0209)	0.7723 (0.0000)	0.5492 (0.0000)
DPPH		0.1796 (0.0013)	0.6508 (0.0000)	0.2995 (0.0000)
DMPD	–0.1324 (0.0178)		0.7312 (0.0000)	0.4311 (0.0000)
ORAC	–0.2420 (0.0000)		0.5234 (0.0000)	0.2850 (0.0000)
FRAP		0.2895 (0.0000)	0.6984 (0.0000)	0.2989 (0.0000)
TPP	0.1709 (0.0307)	–0.1610 (0.0420)		0.1834 (0.0203)

[a] The correlation was evaluated by linear regression using the Statgraphics Plus software. Only linear coefficients with significance lower than 0.05 are shown; (p values)* $\leq$ 0.05 (n = 321).

positively between each other; that is, they are not antagonistic. These results also indicate that in the global antioxidant profile of the wines, only one of these methods could be represented; more recommended is the inhibition of lipid peroxidation.

Correspondence about Methods. It was considered interesting to evaluate the possible correspondence among the results of the different kinds of methods studied, to determine if the most active wines vs TAC were also the most active scavengers or the most protective of biomolecules of damage. Linear correlation was selected as a rapid and simple system to check this fact.

Some significant but very weak correlations were found between the TAC methods and the scavenger ones (**Table 9**). It is interesting to note that HRSA and SRSA showed different correlations. The first only showed negative correlations with HAT methods (DMPD and ORAC), and the latter only showed positive correlations with SET methods (ABTS, DPPH, and FRAP). Furthermore, TPP was positively correlated with the former and negatively with the last one. These results confirm the hypothesis that different mechanisms are involved in capturing both types of radicals or their related compounds. The negative correlation implies that, in general, the wines with the greatest ability to transfer hydrogen atoms are the least effective at stabilizing the hydroxyl radical.

The TAC methods presented significant positive correlations with the oxidative stress biomarker methods. The coefficients of correlation were high, especially with protection to DNA damage, indicating a strong correlation. Then, it is possible to affirm that wines with higher values of TAC also showed the best protective effect of biomolecules affected by oxidative stress.

To the best of our knowledge up until the present, this is the first time that linking among these different antioxidant properties of the wines has been shown. These results are very interesting to the oenological sector but also contribute to the "state of art" about antioxidant role of red wines and will be useful to the approximation to in vivo situation.

Some significant but also weak correlations were found between the scavenger capacities and the biomarkers protection (**Table 10**). Negative correlation was found between HRSA and inhibition of peroxidation (−0.1971; p = 0.0004), which is to say, unlike what may have been expected, the wines with a strong ability to inhibit lipid peroxidation were not the best hydroxyl scavengers. What is more, there could be antagonistic effects judging by the negative value of the correlation. The negative correlation agrees with the negative correlations found earlier between the TAC methods and the HRSA as well as confirming the previously commented strong relations between the TAC methods and the inhibition of lipid peroxidation.

The positive correlation detected between SRSA and inhibition of DNA (0.2031; p = 0.0003) confirms that the protective role of the wines to DNA damages is at least in part linked to the capture of the superoxide radical. Once again, the specific correlations to each scavenger method with the others clearly highlight the differences between each one of them as well as their complementary aspects.

Table 10. Linear Correlation Coefficients among Scavenging Activities and Biomarkers of Oxidative Stress[a]

	HRSA	SRSA
DNA damage		0.2031 (0.0003)
inhibition lipid peroxidation	–0.1971 (0.0004)	

[a] The correlation was evaluated by linear regression using the Statgraphics Plus software. Only linear coefficients with significance lower than 0.05 are shown; (p values)* $\leq$ 0.05 (n = 321).

Conclusions. Given the high number of samples analyzed in this work and their heterogeneity, it is considered that the samples contained sufficient initial variability to establish general conclusions, which could be extrapolated to other red wines. A complete antioxidant profile of the red wines could be established by evaluation: ABTS as TAC methodology, both scavengers activities (hydroxyl and superoxide), which give complementary information, and some type of biomarker methods, preferably the inhibition of lipid peroxidation. The evaluation of TPP content is also recommended, because this method evaluates the reductive power of the wines.

The ABTS method showed good qualities as routine analysis methods due to its cost, speed, easy measurement, reactive stability, high precision, and the fact that it provided similar information to that given by the rest of TAC studied methods. Inhibition of lipid peroxidation is the recommended biomarker method due to the simplicity with which its results are treated and interpreted. ABTS could also be a good system of approximation to the protective effects of the biomolecules, when biomarker methods are not able to be evaluated.

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