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Extending Applicability of the Oxygen Radical Absorbance Capacity (ORAC–Fluorescein) Assay

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The ORAC-fluorescein (ORAC−FL) method recently validated using automatic liquid handling systems has now been adapted to manual handling and using a conventional fluorescence microplate reader. As calculated for Trolox, the precision of the method was <3.0, expressed as percent coefficient of variation. The accuracy of the method was <2.3, expressed as percent variation of the mean. The detection and quantification limits were those corresponding to 0.5- and 1-μM Trolox standard solutions, respectively. The method has been applied to 10 pure compounds (benzoic and cinnamic acids and aldehydes, flavonoids, and butylated hydroxyanisole), to 30 white, rose, and bottled- and oak-aged red wines, and to 7 commercial dietary antioxidant supplements. All samples exhibited a good linear response with concentration. As seen by other methodologies, the chemical structure of a compound determines its antioxidant activity (ORAC−FL value). Of particular interest were the results with oak-aged red wines from different vintages (1989−2002) that confirm influence of vintage, but not origin of the oak, in the antioxidant activity of wines from the same variety. Dietary antioxidant supplements presented a great variability (170-fold difference) in their antioxidant potency. This work proves applicability of the ORAC−FL assay in evaluating the antioxidant activity of diverse food samples.

KEYWORDS: Antioxidant activity; ORAC; fluorescein; phenolics; wine; dietary antioxidant supplements

INTRODUCTION

Attempts to develop methods for assaying antioxidant activity against reactive oxygen species (ROS) are fully justified by the implications of ROS in the pathogenesis of several chronic diseases, and in general, in the aging process. In the late eighties, Glazer (*1*, *2*) developed an assay to measure hydrophilic antioxidant activity against ROS based on the detection of chemical damage to R- or β-phycoerythrins (PEs) through the decrease in their fluorescence emission. PEs were exposed either to peroxyl radicals, generated by thermal decomposition of 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), or to hydroxyl radicals, generated at copper-binding sites on macromolecules in the presence of ascorbate and Cu^{2+}. In the presence of peroxyl radicals, the decay in the fluorescence of PE followed a zero order (i.e., linear with time) kinetic, and the antioxidant capacity of a compound/mixture was related to the increase observed in the lag phase and/or to the decrease in the rate constant. Using β-phycoerythrin as fluorescent probe and AAPH as free radical initiator, Cao and co-workers (*3*, *4*) quantified the protective effect of an antioxidant by assessing the area under the fluorescence decay curve (AUC) of the sample as compared to that of the blank in which no antioxidant was present. Results were expressed as Trolox equivalents. This methodology was named as the oxygen radical absorbance capacity (ORAC) assay, and it is still one of the few methods that combines both inhibition percentage and inhibition time of the reactive species action by antioxidants into a single quantity (*4*). The ORAC assay has been largely applied to the assessment of free radical scavenging capacity of human plasma, proteins, DNA, pure antioxidant compounds and antioxidant plant/food extracts (see *5* for references). The principal drawback of this assay is the PE itself, since it varies from lot to lot, is not photostable, and can be photobleached after an exposure to excitation light (*6*). Additionally, it was observed that PE interactions with polyphenols could cause erroneous ORAC values (*6*). Considering these disadvantages, Ou and co-workers (*6*) developed and validated an improved oxygen radical absorbance capacity assay using fluorescein (FL) as the fluorescent probe (ORAC−FL). FL as compared to PE does not interact with antioxidants, shows an excellent photostability, and reduces the cost of experiments (*6*). The ORAC−FL assay was further extended to lipophilic antioxidants by using methylated β-cyclodextrin as water solubility enhancer (*7*).

The oxygen radical absorbance capacity assay using fluorescein (ORAC−FL) method was developed on a COBAS FARA II centrifugal analyzer with fluorescence-measuring attachment (Roche Diagnostic System Inc., Branchburg, NJ), that could analyze up to nine samples at a single concentration per run (*6*). Recently, the ORAC−FL has been fully automated by using a multichannel liquid handling system (robotic) coupled with a microplate fluorescence reader in 96-well format, that improved the efficiency of the assay 10-fold (*8*). Automation indeed reduces time analysis and improves efficiency in large scale

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analyses, but it is available in few laboratories. Small laboratories from public research Institutions/universities and from quality and control or research and development industry departments would still use manual handling for the ORAC assay, because they do not need to analyze many samples every day. In this paper, we adapted the ORAC-fluorescein assay to manual handling, and using a conventional fluorescence plate reader. The antioxidant activity of several pure compounds and food (solid and liquid) samples was determined by the adapted ORAC−FL method. Wine was chosen as a liquid sample because of its high content in antioxidant phenolics (i.e., anthocyanins, catechins, and flavonols) and dietary antioxidant supplements as solid samples because they contain different antioxidant-type compounds (e.g., vitamins, carotenoids, and flavonoids). The literature contains very few studies about the antioxidant activity measurement by the ORAC method for both wine (*4*) and dietary antioxidant supplements (*9*).

MATERIALS AND METHODS

Chemicals. FL, disodium salt, β-Phycoerythrin (PE), butylated hydroxyanisole (BHA), 3,4-dihydroxycinnamic acid (caffeic acid) and (+)-catechin were purchased from Sigma (St Louis, MO). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH), 3,4-dihydroxybenzoic acid (protocatechuic acid), 4-hydroxy-3-methoxycinnamic aldehyde (coniferyl aldehyde), and 5'-caffeoylquinic acid (chlorogenic acid) were obtained from Aldrich (Milwaukee, WI). 4-Hydroxy-3-methoxycinnamic acid (ferulic acid), 4-hydroxycinnamic acid (*p*-coumaric acid), and 3,4-dihydroxybenzoic aldehyde (protocatechuic aldehyde) were purchased from Fluka A.G (Buchs SG, Switzerland). Quercetin was obtained from Koch-Light Laboratories Ltd. (Colnbrook, England). For ORAC−FL assessment, standards were dissolved in <200 μL of methanol and made up to the final volume with 75 mM phosphate buffer (pH 7.4). A fluorescein stock solution (1.17 mM) was made in 75 mM phosphate buffer (pH 7.4) and was stored at 4 °C for 4 weeks. β-PE solution (27.8 nM), AAPH, and Trolox solutions in 75 mM phosphate buffer (pH 7.4) were prepared daily.

Wines. Two white wines (var. Verdejo from Rueda *appellation contrôlée* (Valladolid, Spain) and var. Albariño from *Rias Baixas appellation contrôlée* (Pontevedra, Spain)) and three rose (var. Cabernet-Sauvignon from *Somontano appellation contrôlée* (Huesca, Spain), var. Tempranillo from *Méntrida appellation contrôlée* (Toledo, Spain), and var. Garnacha from *Navarra appellation contrôlée* (Pamplona, Spain)) wines from the 2002 vintage were obtained from local markets. Twenty-five red wines were provided by EVENA (Viticulture and Enology Station of Navarra, Olite, Navarra, Spain). Three of them corresponded to bottled-aged wines elaborated from grapes of var. Graciano, Tempranillo, and Cabernet-Sauvignon from the 2000 vintage. The rest of them corresponded to oak-aged wines elaborated from grapes of var. Tempranillo and Cabernet-Sauvignon from the 1989, 1992, 1995, 2000, 2001, and 2002 vintages. These wines were aged in barrels from American or French (var. *Allier*) oak for one year, except for 2002 wines that were aged for three (var. Tempranillo) or four (var. Cabernet-Sauvignon) months. For ORAC−FL assessment, wines were diluted as convenient with 75 mM phosphate buffer (pH 7.4). Total polyphenol content in wines was determined according to the Folin−Ciocalteau method (*10*), using gallic acid as a standard. The results were expressed as mg of gallic acid equivalents (GAE) per liter.

Dietary Antioxidant Supplements. Seven commercial dietary antioxidant supplements (named #1−7) were obtained from local markets. According to the manufactures, they contain grape extracts (#1−#5 and #7), vitamin C (#5−#7), vitamin E (#5 and #7), carotenoids (#5 and #7), citrus flavonoids (#6 and #7), yeast rich in selenium (#5 and #7) and others. Different quantities (0.02 g for #4; 0.08 g for #3; 0.1 g for #1, #6, and #7; 0.5 g for #2; 2 g for 5#) of dietary antioxidant supplements were dissolved in methanol and centrifuged (2000 rpm, 10 min), and the supernatant was collected for analysis. For ORAC−FL analysis, supernatants were diluted as convenient with 75 mM phosphate buffer (pH 7.4). Two different batches of each supplement were sampled.

Equipment. A Polarstar Galaxy plate reader (BMG Labtechnologies GmbH, Offenburg, Germany) controlled by the Fluostar Galaxy software version (4.11−0) was used. Black 96-well microplates (96F untreated microwell, Nunc, Denmark) were used. For the ORAC−PE assay, the 544-P excitation and the 584-P emission filters were used. For the ORAC−FL assay, the 485-P excitation and 520-P emission filters were used. For both assays, fluorescence measurement was carried out at 37 °C.

ORAC−FL Assay. The method of Ou et al. (*6*) was modified as follows: The reaction was carried out in 75 mM phosphate buffer (pH 7.4), and the final reaction mixture was 200 μL. Antioxidant (20μL) and fluorescein (120 μL; 70 nM, final concentration) solutions were placed in the well of the microplate. The mixture was preincubated for 15 min at 37 °C. AAPH solution (60 μL; 12 mM, final concentration) was added rapidly using a multichannel pipet. The microplate was immediately placed in the reader and the fluorescence recorded every minute for 80 min. The microplate was automatically shaken prior each reading. A blank (FL + AAPH) using phosphate buffer instead of the antioxidant solution and eight calibration solutions using Trolox (1−8 μM, final concentration) as antioxidant were also carried out in each assay. All the reaction mixtures were prepared in duplicate, and at least three independent assays were performed for each sample.

Raw data were exported from the Fluostar Galaxy software to an Excel (Microsoft, Roselle, IL) sheet for further calculations. Antioxidant curves (fluorescence versus time) were first normalized to the curve of the blank corresponding to the same assay by multiplying original data by the factor $\text{fluorescence}_{\text{blank},t=0}/\text{fluorescence}_{\text{sample},t=0}$. From the normalized curves, the area under the fluorescence decay curve (AUC) was calculated as

$$\text{AUC} = 1 + \sum_{i=1}^{i=80} f_i/f_0$$

where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time i.

The net AUC corresponding to a sample was calculated by subtracting the AUC corresponding to the blank. Regression equations between net AUC and antioxidant concentration were calculated for all the samples. ORAC−FL values were expressed as Trolox equivalents by using the standard curve calculated for each assay. Final results were in μmol of Trolox equivalent/μmol of pure compound for phenolic standards, μmol of Trolox equivalent/mL of wine for wine samples, and μmol of Trolox equivalent/mg of supplement for dietary antioxidant supplements.

ORAC−PE Assay. The assay was based on the previous procedure described by Cao et al. (*4*). The reaction was carried out in 75 mM phosphate buffer (pH 7.0), and the final reaction mixture was 200 μL. Antioxidant (20 μL) and β-phycoerythrin (120 μL; 16.7 nM final concentration) solutions were placed in the well of the microplate. The mixture was preincubated for 15 min at 37 °C. AAPH solution (60 μL; 32 mM, final concentration) was added rapidly using a multichannel pipet. The microplate was immediately placed in the reader and the fluorescence recorded every minute for 80 min. The microplate was automatically shaken prior to each reading. A blank using phosphate buffer instead of the antioxidant solution and 10 calibration solutions using Trolox (3−40 μM final concentration) as antioxidant were also carried out in each assay. All the reaction mixtures were prepared in duplicate, and at least three independent assays were performed for each sample. ORAC−PE values were calculated as described above for ORAC−FL ones.

RESULTS AND DISCUSSION

Method Validation. Figure 1 illustrates the layouts of a 96-well microplate used for the ORAC−FL measurement in the Polarstar Galaxy microplate reader. The fluorescence signal of FL was constant at least over 80 min when no AAPH was added (**A1** and **B1**), indicating that FL was photostable under the

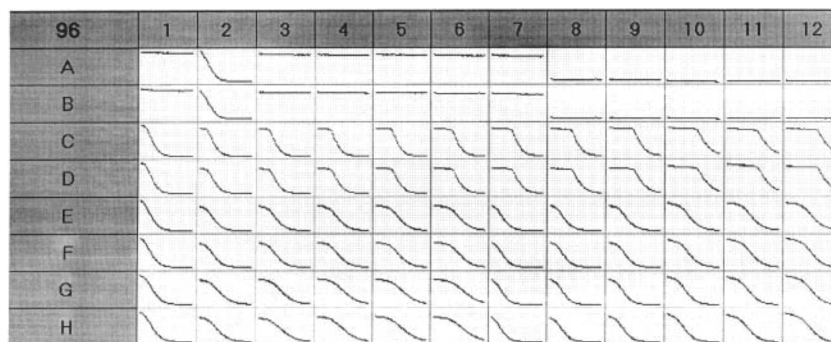

Figure 1. Display of the results obtained by the Polarstar Galaxy microplate reader. **A1** and **B1** correspond to fluorescein (70 nM). **A3**–**A7**, **B3**–**B7** correspond to the mixture of fluorescein and antioxidant (**3**, Trolox (10 μM); **4**, caffeic acid (1.0 μM); **5**, protocatechuic acid (1.0 μM); **6**, bottled-aged red wine var. Graciano (2.0 μL/mL); and **7**, dietary antioxidant supplement #4 (28.6 μg/mL)). **A2** and **B2** correspond to fluorescein and **A8**–**A12** and **B8**–**B12** to the antioxidants (**8**, Trolox; **9**, caffeic acid; **10**, protocatechuic acid; **11**, bottled-aged red wine var. Graciano; and **12**, dietary antioxidant supplement #4; all at the same concentration reported above) in the presence of AAPH (12 mM). **C1**–**C12** and **D1**–**D12** corresponds to the mixtures of fluorescein and Trolox at different concentrations (**1**, 0.5 μM; **2**, 1 μM; **3**, 2 μM; **4**, 3 μM; **5**, 4 μM; **6**, 5μM; **7**, 5.5 μM; **8**, 6 μM; **9**, 7 μM; **10**, 8 μM; **11**, 9 μM; and **12**, 10 μM) in the presence of AAPH (12 mM). **E1**–**E12** and **F1**–**F12** correspond to the mixtures of fluorescein and caffeic acid (**1**, 0.1 μM; **2**, 0.2 μM; **3**, 0.4 μM; **4**, 0.6 μM; **5**, 0.8 μM; **6**, 1.0 μM) or protocatechuic acid (**7**, 0.15 μM; **8**, 0.3 μM; **9**, 0.5 μM; **10**, 0.7 μM; **11**, 0.85 μM; and **12**, 1.0 μM) in the presence of AAPH (12 mM). **G1**–**G12** and **H1**–**H12** correspond to the mixtures of fluorescein and bottled-aged red wine var. Graciano (**1**, 0.33 μL/mL; **2**, 0.66 μL/mL; **3**, 1.1 μL/mL; **4**, 1.43 μL/mL; **5**, 1.66 μL/mL; **6**, 2.0 μL/mL) or dietary supplement #4 (**7**, 2.85 μg/mL; **8**, 6.66 μg/mL; **9**, 10 μg/mL; **10**, 13.3 μg/mL; **11**, 20 μg/mL and **12**, 28.6 μg/mL) in the presence of AAPH (12 mM).

Table 1. Linearity of Trolox Calibration Curves (Net AUC vs Concentration)

run	slope	intercept	r^2
1	4.4685	5.1348	0.9997
2	4.4664	4.7202	0.9977
3	4.6535	4.0831	0.9979
4	4.4981	4.0429	0.9985
5	4.4369	4.1478	0.9964
6	4.4274	4.4938	0.9983
7	4.2389	5.3897	0.9947
8	4.4823	4.5954	0.9967
9	4.4408	4.7249	0.9988
10	4.4719	4.9657	0.9987
11	4.6089	4.3267	0.9985
12	4.4687	4.0152	0.9958
a.v.	**4.4719**	**4.5534**	**0.9976**
SD	0.1005	0.4530	0.0015
% RSD	2.25	9.95	0.15

excitation conditions used. When AAPH was added (blank) (**A2** and **B2**) the fluorecence signal fell completely. In the presence of increasing concentrations of Trolox (**C1**−**C12** and **D1**−**D12**), the decay of the curve was delayed. The same was observed for pure phenolic compounds (**E1**−**E12** and **F1**−**F12**), wines (**G1**−**G6** and **H1**−**H6**) and dietary antioxidant supplements (**G7**−**G12** and **H7**−**H12**). The FL fluorescence response was not affected by the presence of the antioxidant (**A3**−**A7** and **B3**−**B7**), and antioxidant did not exhibit any fluorescence on its own (**A8**−**A12** and **B8**−**B12**). The system views the curves "at real time", which allows the analyst to stop the reaction and to repeat it immediately if an inappropriate antioxidant concentration has been used. For quantitative purposes, the net area under the curve ($AUC_{Sample} - AUC_{Blank}$) was calculated. **Table 1** reports the Trolox (1−8 μM, final concentration) calibration curves (net AUC versus concentration) for twelve independent runs. The coefficient of correlation (r^2) was ≥0.994. The precision of the method calculated for 2, 5, and 7-μM Trolox standard solutions ($n = 5$) was 1.9, 2.9, and 1.5, respectively, expressed as % coefficient of variation. The accuracy of the method calculated for 2, 5, and 7-μM Trolox standard solutions ($n = 5$) was 2.2, 1.7, and 0.7, respectively, expressed as % variation of the mean. The detection and

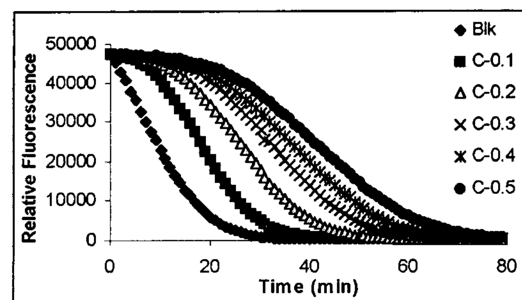

Figure 2. Time course of the reaction of fluorescein with AAPH in the absence (Blk) and in the presence of (+)-catechin (C) at different concentrations (0.1–0.5 μM).

quantification limits were those corresponding to 0.5 and 1-μM Trolox standard solutions, respectively.

ORAC−FL Values for Pure Antioxidants. The antioxidant capacity of different phenolic compounds (benzoic and cinnamic acids and aldehydes, flavonoids, and BHA) was determined by the ORAC−FL method. As an example, **Figure 2** depicts the decay curves for (+)-catechin at different final concentration (0.1−0.5 μM, final concentrion). The linearity between the net AUC and the concentration was checked for all the compounds (**Table 2**). For each standard, solutions of concentration within the linearity range gave to the same ORAC−FL value. Flavonoids quercetin and (+)-catechin showed the highest ORAC−FL values (10.5 and 14.9 Trolox equivalent/μmol of pure compound, respectively) whereas BHA showed the lowest (2.43) (**Table 2**). As expected, caffeic acid was more active than ferulic acid and its esterification with quinic acid (chlorogenic acid) reduced activity. Although aldehydes are not generally studied for their antioxidant activity, this study demonstrates that they exhibit slightly less antioxidant activity than their corresponding acids (**Table 2**). Using automated systems, other authors have reported lower ORAC−FL values for caffeic acid (4.37 Trolox equivalent/μmol of pure compound), chlorogenic acid (3.14), quercetin (7.28), and (+)-catechin (6.76) (*6*), and for ferulic acid (2.52) and protocatechuic acid (5.14) (*8*), although the relative order among them was the same that the one found in this study. No references on ORAC−FL values were found for

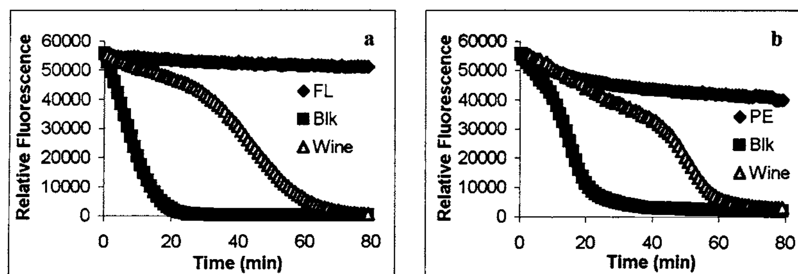

Figure 3. Time course of the reaction of (a) fluorescein (FL) with AAPH in the presence of bottled-aged red wine var. Graciano (1.66 μL/mL) and of (b) β-phycoerythrin (PE) with AAPH in the presence of young red wine var. Graciano (5.0 μL/mL).

Table 2. Trolox Equivalents and Linear Ranges (Net AUC vs Concentration) of Pure Compounds

sample	Trolox equivs[a,b]	conc range (μM)	slope	intercept	r^2
quercetin	10.5 ± 0.4	0.2–0.6	40.987	2.4251	0.9915
(+)-catechin	14.9 ± 0.8	0.2–0.5	50.149	6.2378	0.9921
caffeic acid	6.63 ± 0.24	0.2–1.0	26.134	3.4984	0.9982
protocatechuic aldehyde	6.31 ± 0.30	0.2–0.8	29.724	3.6069	0.9954
protocatechuic acid	6.70 ± 0.31	0.3–1.0	30.206	4.2215	0.9980
chlorogenic acid	5.70 ± 0.15	0.3–1.0	26.139	4.5922	0.9987
ferulic acid	4.47 ± 0.21	0.4–1.3	19.326	3.5041	0.9972
coniferyl aldehyde	4.20 ± 0.11	0.2–1.5	20.341	2.1213	0.9994
p-coumaric acid	4.51 ± 0.23	0.4–1.0	18.531	5.4604	0.9992
BHA	2.43 ± 0.06	1.0–3.0	10.815	4.1739	0.9984

[a] Expressed as μmol of Trolox equivalent/μmol of pure compound. [b] Results are presented as the mean (n = 3) ± SD.

the other compounds studied in this paper. The ORAC−PE assay appears to show even higher variability among laboratories. For example, ORAC−PE value for protocatechuic acid was reported to be 1.07 Trolox equivalent/μmol of pure compound (*11*), 2.06 (*9*), and ~6 (*12*). ORAC−PE value for quercetin was reported to be 2.07 Trolox equivalent/μmol of pure compound (*6*), 3.29 (*9*), and 3.39 (*13*). For the ORAC−PE assay, variability among laboratory results may not be only attributed to different equipment, analytical products and analyst, but also to PE variability among lots (*6*).

Inasmuch as spectrofluorometric approaches are more sensitive than spectrophotometric, the possibility of following a free radical reaction to its completion in the ORAC−FL assay has permitted the evaluation of the antioxidant activity of *p*-coumaric acid, which was quite difficult to achieve by other free-radical methods (*14*).

ORAC−FL Values for Wines. There is increasing evidence that red wine may protect against atherosclerosis and cardiovascular diseases. This beneficial effect of wine may be in part explained by the presence of phenolic compounds exhibiting different biological activities, including antioxidant properties, anti-aggregatory platelet property, and inhibition of vascular smooth muscle cell proliferation (*15*, *16*). With respect to the antioxidant capacity of wine, Cao et al. (*4*) determined the ORAC−PE values of two red and white wines, but to our knowledge, no studies have been carried out by using the ORAC−FL assay. **Figure 3** compares the delay curves of both fluorescein (ORAC−FL) and β-phycoerythrin (ORAC−PE) on their own and in the presence of AAPH with and without the addition of a young red wine, var. Graciano. As reported by other authors (*8*), PE was found to lose almost 23% of its fluorescence intensity in the first 50 min at 37 °C in the absence of AAPH (**Figure 3b**), in contrast to fluorescein (**Figure 3a**). When adding wine as antioxidant, different kinetic curve trends were observed for ORAC−PE and ORAC−FL assays, which was attributed to possible interactions between wine polyphenols and PE as reported before (*6*). The ORAC−PE value found for the young red wine var. Graciano (18.6 ± 1.5 μmol of Trolox equivalent/mL of wine) was close to that reported by Cao et al. (*4*) (12.3 ± 0.5). However, the ORAC−FL assay led to an almost 2-fold higher value (39.9 ± 2.1 μmol of Trolox equivalent/mL of wine). Ou et al. (*6*) also reported higher ORAC−FL values as compared to ORAC−PE (1.6−3.5-fold) for different pure compounds, other beverages, and some natural product extracts.

Once the method proved to be suitable for wine samples, we studied the effect of the type (white, rose and red) on wine antioxidant capacity. The linearity between the net AUC and the concentration (μL of wine/mL of reaction mixture) was checked for all the samples (**Table 3**). ORAC−FL values for bottled-aged red wines were around 3-fold higher than those for rose wines and around 10-fold than those for white wines (**Table 3**). Differences between red and white wines were of the same order as those reported by other authors using the LDL oxidation (*17*), ABTS$^{\bullet+}$ (*18*−*20*), DPPH • (*20*, *21*), DMPH (*22*), or lipid peroxidation (*23*) assays. For the three types of wine, differences in ORAC−FL values among grape varieties were attributed to their different phenolic content (**Table 3**). A good linear correlation was found between the ORAC−FL values and the total polyphenol content (n = 8), r^2 = 0.989. Phenolic composition is known to differ from white (prevalence of hydroxycinnamic acid derivatives, catechins, and procyanidins) to rose and red (a higher content of anthocyanins, flavonol glucosides, and polimeric proanthocyanins) wines.

Several authors have shown the influence of technological procedures such as sulfur dioxide (*24*), skin contact (*25*, *26*), carbonic maceration (*27*), and vinification (*28*) on the antioxidant activity of wines. The effect of aging has been studied by assaying wines from different vintages (*19*, *21*, *24*). Manzocco et al. (*24*) found lower antioxidant activity in a Montepulciano d'Abruzzo red wine from the 1973 (79% loss) and from the 1995 (68% loss) vintages in comparison to that from the 1996 vintage. Landrault et al. (*19*) also found that antioxidant activity of commercial red French wines from 1995 to 1991 vintages was lower (33% loss) than that from 1999 to 1996 vintages, although great variability was observed among samples. Larrauri et al. (*21*) found that the antioxidant activity of commercial red Spanish wines increased with oak-aging, although again samples were from different varieties and winemaking processes. In this study, the influence of aging in wine antioxidant activity was studied by assaying twenty-two wines from Tempranillo and Cabernet-Sauvignon varieties (vintages 2002−1989) vinified in the same manner and aged in barrels made from American and French oak (**Table 4**). ORAC−FL values for oak-aged red wines varied from 35.8 to 63.8 μmol of Trolox equivalent/mL of wine (**Table 4**). Both ORAC−FL and total polyphenol values for oak-aged red wines (**Table 4**) were higher than those reported

Table 3. Trolox Equivalents and Linear Ranges (Net AUC versus Concentration) of the Bottled-Aged Red, Rose and White Wines Studied

	Trolox-equivs[a,b]	conc range (μl/ml)	slope	intercept	r^2	total polyphenol [b,c]
			Bottled-aged Red Wines			
var. Tempranillo	30.8 ± 2.6	0.03–0.17	133.84	3.3192	0.9990	1302 ± 8
var. Graciano	39.9 ± 2.1	0.03–0.2	155.01	4.8383	0.9903	1468 ± 23
var. Cabernet S.	34.7 ± 1.8	0.03–0.2	138.58	3.3680	0.9982	1428 ± 34
			Rose Wines			
var. Cabernet S.	8.95 ± 0.59	0.17–1.0	39.341	6.2376	0.9980	389 ± 7
var. Tempranillo	10.0 ± 0.6	0.17–0.67	42.359	4.2310	0.9934	439 ± 10
var. Garnacha	11.2 ± 0.5	0.17–0.67	42.777	4.7532	0.9960	432 ± 8
			White Wines			
var. Albariño	4.84 ± 0.30	0.25–1.33	20.413	4.7923	0.9979	214 ± 7
var. Verdejo	3.18 ± 0.24	0.50–2.0	14.314	5.7729	0.9990	186 ± 4

[a] Expressed as μmol of Trolox equivalent/mL of wine. [b] Results are presented as the mean ($n = 3$) ± SD. [c] Expressed as mg of gallic acid equivalent (GAE)/L.

Table 4. Trolox Equivalents[a,b] and Total Polyphenols[b,c] of the Oak-Aged Red Wines Studied

	var. *Tempranillo*		var. *Cabernet Sauvignon*	
vintage	American oak	French oak	American oak	French oak
2002	37.4 ± 1.2	36.2 ± 1.0	45.7 ± 1.6	50.8 ± 1.9
	1821 ± 12	1785 ± 16	2001 ± 27	2080 ± 25
2001	37.0 ± 0.6	35.8 ± 2.8	n.a.[d]	n.a.
	1661 ± 14	1657 ± 9		
2000	43.2 ± 1.1	45.8 ± 1.1	41.6 ± 1.1	41.3 ± 1.3
	1981 ± 10	2015 ± 24	2074 ± 5	1921 ± 21
1995	43.6 ± 1.8	48.5 ± 2.0	63.8 ± 0.3	61.3 ± 0.8
	1834 ± 25	1911 ± 28	2350 ± 18	2428 ± 15
1992	38.3 ± 3.5	36.9 ± 2.8	54.1 ± 0.5	56.3 ± 2.0
	1584 ± 29	1605 ± 19	1960 ± 21	2096 ± 11
1989	36.5 ± 1.4	37.2 ± 1.0	53.5 ± 2.0	50.7 ± 1.4
	1427 ± 8	1511 ± 23	2065 ± 4	1953 ± 19

[a] Expressed as μmol of Trolox equivalent/mL of wine. [b] Results are presented as the mean ($n = 3$) ± S. D. [c] Expresed as mg of gallic acid equivalent (GAE)/L. [d] n.a. = not available.

above for bottled-aged red wines from the same grape variety (**Table 3**). As seen for bottled-aged wines, oak-aged wines from Cabernet variety exhibited higher antioxidant activity than those from Tempranillo variety (**Tables 3** and **4**). Analysis of variance (ANOVA) of the ORAC−FL value demonstrated significant ($p < 0.01$) differences between varieties and among vintages, but not between oak origins (**Figure 4**). The 95% confidence intervals corresponding to wines from the 1995 vintage (8 years old) were separated from the rest of the vintage groups for both grape varieties, with the exception of Tempranillo wines from the 2000 vintage (**Figure 4**). It is important to note that intervals corresponding to wines from the vintage 2002 (6 months old) and from the vintage 1989 (13 years old) overlapped in both varieties. Although a certain linear correlation was observed between the ORAC−FL value and polyphenol content ($r^2 = 0.7485$) in oak-aged red wines, the 95% confidence intervals concerning the polyphenol content showed different distribution pattern to that concerning to ORAC−FL value (**Figure 4**). Girotti et al. (*29*) also reported a low correlation between antioxidant activity and total polyphenol content when following a Sangiovese wine during one-year maturation in oak casks. These results show that oak aging effectively increases antioxidant activity of red wines, but particular characteristics of the grape used in each vintage as well as effects of bottle time after oak-aging would lead to differences in the antioxidant activity of wines from different vintages.

ORAC−FL Values for Dietary Antioxidant Supplements. Dietary antioxidant supplements found in the market are formulated with a wide variety of sources such as fruits, vegetables, minerals, and herbals. Standardization of the supplement through the antioxidant capacity measurement is desirable (*30*). By using the ORAC−PE assay, Prior and Cao (*9*) evaluated forty-six commercial preparations of antioxidant related dietary supplements. To evaluate the feasibility of using the ORAC−FL assay for these products, seven commercial dietary antioxidant supplements were evaluated. **Figure 5** depicts the FL and PE decay curves on their own and in the presence of AAPH with and without the addition of commercial dietary antioxidant supplement #4. Different kinetic curve trends were observed for the two assays when adding the supplement as antioxidant, which again may be attributed to possible interactions between supplement constituents and PE (*6*). The ORAC−FL value corresponding to supplement #4 (3.18 ± 0.08 μmol of Trolox equivalent/mg of supplement) was around 2.6-fold higher than the ORAC−PE value (1.22 ± 0.16).

Data for the total antioxidant capacity (ORAC−FL values) and linear ranges for the seven dietary supplements studied are presented in **Table 5.** ORAC−FL values varied from 0.018 to 3.18 μmol of Trolox equivalent/mg of supplement, which meant a ~170-fold maximum-minimum difference. The relative order for supplements was: $4 > 7 > 6 > 1 > 3 > 2 > 5$, that seemed not to be related with their composition (see Materials and Methods). The same supplements assayed for their antiradical activity against a synthetic radical (2,2-diphenyl-1-picrylhydrazyl, DPPH•) followed a different relative order: $7 \geq 4 > 1 > 6 \geq 3 > 2 > 5$ (*31*). The nature of the radical clearly conditioned the response of the antioxidant/s present in each supplement. In our opinion, the ORAC procedure is more convenient for evaluating antioxidant activity of dietary supplements because it involves peroxyl radicals, which are the most abundant radicals in biological systems.

Final Remarks. The increasing interest in the evaluation of the antioxidant activity in complex matrixes has been aimed at the development of new methodologies and/or the improvement of the known ones. The ORAC method takes into account both inhibition percentage and inhibition time of the peroxyl radical action by antioxidants. Deficiencies of the ORAC assay associated with the use of β-phycoerythrin as fluorescent have been overcome by the use of fluorescein (FL). This paper proves suitability of manual handling and using a conventional fluorescence microplate reader for performing the ORAC−FL assay on pure compounds and food samples. Useful ORAC−FL data for benzoic and cinnamic acids and aldehydes, flavonoids, BHA, wines, and dietary antioxidant supplements are given. The results confirm that wine antioxidant activity is influenced by grape variety and enological practices. Oak aging

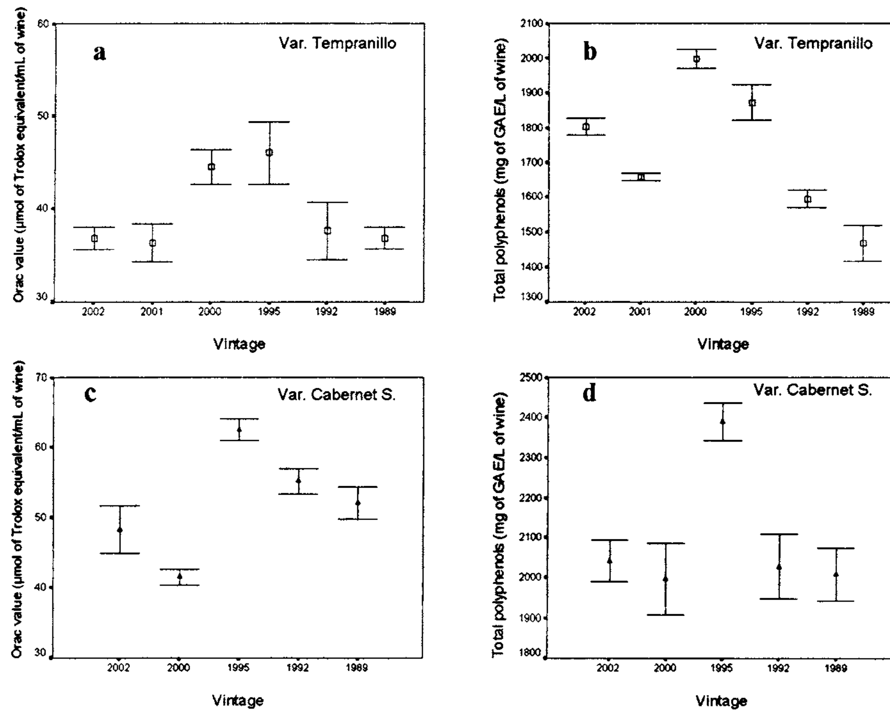

Figure 4. ANOVA analysis of the ORAC value (a, c) and the total polyphenol content (b, d) corresponding to red oak-aged wines from Tempranillo (n = 12) and Cabernet Sauvignon (n = 10) varieties from different vintages (1989–2002). Error bars represent the 95% confidence intervals for mean.

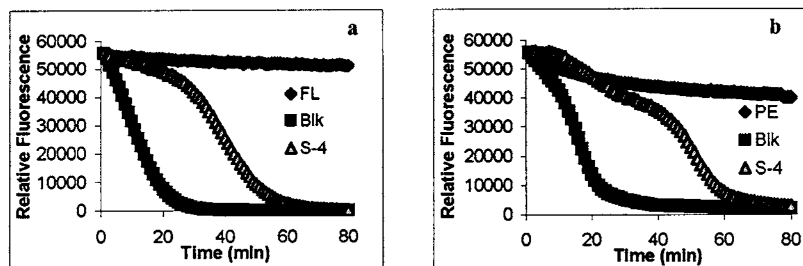

Figure 5. Time course of the reaction of (a) fluorescein (FL) with AAPH in the presence of commercial antioxidant supplement #4 (20 μg/mL), and of (b) β-phycoerythrin (PE) with AAPH in the presence commercial antioxidant supplement #4 (65 μg/mL).

Table 5. Trolox Equivalents and Linear Ranges (Net AUC vs Concentration) of the Commercial Dietary Antioxidant Supplements Studied

supplement	Trolox-equivs[a,b]	conc range (μg/mL)	slope	intercept	r^2
1	1.47 ± 0.08	16.7–66.7	0.5712	8.4502	0.9942
2	0.079 ± 0.003	166–1000	0.0364	3.7752	0.9966
3	0.704 ± 0.035	26.7–114	0.2936	4.1812	0.9959
4	3.18 ± 0.08	6.66–28.6	1.1571	5.7536	0.9954
5	0.018 ± 0.001	1000–4000	0.0085	2.4959	0.9926
6	2.12 ± 0.08	8.30–40.0	0.9008	3.2659	0.9920
7	2.27 ± 0.08	10.0–40.0	0.8578	5.0344	0.9921

[a] Expressed as μmol of Trolox equivalent/mg of dietary supplement. [b] Results are presented as the mean (n = 3) ± SD.

increases the antioxidant activity of wines, but grape annual characteristics as well as bottle-aging effects can induce differences among vintages. The antioxidant capacity of the dietary antioxidant supplements studied is variable, ranging from 0.018 to 3.18 μmol of Trolox equivalent/mg of supplement, which, in any case, is considerably lower than the values found for pure flavonoids such as (+)-catechin (51.3 μmol) and quercetin (34.6 μmol). Based on the results, we propose the ORAC−FL assay as a simple and sensitive method for evaluating the antioxidant activity of both wine and antioxidant dietary supplement samples.

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