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ORAC- FLUORESCEIN AS A MODEL FOR EVALUATING ANTIOXIDANT ACTIVITY OF WINES

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In this paper an ORAC-fluorescein (ORAC-FL) protocol for evaluating antioxidant activity of wines has been developed. Fluorescein and radical initiator (AAPH) concentrations were fixed in 70 nmol/L and 12 mmol/L, respectively. Wine concentration that gave a linear response antioxidant activity versus concentration was in the range 0.50-2.0 μL of wine/mL of reaction mixture for white wines, 0.15-0.65 for rose wines, and 0.033-0.20 for red wines. The method was applied to 33 Spanish white, rose, young red, bottled-aged red and oak-aged red wines from different vintages. ORAC-FL values were higher for oak-aged red wines (35.8-63.8 μmol of Trolox equivalent/mL of wine), followed by young and bottled--aged red wines (30.8-40.7), rose wines (8.95-11.2) and white wines (3.18-4.84). Grape variety as well as vintage influenced wine antioxidant activity.

INTRODUCTION

Several studies have shown health benefits, particularly in relation to cardiovascular diseases (CVDs), from a moderate red wine consumption [Renaud & De Lorgeril, 1992; Serafini *et al.*, 1998; Gronbaek *et al.*, 1995]. These effects are in part due to the presence of polyphenols [Gronbaek, 2002] (mainly phenolic acids, flavonols, catequins and anthocyanins), which are known to exhibit antioxidant properties [Teissedre *et al.*, 1996; Satue-Garcia *et al.*, 1997]. Wine polyphenols are postulated to be involved primarily with LDL oxidation, although other mechanisms that could explain their protective effects against CVDs are not discarded [Wollin & Jones, 2001].

In the last years, measurement of the wine antioxidant potential as a whole has gained attention by the scientific community and also by different wine international organizations, such as the *Office International de la Vigne et du Vin* (l'OIV) [OIV, 2003]. Several approaches have been conducted to evaluate wine antioxidant activity: DPPH$^{\bullet}$ assay, ABTS$^{\bullet+}$ assay, Cu^{2+} ion-dependent LDL oxidation assay, Fremys' radical reduction assay, methyl linoleate autoxidation assay, *etc.* [Burns *et al.*, 2000; Larrauri *et al.*, 1999; Landrault *et al.*, 2001; Fuhrman *et al.*, 2001; Dávalos *et al.*, 2003]. The oxygen radical antioxidant capacity (ORAC) assay evaluates the decrease in the fluorescence of a fluorescent probe (β-phycoerythrin) due to the action of peroxyl radicals generated by thermal decomposition of 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH) [Cao *et al.*, 1995]. In the presence of an antioxidant, radicals are scavenged and the decay of the fluorescence curve is retarded. The difference between the "area under the fluorescence decay curve" (AUC) corresponding to the mixture with antioxidant and the AUC corresponding to the mixture without antioxidant (net AUC), was translated into a Trolox standard calibration curve to express the antioxidant activity as mmol of Trolox equivalent/mmol of antioxidant. The ORAC assay provides a unique assessment in which the inhibition time and the degree of inhibition are taken into account as the reaction goes to completion [Prior *et al.*, 1995]. The ORAC approach has been previously used for the evaluation of the antioxidant activity of a wide variety of samples such as tea, fruits, honey, vegetables, dietary supplements, botanicals, human plasma, *etc.* [Prior & Cao, 1999], but the literature contains very few studies regarding the antioxidant capacity of wine by the ORAC method [Cao *et al.*, 1995].

Recently, some limitations for the use of β-phycoerythrin as fluorescent probe in the ORAC assay have been reported [Ou *et al.*, 2001]. Other fluorescent substance, flourescein (FL), has been postulated as a better probe for this assay [Ou *et al.*, 2001]. The purpose of this work was to adapt the ORAC-fluorescein method (ORAC-FL) to wine samples and to determine the antioxidant activity (ORAC-FL values) of wines from different grape varieties and vintages.

MATERIALS AND METHODS

Chemicals. Fluorescein (FL) disodium was purchased from Sigma (St Louis, MO, USA). 2,2'-Azobis (2-methyl-propionamidine) dihydrochloride (AAPH), and 6-hydroxy--2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were obtained from Aldrich (Milwaukee, WI).

Wines. A total of 33 Spanish wines were assayed. Two white (var. Verdejo and Albarino) and three rose (var. Cabernet-Sauvignon, Tempranillo and Garnacha) wines

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were obtained from local markets. A young red wine from grapes (var. Merlot) of the 2002 vintage and three bottled-aged red wines from grapes (var. Graciano, Tempranillo, Cabernet-Sauvignon) of the 2000 vintage were elaborated at the Viticulture and Enology Station of Navarra (EVENA, Olite, Navarra, Spain). Twenty-four oak-aged red wines were elaborated from grapes of the 1989 (var. Tempranillo and Cabernet-Sauvignon), 1992 (var. Tempranillo and Cabernet-Sauvignon), 1995 (var. Tempranillo and Cabernet-Sauvignon), 2000 (var. Tempranillo and Cabernet-Sauvignon), 2001 (var. Tempranillo) and 2002 (var. Tempranillo, Cabernet--Sauvignon and Merlot) vintages at the Viticulture and Enology Station of Navarra (EVENA, Olite, Navarra, Spain). They were aged in both French (var. Allier for all wines expect for Merlot 2002 that was in var. Nevers) and American oak for 1 year, expect wines from the 2002 vintage that were aged for three (var. Tempanillo and Merlot) or four months (var. Cabernet-Sauvignon).

ORAC-FL assay. The method of Ou *et al.* [2001] was adapted to manual handling and to the use of a Polarstar galaxy (BMG Labtechnologies GmbH, Offenburg-Germany) plate reader. The reaction was carried out in 75 mmol/L phosphate buffer (pH 7.4) and the final reaction mixture was 200 μL. Antioxidant (20 μL) and fluorescein (120 μL; 70 nmol/L final concentration) solutions was placed in the well of the microplate (black 96-well plates, Nunc™ black microwell, Denmark). The mixture was preincubated for 15 min at 37°C prior. AAPH solution (60 μL; 12 mmol/L final concentration) was added rapidly using a multichannel pipette. The plate was immediately placed in the reader and the fluorescence recorded every minute for 80 min. The plate was automatically agitated prior each reading. Fluorescence measures were carried out at 37°C. Excitation and emission filters were 485-P and 520-P, respectively. A blank using phosphate buffer instead of the antioxidant solution and eight calibration solutions using Trolox (1-8 μmol/L final concentration) as antioxidant were also carried out in the same run. AAPH and Trolox solutions in 75 mmol/L phosphate buffer (pH 7.4) were prepared daily and fluorescein was diluted from a stock solution (1.17 mmol/L) in 75 mmol/L phosphate buffer (pH 7.4) that was stored at 4°C for weeks. All reaction mixtures were prepared by duplicate and at least three independent runs 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 by multiplying original data by the factor fluorescence $_{\text{blank, t=0}}$/ fluorescence $_{\text{sample, t=0}}$. From the normalized curves, the area under the fluorescence decay curve (AUC) and the net AUC were calculated as follows:

$$AUC = 1 + \sum_{i=1}^{i=80} f_1 / f_0 \quad (1)$$

$$\text{net AUC} = \text{AUC}_{\text{antioxidant}} - \text{AUC}_{\text{blank}} \quad (2)$$

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

Linear regression equations between net AUC and antioxidant concentration were calculated for all the samples. Antioxidant activity (ORAC value) was calculated by using a Trolox calibration curve:

$$\text{ORAC-FL value (Trolox eq.)} = 4.55 + 4.47 \times \text{net AUC} \quad (3)$$

Final results were in mmol of Trolox equivalent/mL of wine.

RESULTS AND DISCUSSION

Figure 1 depicts the time course of the reaction of fluorescein with AAPH in the absence and in the presence of a wine under the conditions reported in Materials and Methods. The net AUC calculated by equations (1) and (2) is graphically indicated in Figure 1. The wine concentration (μL of wine/mL of reaction mixture) ranges that ensured linearity between net AUC and wine concentration were established as: 0.5-2.0 μL of wine/mL of reaction mixture for white wines, 0.15-0.65 for rose wines, and 0.033-0.20 for red wines (Figure 2). These concentration ranges corresponded to net AUC values between ~1 and ~3-fold the AUC corresponding to the blank. Wine concentrations within these linear ranges led to the same ORAC-FL value after applying the Trolox calibration curve (equation 3) and dividing by wine concentration.

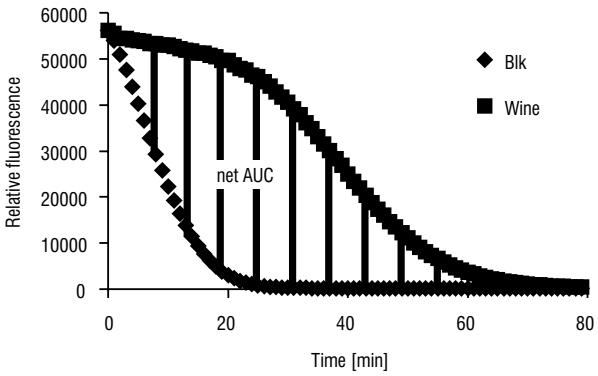

FIGURE 1. Time course of the reaction of fluorescein (FL) with AAPH in the absence (Blk) and in the presence of the young red wine var. Tempranillo.

Winemaking process influences the antioxidant activity of wine. Figures 3 and 4 show the ORAC-FL values of the 33 samples analyzed corresponding to white, rose, young red, bottle-aged red and oak-aged red wines. ORAC-FL values were higher for oak-aged red wines (35.8-63.8 μmol of Trolox equivalent/mL of wine), followed by young/ /bottled-aged red wines (30.8-40.7), rose wines (8.95-11.2) and white wines (3.18-4.84). A ~ 20-fold difference was found between the lowest and the highest ORAC-FL value. Differences in antioxidant activity among wines were attributed to qualitative and quantitative differences in their phenolic composition that is influenced by the phenolic composition of the grapes used and by the winemaking process. Besides the phenolic and cinnamic acids and derivatives, and the catechins mainly presented in white grapes, red grapes contain anthocyanins, which are absent from the formers. Proanthocyanins are present in both grape types, but they show structural (composition and

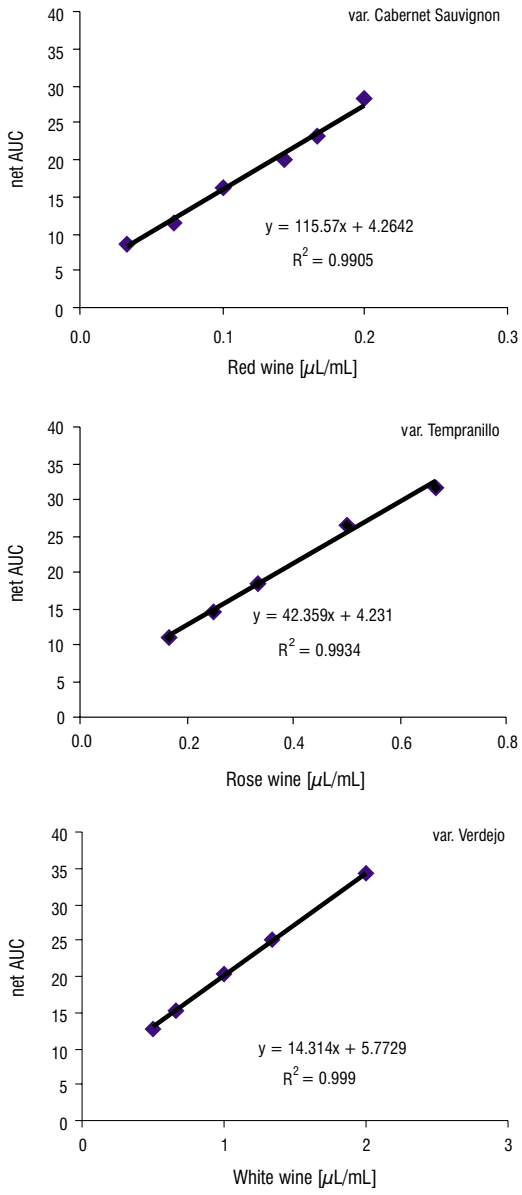

FIGURE 2. Regression analysis of net "area under the fluorescence decay curve" (AUC) against wine concentration.

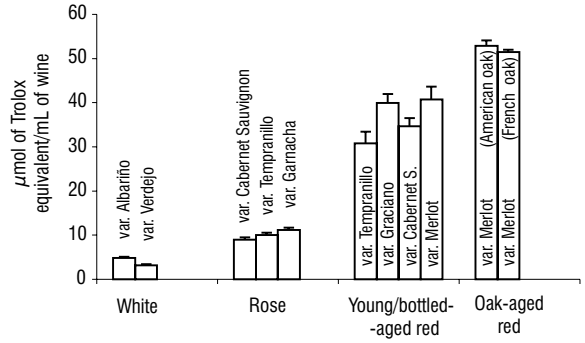

FIGURE 3. ORAC-fluorescein (ORAC-FL) values for white, rose, young/bottled-aged red and oak-aged red wines from different grape varieties.

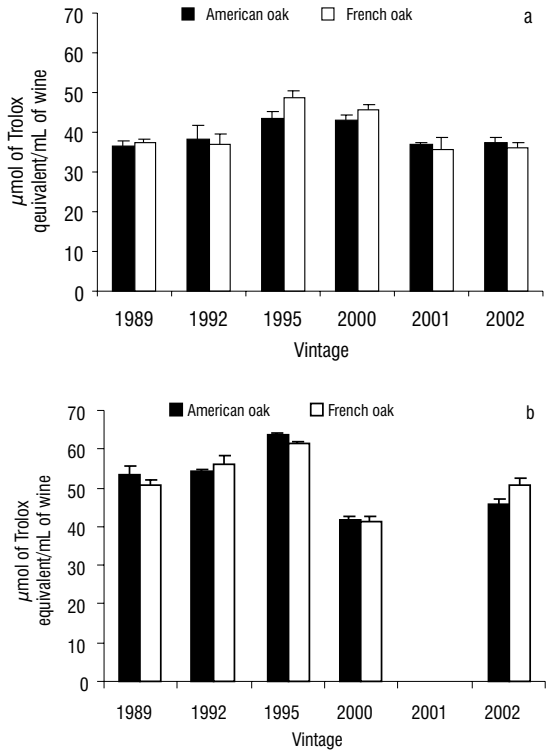

FIGURE 4. ORAC-fluorescein (ORAC-FL) values for oak-aged red wines from different vintages.

degree of polymerization) differences. Since anthocyanins and proanthocyanins are transferred from the solid parts of the grape (skins, seeds and stems) into the must during winemaking operations, the way and the time of the solid--liquid contact influences wine phenolic composition: *i.e.* rose wines (in which fermentation is performed in the absence of solid parts) show lower anthocyanin and proanthocyanidin content than red wines. Phenolic composition of oak-aged red wines is enriched by the compounds extracted from the wood and by the reactions of oxidation, condensation and polymerization that take place during the ageing process.

Concerning oak-aged wines, their antioxidant activity was influenced by grape variety and by vintage, but not by oak origin (Figures 3 and 4). ORAC-FL values for oak-aged red wines were higher than those from young/bottled--aged red wines from the same grape variety. As seen for young/bottled-aged red wines vinified in the same manner (Figure 3), ORAC-FL values of oak-aged wines were in the order var. Merlot>var.Cabernet-Sauvignon>Tempranillo for all the vintages studied (Figure 4). For var. Tempranillo, wines from the vintage 2000 and 1995 (2 and 7 years old, respectively) showed higher antioxidant activity (~1.2-fold) than either younger wines (vintages 2002 and 2001, 6 months and 1 years old, respectively) or older wines (vintages 1992 and 1989, 10 and 13 years old, respectively) (Figure 4). Similarly, for var. Cabernet-Sauvignon, wines from the vintage 1995 (7 years old) showed higher antioxidant activity (~1.2-fold) than either younger wines (vintage 2002) or older wines (vintages 1992 and 1989, 10 and 13 years old, respectively), with the exception of vintage 2000 (~1.5-fold lower activity than vintage 1995) (Figure 4). The literature reports very few studies about the influence of vintage in the antioxidant activity of wines. Larrauri *et al.* [1999] determined the antioxidant activity by the DPPH. method of "crianza" and "reserva" wines from four different Spanish *Appleation Controlé*, but they did not

found a general trend, possibly due to the diversity of grape varieties, winemaking processes and ageing time specifications of the *Appleation Controlé*. Landrault *et al.* [2001] found that the antioxidant activity determined by the ABTS method of French commercial wines from vintages 1996-1999 were higher than those from vintages 1995-1991, but again wines from different grape varieties and wineries were considered. Our results confirm differences in the antioxidant activity of oak-aged wines from different vintages but from the same grape variety, and they were of the same order than those among grape varieties. Older wines seem not to mean higher antioxidant activities. Thus, it might be an optimum ageing time for antioxidant activity of a wine that could vary with grape variety. More studies including other grape varieties, vintages and winemaking processes should be carried out to complete these observations.

CONCLUSIONS

An ORAC-fluorescein (ORAC-FL) protocol for the evaluation of the antioxidant capacity of wines has been developed. The ORAC assay is a sensitive method based on the detection of chemical damage to fluorescein through the decrease in its fluorescence emission by peroxyl radicals that are generated *in situ* by the thermal decomposition of the free radical initiator AAPH [Glazer, 1990]. Advantages of this assay include not only the higher sensibility of fluorescence methods compared to spectrophotometric ones, but also the fact that combines both inhibition time and the degree of inhibition. To our knowledge, this paper reports one of the few approaches that include the evaluation of the antioxidant activity of different type of wines (white, rose, young red, bottled-aged red and oak-aged red) elaborated from different grape varieties and vintages. Grape variety as well as winemaking process (ageing) do influence wine antioxidant activity. Old red wines greatly appreciated by their sensory properties may not exhibit the maximum potential of their antioxidant activity. All these results let us to propose the ORAC-fluorescein assay as an appropriate method for the evaluation of wine antioxidant activity.

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