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Determination of Antioxidant Activity of Wine Byproducts and Its Correlation with Polyphenolic Content

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It has been demonstrated that wine and other products derived from the grape have a high antioxidant capability; as a possible consequence of this, they may have potential benefits for health. The byproducts of the winemaking process represent a source of antioxidant compounds that has been relatively unexploited to date, but that is now the subject of increasing industrial interest. This article describes an approach to the study of the antioxidant activity of grape marcs, stalks, and dregs of both white and red varieties. This activity is compared with the measurements of their content of total polyphenols and of individual polyphenolic compounds, identified and quantified by HPLC. From the results we have been able to establish a positive correlation between the antioxidant activity and the total polyphenolic content of samples, but not with specific compounds.

KEYWORDS: Antioxidant activity; wine byproducts; electrochemical method; polyphenols

INTRODUCTION

Free radicals have been implicated in over a hundred disease conditions in humans, including arthritis, atherosclerosis, advancing age, Alzheimer's and Parkinson's diseases, gastrointestinal disfunctions, tumor promotion and carcinogenesis, and AIDS. Antioxidants are potent scavengers of free radicals and serve as inhibitors of neoplastic processes. A large number of synthetic and natural antioxidants have been demonstrated to induce beneficial effects on human health and disease prevention. Epidemiological studies have demonstrated that five to seven servings of fresh fruit and vegetables and two glasses of red wine per day can lead to a prolonged healthy life. This capacity has been attributed to wine because of its content of polyphenols, which are well-known antioxidant compounds (*1*−*6*).

Coronary heart disease (CHD) is a major cause of mortality and morbidity in the developed countries of the world. The oxidation of low-density lipoproteins (LDLs) by free radicals is associated with initiation of atherosclerosis, and, therefore, development of CHD. LDLs are protected from oxidation by antioxidants, and increased consumption of red wine containing polyphenols is thought to account for the lower incidence of CHD in Mediterranean countries (*7*, *8*).

The polyphenolic compounds present in the grape pass to the wine more or less depending on the characteristics of the winemaking process. But independently of this transfer, and bearing in mind that the major part of these compounds is found in the grape solids, a high proportion remains in the vinification residues or wastes. The anthocyanins extracted from winemaking byproducts, known as "enocyanin", have been commercialized since 1879. Nowadays, a very opportunistic and viable business has emerged within the wine industry. This industry produces grape seed and grape skin extracts that are finding increasing applications as food lipid antioxidants (*9*) or dietary supplements for disease prevention (*1*, *6*). Recently, even a new family of antioxidants has been obtained from a residual fraction of polymeric polyphenols of grape origin (*10*).

Extracts from residues left in the production of wine are used as active substance combinations for producing cosmetic and pharmaceutical compositions. Such compositions are used in skin and hair products (*11*, *12*), hemorrhoid treatments (*13*), or for reducing plasma triglycerides, platelet aggregation, and oxidative capacity (*14*). Also, a preservative solution for peeled fruits and vegetables, juices, and cut flowers has been patented which includes flavonoids provided from grape seed oil (*15*).

Then, the recovery of antioxidant compounds from these residues could represent a significant advance in maintaining the environmental equilibrium, because in grape and wine-producing zones large quantities of residues are generated, and this presents problems of storage, transformation, or elimination, in both ecological and economic terms.

This situation explains the increasing interest that has arisen in exploiting these byproducts of vinification, particularly for their content of antioxidant compounds. In this article we study the marcs, stalks, and dregs of certain white and red varieties of grape.

MATERIALS AND METHODS

Reagents. The Folin reagent (Sigma-Aldrich, Madrid, Spain) and sodium carbonate (Panreac, Barcelona, Spain) were employed for the

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measurement of the Folin−Ciocalteu total polyphenolic index. The calibration curve was constructed with gallic acid (Merck, Darmstadt, Germany).

For the antioxidant activity measurement, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Sigma-Aldrich) in a phosphate buffer medium (pH = 6; I = 0.05), prepared from solutions of KH_2PO_4 and Na_2HPO_4 (Fluka, Buchs, Switzerland), and zinc acetate (Panreac) were used. The calibration curve was constructed with 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) supplied by Sigma-Aldrich.

The solvents employed for the HPLC analysis were prepared with methanol and acetic acid of HPLC quality (Scharlau, Barcelona, Spain) and water purified in a Milli-Q system (Millipore, Bedford, MA). The solutions were filtered through cellulose acetate membranes (solvent A) and Teflon membranes (solvent B) of 0.45-μm pore size (Micron Separation, Westboro, MA) and were degasified in an ultrasound bath.

Calibration curves were constructed for the following polyphenols: *p*-hydroxyphenethyl alcohol, furfural, ferulic acid, catechin, and epicatechin (Sigma-Aldrich), gallic acid and *trans*-*p*-coumaric acid (Merck), caffeic acid, vanillic acid, isovanillin, and syringialdehyde (Fluka), syringic acid (Eastman Kodak, Rochester, NY) and chlorogenic acid (Sarsynthèse, Merignac, France). Caftaric, *cis*-*p*-coumaric, *cis*-coutaric, and *trans*-coutaric acids are not commercialized, so the former was quantified using the caffeic acid calibration curve and the rest were quantified by using the *trans*-*p*-coumaric acid curve (*16*).

Samples. The samples studied were the following: marcs, stalks, and dregs of three red varieties (Cabernet Sauvignon, Syrah, and Tempranillo), and marcs and dregs of two white varieties (Palomino Fino and Moscatel). The marcs are the residues of the harvested grapes after the grape juice has been obtained by the pressing, and consist of peels, stalks, and seeds. In the case of the red varieties, the grapes are de-stalked as one of the initial processes in the winemaking, and thus are available separately from the other residues. Besides, the red marcs suffer a maceration process in which a lot of polyphenols are extracted to the wine. The dregs are the thick solids that fall to the bottom of the vessels in the course of the fermentations; they are separated from the wine by the racking.

The samples were taken during the grape harvests of 1999 and 2000, and in all cases the samples studied were from grapes cultivated under both irrigated and nonirrigated conditions. Drip irrigation supplied 150 L/m^2 between April and July, the hottest months of the year. All the samples were obtained from the Agricultural Research Centre "Rancho de la Merced" (Jerez de la Frontera, Spain).

Lyophilization and Extraction Assisted by Ultrasonics. All the samples were lyophilized in an EZ585Q lyophilizer of FTS Systems (NY). Initial quantities of between 50 and 100 g of the marcs and the stalks (solid samples) were weighed, and volumes of about 25 mL of the dregs (liquid samples) were measured.

To extract the polyphenolic compounds from the samples prior to their study, a quantity of 0.5 g of each sample lyophilized was weighed. A volume of 5 mL of methanol was then added, and after being well agitated, the samples were placed in an ultrasonic bath for 15 min. They were then centrifuged (2 min) to separate out the liquid fraction containing the polyphenols extracted.

Total Polyphenolic Index: Folin−Ciocalteu Method. Observing the sequence specified here, the following are introduced into a calibrated 25-mL flask: 250 μL of sample, 12.5 mL of distilled water, 1250 μL of Folin−Ciocalteu reagent, and 5 mL of a solution of sodium carbonate at 20% and distilled water to make up the total volume of 25 mL. The solution is agitated to homogenize it and left to stand for 30 min for the reaction to take place and stabilize. The absorbance at 750 nm is determined in a cuvette of 1 cm (*17*).

Measurement of the Antioxidant Activity. The device used for measurement of the antioxidant power consisted of an 80-mL beaker (cathode) inside of which a 30-mL filtering crucible, pore size 4, is set (anode). A flat platinum electrode (30 × 60 mm) is introduced in the cathode and a cylindrical platinum mesh (h = 22 mm, d = 22 mm) is introduced in the anode. The feed source used (FAC-307C from Promax) allows the working conditions to be set in constant intensity mode. An UV−Vis transmission probe coupled to a PC2000 miniaturized spectrophotometer from Ocean Optics, Inc. (Eerbeek, The Netherlands) with a DH-2000 halogen−deuterium light source from Top Sensor Systems (Eerbeek, The Netherlands) was used to monitor the reaction.

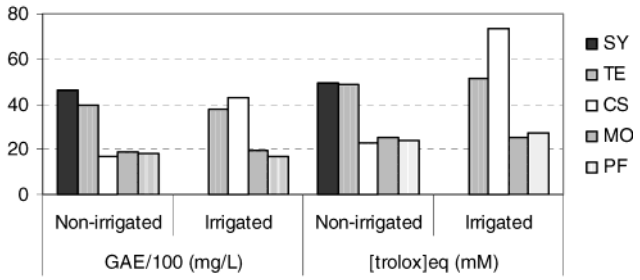

Figure 1. Total polyphenolic index (GAE) and antioxidant activity ([trolox]$_{eq}$) of the marcs samples.

The test, previously developed (*18*), consisted of oxidation by means of the electrolytic system described, of a solution of ABTS 50 μM, to which the sample to be tested was added. The procedure is as follows: First, the platinum electrodes have to be washed with nitric acid 60% and flame calcined. One is placed in the cathode immersed in 30 mL of a saturated solution of $Zn(CH_3COO)_2$, and the other is placed in the anode with 25 mL of a solution of ABTS 50 μM (pH 6). Aliquots of the samples are added to the anode, which is continuously agitated by a magnetic stirrer. To start the experiment, a constant intensity of 2 mA is applied while the spectrophotometer is continuously recording the absorbance at 414 and 734 nm versus time. The response function utilized is the coulombs employed in the oxidation of the samples added, calculated from the percentage of variation of the function (absorbance at 414 nm/absorbance at 734 nm) versus time. When the variation between two consecutive measurements of this quotient falls below 10%, it is considered that the oxidation of the ABTS has begun and, therefore, that the oxidation of the antioxidant or sample added has concluded.

Analysis by High-Performance Liquid Chromatography (HPLC). The analysis was performed using a Waters HPLC system (Waters/Millipore, Milford, MA) consisting of a model 616 pump, a model 600S gradient controller, a model 717 automatic sampler, and a model 996 photodiode detector.

Separation of the polyphenols was conducted in a LiChrospher 100 RP-18 column (Merck), 5-μm particle size, 25-cm length, and 3-mm i.d. The chromatographic conditions were the following: 0.4 mL/min flow rate; 80 μL injection volume; and eluents, A (10% methanol, 2% acetic acid, 88% Milli-Q water) and B (90% methanol, 2% acetic acid, 8% Milli-Q water). The detection by UV absorption was conducted by scanning between 250 and 600 nm, with a resolution of 1.2 nm, and the identification and quantification were conducted at 320 nm for the derivatives of cinnamic acid and at 280 nm for the rest of the polyphenols. The data acquisition and treatment were conducted using the Millenium 2010 version 2.21 software.

RESULTS AND DISCUSSION

First, all the samples were lyophilized to eliminate all the water they contained and to facilitate extraction of the polyphenols. After lyophilization, weight losses of 60−70% were recorded for marcs and stalks (solid samples). Dregs (liquid samples) showed weight losses of 80−90%.

After that, the liophilized samples were extracted with methanol. The total polyphenolic index (TPI) and the antioxidant activity (AA) of the resulting extracts were measured and their polyphenolic content was studied by HPLC. **Figures 1**, **2**, and **3** show the results of the TPI, as equivalents of gallic acid (mg/L) per gram of sample lyophilized, and the AA, as concentration of Trolox equivalent per gram of sample lyophilized.

In general terms, it is observed that marcs and stalks present a higher polyphenolic content and antioxidant activity than the dregs. This is a logical finding considering both the stalks and the peels and seeds (that comprise the marcs) are the parts of

Table 1. Polyphenols Quantified (in mg/L) in the Marcs Samples

	Syrah	Tempranillo		Cabernet Sauvignon		Moscatel		Palomino Fino	
	nonirr.	nonirr.	irr.	nonirr.	irr.	nonirr.	irr.	nonirr.	irr.
gallic acid	1.14	2.26	1.85		0.84	0.66			
furfural		0.30	0.27						
p-OH-phenethyl alcohol	1.85		1.35			4.44	8.08		
catechin		8.86	6.97	1.52	14.87			9.66	5.56
vanillic acid				1.45					
syringic acid	4.32			2.05					
epicatechin	21.74	4.54	3.44					3.23	2.37
caftaric acid						2.36	2.50	2.34	1.12
trans-coutaric acid							0.25	0.60	
clorogenic acid				0.37					
trans-p-coumaric acid				0.11					

Table 2. Polyphenols Quantified (in mg/L) in the Stalks Samples

	Syrah		Tempranillo		Cabernet Sauvignon	
	nonirr.	irr.	nonirr.	irr.	nonirr.	irr.
gallic acid		0.76				0.69
p-OH-phenethyl alcohol	60.05	23.04	2.97	11.97	3.83	11.51
catechin			67.44	28.08		36.88
syringic acid					1.14	1.47
epicatechin		2.41	33.85		0.76	
caftaric acid	9.56	1.25	6.69	3.15	3.08	2.03
trans-coutaric acid	3.80		3.28	1.63	0.27	1.17
trans-p-coumaric acid	0.90		0.09		0.14	0.81

Table 3. Polyphenols Quantified (in mg/L) in the Dregs Samples

	Syrah		Tempranillo		Cabernet Sauvignon		Moscatel		Palomino Fino	
	nonirr.	irr.	nonirr.	irr.	nonirr.	irr.	nonirr.	irr.	nonirr.	irr.
p-OH-phenethyl alcohol							2.09			
catechin	1.36									
vanillic acid	2.93	1.79				1.39				
syringic acid	3.22	1.67			1.47	2.14				
isovanillin	1.00									
caftaric acid	6.23	4.03					0.95	4.67		4.17
cis-coutaric acid		0.37					0.36	0.30		0.18
trans-coutaric acid	1.93	0.55								0.22
clorogenic acid	0.59	1.07				1.94			2.63	0.80
caffeic acid	11.59	1.39					3.86	1.39		
cis-p-coumaric acid	0.38			1.14						
syringialdehyde	0.54	0.28	0.48	0.64						
trans-p-coumaric acid	2.87	0.59	0.30	0.57						
ferulic acid	0.44	2.01	0.56	0.62	0.49	1.10				

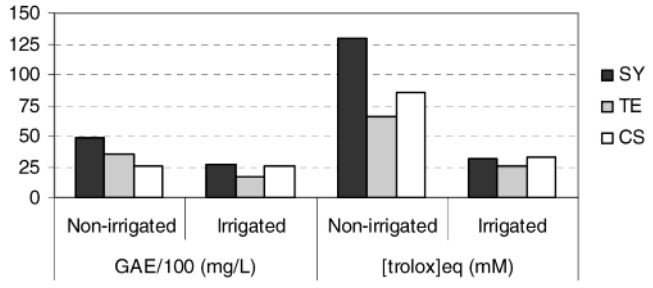

Figure 2. Total polyphenolic index (GAE) and antioxidant activity ($[trolox]_{eq}$) of the stalks samples.

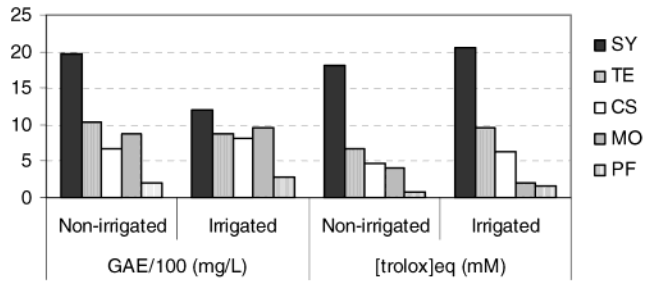

Figure 3. Total polyphenolic index (GAE) and antioxidant activity ($[trolox]_{eq}$) of the dregs samples.

the grape that contain most of the polyphenolic material. Also, in general terms, the red varieties present higher values than the white. Of the five varieties studied, the Syrah stands out clearly from the others for its high polyphenolic content and antioxidant power, in all the types of samples. The variety presenting the lowest values is the white Palomino Fino.

To compare the results of the TPI and AA, the coefficients of correlation were determined for the three types of sample, obtaining 0.9069, 0.8438, and 0.7915 for the marcs, stalks, and dregs, respectively. These results show a good correlation between both parameters, i.e., between the polyphenolic content of the samples and their antioxidant activity. Previous studies conducted with wines have also found a positive correlation

between the Folin−Ciocalteu Index and the antioxidant activity measured using the electrochemical method (*19*) and other methods (*20*, *21*).

With respect to the comparison between cultivation conditions with and without irrigation, previous studies conducted with two varieties of grapes, red and white, have found that irrigation leads to increase of the grapes volume because of higher water content. Then the grapes content is diluted, and as a result the TPI and AA decrease, in comparison with those grapes from nonirrigation (*22*).

Regarding the byproducts, the same effect was observed for the stalks; that is, higher values of TPI and AA for the nonirrigated conditions. As for marcs and dregs, similar values were found for each variety under both conditions. The only notable case is that of the Cabernet Sauvignon marcs, which present a much higher antioxidant activity and polyphenolic content in irrigated conditions.

Marcs and dregs are byproducts obtained from grapes after several enologycal processes in the winemaking. The winemaking process of each wine is different and this has a big influence on the byproducts composition. Consequently, the cultivation conditions effect cannot be observed or is not significant. This effect is observed only in the stalks, which have not suffered any process because they were separated from grapes before the winemaking.

To complete the study, the samples were analyzed using HPLC. Some polyphenolic compounds were identified, and calibration curves were constructed to quantify them. Analytical sensitivity (AS) and linearity (LOL) of these curves were calculated using the ALAMIN program (*23*): gallic acid (AS, 0.1149; LOL, 99.436%), furfural (1.0319; 96.288%), *p*-hydroxyphenethyl alcohol (0.2288; 98.934%), catechin (1.5217; 90.602%), vanillic acid (1.1325; 94.234%), syringic acid (0.2304; 98.953%), epicatechin (0.0439; 99.783%), isovanillin (0.2291; 99.248%), chlorogenic acid (0.2171; 98.874%), caffeic acid (0.1356; 99.425%), syringialdehyde (0.1087; 99.539%), *trans*-*p*-coumaric acid (0.1371; 99.438%), and ferulic acid (0.1584; 99.401%). The quantified compounds in the samples are shown in **Tables 1**, **2**, and **3**.

It can be observed that, in the marcs, in general terms, the most abundant compounds are gallic acid, catechine, and epicatechine. The presence of these compounds is notable in the samples with greater antioxidant activity (Syrah, Tempranillo, and irrigated Cabernet Sauvignon). The only compound found in all samples from white grape varieties was caftaric acid.

In the stalks, the presence of caftaric acid, *p*-hydroxyphenethyl alcohol, and *trans*-coutaric acid was notable. Other compounds found in some but not all of the stalk samples were catechine, epicatechine, and *trans*-*p*-coumaric acid.

Regarding the dregs, the samples of Syrah showed much higher AA values compared with the rest, and a great number of polyphenols were found in them.

From an analysis of all the samples together, no correlation was found between any of the specific polyphenols identified and the AA of the samples. This would indicate that the AA is related to the total polyphenolic content but not with particular compounds, despite some individual polyphenolic compounds contributing more than others to the total AA measured for each type of sample.

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