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Flavanol Content and Antioxidant Activity in Winery Byproducts

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Proanthocyanidins, particularly those coming from wine and grape products, have became of interest to nutritionists. Particular attention is currently being paid to the exploitation of this kind of grape byproducts for obtaining bio-active phenolic compounds with potential application as food antioxidants and preventive agents against cancer and other diseases. In this work, the flavanol composition of various winery byproducts submitted to different degrees of industrial exploitation has been studied and their antioxidant activity determined using two different methods (TBARS and TEAC) to evaluate their interest as suitable sources for the preparation of flavanol-rich antioxidant extracts. All the byproducts studied were still good flavanol sources no matter their exploitation degree. An important conclusion was that dried grape seeds, obtained as an end byproduct after the color extraction and alcohol distillation of the wine pomace, still kept important flavanol concentrations and significant antioxidant activity, even if they were submitted to high temperatures. These byproducts can be considered a cheap source for the extraction of antioxidant flavanols, which can be used as dietary supplements or in the production of phytochemicals.

KEYWORDS: Winery byproducts; flavanols; antioxidant activity; TEAC; TBARS

INTRODUCTION

Numerous studies indicate that oxidative stress is associated to the development of a great number of chronic ailments, such as arthritis, dementias, cardiovascular illnesses, and cancer, and that antioxidants in the diet can play an important role in the prevention of these illnesses (*1*). Thus, different epidemiological studies have demonstrated the association between a diet rich in polyphenols and the decrease in the risk of suffering certain diseases, especially cardiovascular ones and certain types of cancer (*2*−*4*).

The phenolic compounds of wine, and particularly the flavanols (e.g., catechins, proanthocyanidins), have been the center of attention of recent studies since their relation to the beneficial effects attributed to a moderate consumption of wine was observed (*5*, *6*). These compounds have their origin in the grape, and only a part is transferred to the must during the elaboration of the wine, which is why important quantities still remain in the grape pomace. For this reason, at present, a particular interest exists in the exploitation of this type of byproducts of the grape to obtain potentially bio-active phenolic compounds (*7*−*9*).

In recent years, the use of grape seed extracts (GSE) has begun to become popular as a nutritional supplement that also has antioxidant activity. These extracts contain a heterogeneous mixture of monomers, oligomers, and polymers formed by subunits of flavan-3-ol (*10*, *11*). With regard to their pharmacological properties, these phenols have shown themselves active, in in vitro studies, against the oxidation of the low-density lipoproteins, at the same time as they appear to demonstrate antiulcer, anticarcinogenic, antimutagenic, and antiviral activity (*12*−*15*).

Moreover, at present, there is a growing interest in the exploitation of the residues generated by the food industry. In particular, in the zones of grape and wine production, a great quantity of residues are generated whose storage, transformation, or elimination pose problems both in ecological and economic terms. For this reason, the recovery of the antioxidant compounds present in these byproducts could represent an interesting advance in the maintenance of the environmental equilibrium (*16*, *17*).

The objective of this work was to determine the flavanol composition and the antioxidant activity of byproducts of winemaking from different varieties of grapes. The samples were collected directly from cellars and also at different stages of a process of industrial exploitation of pomaces destined to the extraction of alcohol and anthocyanins from the byproducts, with the objective of testing how this process affected the composition of the pomaces and their corresponding seeds and evaluating the interest of these byproducts as possible sources for the preparation of flavanol-rich antioxidant extracts.

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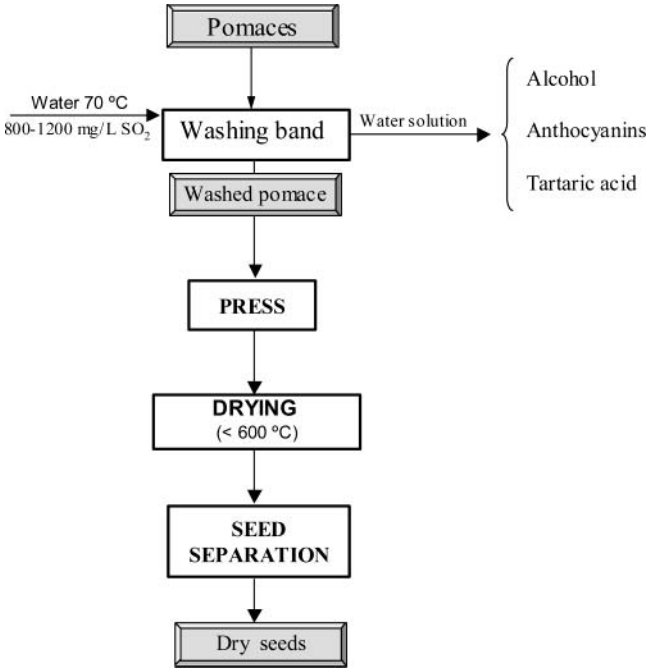

Figure 1. Scheme of the process of industrial winery exploitation of the wine-making byproducts (the points where the samples were collected are indicated in gray).

MATERIAL AND METHODS

Samples. Eighteen samples of wine-making byproducts (nine corresponding to wine pomaces and nine to grape seeds) derived from six grape varieties, four red (Cabernet-Sauvignon, Merlot, Tempranillo, and Garnacha) and two white (Albillo and Viura), were collected from different Spanish cellars and analyzed to evaluate their suitability to be used as sources for the extraction of antioxidant flavanols.

On the other hand, samples from wine pomaces of three different origins but all of them derived from Tempranillo red grapes were also collected in a winery before and after their washing with hot sulfited water for anthocyanin and alcohol production. In these samples, the flavanol composition and the antioxidant activity were analyzed in the raw pomaces before and after washing and also in the grape seeds manually separated from them. Likewise, the dried grape seeds obtained as a final waste from the winery processing (**Figure 1**) of each of the samples were also studied with the purpose of establishing the effect of the drying treatment on the flavanol contents and antioxidant activity.

Sample Preparation. All the samples were lyophilised and their moisture calculated by weight difference before and after lyophilisation. Freeze-dried samples, 2 g for the grape seeds and 5 g for the pomaces, were ground to obtain a homogeneous powder; 75% methanol was added; and the mixture was homogenized and then maintained for 15 min in an ultrasonic bath to assist the extraction. Afterward, it was centrifuged for 10 min and the supernatant was collected. The extraction process was repeated twice more. The methanolic extracts were combined and concentrated at low pressure until an aqueous extract was obtained, which was made up to a final volume of 10 mL with water. Two milliliters of ethyl acetate was added to 2 mL of the aqueous extract, mixed for 2 min, and further centrifuged for 5 min. The ethyl acetate phase was collected and the aqueous phase was submitted to the same extraction process twice more (*18*). The ethyl acetate phases from each sample were combined, dried, and redissolved in 2 mL of water.

High-Performance Liquid Chromatography (HPLC) Analysis. The analysis of flavanols was carried out using a Hewlett-Packard 1100 chromatographic system. The separation of the flavanols was performed using a Spherisorb S3 ODS2 reverse phase C18 column, 3-μm particle size, 150 × 4.6 mm i.d. (Waters). The chromatographic conditions were as follows: flow, 0.5 mL/min; volume of injection, 100 μL; and solvents, A, 2.5% acetic acid, B, 2.5% acetic acid/acetonitrile (90:10), and C, gradient grade acetonitrile. The gradient consisted of 0−100% of B in A for 5 min, 0−15% of C in B for 25 min, 15−50% of C in B for 5 min, and 50% C isocratically for 5 min. Detection was carried out in a diode array detector (Hewlett-Packard 1100) using 280 nm as preferred wavelength.

The chromatographic system was calibrated for the quantification of flavanols with standards previously isolated in our laboratory (19): catechin (C); epicatechin (EC); epicatechin-3-*O*-gallate (ECG); and proanthocyanidin dimers B1 (EC−C), B2 (EC-EC), B3 (C−C), and B4 (C-EC). The concentration of flavan-3-ols in seeds and pomaces was expressed in mg/100 g of dry weight.

Measurement of the Antioxidant Activity. *Lipid Phase Antioxidant Activity*. TBARS Method (*20*). Phosphatidyl choline (Sigma, St. Louis, MO) at a final concentration of 1 mg/mL was suspended in 150 mM KCl (Panreac Química SA, Barcelona, Spain) containing 0.2 mM $FeCl_3$ (Panreac Química SA), and the samples were assayed at a range of concentrations. Peroxidation was started by adding ascorbate at a final concentration of 0.05 mM in a final volume of 0.4 mL. Samples were incubated at 37 °C for 40 min and the reaction terminated by addition of 0.8 mL of 20% trichloroacetic acid (TCA) (Fluka Chemie, Buchs, Switzerland)/0.4% thiobarbituric acid (TBA) (Sigma)/0.25 N HCl, and 0.01 mL of butylated hydroxytoluene (BHT) (Aldrich, Milwaukee, WI) in ethanol. The production of thiobarbituric acid reactive substances (TBARS) was measured spectrophotometrically at 535 nm after incubation at 80 °C for 20 min and expressed as dry weight (μg), causing 50% inhibition (IW_{50}).

Aqueous Phase Antioxidant Activity. TEAC Method (*21*, *22*). The TEAC assay is based on the scavenging of the relatively stable blue/green ABTS radical (2,2′-azinobis (3-ethyl-benzothiazoline-6-sulfonate), Fluka Chemie, Buchs, Switzerland), converting it into a colorless product (*23*). The degree of this decolorization reflects the amount of $ABTS^{\bullet+}$ that has been scavenged. In this assay, the radical is generated by the interaction of ABTS with the ferrylmyoglobin radical species, generated by the activation of metmyoglobin (Sigma) in the presence of hydrogen peroxide (Panreac Química SA) and can easily be detected spectrophotometrically at 734 nm. A TEAC value can be assigned to all compounds able to scavenge the $ABTS^{\bullet+}$ cation by comparing their scavenging capacity to that of Trolox C (Fluka Chemie), a water-soluble vitamin E analogue. Results are expressed as TEAC value.

Statistical Analysis. To assay the possible correlation between the content in flavanols of the extracts and their antioxidant activity, an analysis of variance (ANOVA) was applied using the Statview 4.1 statistical software. Differences of $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Quantitative Analysis of Flavanols by HPLC. All the samples were analyzed by HPLC to quantify seven different flavanols: C, EC, ECG, B1, B2, B3, and B4 (**Figure 2**). The global content of catechins and proanthocyanidins was calculated from the sum of the individual concentrations of each of the flavanols quantified. It can be observed (**Figure 3**) that the total flavanol content in the pomaces derived from different grape varieties is very different among samples (with values ranging between 29 and 199 mg/100 g of dry weight), as well as in the seeds separated from them (136−719 mg/100 g of dry weight).

The analysis of the samples derived from different grape varieties and wine-making process (**Figure 3**) revealed that differences exist not only among varieties, but also within the same variety. Although the results obtained do not allow definitive conclusions to be drawn, given the limits of the sampling, this leads to the supposition that the content of flavanols is conditioned not only by the type of grape but also by edapho-climatic factors linked to the grape origin. On the other hand, it could be observed that the pomaces from red wine making continued to have relevant flavanol amounts, and they are still good sources for their extraction.

As it could be expected, in all the cases, the seeds presented greater concentration in total and individual proanthocyanidins than the complete pomaces. Nevertheless, in the case of the

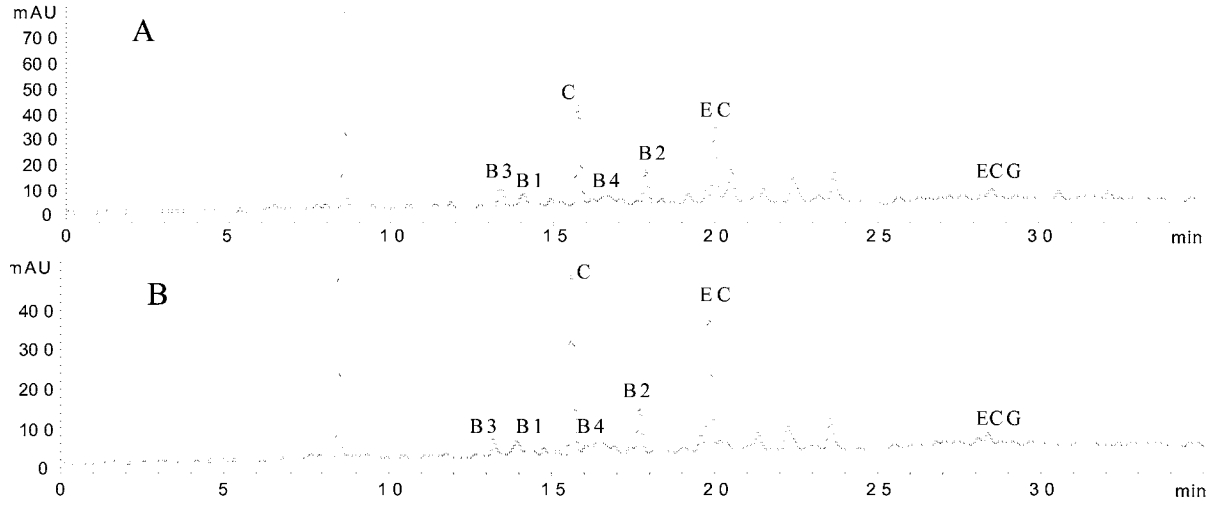

Figure 2. HPLC chromatograms recorded at 280 nm, corresponding to samples of a Tempranillo red wine pomace (A) and the grape seeds manually separated from it (B). See Materials and Methods for sample preparation.

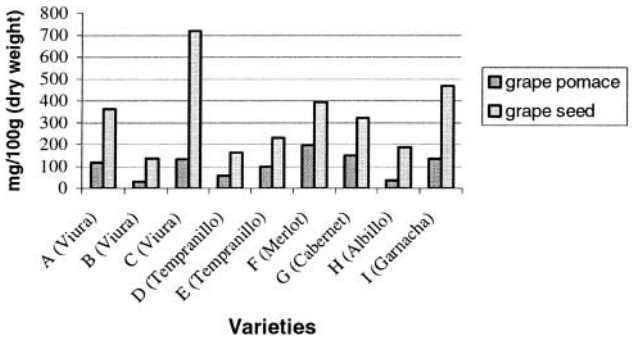

Figure 3. Total flavanol content in byproducts from the wine making of different grape varieties.

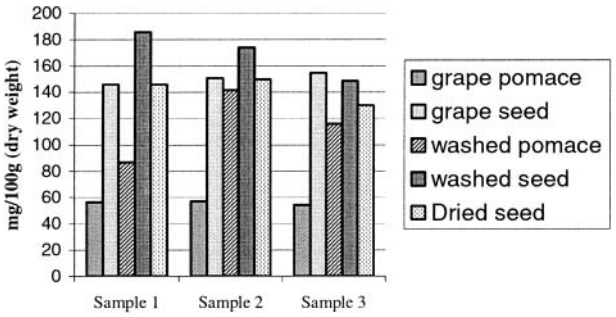

Figure 4. Total flavanol content in byproducts collected at different stages of a process of industrial exploitation of wine pomaces.

pomaces and seeds collected in the winery after extraction with hot sulfited water (**Figure 4**), the differences in the flavanol content between pomaces and seeds were not so important. This could simply be due to an increase of the proportion of seeds present in the pomace after the industrial extraction due to the loss of pulp and skin, fundamentally, with respect to the original pomace.

It could be also observed that the concentration of flavanols determined in the washed pomaces was greater than in the raw wine pomaces. A possible explanation is that the industrial treatment with hot sulfited water improves the extractability of some proanthocyanidins linked to matrix structures. Furthermore, a cleavage of proanthocyanidins of greater degree of polymerization to those analyzed in this work can also occur by effect of the temperature and the acidity, thus increasing the amount of quantifiable flavanols.

A relevant finding was that the dried seeds obtained as a final winery waste showed only a small decrease (approximately 10%) in the content of total analyzed flavanols with respect to that determined in the original seed (**Figure 4**), thus they still retain important quantities of flavanols despite the high temperatures used in the drying process.

In general, in all the samples analyzed, either seeds or pomaces before or after the industrial process, the major flavanols determined were the monomers catechin and epicatechin. Regarding the oligomers, it is worth indicating that the principal component, in all cases, was the procyanidin dimer B2 followed by B4 in the raw pomaces and B1 in the washed pomaces. It has been shown that proanthocyanidin polymers in the grape seed fundamentally consist of catechin as terminal and epicatechin as extension subunits (*24*). Therefore, the procyanidin dimer B1 (EC-C) would be expected to be a major product of their breakdown, and thus, the increase in the relative content of B1 observed in the washed pomaces would support that a cleavage of proanthocyanidins polymers takes place during the process of industrial extraction.

Antioxidant Activity. A great number of methods can be used to evaluate the antioxidant capacity. Each method can be applied both to determine the antioxidant capacity of biological matrixes and to evaluate individual compounds, certain components of foodstuffs, or even extracts from the foodstuffs. Because foodstuffs contain both oil-soluble and water-soluble compounds with antioxidant capacity, in this work, two different methods have been selected for the evaluation of the antioxidant capacity. One determines the inhibition of the peroxidation, induced by the ascorbate/iron complex, of the phosphatidylcholine substrate (TBARS assay); the other, TEAC (Trolox equivalent antioxidant capacity) assay, measures the capacity of a compound to capture the radical cation ABTS (2,2′-azinobis(3-ethyl-benzothiazoline-6-sulfonate)) compared with Trolox C, a hydrosoluble analogue of vitamin E. The antioxidant activity was determined in the same aqueous extracts previously used for the quantification of flavanols.

In **Table 1** the results of antioxidant capacity obtained using the TBARS method in the samples derived from different grape varieties are presented. The lowest value of inhibitory weight 50 (IW_{50}), thus the greatest antioxidant capacity, was found for grape seeds, especially in the cases of samples from cellars C (71 μg) and A (146 μg), both belonging to the Viura variety. On the contrary, the greatest IW_{50} was presented by the pomaces

Table 1. Values (IW_{50}) of Antioxidant Activity Obtained in the TBARS Assay for the Samples Collected after Wine Making of Different Grape Varieties

variety	cellar	IW_{50} (μg) grape pomace	IW_{50} (μg) grape seed
Viura	A	250	146
	B	543	349
	C	586	71
Tempranillo	D	470	185
	E	444	222
Merlot	F	232	430
Cabernet Sauvignon	G	653	170
Albillo	H	751	185
Garnacha	I	248	205

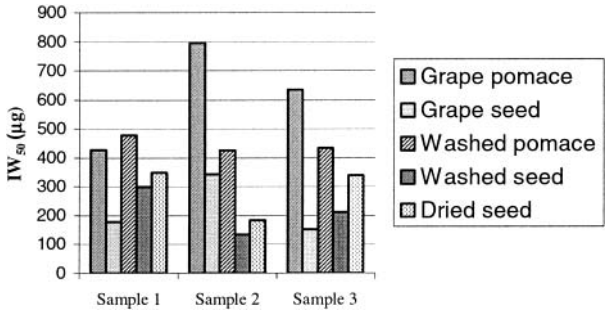

Figure 5. Antioxidant activity of the extracts obtained from different byproducts in the TBARS assay.

collected from cellars H (751 μg) and G (653 μg), corresponding to the varieties Albillo and Cabernet Sauvignon, respectively. In general, the results obtained are very different even within the samples of a same variety, which makes it impossible to establish a good correlation between the antioxidant activity and the type of grape analyzed.

When this same assay was performed for the samples obtained at different stages of the process of industrial exploitation (**Figure 5**), it was observed that, in general, a lower IW_{50} value was obtained in the washed pomaces than in the raw grape pomaces. This could be due, as it has been indicated above, to an enrichment in the proportion of seeds in the pomace but also to a cleavage of proanthocyanidin polymers or a simple diffusion of active components from the seed to the pomace. On the contrary, the process of industrial extraction supposes a small loss in the antioxidant capacity of the seeds in this assay, which would support the existence of a partial diffusion.

When the TEAC assay was applied to samples of different grape varieties (**Table 2**), it was found that the values obtained were positive in all the samples, and therefore, their antioxidant capacities were superior to that of a solution of 1 mM Trolox C. In general terms, it was observed that the activity of the extracts of grape seeds was superior to that of the pomaces, which could be explained by their greater content in flavanols but also by the fact that the seeds are rich in galloylated flavanols, compounds that have an antioxidant activity in aqueous medium superior to that of their nongalloylated homologues (*13*). The TEAC values obtained for the pomaces were lower and oscillated in a narrower range (between 10 and 42) than in the case of the seeds (between 45 and 140).

In the case of the winery samples before and after the industrial exploitation process (**Figure 6**), the results obtained in the TEAC assay are similar to those found in the TBARS assay: A large increase was produced in the TEAC values of

Table 2. TEAC Values Obtained for the Samples Collected after Wine Making of Different Grape Varieties

variety	cellar	TEAC grape pomace	TEAC grape seed
Viura	A	30	70
	B	30	70
	C	38	125
Tempranillo	D	36	80
	E	38	55
Merlot	F	42	140
Cabernet Sauvignon	G	24	80
Albillo	H	10	45
Garnacha	I	38	90

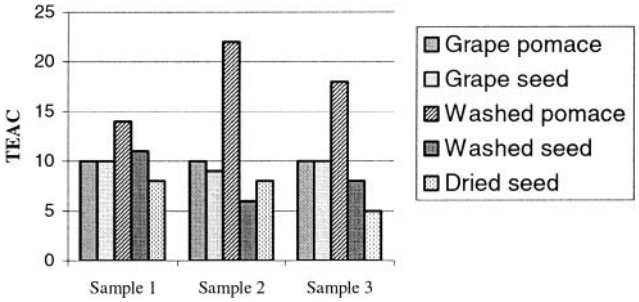

Figure 6. Antioxidant activity of the extracts obtained from different byproducts in the TEAC assay.

the washed pomaces with regard to the raw pomaces and a loss in the processed seeds. A possible explanation, as previously mentioned, is that a partial diffusion of active compounds from the seed to the pomace occurs.

In conclusion, we can state that the byproducts of wine making, either seeds or pomaces, are good sources for the extraction of flavanols. Similar conclusions have been reached for other authors (*16*, *17*), but only for the raw material after the wine making. However, in our study, we show that the byproducts obtained after winery exploitation continue to have relevant amounts of flavanols and important antioxidant activity. What is more, the industrial washing with hot sulfited water applied in the winery could even favor the extraction of flavanols in the case of the pomaces. On the other hand, the dried seeds obtained as a final waste of the exploitation process still retain important amounts of flavanols. These byproducts would constitute a very cheap source for the extraction of antioxidant flavanols, which can be used as dietary supplements, or in the production of phytochemicals, which supposes an important economic advantage.

Correlation Between the Flavanol Content and Antioxidant Activity. With the objective of determining the possible relationship between the variables antioxidant activity and flavanol content of all the byproducts analyzed, the coefficients of correlation and the corresponding models of regression for the TEAC and the IW_{50} were calculated considering three groups of samples (only seeds, only pomaces, and the grouping of both types of samples).

When the flavanol contents, both total and individual, are related to the values obtained in the TEAC assay, the best correlations are found when only the grouping of seeds showing coefficients greater than 0.8 (e.g., with C, EC, and the total content of flavanols) are considered. The relationship between the TEAC values and the total contents of flavanols explains 55.7% of the variability when the grouping of all the samples

is taken into account, which can be considered statistically significant ($p < 0.001$), although it is a fact that the significance is mainly determined by the samples of seeds.

When an attempt is made to establish the possible relationship between the value of IW_{50} and the total content of flavanols, the model proposed is similar to the former and presents coefficients of correlation near −0.50 for all the variables, in this case, the influence of the seeds being the greatest. In both cases, the greatest correlation found when only the group of seeds is considered can be explained by the presence, in the pomace, of other compounds with antioxidant activity (e.g., flavanols, phenolic acids, and anthocyanins) which have not been quantified in our work. Some authors (*16*) have made studies of correlation similar to ours, finding coefficients of correlation superior to those found in our work, although it is necessary to take into account that those authors determined total phenolics using the reagent of Folin−Ciocalteu and not exclusively proanthocyanidins, which could explain the lower correlations obtained in our case.

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