JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Assessment of the Production of Antioxidants from Winemaking Waste Solids

JOSÉ MANUEL CRUZ, HERMINIA DOMÍNGUEZ,* AND JUAN CARLOS PARAJÓ

Departamento de Enxeñería Química, Edificio Politécnico, Universidade de Vigo (Campus Ourense), As Lagoas, 32004 Ourense, Spain

Winemaking waste solids (WS, resulting from red grapes after fermentation and distillation to recover spirits) were subjected to various processing schemes for isolating fractions with antioxidant activity. The liquors entrapped in WS as received were separated by pressing and freeze-dried to yield a fraction with antioxidant activity (measured as DPPH radical scavenging capacity) comparable to those of synthetic antioxidants. A second approach based on the direct processing of raw WS in sulfuric acid medium under fixed operational conditions and further extraction of hydrolysis liquors with ethyl acetate enabled the isolation of a fraction with higher antioxidant ability at an improved yield. The most favorable approach started with a washing stage leading to liquors (which were directly freeze-dried to yield 1.20 g of extract/100 g of oven-dry WS and presented an EC_{50} of 0.41 g of extract/L) and washed solids, which were dried and subjected to hydrolytic processing (i) with water as a reactive in an autocatalyzed reaction (autohydrolysis) or (ii) with sulfuric acid solutions to give an ethyl acetate-soluble fraction with improved antioxidant properties (EC_{50} in the range of 0.18−0.40 g/L). Samples from washing liquors and processing of washed solids in aqueous medium were subjected to chromatographic fractionation and analysis to give isolates with remarkable antioxidant activity (with EC_{50} as low as 0.07 g/L) and to identify their major components.

KEYWORDS: Acid hydrolysis; analysis; antioxidant activity; autohydrolysis; ethyl acetate; red grape; solid wastes; winemaking

INTRODUCTION

Substitution of synthetic antioxidants by natural ones has gained interest over the past few years in the food industry due to health and safety concerns. In this context, plant-derived materials have been tested as sources for active antioxidants. Residual biomass (from agricultural or industrial activities) is a favorable raw material for chemical processing due to its low cost and the possibility of avoiding environmental problems caused by its disposal (*1*−*5*). On the other hand, the external fibrous and lignified parts of plants (such as peels, hulls, or seeds) appearing in wastes present higher contents of phenolics potentially useful as antioxidants than the corresponding inner parts.

Grape- and wine-derived byproducts are largely available and show high phenolic content. This hinders their direct utilization in agriculture, but could be favorable for processes intending the selective separation and recovery of natural compounds with antioxidant activity.

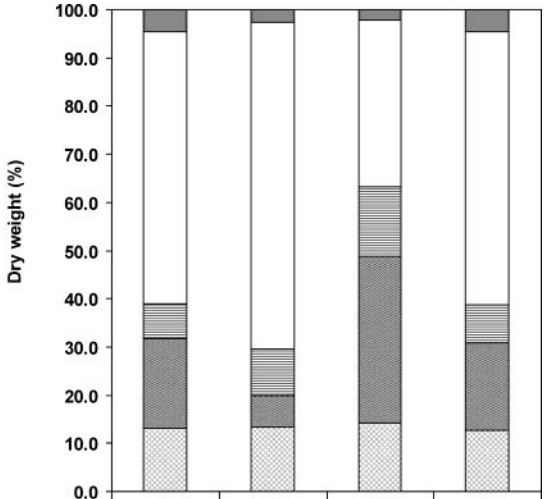
Waste solids from winemaking are heterogeneous, and the various materials present in them have different compositions (*6*). The major phenolic compounds in *Vitis vinifera* grape seeds are epicatechin (accounting for 60% of the monomers) followed by catechin and gallic acid (*7*−*9*), whereas the most abundant phenolics in grape peels are epicatechin, epigallocatechin, gallic acid, and catechin (*10*−*13*). The presence of phenolic acids such as caftaric and coutaric, catechin, epicatechin, astilbin, and engeletin as well as myricetin, kaempferol, and quercetin glucosides in stalks has been reported (*11*).

The antioxidant activity of grape seed extracts against several free radicals (*9*) has been reported (*14*). Other biological activities, such as the inhibition of carcinoma cells (*15*), the amelioration of cytotoxic effects caused by chemotherapeutic agents (*16*), vasorelaxing capacity (*17*), and antiulcer activity (*7*), have been noted. Grape seed extracts also present antimicrobial and agrochemical properties (*8*). On the other hand, oral administration of grape seed extracts did not induce toxicity in rats (*18*), and their utilization in a number of antioxidant formulations has been claimed (*19*−*21*).

Although grape seeds are a potential source of different valuable components (including antioxidants), a more practical alternative could be based on the utilization of the whole cake of pressed grapes (*22*) or its ethanolic extracts (*4*). However, winemaking factories employ pressed grapes for producing distilled spirits, and the real solid waste from this kind of process is the product resulting from the distillation stage, here denoted waste solids (WS). WS are not used currently in Spain and are considered as the starting material in the present work.

* Author to whom correspondence should be addressed (telephone +34988387082; fax +988387001; e-mail herminia@uvigo.es).

10.1021/jf049376c CCC: $27.50 © 2004 American Chemical Society
Published on Web 08/17/2004



	skin	seed	stem	mix
Acid soluble lignin	4.6	2.7	2.2	4.4
Acid insoluble residue	56.4	67.7	34.5	56.7
Hemicellulose	7.2	9.6	14.5	8.0
Cellulose (as glucan)	18.6	6.7	34.6	18.2
Others	13.2	13.3	14.2	12.6

Figure 1. Composition of the feedstock (denoted waste solids, WS).

Mild hydrolytic processing of plant biomass solubilizes extractives, hemicelluloses (as hemicellulosic sugars), and phenolic compounds. The phenolics linked to the hemicelluloses and to the acid-soluble lignin fraction are active antioxidants, as reported for the liquors from acid hydrolysis of corn bran (*23*), *Eucalyptus* wood, corn cob and leaves, barley bran (*24*, *25*), and barley grains (*26*). The release of phenolic compounds with antioxidant activity has been reported for hydrolytic processes such as steam explosion (*27*, *28*) or treatments with hot, compressed water (*29*). To our knowledge, no data are available on the acid hydrolysis of winemaking waste solids. Because this material has a significant content of lignin (*2*) and tannins (*30*), the release of phenolic compounds with antioxidant activity during their hydrolytic processing is expected.

The aim of the present work was to assess the potential of WS for the production of antioxidants. Operational schemes involving pressing, washing, and acid hydrolysis or autohydrolysis followed by ethyl acetate extraction for obtaining compounds with antioxidant activity have been considered. Chromatographic fractionation and identification of the major compounds obtained from selected processing schemes are also considered in this work.

MATERIALS AND METHODS

Material. Waste solids (WS) leaving the spirit distillation stage in winemaking manufacture from red grapes were kindly supplied by Cooperativa Vitivinícola do Ribeiro (Ourense, Spain). WS samples were stored in sealed plastic bags and kept at −80 °C. The composition of the feedstock was determined by quantitative acid hydrolysis (*31*) followed by UV and HPLC analyses of the liquors (to quantify the acid-soluble lignin and polysaccharide fractions, respectively) and by gravimetric analysis of the residual solid after oven-drying (to determine the acid-insoluble residue). The compositions of the different WS fractions are summarized in **Figure 1**.

Processing of WS. Figure 2 shows the processing of WS according to three different alternatives (processes a−c), which led to extracts I−IV with antioxidant activity. Extract I was obtained from the liquors released by pressing WS at 20 bar as indicated for process a in **Figure 2**. Extract II was obtained according to process b in **Figure 2**: WS as received were subjected to a hydrolytic stage with sulfuric acid at a liquid/solid ratio of 8 kg/kg (oven-dry basis) under fixed conditions of temperature and acid concentration (see below). After the hydrolytic stage, solid−liquid separation was accomplished by vacuum filtration, and the resulting liquors were extracted with ethyl acetate under previously reported conditions (*24*, *32*). The organic phase was vacuum evaporated to recover the solvent, and the extract was freeze-dried to recover extract II. Extract III was obtained from WS washing liquors (in multistep washing carried out with tap water at a liquid-to-solid ratio of 25 g/g during 1 h at 60 °C) by direct freeze-drying according to process c in **Figure 2**. Extract IV was obtained from washed and dried WS (denoted WDWS) and autoclave treatment with water or with sulfuric acid solutions at a liquid/solid ratio of 8 kg/kg under a variety of operational conditions and further extraction with ethyl acetate, as indicated for process c in **Figure 2**. In autoclave treatments, the processing time was measured as the duration of the isothermal reaction stage. As the heating and cooling periods are not considered in the reaction time, the joint effects caused by them can be measured in the samples collected at $t = 0$.

Chromatography Fractionation. Extracts (~0.2 g) were redissolved in methanol (5 mL) and loaded onto a 40 cm × 3.5 cm column containing Sephadex LH-20 (from Amersham Biosciences, Uppsala, Sweden), which was eluted with methanol at a flow rate of 3 mL/min. Fractions were collected using a Gilson FC 203B fraction collector with 3 mL test tubes. Absorbances (280 nm) were recorded on-line using an Agilent 8453 spectrophotometer.

Analysis of Extracts. The yields in solids recovered in extracts I−IV of **Figure 2** were determined gravimetrically and expressed as percentages of the initial oven-dry weight of WS. The phenolic content of the various extracts was determined with the Folin−Denis method (*33*) using gallic acid as a standard, and the corresponding concentrations were used to calculate the yield in phenolics (expressed as grams of gallic acid equivalents per gram of substrate). The reported data are average values of three replicates.

α,α-Diphenyl-β-picrylhydrazyl (DPPH) Radical Scavenging Activity. A minor modification of the method described by von Gadow et al. (*34*), consisting of adding 2 mL of a 3.6×10^{-5} M methanolic solution of DPPH to 50 μL of a methanolic solution of the antioxidant instead of the amounts proposed in the original method, was used in this work. The decrease in absorbance at 515 nm was recorded for 16 min. The volumetric activity of isolates was measured in terms of the parameter ED_{50}, which was calculated as the volume ratio V'/V (where V' is the volume of the methanolic solution employed in the assay containing the desired amount of extracts and V is the volume of the original aqueous solution before extraction containing the same amount of extracts) necessary to cause 50% inhibition of the DPPH radical. According to this definition, higher antioxidant activity results in increased values of ED_{50} and in decreased values of EC_{50}.

All tests and analyses were run in duplicate or in triplicate, and the average values are presented. Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) were used as reference antioxidants.

Analysis of Phenolic Acids by HPLC. Extracts from 25 mL of the considered solution were dissolved in 10 mL of methanol and analyzed by high-performance liquid chromatography (HPLC) using an Agilent 1050 instrument fitted with a 1050 DA detector. Separation was performed at room temperature with a Supelcosil LC-18 (5 μm) using a 4.6 mm × 25 cm column (Supelco). Gradient elution was performed at a flow rate of 1.0 mL/min by mixing 0.01 M sodium citrate buffer (A) (at pH 5.4, adjusted with 50% acetic acid) and methanol (B). The gradient was programmed as follows: from 0 to 12 min, B increased from 2 to 4%; from 12 to 20 min, B increased from 4 to 13%; from 20 to 22 min, B was kept constant at 13%; from 22 to 26 min, B decreased from 13 to 2%; from 26 to 30 min, B was kept constant at 2%.

GC-MS analysis. Samples were derivatized as reported by Quesada et al. (*35*): ~50 mg of standard reagents or extracts was weighed into a 25 mL round-bottom flask and trimethylsilylated by adding 200 μL of pyridine, 1 mL of BSTFA, and 50 μL of TMCS. The round-bottom flask was sealed, shaken vigorously, kept at 60 °C under stirring for 30 min in a water bath, and cooled to room temperature before GC-MS analysis (injection volume = 1.5 μL). Derivatized samples were analyzed using a Hewlett-Packard 5989 chromatograph fitted with a Hewlett-Packard 5972 mass spectrometer. A capillary silica column (60 m × 0.25 mm) packed with HP-5MS was employed for separation,

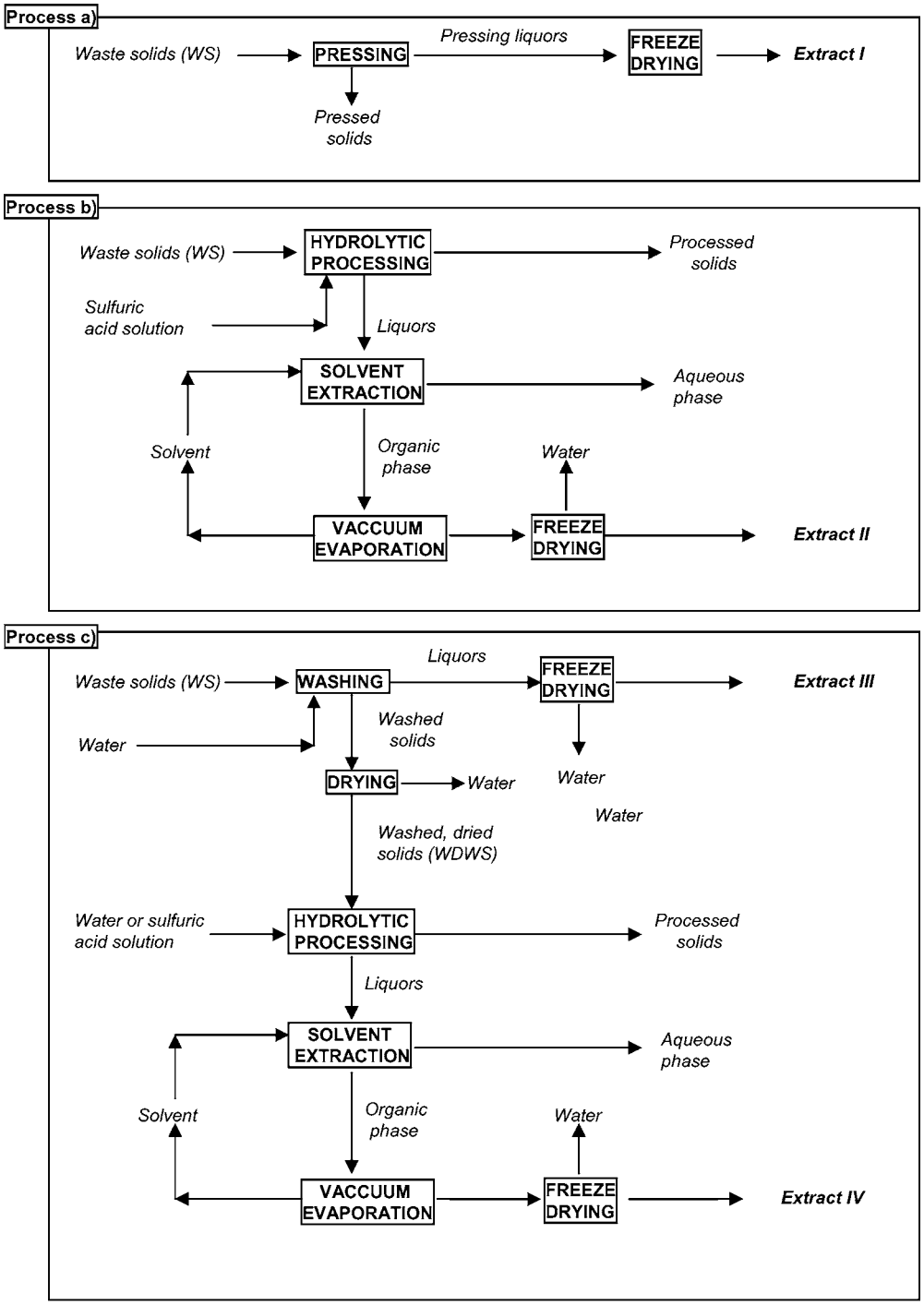


Figure 2. Flow diagram of the processes used to produce and recover extracts with antioxidant activity from the feedstock.

and helium was used as a carrier gas (flow rate = 1 mL/min). Other features of the method are as follows: injector temperature, 250 °C; detector temperature, 280 °C; temperature gradient, isothermal operation at 90 °C from 0 to 10 min, heating at 5 °C/min to 205 °C, heating at 8 °C/min to 250 °C, isothermal operation at 250 °C for 15 min. The identity of the compounds was confirmed by comparing both the retention time and the mass spectral data with those of pure compounds.

RESULTS AND DISCUSSION

Production of Antioxidants by Processes a and b. As WS are expected to contain soluble phenolics with antioxidant activity, a simple approach consisting of pressing WS and freeze-drying the pressing liquors was first employed to obtain extract I according to process a in **Figure 2**. This operational procedure led to a yield of 0.468 g of extract I/100 g of oven-dry WS, and the isolate showed a moderate specific antioxidant activity (EC_{50} = 0.719 g/L).

Solids leaving the winemaking fermentation step contain cell wall-linked phenols, proteins, and condensed tannins linked together in an insoluble lignin−protein−tannin complex (*30*, *36*). As hemicelluloses and lignin present in WS are partially depolymerized by acid hydrolysis, the release of soluble compounds and their antioxidant activity was assessed. Following this philosophy, the WS (as received) were subjected to acid hydrolysis under fixed, medium-severity conditions (treatment with 5% sulfuric acid concentration at 115 °C for

20 min using a liquor/solid ratio of 8 g/g), and the hydrolysis liquors were extracted with ethyl acetate to recover extract II according to process b in **Figure 2**. In comparison with process a, the hydrolytic treatment enabled higher extract yield (0.92 g of extract II/100 g of oven-dry WS) and better specific antioxidant activity of the isolate (shown by the decreased value determined for the parameter EC_{50}, 0.417 g/L). However, parallel experiments carried out following process c in **Figure 2** proved that the implementation of washing stages before the hydrolytic processing enabled better yields and improved antioxidant activity. Because of this, no further investigation on process b was carried out.

Production of Antioxidants by Process c. Process c in **Figure 2**, involving stages of washing, drying, and hydrolytic processing, allows the recovery of active extracts from both washing liquors (extract III) and washed solids (extract IV, the ethyl acetate soluble solids present in liquors coming from the hydrolytic processing of washed and dried WS).

Freeze-drying WS washing liquors resulted in a favorable yield in extract III (1.206 g of extract/100 g of oven-dry WS), which presented a good specific antioxidant activity (EC_{50} = 0.409 g/L). The facts that the recovery yield and antioxidant activity were higher for extract III than for extract II suggest that the sulfuric acid treatment caused condensation of solutes present in the original WS liquid phase and that the compounds produced in condensation reactions were less active than the original ones. Both findings confirm the comparative advantage of process c over process b for the purposes of this work.

According to process c in **Figure 2**, the solid phase leaving the washing step was dried (in order to facilitate its handling, to improve the reproducibility of data, and to extend its conservation) and employed for obtaining extract IV by hydrolytic processing and solvent extraction.

In a first set of experiments, the effects caused by oven-drying at 50 °C for 2 days on the yield and antioxidant activity of phenolic compounds released upon acid hydrolysis of washed WS were assessed. For this purpose, dried and nondried samples of washed WS were subjected to acid hydrolysis under operational conditions (5% sulfuric acid, 115 °C, 20 min, 8 kg of liquid/kg of solid) selected on the basis of previous results (*24*), and the liquors from the hydrolysis stage were extracted in ethyl acetate. The yields in extracts and the antioxidant activity of isolates were not affected by the drying stage, reaching average values of 0.54 g of extract/100 g of oven-dry substrate for the yield and 0.662 g/L for the parameter EC_{50}. This finding confirmed that the mild drying conditions employed avoided thermal decomposition. Similar behavior was reported by Larrauri et al. (*37*) on the antioxidant activity of red grape marc dried at 60 °C, whereas drying at 100 and 140 °C reduced the antioxidant activity by 28 and 50%, respectively. On the basis of these results, the rest of the experiments were performed with washed, dried WS (denoted WDWS, see **Figure 2**).

Effect of Operational Conditions in Hydrolytic Processing with Sulfuric Acid Solutions. New experiments were carried out to assess the effects of the hydrolytic stage conditions on the yield and properties of antioxidant extracts. Treatments with sulfuric acid solutions or water were carried out at a fixed liquid/solid ratio (8 kg/kg), and the effects caused by the rest of the variables affecting the process severity (temperature, time, and sulfuric acid concentration in the case of experiments with externally added catalyst) were considered as operational variables.

The effect of the sulfuric acid concentration was assessed at 130 °C in experiments lasting 1 h using sulfuric acid concentrations at up to 8%, and experimental results were determined for the yield in extract IV, volumetric antioxidant activity of liquors (measured by ED_{50}), and specific antioxidant activity of extracts (measured by EC_{50}). The experimental data in **Figure 3** show that the extraction yield and the parameter ED_{50} presented a closely related dependence on the sulfuric acid concentration, with a marked increase when the sulfuric acid concentration increased from 2.5 to 4% and variations of minor importance when the sulfuric acid concentration increased from 4 to 8%. As expected from this relationship, EC_{50} was almost independent of the acid concentration. Because the maximum yield in extract IV (1.6 g of extract/100 g of oven-dry WDWS) was achieved in the experiment with 4% sulfuric acid, and that considering higher acid concentrations did not result in improved antioxidant activity, 4% sulfuric acid can be considered a practical upper limit for catalyst concentration. HPLC chromatograms of extracts produced at 130 °C showed a major peak at 5.0 min (accounting for 30% of total area) with retention time and spectral data (recorded with the DA detector) coincident with those of gallic acid. The variation pattern of the peak area with the catalyst concentration was closely related to the one determined for ED_{50}.

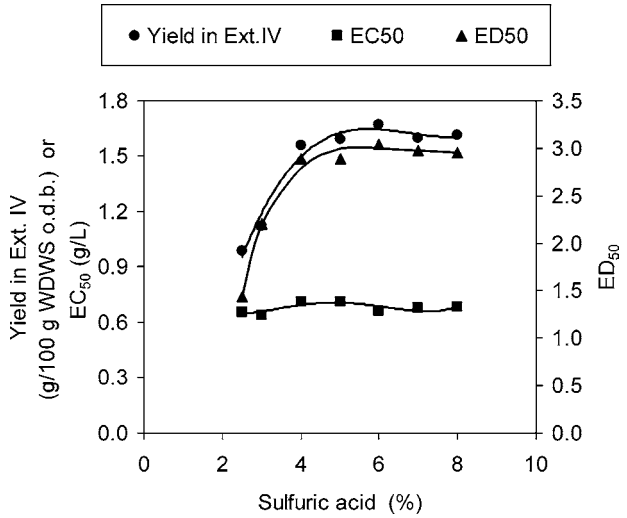


Figure 3. Effect of sulfuric acid concentration on the yield in extract IV, EC_{50}, and ED_{50} in experiments carried out at 130 °C for 1 h.

According to the above findings, 4% sulfuric acid concentration was selected for assessing the effect of the reaction time (up to 120 min) in treatments at 130 °C. The experimental results in **Figure 4** show that the maximum yields in both extract IV and phenolics (1.61 g/100 g of WDWS and 0.21 g of gallic acid equiv/100 g of WDWS, respectively) were achieved in the treatment lasting 90 min. The volumetric antioxidant activity ED_{50} also showed a maximum under these conditions (4.63), and the specific activity (EC_{50} = 0.43 g/L) was satisfactory, particularly when compared with the results achieved at shorter processing times.

Effect of Operational Conditions in Hydrolytic Processing Using Aqueous Media. Recovery of extract IV from aqueous media (autohydrolysis treatments, performed without external acid addition, where the organic acids generated in the medium act as catalysts) was assessed in a preliminary experiment carried out at 100 °C for 5 h using 8 kg of water/kg of oven-dry WDWS. The yield in extract IV was 0.8 g/100 g of WDWS, and the isolate presented a high antioxidant activity, with a reduced EC_{50} (0.2 g/L). As these results were promising, and in order to get further insight into the process, additional

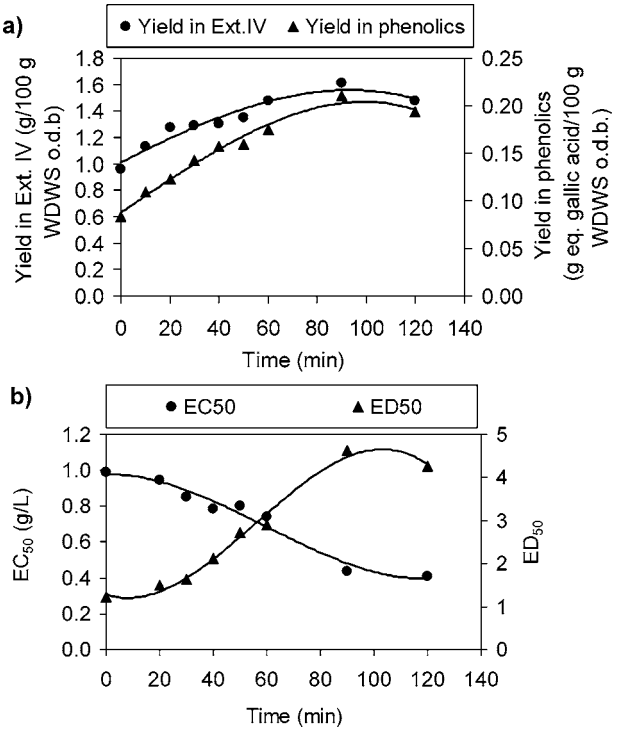


Figure 4. Time course of the yield in extract IV, yield in phenolics, EC_{50}, and ED_{50} in extract IV obtained in hydrolysis experiments carried out with 4% sulfuric acid at 130 °C.

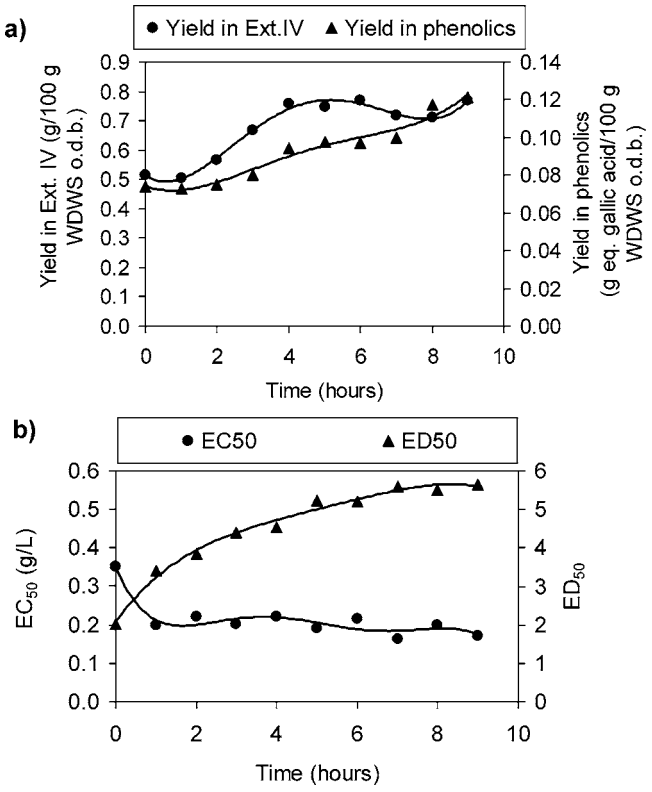


Figure 5. Time course of the yield in extract IV, yield in phenolics, EC_{50}, and ED_{50} in extract IV obtained in autohydrolysis experiments performed at 100 °C.

autohydrolysis treatments lasting up to 9 h were carried out at the same temperature. The experimental results in **Figure 5** show that the yield in extract IV increased with time to reach a stable value after 4 h, whereas the yield in phenolics was stable between 4 and 7 h (0.1 g of gallic acid equiv/100 g of WDWS)

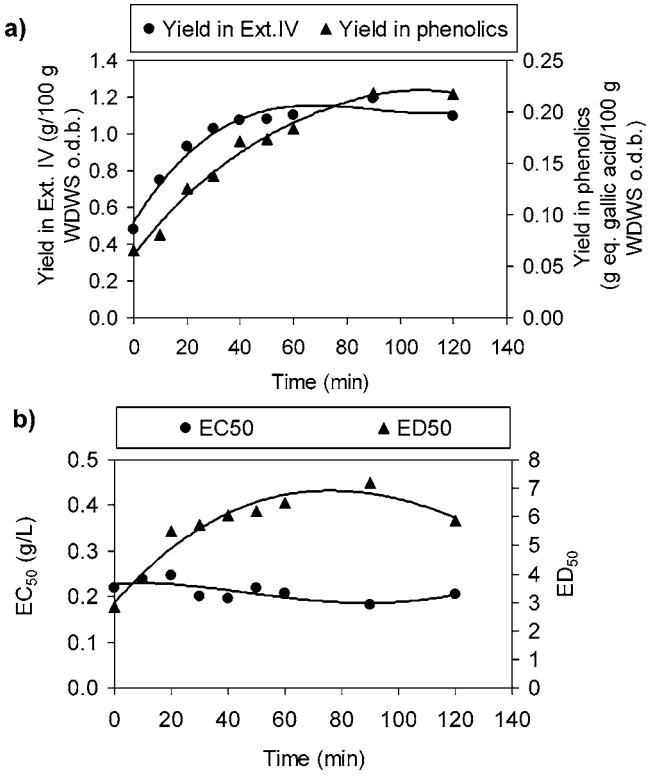


Figure 6. Time course of the yield in extract IV, yield in phenolics, EC_{50}, and ED_{50} in extract IV obtained in autohydrolysis experiments performed at 130 °C.

and increased by 20% during the last 2 h. A relationship between the extract yield and the yield in phenolics was observed. The increase in the extraction yield resulted in higher ED_{50}, whereas the specific antioxidant activity also increased with time, as is shown by the slightly decreasing trend observed for EC_{50} (**Figure 5b**). Even though the specific activity of the extracts was comparable to or higher than those of synthetic antioxidants (for example, BHA and BHT presented EC_{50} values of 0.241 and 2.795 g/L, respectively), the extraction yields were lower than in the previous cases (see **Figures 3** and **4**), and harsher operational conditions were explored to improve the experimental results.

Autohydrolysis experiments were carried out at 130 °C for reaction times up to 120 min (see **Figure 6**). The maximum yield in extract IV (1.2 g of extract/100 g of WDWS) was reached after 90 min and remained almost constant for longer treatments. The yield in phenolics increased with the duration of treatments to reach a maximum value of 0.22 g/100 g of WDWS after 120 min (**Figure 6a**). The specific antioxidant activity showed only minor variation, with EC_{50} of ~0.2 g of extract/L, whereas ED_{50} increased during the first 90 min of treatment and then decreased, with a variation pattern related to the one of the extract yield (**Figure 6b**).

For further studies extract III from washing liquors and extract IV from autohydrolysis during 90 min at 130 °C were selected.

Chromatographic Separation and Composition of Extracts III and IV. Figure 7a shows the spectrophotometric elution profile of extract III (obtained by freeze-drying of washing liquors) in Sephadex LH-20. Five fractions (F1−F5) were separated from the crude solution, and the eluates before F1, between F1 and F2, between F2 and F3, and after F5 were discarded. The crude solids from washing liquors were colored and powdered, whereas F1−F5 were manageable and presented a wide range of colors.

Table 1. Properties of Extract III: Mass Fraction of Phenolics and EC_{50} and Properties of Fractions Obtained by Sephadex LH-20 Isolated from Extract III; Recovery Yield, Mass Fraction of Phenolics, and EC_{50} (See **Figure 2** for Nomenclature)

	extract III	F1	F2	F3	F4	F5	discarded fraction
recovery yield (g/100 g of extract III)		19.36	10.00	5.50	5.78	5.40	53.96
mass fraction of phenolics (g of gallic acid equiv/g of each fraction)	0.15	0.02	0.08	0.17	0.23	0.25	0.12
EC_{50} (g/L)	0.41	0.6	1.06	0.31	0.28	0.16	0.71

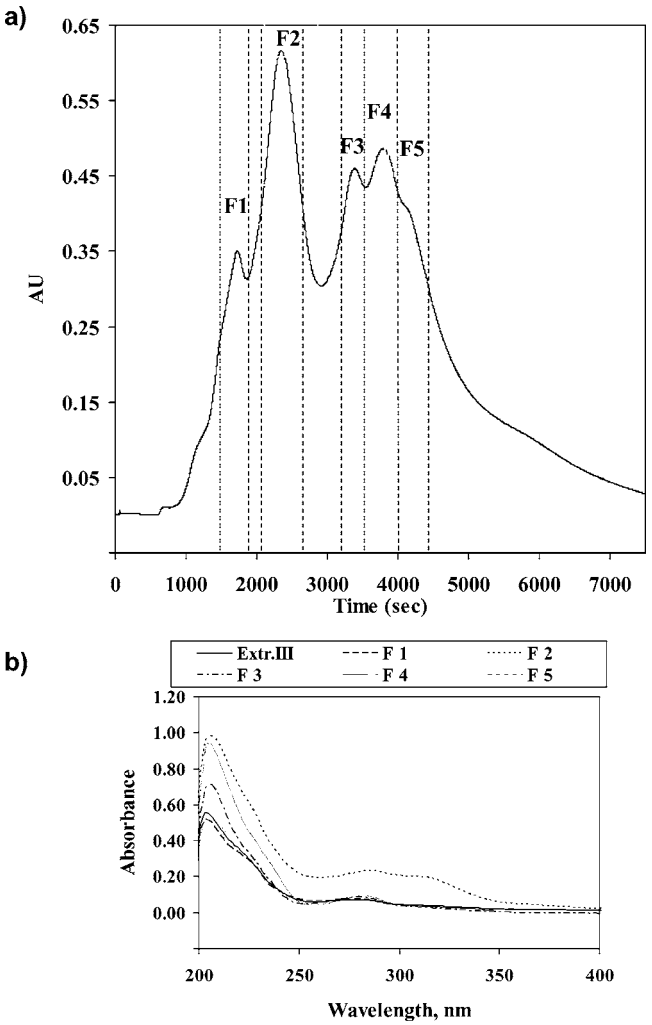


Figure 7. (**a**) Chromatographic profile of extract III and (**b**) UV spectra of extract III and fractions F1–F5.

Table 1 lists the recovery yields determined for each fraction, their phenolic contents (expressed as mass fractions), and their antioxidant activities (expressed in terms of the parameter EC_{50}). The major fraction in terms of recovery was F1 (which contained 20% of the solids contained in the crude washing liquors) followed by F2, whereas similar amounts were recovered in F3−F5. The mass fraction of phenolic compounds in the separated fractions increased with the elution time. The various fractions showed different EC_{50} values, F5 being the most active and F2 the least. The antioxidant activities of F3, F4, and F5 were comparable to or higher than that of BHA (EC_{50} = 0.241 g/L) and higher than that of BHT (EC_{50} = 2.794 g/L). Extract III and the various fractions presented similar UV spectra (see **Figure 7b**), suggesting that their respective components had a related chemical nature. All of the fractions showed an absorption maximum at 210 nm, whereas F3, F4, and F5 showed an absorption maximum at 280 nm (characteristic of soluble lignin) slightly more intense than in the crude extract or F1. Interestingly, the fact that F2 also absorbs at 320 nm suggests its ability to act as a screen for UV-B radiation in cosmetics and sunscreens.

Table 2. Compounds Identified by GC-MS and HPLC in Fractions F1–F5

compound	fraction
phenolic compounds	
gallic acid	F1; F2; F3; F4; F5
protocatechuic acid	F4; F5
3,4-dihydroxybenzaldehyde	F4; F5
vanillic acid	F2; F3
syringic acid	F2; F3; F4
p-coumaric acid	F3
aliphatic fatty acids	
propanoic acid	F1; F3
dodecanoic acid	F3
butadecanoic acid	F2; F3
dodecanoic acid	F2
tetradecanoic acid	F2; F4
nonadecanoic acid	F1
hexadecanoic acid	F1; F2; F3; F4; F5
9,12-octadecadienoic acid	F1; F2; F3; F4; F5
oleic acid	F1; F2; F3; F4; F5
octadecanoic acid	F2; F3; F4; F5
other compounds	
glycerol ether	F1; F3
decane	F5
xylitol	F1
mannose	F1
D-ribose	F1
arabitol	F1
glucose	F1
D-galactose	F1
nonadecane	F2; F3; F4; F5
pentacosane	F5

The compounds identified by GC-MS and HPLC in fractions F1−F5 are classified in **Table 2** as phenolics, aliphatic fatty acids, and other compounds. The main phenolic compounds were gallic acid, protocatechuic acid, 3,4-dihydroxybenzaldehyde, vanillic acid, syringic acid, and *p*-coumaric acid. Phenolic and cinnamic acids have been identified in solids from grape pressing, together with catechins, monomeric and oligomeric flavanols, and glycosylated flavonols (*38*−*40*). When the solids from grape pressing are subjected to distillation, new compounds can appear by thermal decomposition of the original ones (*41*). Gallic acid, found in fractions F1−F5, is a potent antioxidant with the ability to scavenge alkyl radical (*42*) and DPPH radical (*43*−*45*). The activity of gallic acid in emulsion is lower than those of synthetic antioxidants (*46*) but higher than those of chlorogenic acid, ferulic acid, and caffeic acid (*45*), and it is suitable for protecting a variety of molecules against oxidation (*43*, *47*−*49*) and for inhibiting the formation of reactive oxygen species (ROS) in human and rat liver microsomes (*50*). Vanillic acid, with characteristic absorption maxima at 220, 260, and 290 nm, was detected in F2 and F3. The absorbance peaks at

Table 3. Properties of Extract IV: Mass Fraction of Phenolics and EC_{50} and Properties of Fractions Obtained by Sephadex LH-20 Isolated from Extract IV; Recovery Yield, Mass Fraction of Phenolics, and EC_{50} (See **Figure 2** for Nomenclature)

	extract IV	F′1	F′2	F′3	F′4	F′5	discarded fraction
recovery yield (g/100 g of extract IV)		24.48	4.01	9.74	5.62	9.37	46.78
mass fraction of phenolics (g of gallic acid equiv/g of each fraction)	0.16	0.01	0.21	0.15	0.31	0.28	0.09
EC_{50} (g/L)	0.18	16.82	0.65	0.35	0.07	0.08	0.12

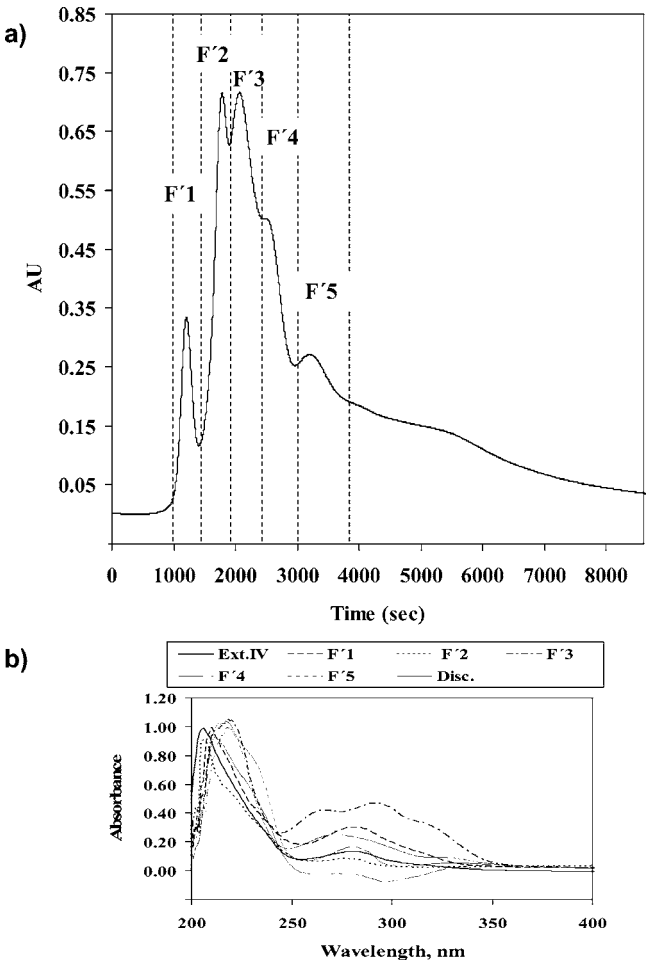


Figure 8. Data concerning extract IV obtained in autohydrolysis treatments at 130 °C during 90 min: (**a**) chromatographic elution profile; (**b**) UV spectra of extract IV fractionation and fractions F′1–F′5.

260 and 294 nm found in fractions F4 and F5 are ascribed to the presence of protocatechuic acid. Syringic acid, identified in fractions 2−4, presented absorption maxima at 220 and at 275 nm. Among fatty acids, hexadecanoic, nonadecanoic, octadecanoic, and oleic acid were abundant in the various fractions. Palma et al. (*8*) reported their presence in the extracts produced by supercritical CO_2 extraction of grape seeds, coming from the seed oil.

Figure 8a shows the spectrophotometric elution profile obtained in the Sephadex fractionation of extract IV (obtained as the ethyl acetate-soluble fraction of autohydrolysis of WDWS carried out under optimal conditions). The separated fractions (F′1−F′5) showed darker color than those obtained from washing liquors and presented analogous texture and manageability.

Table 3 lists data on the extract yield (which varied in the order discarded fraction > F′1 > F′3 > F′5 > F′4 > F′2) and on the mass fractions of phenolics (which were higher for F4 and F5). The same table presents data on the DPPH radical scavenging capacity of the crude extract IV, fractions F′1−F′5, and the discarded fraction. The crude extract and the discarded fraction showed similar activity, whereas F′1 showed a low proportion of phenolics and presented 90 times lower antioxidant activity than the crude extract. Because of this, removal of F′1 from the extract IV is a suitable method for enhancing the antioxidant activity of the resulting isolate. Fractions F′2 and F′3 showed antioxidant activities lower than that of the crude extract, but within the range reported for ethyl acetate extracts of liquors from *Eucalyptus* wood hydrolysis (*24*), *Eucalyptus* wood autohydrolysis (*29*), or corn cob autohydrolysis (*29*). Fractions F′4 and F′5 showed low EC_{50}, with higher radical scavenging activity than other commercial synthetic antioxidants such as BHA. Even though the recovery yields for both fractions are limited, their EC_{50} values suggest that F′4 and F′5 could be of special interest for applications requiring a limited dosage of antioxidants.

Figure 8b shows the UV spectra recorded for extract IV and fractions obtained by Sephadex LH-20 fractionation. Extract IV and F′1 showed maxima at 210 nm and at 270 nm, whereas the compounds in F′2 did not absorb at wavelengths >300 nm. The spectral features of F′3 (which absorbed at wavelengths between 210 and 220 nm and presented absorption maxima at 260, 290, and 310 nm) suggest its possible application as sunscreen. F′4 showed maxima at 210−220 and 270 nm and a shoulder near 330 nm, the latter not appearing in F′5. The discarded fraction did not absorb at 270 nm, in the typical range for soluble lignin fragments.

Table 4 summarizes the compounds identified with GC-MS and HPLC in fractions F′1−F′5. Gallic acid, protocatechuic acid, 3,4-dihydroxybenzaldehyde, vanillic acid, syringic acid, *p*-coumaric acid, 3-hydroxybenzoic acid, isovanillic acid, and cinnamic acid were identified. Cinnamic acid, an efficient antioxidant for protecting lipids (*51*) and an active agent against microbial contamination (*52*), has been also found in poplar wood hydrolysates (*53*). Isovanillic acid has been detected in *Ginkgo biloba* (*54*). Some phenolic acids, particularly cinnamic acids, *p*-coumaric, caffeic, ferulic, gentisic, protocatechuic, syringic, and isovanillic, show cytostatic activity against carcinoma cells (*55*) and antiinflamatory activity (*56*). F′2 absorbed at 275 nm, a feature characteristic of syringic acid. F′4 contained protocatechuic and cinnamic acids, with absorbance peaks at 220 and 260 nm characteristic of protocatechuic acid. The antioxidant potency of protocatechuic acid as a DPPH radical scavenger (*34*) and that of cinnamic acid as an antioxidant (*51*, *57*) could explain the high antioxidant activity of this fraction. In F′3 *p*-coumaric acid (showing a maximum at 244 nm and another at 323 nm) was identified. The presence of syringic acid in F′2, F′3, F′4, and F′5 was also confirmed by GC-MS. The peak at 275 nm (characteristic of syringic acid) in F′2 was also found in F′3, F′4, and F′5.

In conclusion, the winemaking waste solids can be subjected to alternative processing schemes for isolating fractions with

Table 4. Compounds Identified by GC-MS and HPLC in Fractions F′1–F′5

compound	fraction
phenolic compounds	
gallic acid	F′1; F′2; F′3; F′4
protocatechuic acid	F′2; F′3; F′4
3,4-dihydroxybenzaldehyde	F′2; F′5
vanillic acid	F′2; F′3
syringic acid	F′2; F′3; F′4; F′5
p-coumaric acid	F′1; F′3; F′4
3-hydroxybenzoic acid	F′3
isovanillic acid	F′2; F′3
cinnamic acid	F′3; F′4
aliphatic fatty acids	
propanoic acid	F′1; F′2
propanodioic acid	F′2
tetradecanoic acid	F′2; F′3
hexadecanoic acid	F′1; F′2; F′3; F′4; F′5
9,12-octadecadienoic acid	F′1; F′2; F′3
oleic acid	F′1; F′2; F′3; F′4
octadecanoic acid	F′1; F′2; F′3; F′4; F′5
other compounds	
eicosane	F′3; F′5
nonadecane	F′2; F′3; F′5
pentacosane	F′3; F′5
octadecane	F′5

antioxidant activity. The liquors entrapped in the solids contain radical scavengers as potent as synthetic antioxidants. The application of a hydrolytic step, either with water or with sulfuric acid solutions, produced an ethyl acetate-soluble fraction with antioxidant activity. Further fractionation of the crude extracts from the ethyl acetate solubles allowed concentration of the antioxidant activity in some fractions.

ACKNOWLEDGMENT

We are grateful to M. Jesús Rey and Montserrat González for technical support.

LITERATURE CITED

(1) Duh, P. D.; Yen, W. J.; Du, P. C.; Yen, G. C. Antioxidant activity of mung bean hulls. *J. Am. Oil Chem. Soc.* **1997**, *74*, 1059−1063.

(2) Meyer, A. S.; Jepsen, S. M.; Sørensen, N. S. Enzymatic release of antioxidants for human low-density lipoprotein from grape marc. *J. Agric. Food Chem.* **1998**, *46*, 2439−2446.

(3) Azizah, A. H.; Nik Ruslawati, N. M.; Swee Tee, T. Extraction and characterization of antioxidant from cocoa by-products. *Food Chem.* **1999**, *64*, 199−202.

(4) Bonilla, F.; Mayen, M.; Merida, J.; Medina, M. Extraction of phenolic compounds from red grape marc for use as food lipid antioxidants. *Food Chem.* **1999**, *66*, 209−215.

(5) Moure, A.; Cruz, J. M.; Franco, D.; Domínguez, J. M.; Sineiro, J.; Domínguez, H.; Núñez, M. J.; Parajó, J. C. Natural antioxidants from residual sources, a review. *Food Chem.* **2001**, *72*, 145−171.

(6) Macheix, J. J.; Fleuriet, A.; Billot, J. In *Fruit Phenolics*; CRC Press: Boca Raton, FL, 1990.

(7) Saito, M.; Hosoyama, H.; Ariga, T.; Kataoka, S.; Yamaji, N. Antiulcer activity of grape seed extract and procyanidins. *J. Agric. Food Chem.* **1998**, *46*, 1460−1464.

(8) Palma, M.; Taylor, L. T.; Varela, R. M.; Cutler, S. J.; Cutler, H. G. Fractional extraction of compounds from grape seeds by supercritical fluid extraction and analysis for antimicrobial and agrochemical activities. *J. Agric. Food Chem.* **1999**, *47*, 5044−5048.

(9) Yamaguchi, F.; Yoshimura, Y.; Nakazawa, H.; Ariga, T. Free radical scavenging activity of grape seed extract and antioxidants by electron spin resonance spectrometry in an H_2O_2/NaOH/DMSO system. *J. Agric. Food Chem.* **1999**, *47*, 2544−2548.

(10) Hurtado, I.; Caldo, P.; Gonzalo, A.; Ramon, J. M.; Minguez, S.; Fiol, C. Antioxidative capacity of wine on human LDL oxidation in vitro: effect of skin contact in winemaking of white wine. *J. Agric. Food Chem.* **1997**, *45*, 1283−1289.

(11) Souquet, J.-M.; Labarbe, B.; Le Guernevé, C; Cheynier, V.; Moutounet, M. Phenolic composition of grape stems. *J. Agric. Food Chem.* **2000**, *48*, 1076−1080.

(12) Sun, R. C.; Sun, X. F.; Zhang, S. H. Quantitative determination of hydroxycinnamic acids in wheat, rice, rye, and barley straws, maize stems, oil palm frond fiber, and fast-growing poplar wood. *J. Agric. Food Chem.* **2001**, *49*, 5122−5129.

(13) Pastrana-Bonilla, E.; Akoh, C. C.; Sellappan, S.; Krewer, G. Phenolic content and antioxidant capacity of Muscadine grapes. *J. Agric. Food Chem.* **2003**, *51*, 5497−5503.

(14) Gabrielska, J.; Oszmianski, J.; Lamer-Zarawska, E. Protective effect of plant flavonoids on the oxidation of lecithin liposomes. *Pharmazie* **1997**, *52*, 2−3.

(15) Agarwal, C.; Sharma, Y.; Zhao, J.; Agarwal, R. A polyphenolic fraction from grape seeds causes irreversible growth inhibition of breast carcinoma MDA-MB468 cells by inhibiting mitogen-activated protein kinases activation and inducing G1 arrest and differentiation. *Clin. Cancer Res.* **2000**, *6*, 2921−2930.

(16) Joshi, S. S.; Kiszynski, C. A.; Benner, E. J.; Bagchi, M.; Bagchi, D. Amelioration of the cytotoxic effects of chemotherapeutic agents by grape seed proanthocyanidin extract. *Antioxid. Redox Signaling* **1999**, *1*, 563−570.

(17) Fitzpatrick, D. F.; Fleming, R. C.; Bing, B.; Maggi, D. A.; O'Malley, R. M. Isolation and characterization of endothelium-dependent vasorelaxing compounds from grape seeds. *J. Agric. Food Chem.* **2000**, *48*, 6384−6390.

(18) Wren, A. F.; Cleary, M.; Frantz, C.; Melton, S.; Norris, L. 90-day oral toxicity study of a grape seed extract (IH636) in rats. *J. Agric. Food Chem.* **2002**, *50*, 2180−2192.

(19) Nkiliza, J.; Marzelle, J. C. Procédé de préparation d'extraits contenant des composes polyphénoliques oligomères de type catéchiques à partir de sources végétales et extraits obtenus. EP 0 692 480 A1, 1995.

(20) Wulff, H. P. Natural antioxidant composition. WO 97/39632, 1997.

(21) Pointer, R. L. Anti-microbial compound. WO 02/09722 A1, 2000.

(22) Saura-Calixto, F. Antioxidant dietary fiber product, a new concept and a potential food ingredient. *J. Agric. Food Chem.* **1998**, *46*, 4303−4306.

(23) Ohta, T.; Yamasaki, S.; Egashira, Y.; Sanada, H. Antioxidative activity of corn bran hemicellulose fragments. *J. Agric. Food Chem.* **1994**, *42*, 653−656.

(24) Cruz, J. M.; Domínguez, J. M.; Domínguez, H.; Parajó, J. C. Antioxidant and antimicrobial effects of extracts from hydrolysates of lignocellulosic materials. *J. Agric. Food Chem.* **2001**, *49*, 2459−2464.

(25) Gónzalez, J.; Cruz, J. M.; Domínguez, H.; Parajó, J. C. Production of antioxidants from *Eucalyptus globulus* wood by solvent extraction of hemicellulose hydrolysates. *Food Chem.* **2004**, *84*, 243−251.

(26) Yu, J.; Vasanthan, T.; Temelli, F. Analysis of phenolic acids in barley by high-performance liquid chromatography. *J. Agric. Food Chem.* **2001**, *49*, 4352−4358.

(27) Felizón, B.; Fernández-Bolaños, J.; Heredia, A.; Guillén, R. Steam-explosion pre-treatment of olive cake. *J. Am. Oil Chem. Soc.* **2000**, *77*, 15−22.

(28) Fernández-Bolaños, J.; Felizón, B.; Brenes, M.; Guillén, R.; Heredia, A. Hydroxytyrosol and tyrosol as the main compounds found in the phenolic fraction of steam-exploded olive stones. *J. Am. Oil Chem. Soc.* **1998**, *75*, 1643−1649.

(29) Garrote, G.; Cruz, J. M.; Domínguez, H.; Parajó, J. C. Valorisation of waste fraction from autohydrolysis of selected lignocelullosic materials. *J. Chem. Technol. Biotechnol.* **2003**, *78*, 392−398.

(30) Saura-Calixto, F.; Goñi, I.; Manas, E.; Abia, R. Klason lignin, condensed tannins and resistant protein as dietary fiber constituents: determination in grape pomaces. *Food Chem.* **1991**, *39*, 299−309.

(31) Browning, B. L. In *Methods of Wood Chemistry*; Wiley: New York, 1967.

(32) Cruz, J. M.; Domínguez, J. M., Domínguez, H.; Parajó, J. C. Solvent extraction of hemicellulosic wood hydrolysates: a procedure useful for obtaining both detoxified fermentation media and polyphenols with antioxidant activity. *Food Chem.* **1999**, *67*, 147−153.

(33) AOAC. *Official Methods of Analysis of AOAC International*, 16th ed., 3rd rev; Cunniff, P., Ed.; AOAC International: Gaithersburg, MD, 1997; Method 952.03.

(34) von Gadow, A.; Joubert, E.; Hansmann, C. F. Comparison of the antioxidant activity of aspalathin with that of other plant phenols of rooibos tea (*Aspalathus linearis*), α-tocopherol, BHT and BHA. *J. Agric. Food Chem.* **1997**, *45*, 632−638.

(35) Quesada, J.; Rubio, M.; Gomez, D. Chemical characterization of ozonated lignin solutions from corn (*Zea mays*) stalk and poplar (*Populus deltoides*) wood by capillary gas chromatography. *J. High Resolut. Chromatogr*. **1997**, *20*, 565−568.

(36) Valiente, C.; Arrigoni, E.; Esteban, R. M.; Amado, R. Grape pomace as a potential food fiber. *J. Food Sci*. **1995**, *60*, 818−820.

(37) Larrauri, J. A.; Rupérez, P.; Saura-Calixto, F. Effect of drying temperature on the stability of polyphenols and antioxidant activity of red grape pomace peels. *J. Agric. Food Chem*. **1997**, *44*, 1390−1393.

(38) Alonso, A. M.; Domínguez, C.; Guillén, D. A.; Barroso, C. G. Determination of antioxidant power of red and white wines by a new electrochemical method and its correlation with polyphenolic content. *J. Agric. Food Chem*. **2002**, *50*, 3112−3115.

(39) Torres, J. L.; Bobet, R. New flavanol derivatives from grape (*Vitis vinifera*) byproducts. Antioxidant aminoethylthio-flavan-3-ol conjugates from a polymeric waste fraction used as a source of flavanols. *J. Agric. Food Chem*. **2001**, *49*, 4627−4634.

(40) Torres, J. L.; Varela, B.; García, M. T.; Carilla, J.; Matito, C.; Centelles, J. J.; Cascante, M.; Sort, X.; Bobet, R. Valorization of grape (*Vitis vinifera*) byproducts. Antioxidant and biological propierties of polyphenolic fractions differing in procyanidin composition and flavonol content. *J. Agric. Food Chem.* **2002**, *50*, 7548−7555.

(41) Cadahia, E.; Varea, S.; Muñoz, L.; Fernández de Simón, B.; Garcia-Vallejo, M. C. Evolution of ellagitanins in Spanish, French, and American oak woods during natural seasoning and toasting. *J. Agric. Food Chem.* **2001**, *49*, 3677−3684.

(42) Millic, B. L.; Djilas, S. M.; Sonja, M.; Canadanovic-Brunet, J. M. Antioxidative activity of phenolic compounds on the metal-ion breakdown of lipid peroxidation system. *Food Chem.* **1998**, *62*, 443−447.

(43) Cuvelier, M. E.; Bondet, V.; Berset, C. Behavior of phenolic antioxidants in a partitioned medium: structure−activity relationship. *J. Am. Oil Chem. Soc*. **2000**, *73*, 645−652.

(44) Chung, H. S.; Chang, L. C.; Lee, S. K.; Shamon, L. A.; van Breemen, R. B.; Mehta, R. G.; Farnsworth, N. R.; Pezzuto, J. M.; Douglas, K. A. Flavonoids constituents of *Chorizanthe diffusa* with potential cancer chemopreventive activity. *J. Agric. Food Chem*. **1999**, *47*, 36−41.

(45) Fukumoto, L. R.; Mazza, G. Assessing antioxidant and prooxidant activities of phenolic compounds. *J. Agric. Food Chem*. **2000**, *48*, 3597−3604.

(46) Subba Rao, M. V.; Muralikrishna, G. Evaluation of the antioxidant properties of free and bound phenolic acids from native and malted finger millet (Ragi, *Eleusine coracana* Indaf-15). *J. Agric. Food Chem.* **2002**, *50*, 889−892.

(47) Brand-Williams, W.; Cuvelier, M. E.; Berset, C. Use of a free radical method to evaluate antioxidant activity. *Lebensm. Wiss. Technol.* **1995**, *28*, 25−30.

(48) Andreasen, M. F.; Landbo, A. K.; Christensen, L. P.; Hansen, A.; Meyer, A. S. Antioxidant effects of phenolic rye (*Secale cereale L.*) extracts, monomeric hydroxycinnamates, and ferulic acid dehydrodimers on human low-density lipoproteins. *J. Agric. Food Chem.* **2001**, *49*, 4090−4096.

(49) Kikuzaki, H.; Hisamoto, M.; Hirose, K.; Akiyama, K.; Taniguchi, H. Antioxidant properties of ferulic acid and its related compounds. *J. Agric. Food Chem.* **2002**, *50*, 2161−2168.

(50) Stupans, I.; Tan, H. W.; Kirlich, A.; Tuck, K.; Hayball, P.; Murray, M. Inhibition of CYP3A-mediated oxidation in human hepatic microsomes by the dietary derived complex phenol, gallic acid. *J. Pharm. Pharmacol*. **2002**, *54*, 269−275.

(51) Camire, M. E.; Dougherty, M. P. Added phenolic compounds enhance stability in extruded corn. *J. Food Sci.* **1998**, *63*, 516−518.

(52) Roller, S.; Seedhar, P. Carvacrol and cinnamic acid inhibit microbial growth in fresh-cut melon and kiwifruit at 4° and 8 °C. *Lett. Appl. Microbiol.* **2002**, *35*, 390−394.

(53) Ando, S.; Arai, I.; Kiyoto, K.; Hanai, S. Identification of aromatic monomers in steam-exploded poplar and their influences on ethanol fermentation by *Saccharomyces cerevisiae*. *J. Ferment. Technol.* **1986**, *64*, 567−570.

(54) Zheng, W.; Wang, S. Y. Antioxidant activity and phenolic compounds in selected herbs. *J. Agric. Food Chem.* **2001**, *49*, 5165−5170.

(55) García, M. D.; Ahumada, M. C.; Saenz, M. T. Cytostatic activity of some phenolic acids of *Scrophularia frutescens* var. *frutescens*. *Bioscience* **1998**, *53*, 1093−1095.

(56) Fernández, M. A.; Saenz, M. T.; García, M. D. Anti-inflamatory activity in rats and mice of phenolic acids isolated from *Scrophularia frutescens*. *J. Pharm. Pharmacol*. **1998**, *50*, 1183−1186.

(57) Niwa, T.; Doi, U.; Kato, Y.; Osawa, T. Antioxidative properties of phenolic antioxidants isolated from corn steep liquor. *J. Agric. Food Chem.* **2001**, *49*, 177−182.

Received for review April 18, 2004. Revised manuscript received July 1, 2004. Accepted July 5, 2004. We are grateful to MCYT-FEDER (PPQ2000-0688-C05-02) and to Xunta Galicia (PGIDT, 01PXI38302PN) for financial support of this work.

JF049376C