JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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pubs.acs.org/JAFC

Comparison of Accelerated Methods for the Extraction of Phenolic Compounds from Different Vine-Shoot Cultivars

M. Pilar Delgado-Torre,[†,‡] Carlos Ferreiro-Vera,[†,‡] Feliciano Priego-Capote,[†,‡] Pedro M. Pérez-Juan,[§] and María Dolores Luque de Castro*[,†,‡]

[†]Department of Analytical Chemistry, Annex Marie Curie Building, Campus of Rabanales, University of Córdoba, Córdoba, Spain
[‡]Institute of Biomedical Research Maimónides (IMIBIC), Reina Sofía Hospital, University of Córdoba, E-14071 Córdoba, Spain
[§]Bodega y Viñedos Vallebravo, Sierra de Segura, E-23350 Puente de Genave, Jaén, Spain

Supporting Information

ABSTRACT: Most research on the extraction of high-priced compounds from vineyard/wine byproducts has traditionally been focused on grape seeds and skins as raw materials. Vine-shoots can represent an additional source to those materials, the characteristics of which could depend on the cultivar. A comparative study of hydroalcoholic extracts from 18 different vineyard cultivars obtained by superheated liquid extraction (SHLE), microwave-assisted extraction (MAE), and ultrasound-assisted extraction (USAE) is here presented. The optimal working conditions for each type of extraction have been investigated by using multivariate experimental designs to maximize the yield of total phenolic compounds, measured by the Folin−Ciocalteu method, and control hydroxymethylfurfural because of the organoleptic properties of furanic derivatives and toxicity at given levels. The best values found for the influential variables on each extraction method were 80% (v/v) aqueous ethanol at pH 3, 180 °C, and 60 min for SHLE; 140 W and 5 min microwave irradiation for MAE; and 280 W, 50% duty cycle, and 7.5 min extraction for USAE. SHLE reported better extraction efficiencies as compared to the other two approaches, supporting the utility of SHLE for scaling-up the process. The extracts were dried in a rotary evaporator, reconstituted in 5 mL of methanol, and finally subjected to liquid−liquid extraction with *n*-hexane to remove nonpolar compounds that could complicate chromatographic separation. The methanolic fractions were analyzed by both LC-DAD and LC-TOF/MS, and the differences in composition according to the extraction conditions were studied. Compounds usually present in commercial wood extracts (mainly benzoic and hydroxycinnamic acids and aldehydes) were detected in vine-shoot extracts.

KEYWORDS: *phenolic compounds, vine-shoots, superheated liquid extraction, microwave-assisted extraction, ultrasound-assisted extraction, agricultural byproduct*

INTRODUCTION

Tons of agricultural waste and byproducts from the agrofood industry with no economic value are produced every year all over the world. Some examples of these materials from the Mediterranean basin are olive-trimmings, vine and olive leaves, wine lees, and vine-shoots. Most of these materials have traditionally been used mainly as a heating source or cast upon the ground to rot. However, these uses have drawbacks associated with transportation costs and environmental contamination.

Spain is the country with the largest area in the world dedicated to vineyards, with approximately 1.1 million hectares, being the third wine-producing country, following France and Italy. Thus, the huge amount of vine-shoots produced every year has led to a growing interest in exploitation of this residue. Most research on vine-shoots has been focused on the production of paper pulp and ethanol, the former requiring in-depth studies to improve production as vine-shoots provide pulp of lower quality than other agricultural residues such as wheat straw.[1] Some other methods of vine-shoot exploitation, such as tanning and dyeing of leather;[2] production/extraction of phenols[3,4], volatile compounds,[5] activated carbon for wine treatment,[6] lactic acid,[7,8] biosurfactants,[7] and ferulic and coumaric acids;[9] and production of smoke flavorings[10−12] have been investigated.

The composition of vine-shoots is characterized by three main fractions: cellulose, hemicellulose, and lignin; the content of holocellulose is around 68% and that of lignin around 20% (dry weight). Lignin, a well-known component of secondary cell walls, is a high molecular mass cross-linked polymer, which is built up by random oxidative coupling of three major C6−C3 (phenylpropanoid) units (monolignols) due to the lack of enzymatic control. These units [namely, *trans-p*-coumaryl (4-hydroxycinnamyl), coniferyl (4-hydroxy-3-methoxycinnamyl, forming guayacyl units), and sinapyl (3,5-dimethoxy-4-hydroxycinnamyl, forming syringyl units)], are characterized by a phenolic structure.

As lignin can be hydrolyzed to release aromatic phenolic compounds such as low molecular mass alcohols, aldehydes, ketones, or acids, vine-shoots can be a suitable phenolic source. The abundance and richness of vine-shoots make their exploitation highly interesting in economic terms as this raw

Received: September 1, 2011
Revised: February 7, 2012
Accepted: February 29, 2012
Published: February 29, 2012

dx.doi.org/10.1021/jf205078k | J. Agric. Food Chem. 2012, 60, 3051−3060

material can be very useful to obtain products of a high-added value in the nutraceuticals, cosmetics, pharmacological, enological, and food additive industries. Thus, the phenol extracts from vine-shoots have proved their effectiveness in animals by reducing proliferation of leukemic cells,[13] against epilepsy,[14] and for the prevention of aging and diseases such as atherosclerosis, diabetes, and inflammatory processes[15] as a result of the antioxidant properties of phenols and their ability to act as efficient free radical scavengers.

Conventional methods for the extraction of phenolic compounds from solid samples have traditionally been based on stirring. Presently, the use of auxiliary energies such as microwaves or ultrasound has provided dramatic acceleration of the extraction process.[16,17] Also, superheated liquids are an attractive alternative for extraction, with two fundamental advantages over conventional techniques: (a) Raising the temperature above the boiling point of the solvent (but keeping it in liquid state by increasing the pressure as required) increases the diffusion rate, solubility and mass transfer of the compounds and decreases the viscosity and surface tension of the solvent. These changes improve the contact of the compounds with the solvent and enhance extraction, which can then be done more rapidly and with less solvent consumption as compared with conventional methods. (b) The absence of light and air significantly reduces both degradation and oxidation of the target compounds during extraction.[18] Toxic solvents such as methanol−water mixtures[5,19] have traditionally been reported for the extraction of phenols from vine byproducts; nevertheless, the increased human use of these compounds makes mandatory the development of methods based on nontoxic extractants such as ethanol−water mixtures.

Furanic compounds are a family of compounds to be taken into account in the content of cellulose and hemicellulose in vine-shoots. The extraction temperature can enhance the degradation of sugars released from vine-shoots wood and promote the formation of furans.[20] The contribution of two furanic compounds, furfural and hydroxymethylfurfural (HMF), to flavoring in food processed by heating is well-known.[21] Most of the research facilities around the world, including the U.S. Food and Drug Administration, have examined furans not only as flavor compounds but also as novel harmful substances in food that undergo a thermal treatment. The European Food Safety Authority (EFSA) has articulated that furan is obviously carcinogenic in rats and mice, probably due to the combination of a genotoxic mechanism[22,23] and hepatotoxicity.[24] From a safety perspective and for food quality assurance, HMF legal limits have been already issued for some foodstuffs. In the particular case of concentrated rectified grape must, EC Regulation No. 1493/99 sets a limit of 25 mg/kg.[25] Within these premises, this fraction should be minimized for a proper exploitation of vine-shoot extracts.

On the basis of this background, the present study was aimed at (i) demonstrating the feasibility of using vine-shoots to obtain extracts with high phenolic content, (ii) comparing the suitability of extraction techniques such as superheated liquid extraction (SHLE), ultrasound-assisted extraction (USAE), and microwave-assisted extraction (MAE) for the isolation of nutraceutical extracts, and (iii) showing the variability of vine-shoot cultivars in terms of concentration of monitored phenols.

MATERIALS AND METHODS

Samples. Vine-shoots of different *Vitis vinifera* cultivars were sampled in Sierra de Segura (Spain). The studied cultivars were Airén, Baladí, Bobal, Cabernet franc, Cabernet Sauvignon, Charnonnay, Garnacha tinta, Garnacha tintorera, Malbec, Mazuelo, Merlot, Montepila, Moscatel, Pedro Ximénez, Petit Verdot, Sauvignon blanc, Syrah and Tempranillo, which constitute a mixture of traditional and new cultivars in Spain. All of them have been cultivated under the same conditions of soil, climate, hydric regime, etc. The samples were taken in autumn, after leaf-fall, by making a selection of 10 similar stocks of each cultivar. A piece of 10 cm of vine-shoot at the hight of the first leaf bud was taken in all cases. All species were dried for 72 h at 35 °C, milled to get a homogeneous 40 mesh particle size (<0.42 mm diameter), and kept at −20 °C until use.

Reagents. Ethanol (96% v/v) PA from Panreac (Barcelona, Spain) and distilled water were used to prepare the different ethanol−water mixtures. Methanol (HPLC grade) and phosphoric acid (85%, v/v) (both supplied by Panreac) were used to prepare the HPLC mobile phases. Deionized water (18 MΩ·cm) was obtained from a Millipore (Bedford, MA, USA) Milli-Q plus system, and *n*-hexane (LiChrosolv, Merck, Darmstadt, Germany) was used for liquid−liquid extraction.

Fluorescein (3′,6′-dihydroxyspiro[isobenzofuran-1[3*H*],9′[9*H*]-xanthen]-3-one) and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Fluka (Buchs, Switzerland). Folin−Ciocalteu (F−C) reagent, sodium carbonate, gallic acid, and 2,2′-azobis(2-methylpropanimidamide dihydrochloride) (AAPH) were from Sigma. Calibration curves were run for 5-hydroxymethylfurfural (5-hydroxymethyl-2-furancarboxaldehyde) and for the following phenols: (+)-catechin, C6 phenols; pyrogallol (1,2,3-trihydroxybenzene) and pyrocatechol (1,2-dihydroxybenzene), C6−C1 phenols; acetovanillone (1-(4-hydroxy-3-methoxyphenyl)ethanone, vanillin (4-hydroxy-3-methoxybenzaldehyde), guaiacol (2-methoxyphenol), and gallic (3,4,5-trihydroxybenzoic acid), protocatechuic (3,4-dihydroxybenzoic acid), *p*-hydroxybenzoic, vanillic (4-hydroxy-3-methoxybezoic acid), and syringic (4-hydroxy-3,5-dimethoxybenzoic acid) acids, C6−C3 phenols; coniferaldehyde (4-hydroxy-3-methoxycinnamaldehyde), sinapaldehyde (4-hydroxy-3,5-dimethoxycinnamaldehyde), and *p*-coumaric (4-hydroxycinnamic acid), ferulic (4-hydroxy-3-methoxycinnamic acid), and sinapic (4-hydroxy-3,5-dimethoxycinnamic acid) acids. Standards were acquired from Sigma-Aldrich (St. Louis, MO, USA), and *p*-cresol (1-hydroxy-4-methylbenzene) was used as external standard.

Apparatus. Vine-shoots were milled with a ball grinder (Restch MM301, Haan, Germany). Superheated liquid extractions were performed by a laboratory-made dynamic extractor,[12] consisting of the following units: (a) an extractant supply; (b) a high-pressure pump (Shimadzu LD-AC10) that propels the extractant through the system; (c) a switching valve placed next to the pump to develop static extractions when switched off; and (d) a stainless steel cylindrical extraction chamber (550 mm × 10 mm i.d., 4.3 mL internal volume) where the sample is placed (this chamber is closed at both ends with screws having caps that contain cotton-made filters to ensure the sample is not carried away by the extractant); (e) a restriction valve to maintain the desired pressure in the system; (f) a cooler made of a stainless steel tube (1 m length, 0.4 mm i.d.) and refrigerated with water; (g) a gas chromatograph oven (Konix, Cromatix KNK-2000) where the extraction chamber is placed and heated.

A Microdigest 301 digestor of 200 W maximum power (Prolabo, Paris, France), furnished with a microprocessor programmer (Prolabo) to control the microwave unit was used to enhance microwave-assisted extraction, and a Branson 450 digital sonifier (20 kHz, 450 W, duty cycle (fraction of time ultrasound irradiation is applied/s) equipped with a cylindrical titanium alloy probe (12.7 mm in diameter) were used for MAE and USAE. An R-220 rotary evaporator from Büchi (Flawil, Switzerland) working with a 50 mL balloon flask was used to concentrate the liquid extracts.

The absorbance of the extracts was monitored by a spectrometer Termo Spectronic Helios Gamma (Waltham, MA, USA). Fluorometric monitoring of the ORAC assay was performed by an F-2500

Hitachi fluorescence spectrophotometer (Pleasanton, Canada) equipped with a 10 mm path length cuvette.

Shaking and centrifugation of the extracts were carried out by an MS2 minishaker (IKA, Germany) Vortex and a Mixtasel (Selecta, Barcelona, Spain) centrifuge, respectively.

Individual separation of phenolic compounds and carbohydrate derivatives was carried out by a liquid chromatograph (LC) consisting of a ProStar 410 autosampler equipped with a 0.5 mL sample loop (Varian, Palo Alto, CA, USA) connected online with a liquid chromatograph pump (Varian, 240 pump). A 330 Varian diode array detector (DAD) was used for monitoring the chromatographic eluate at the optimal wavelength for each analyte. Data processing was carried out using Star Chromatography Workstation version 5.52 software running on a personal computer.

Polyview-2000 software (Varian) was used for both characterization of the spectra and assessment of peak purity. This software allows examination and analysis of spectra, including plots of purity parameter, setting of absorbance ratios, and determination of maximum absorbance. Determination of the purity of chromatographic peaks and recalculation of the peak at different wavelengths, and integration parameters, which allow exchange signal-to-noise ratio in a diode array data file, is also provided by this software.

Statgraphics Centurion XV version 15.1.02 for Windows was used for multivariate analysis of generated data.

Superheated Liquid Extraction (SHLE). One gram of milled vine-shoots was placed into the extraction cell that was inserted into the gas chromatograph oven. Then, a relatively high flow rate (7 mL/min) was used for 1 min to fill the cell rapidly. To ensure the absence of air inside the extraction cell, the restrictor valve was kept opened until the first drop of extractant appeared at its end. At that moment, the restrictor valve was closed, and when the desired pressure was reached, the switching valve was closed, the pump was turned off, and the oven was switched on. While the temperature rose, the switching valve had to be opened at short intervals to prevent the pressure from surpassing the working value. Once the selected temperature and pressure were reached, static extraction was performed for a preset time. Finally, the oven was switched off, the chamber was cooled below the boiling point of ethanol and, then, the switching valve and the restrictor valve were switched to enable new extractant to flow through the cell and flush out the extract. The extractant used was 80% (v/v) aqueous ethanol at pH 3, and the extraction time was 1 h.

Microwave-Assisted Extraction (MAE). One gram of milled vine-shoots was placed into the extraction vessel with 20 mL of 80% (v/v) aqueous ethanol at pH 3. The vessel was positioned at the suitable zone for irradiation with focused microwaves. Auxiliary energy was applied at 140 W irradiation power for 5 min, after which the solid residue was removed by centrifugation prior to analysis of the extract.

Ultrasound-Assisted Extraction (USAE). One gram of milled vine-shoots was placed into the extraction vessel with 20 mL of 80% (v/v) aqueous ethanol at pH 3. The ultrasonic probe was immersed into the extraction mixture for sonication at 280 W irradiation power for 7.5 min with a duty cycle of 70% (0.7 s/s irradiation cycles). After that, the extract was isolated by centrifugation prior to analysis.

Determination of Total Phenols by the Folin−Ciocalteu Method. The amount of total phenolic compounds was measured by the F−C method using gallic acid as calibration standard. The calibration curve was carried out with solutions of 100, 200, 300, 400, 500, and 600 mg/L of this compound ($y = 0.0009x + 0.0081$, $R^2 = 0.9978$). A 0.5 mL aliquot of extract, 10 mL of distilled water, 1 mL of F−C reagent, and 3 mL of Na_2CO_3 (20%, w/v) were mixed, made up to 25 mL with distilled water, and heated at 50 °C for 5 min. After heating, the samples were kept at room temperature for 30 min and, finally, the absorbance was measured at 765 nm against a blank solution containing distilled water instead of extract. The concentration of phenolic compounds thus obtained was multiplied by the dilution factor of the extract volume and divided by the amount of vine-shoots used. The results were expressed as equivalent to milligrams of gallic acid per gram of vine-shoot extract (mg GAE/g vine-shoots).

Determination of the Antioxidant Capacity by the Oxygen Radical Absorbance Capacity (ORAC) Assay. The antioxidant capacity of the extracts was measured following the ORAC assay, based on inhibition of the peroxyl radical-induced oxidation initiated by thermal decomposition of azo compounds such as AAPH. In addition to AAPH as a peroxyl radical generator, fluorescein as a fluorescent probe (which acts as a target for the peroxyl radicals generated by AAPH, which quench the fluorescein emission) and Trolox as an antioxidant standard were used. All solutions were prepared in phosphate buffer (10 mM, pH 7.4). Excitation and emission wavelengths were set at 485 and 520 nm, respectively. An amount of 0.625 mL of diluted extract, blank, or Trolox calibration solution (12.5, 6.25, 3.13, and 1.56 μM final concentrations) was mixed with fluorescein solution (3.75 mL, 10 nM) for 30 min at 37 °C without shaking. Then, the AAPH solution (0.625 mL, 240 mM) was added to the mixture, and the fluorescence was monitored every 5 min for 85 min.

Treatment of Extracts. The extracts from SHLE, MAE, or USAE were dried in a rotary evaporator and then reconstituted in 5 mL of methanol (methanolic fractions, MF). The extracts were subjected to liquid−liquid extraction with *n*-hexane (10 mL, 5 min shaking and 6 min centrifugation at 855*g*) to remove nonpolar compounds, which could complicate the chromatographic separation. For obtainment of the aqueous fraction (AF) of the extracts, 2 mL of MF was subjected to rotary evaporation to a final volume of 200 μL. Finally, this fraction was taken to a volume of 650 μL with the chromatographic mobile phase A and filtered using a 0.45 μm pore size filter before injection into the chromatograph.

LC-DAD Analysis. The separation of analytes was performed on an Inertsil ODS-2 column (250 mm × 4.6 mm i.d., 5 μm particle, Análisis Vínicos, Tomelloso, Ciudad Real, Spain) using an injection volume of 20 μL and a flow rate of 1 mL/min. Mobile phase A consisting of 0.2% (v/v) phosphoric acid aqueous solution and mobile phase B consisting of methanol were used. The gradient method was as follows: from 96 to 82% mobile phase A in 20 min, held for 20 min, from 82 to 74% mobile phase A in 24 min, and from 74 to 50% mobile phase B in 9 min. The analytes were identified by comparing both their retention times and UV spectra with those of the corresponding standards and quantified by interpolation in the corresponding calibration curves. The absorption wavelengths were set at 260 nm for ellagic acid; at 280 nm for hydroxybenzoic acids, catechin, and phenolic aldehydes; at 320 nm for hydroxycinnamic acids; and at 360 nm for hydroxycinnamic aldehydes.

LC-TOF/MS Confirmatory Analysis. The analyses to confirm the identity of the studied compounds were performed by an Agilent 1200 series LC system (consisting of a binary pump, a vacuum degasser, an autosampler, and a thermostated column compartment) interfaced to an Agilent 6540 UHD Accurate-Mass TOF LC/MS detector (Palo Alto, CA, USA) equipped with an Agilent Jet Stream Technology electrospray ion source operating in the negative and positive ion mode. Chromatographic separation was performed using an Inertsil ODS-2 column (250 mm × 4.6 mm i.d., 5 μm particle, Análisis Vínicos), kept at 25 °C. Mobile phases were water (phase A) and acetonitrile (phase B), both LC-MS/MS grade and with 0.1% formic acid as ionization agent. The HPLC pump was programmed with a flow rate of 1 mL/min, and the following gradient elution was developed: from 4 to 18% mobile phase B in 20 min, held for 20 min, from 18 to 26% mobile phase B in 44 min, from 26 to 50% mobile phase B in 26 min, and from 50 to 100% phase B in 30 min. The injection volume was 10 μL, and the injector needle was washed five times with 70% methanol. Furthermore, the needle seat back was flushed for 12 s at a flow rate of 4 mL/min with 70% methanol to clean it.

The operating conditions of the mass spectrometer were as follows: gas temperature, 350 °C; drying gas, nitrogen at 10 L/min; nebulizer pressure, 35 psi; sheath gas temperature, 380 °C; sheath gas flow, nitrogen at 10 L/min; capillary voltage, 3250 V; skimmer, 65 V; octopole radiofrequency voltage, 750 V; focusing voltage, 90 V. Data acquisition (2.5 Hz) in both centroid and profile modes was governed via Agilent MassHunter Workstation software. The mass range and

detection window were set at m/z 100−1100 and 100 ppm, respectively. The instrument was calibrated and tuned according to procedures recommended by the manufacturer. To ensure the desired mass accuracy of recorded ions, continuous internal calibration was performed during analyses with the use of signals at m/z 121.0509 (protonated purine) and m/z 922.0098 [protonated hexakis-(1*H*,1*H*,3*H*-tetrafluoropropoxy)phosphazine or HP-921] in positive ion mode. In negative ion mode, ions with m/z 119.0362 (proton abstracted purine) and m/z 966.000725 (formate adduct of HP-921) were used. Analytes were identified by mass accurate detection. MassHunter Workstation software (version 3.01 Qualitative Analysis, Agilent Technologies, Santa Clara, CA, USA) was used for processing all data obtained with LC-TOF/MS in full single MS mode. The feature extraction algorithm took into account all ions exceeding 5000 counts with a charge state equal or above 1, and a feature had to be composed of two or more ions to be valid (e.g., two ions in the isotope cluster). Within the algorithm employed for full single MS data, ions with identical elution profiles and related m/z values (representing different adducts or isotopes of the same compound) were extracted as molecular features (MFs) or entities characterized by retention time (RT), intensity in apex of chromatographic peak, and accurate mass.

RESULTS AND DISCUSSION

Extraction Protocols for Isolation of Polar Compounds. Three different extraction protocols were tested for isolation of interesting compounds from vine-shoots because of their potential applicability associated with their nutraceutical properties. Optimization of the three extraction protocols was carried out with Pedro Ximénez cultivar vine-shoots because of its geographical relevance in the area where the study has been developed.

Table 1. Ranges of the Variables Studied by the Different Extraction Methods

extraction method	variable	range	optimum value
USAE	power (W)	280−40	280
	duty cycle (%)	70−30	50
	time (min)	5−15	7.5
MAE	power (W)	140−60	140
	time (min)	3−15	5
SHLE	temperature (°C)	240−160	180
	time (min)	5−90	60

Solid−liquid extraction was the critical step of sample preparation scheme for selective separation of the target compounds. The three extraction approaches were SHLE, to benefit from solvent properties in superheated state, and MAE and USAE, to benefit from the assistance of auxiliary energies to enhance the leaching process. The extractant composition in the case of SHLE was set according to previous studies reported in the literature.[3,12,17] Ethanol−water mixtures (60:40, v/v) acidified at pH 3 were used as extractant media also in the case of MAE and USAE to compare the leaching efficiency with that of SHLE. Apart from the extractant composition, the main variables involved in each leaching technique were optimized to evaluate their incidence on the leaching efficiency. These variables were temperature for SHLE, irradiation power for both MAE and USAE, and duty cycle (defined as the fraction of a second during which ultrasonic energy is applied) only for USAE. The extraction time was also optimized in the three extraction protocols. The ranges studied were set according to the literature and preliminary experiments. The ranges of study for each type of extraction and the optimal values are shown in Table 1. Two parameters were selected as independent response variables: the concentration of total phenols estimated by the F−C test, which should be maximized, and the concentration of 5-hydroxymethylfurfural estimated by chromatographic analysis to evaluate the degradation of carbohydrates, which should be minimized.

In the case of SHLE, the pressure was not included in the multivariate optimization due to its null effect on extraction. Thus, this variable was simply set at a value high enough (10 bar) to ensure the liquid state of the ethanol−water mixtures during extraction. As Figure 1 shows, extraction tests at 160 and 180 °C did not report statistical differences in the concentration of total phenols measured by the F−C test, whetrsd there was a significant increase in the response of the F−C test provided by the extract obtained at 200, 220, and 240 °C. However, extraction temperatures from 200 to 240 °C involved a significant burnt wood smell that was indicative of qualitative alteration of the extract. Additionally, the concentration of hydroxymethylfurfural in the extract reached the maximum level at temperatures above 200 °C. Attending to these results, the maximum concentration of phenolic compounds in the extract with minimum level of hydroxymethylfurfural was attained in the range of 160−180 °C. Temperatures lower than 160 °C yielded extracts with reduced total phenolic concentration (data not shown). Attending to these results, two kinetics experiments were carried at 160 and 180 °C with extraction times from 5 to 90 min. In the SHLE developed at 180 °C, a plateau of the efficiency was reached

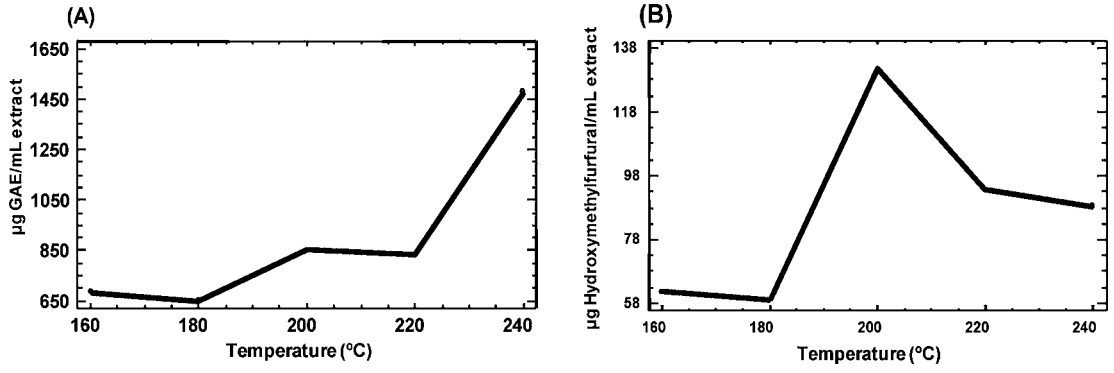

Figure 1. Influence of temperature on both the concentration of phenolic compounds in SHLE extracts estimated by the Folin−Ciocalteu test (A) and that of hydroxymethylfurfural calculated after chromatographic separation by LC-DAD (B).

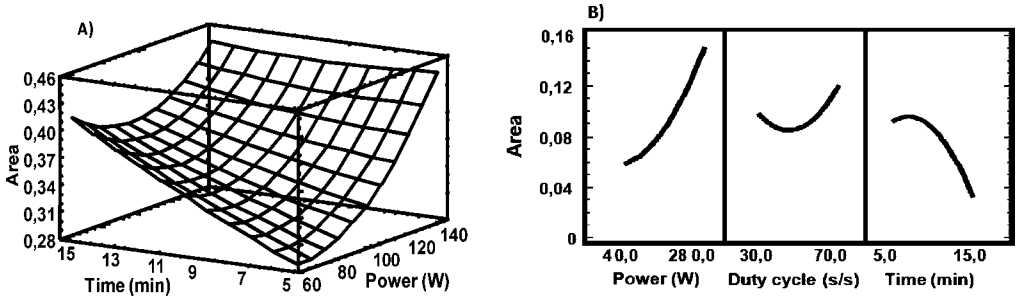

Figure 2. Influence of the main variables involved in MAE (A) and USAE (B) of phenolic compounds from vine-shoots.

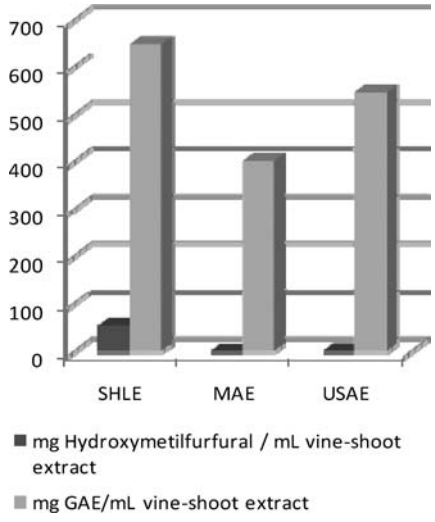

Figure 3. Hydroxymethylfurfural content (μg/mL) and total concentration of phenols expressed as μg equivalent to gallic acid per mL of vine-shoot extract obtained by the Folin−Ciocalteu method for the three extraction approaches.

after 60 min (95% confidence level, data not shown). On the other hand, leaching at 160 °C for 60 min yielded extracts with lower concentration of compounds such as coniferaldehyde, sinapic acid, or sinapaldehyde (see Supplementary Figure 1 in the Supporting Information). Therefore, 180 °C was selected as the optimum extraction temperature.

Surface response designs were applied to optimize USAE and MAE. The microwave power had a positive effect on MAE at short extraction times, but it was practically null at 15 min, as shown by the surface response in Figure 2A (95% confidence level). At high irradiation power (140 W, the maximum without ejections) the extraction time was not an influential variable for times longer than 5 min. The shorter extraction time at which a constant total concentration of phenol compounds was obtained (5 min) was selected for subsequent experiments.

Both power and duty cycle presented positive effects on USAE, as can be seen in Figure 2B; in contrast, the increase of the extraction time proved to have a opposite influence, as the total concentration of phenols in the extract was decreased 70% from 5 to 15 min; consequently, the highest values of power (280 W) and duty cycle (70%) were chosen together with 7.5 min as extraction time.

The effect of both types of energy (microwaves and ultrasound) on the extraction of the target compounds clearly differs from that exerted when the target compounds have been extracted from other raw materials, such as olive tree leaves,[19,26] vine lees,[15,27] or alperujo.[12,28]

Once the three extraction protocols were optimized, their leaching efficiencies were compared in terms of concentration of total phenols estimated by the F−C test and that of hydroxymethylfurfural. Attending to these results, SHLE provided the highest concentration of phenolic compounds expressed as micrograms of gallic acid per gram of initial solid vine-shoots (n = 3, p = 0.00022), 650.4 μg/mL, versus 546.4 and 401.4 μg/mL obtained with USAE and MAE, respectively (see Figure 3). With regard to hydroxymethylfurfural, only SHLE extracts contained significant levels of this furanic aldehyde, whereas MAE and USAE extracts reported very low concentrations. The concentration of hydroxymethylfurfural in

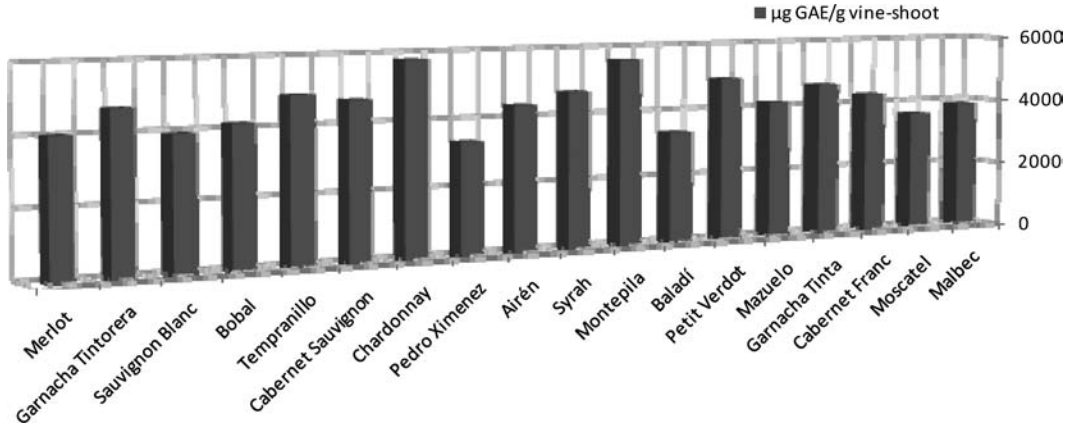

Figure 4. Total concentration of phenols in the SHLE extracts from different vine-shoot cultivars expressed as μg equivalent to gallic acid per gram of vine-shoots obtained by the Folin−Ciocalteu method.

dx.doi.org/10.1021/jf205078k | J. Agric. Food Chem. 2012, 60, 3051−3060

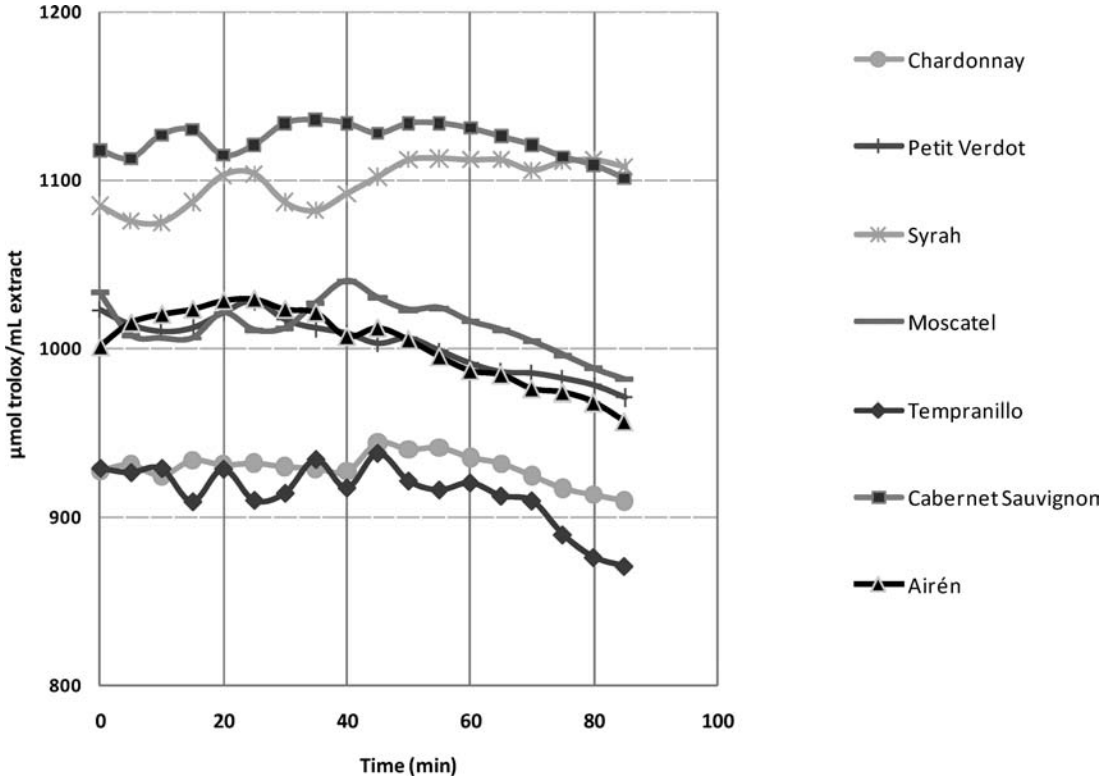

Figure 5. Comparison of the antioxidant capacity of different vine-shoot SHLE extracts measured by the ORAC assay.

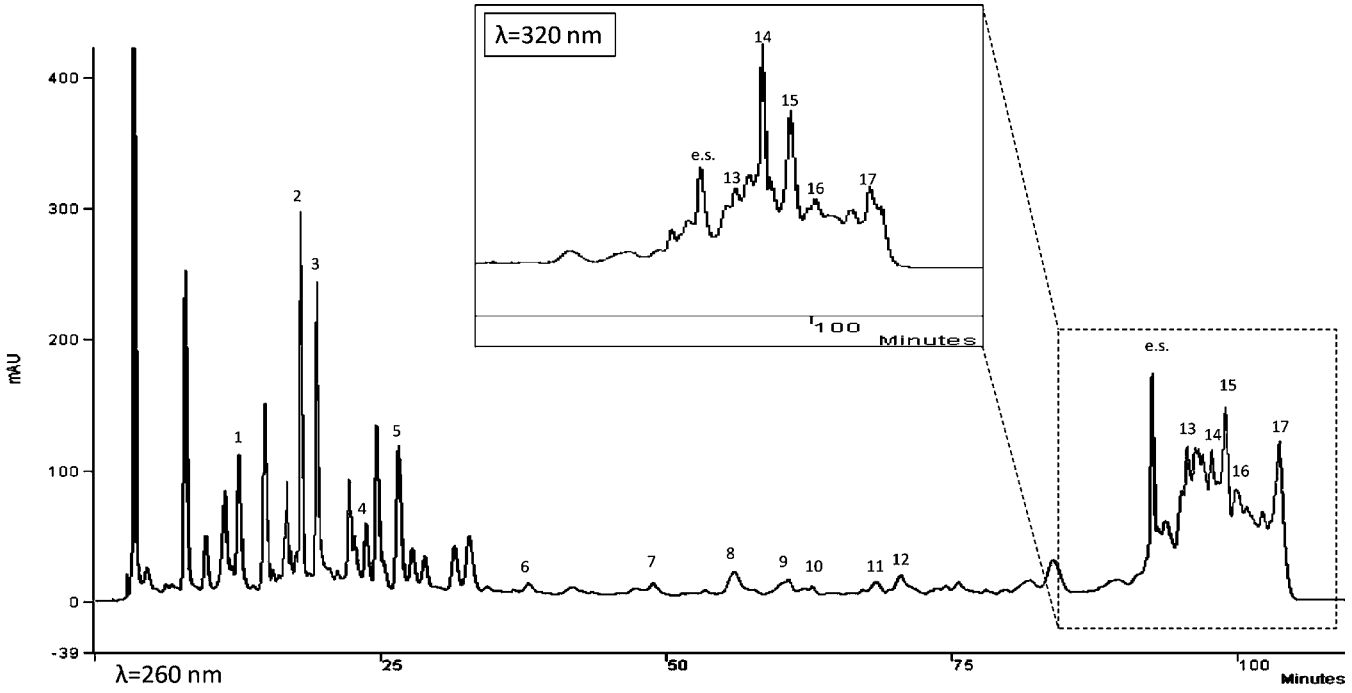

Figure 6. DAD chromatogram at 260 and 320 nm corresponding to a vine-shoot extract from Chardonnay cultivar with identification of interesting compounds. Peaks: 1, pyrogallol; 2, gallic acid; 3, hydroxymethylfurfural; 4, pyrocatechol; 5, protocatechuic acid; 6, hydroxybenzoic acid; 7, catechin; 8, vanillic acid; 9, guaiacol; 10, vanillin; 11, syringic acid; 12, acetovanillone; 13, coumaric acid; 14, ferulic acid; 15, coniferaldehyde; 16, sinapic acid; 17, sinapaldehyde; e.s., *p*-cresol.

the SHLE extract at 180 °C for this cultivar was below 60 μg/mL, whereas this level surpassed 130 μg/mL at 200 °C as shown in Figure 1B. Under controlled conditions, furanic derivatives are flavor correctors, which is quite interesting for enological applications. For this reason, SHLE extracts seem to be more interesting than those provided by USAE and MAE from an organoleptic point of view.

Comparison of the Antioxidant Potential of Different Vine-Shoot Cultivars. After selection of SHLE as the most suitable approach for the isolation of phenols, SHLE was

Table 2. Concentrations and Limits of Detection of a Panel of Selected Compounds in SHLE Extracts from Different Cultivars of Vine-Shoots (Expressed as mg/g Vine-Shoots (n = 3 Analytical Replicates))

	compound[a]																
cultivar	Pg	G.Ac	Hf	Py	P.Ac	H.Ac	C	V.Ac	G	V	S.Ac	Av	C.Ac	F.Ac	Cf	S.Ac	S
Tempranillo	5816	269	386	9	215	21	6735	nd[b]	62	13	nd	35	31	23	12	241	301
Airén	5661	262	311	52	118	nd	589	3	55	9	22	28	nd	32	39	1464	2
Cabernet Sauvignon	12307	570	456	38	302	nd	6233	nd	169	31	26	41	nd	34	16	56	207
Baladí	4961	229	729	120	182	28	7467	65	19	32	1	15	28	45	13	2283	10
Cabernet franc	7475	346	153	33	215	19	405	nd	33	nd	88	19	31	34	16	63	130
Syrah	1206	55	40	49	40	nd	1824	8	8	7	0.6	5	nd	12	nd	104	2
Bobal	3629	167	183	17	139	19	1982	nd	5	nd	5	18	12	41	29	112	106
Chardonnay	5522	255	208	40	129	19	512	68	39	4	22	1	25	9	35	26	19
Garnacha tintorera	4708	217	170	45	136	25	1764	4	2	18	2	30	23	33	23	161	476
Garnacha tinta	1504	69	128	25	106	95	1315	61	41	36	4	39	32	650	15	44	197
Malbec	6477	300	79	116	217	nd	1033	19	12	11	18	17	25	68	23	1537	74
Mazuelo	5254	243	179	57	293	44	5141	nd	19	24	5	79	20	59	10	178	120
Merlot	5208	241	127	232	111	13	1048	13	16	39	11	25	30	36	31	78	46
Montepila	6746	312	662	44	197	23	4914	28	38	53	25	10	12	356	16	77	89
Moscatel	3435	158	115	135	93	23	3624	11	13	6	22	11	28	42	40	38	51
Pedro Ximénez	2361	109	247	66	49	12	1133	10	26	13	4	20	26	74	37	1247	81
Petit Verdot	6793	314	124	91	155	23	2843	152	18	18	18	10	27	10	38	158	49
Sauvignon blanc	5148	238	183	182	150	70	1167	28	38	30	11	6	18	528	40	324	112
LOD	0.8548	0.5415	0.5704	0.5742	0.6051	0.5682	1.4430	0.0739	0.0922	0.6340	0.4918	0.4229	0.6045	0.1822	2.2824	3.1373	0.9259

[a]Pg, pyrogallol; G.Ac, gallic acid; Hf, hydroxymethylfurfural; Py, pyrocatechol; P.Ac, protocatechuic acid; H.Ac, *p*-hydroxybenzoic acid; C, catechin; V.Ac, vanillic acid; G, guaiacol; V, vanillin; S.Ac, syringic acid; Av, acetovanillone; C.Ac, coumaric acid; F.Ac, ferulic acid; Cf, coniferaldehyde; S.Ac, sinapic acid; S, sinapaldehyde. [b]Not detected.

dx.doi.org/10.1021/jf205078k | J. Agric. Food Chem. 2012, 60, 3051−3060

Table 3. Confirmatory Analysis of Monitored Compounds by LC-TOF/MS in Vine-Shoot Extracts

compound	retention time (min)	ion	theor mass	formula	exptl mass	error (ppm)
5-hydroxymethylfurfural	10.1	$(M - H)^-$	126.0317	$C_6H_6O_3$	126.0309	−6.09
gallic acid	12.8	$(M - H)-$	170.0215	$C_7H_6O_5$	170.0202	−7.62
ferulic acid	17.5	$(M + CHO_2)^-$	194.0579	$C_{10}H_{10}O_4$	194.0584	2.62
pyrocatechol	20.1	$(M - H)^-$	110.0368	$C_6H_6O_2$	110.0359	−8.18
protocatechuic acid	20.1	$(M - H)^-$	154.0266	$C_7H_6O_4$	154.0256	−6.46
pyrogallol	21.0	$(M + H)^+$	126.0317	$C_6H_6O_3$	126.0314	−2.6
guaiacol	21.4	$(M - H)^-$	124.0524	$C_7H_8O_2$	124.0518	−5.09
vanillic acid	26.3	$(M + H)^+$	168.0423	$C_8H_8O_4$	168.0412	−6.51
syringic acid	26.8	$(M + H) + [-H_2O]$	198.0528	$C_9H_{10}O_5$	198.051	−9.08
4-hydroxybenzoic acid	27.0	$(M + CHO_2)-$	138.0317	$C_7H_6O_3$	138.0323	4.56
catechin	27.8	$(M - H)^-$	290.079	$C_{15}H_{14}O_6$	290.0803	4.43
o-coumaric acid	28.2	$(M - H)^-$	164.0473	$C_9H_8O_3$	164.0474	0.32
sinapic acid	36.0	$(M + H)^+$	224.0664	$C_{11}H_{12}O_5$	224.0685	−9.24
vanillin	37.4	$(M + CHO_2)^-$	152.0473	$C_8H_8O_3$	152.0486	8.22
acetovanillone	38.47	$(M + H)^+$	166.0630	$C_9H_{10}O_3$	166.0625	−2.92
coniferaldehyde	42.1	$(M + CHO_2)-$	178.063	$C_{10}H_{10}O_3$	178.0647	9.49
sinapaldehyde	44.8	$(M - H) - [-H_2O]$	208.0736	$C_{11}H_{12}O_4$	208.0742	3.11

applied to 18 vine-shoot cultivars. The purpose was to compare the antioxidant potential of the different cultivars with a view to further application to qualitative characterization. As Figure 4 shows, the total concentration of phenols was quite homogeneous among the different vine-shoot cultivars, the highest values corresponding to Chardonnay, Montepila, Petit Verdot, and Tempranillo (ranging between 5775.55 and 4864.44 μg GAE/g of vine-shoots for Chardonnay and Tempranillo, respectively) and the lowest to Pedro Ximénez and Baladí, with 3323.70 and 3401.70 μg.

The ORAC assay was applied to compare the antioxidant capacity of the SHLE extracts from the different vine-shoot cultivars. The high antioxidant power of the vine-shoot extracts demanded for dilution of the extracts to detect a significant kinetic decay. Thus, the extracts were 1:10 diluted prior to the measurement of the ORAC activity. Figure 5 shows the kinetic curves for five vine-shoot varieties representative of the different varieties studied. As can be seen, the curves are characterized by a slight decay along the ORAC experiment. By calculating the difference between the fluorescence signal at the beginning of the experiment and after 85 min, a calibration curve of this parameter versus the concentration of Trolox was plotted fitting a second-degree polynomial function ($y = -0.3274x^2 + 6.9431x + 64.071$; $R^2 = 0.9904$). The ORAC antioxidant activity of vine-shoot extracts was estimated by the calibration curve, resulting in a capacity ranging from 5.7 to 6.8 μM equivalents of Trolox for Airén and Chardonnay varieties, respectively (data not shown). These results are quite consistent with those provided by the F−C test because Chardonnay vine-shoots showed the highest antioxidant capacity and, at the same time, the highest total phenolic content.

Composition of Extracts from Different Vine-Shoot Cultivars. Vine-shoots from 18 cultivars were extracted by SHLE under the selected operation conditions to evaluate the content of interesting compounds from enological and nutraceutical points of view. The same panel of compounds (composed by the standards described under Materials and Methods) was determined in SHLE extracts. Figure 6 shows a DAD chromatogram at 260 nm corresponding to a vine-shoot extract from Chardonnay cultivar with identification of interesting compounds. Table 2 lists the concentrations of representative compounds in extracts from different varieties of vine-shoots. The identity of these compounds was confirmed by LC-MS in high resolution by a TOF mass analyzer. For this purpose, SHLE extracts and standards were analyzed. Identification of target compounds was supported on the chromatograms corresponding to monoisotopic masses and retention times. Search parameters were mass accuracy cutoff below 10 ppm and a peak spacing tolerance of m/z 0.0025 plus 7 ppm. Retention times, formulas, experimental and theoretical masses, and errors, expressed as ppm and obtained by accurate mass measurements of monitored compounds, are shown in Table 3. Hydroxymethylfurfural, a degradation product from hexoses, was found in all extracts, with the highest concentrations of this furanic aldehyde responsible of light creamy toast and toffee flavor found in the varieties Baladí and Montepila (729 and 662 μg/g, respectively). The rest of the cultivars could be discriminated on the basis of the hydroxymethylfurfural concentration in the extracts from their vine-shoots (below and above 200 μg/g) with a 95% confidence level. Thus, extracts from Tempranillo, Airén, Cabernet Sauvignon, Chardonnay, and Pedro Ximénez vine-shoots were characterized by a high content of hydroxymethylfurfural ranging from 208 to 456 μg/g. On the other hand, the rest of the varieties provided a content ranging from 40.0 to 183 μg/g for Syrah and Bobal, respectively.

One of the most characteristic phenolic compounds found in the extracts from all studied cultivars was gallic acid, a final product in the hydrolysis of ellagitannins, which contributes to the astringency character of wines. Gallic acid concentration was in the range of 55−570 μg/g for Syrah and Cabernet Sauvignon varieties, respectively. Pyrogallol, formed from gallic acid decarboxylation, was in all cultivars, covering a wide concentration range: from 1.2 mg/g for Syrah to 12.3 mg/g found in Cabernet Sauvignon cultivar. The similarity between concentrations of pyrogallol and gallic acid in each cultivar demonstrated that both compounds are connected through a biochemical pathway. This similarity was not found in the case of protocatechuic acid and pyrocatechol (decarboxylated product of protocatechuic acid). Thus, the concentration of protocatechuic acid ranged from 40 μg/g in Syrah extract to 302 μg/g for Cabernet Sauvignon, whereas pyrocatechol ranged from 17 μg/g in Bobal to 232 μg/g in Merlot. Catechin, the

building block for tannin synthesis, was found at significant concentrations from 0.4 to 7.4 mg/g in Cabernet franc and Baladí, respectively. This high concentration is indicative of an important effect of the extraction process on the hydrolysis of tannins. Acetovanillone was detected in all varieties of vine-shoots. Other compounds with organoleptical incidence were coniferaldehyde and sinapaldehyde. The former was found in all cultivars except Syrah, in concentrations ranging from 10 to 40 μg/g, whereas sinapaldehyde was found in all extracts in concentrations ranging from 2 to 476 μg/g.

From an enological point of view, the concentration of representative phenols in extracts from vine-shoots makes foreseeable their use to improve wine quality through oxidation/reduction reactions, because they could determine to a large extent its color, flavor, and aroma, acting similarly to wine aging either in contact with oak chips or in oak barrels.[13] In relation to health benefits attributed to phenolic compounds, the nutraceutical interest of these extracts should be evaluated.

■ ASSOCIATED CONTENT

Supporting Information

DAD chromatograms at 280 and 320 nm corresponding to SHLE extracts from Pedro-Ximenez vine-shoots at 160 °C (A) and 180 °C (B) with identification of interesting compounds. Peaks: 1, pyrogallol; 2, gallic acid; 3, hydroxymethylfurfural; 4, pyrocatechol; 5, protocatechuic acid; 6, catechin; 7, vanillic acid; 8, vanillin; 9, coumaric acid; 10, ferulic acid; 11, coniferaldehyde; 12, sinapic acid; 13, sinapaldehyde; e.s., *p*-cresol. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Funding

The Spanish Ministerio de Ciencia e Innovación (MICINN) and FEDER program are acknowledged for financial support through Project CTQ2009-07430. F.P.-C. is grateful to MICINN for a Ramón y Cajal contract (RYC-2009-03921).

Notes

The authors declare no competing financial interest.

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