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Methods for Preparing Phenolic Extracts from Olive Cake for Potential Application as Food Antioxidants

MANUEL SUÁREZ, MARIA-PAZ ROMERO, TOMÁS RAMO, ALBA MACIÀ, AND MARIA-JOSÉ MOTILVA*

Department of Food Technology, XaRTA-TPV, Escuela Técnica Superior de Ingeniería Agraria, University of Lleida, Alcalde Rovira Roure 191, 25198 Lleida, Spain

Olive cake, the most important byproduct of olive oil extraction by the two-phase centrifugation system, was used to obtain phenolic extracts. The extracts were obtained using the two constituents of this waste, vegetative water and solid residue, to maximize the extraction of all phenolic compounds. Different extraction procedures were studied, a simple and rapid extraction procedure being developed from the solid residue using an accelerated solvent extractor (ASE). Afterward, the phenolic extracts were fractionated using semipreparative HPLC to study the antioxidant activity of the different components. The identification of the phenolic compounds was carried out with an ultraperformance liquid chromatograph coupled to tandem mass spectrometry equipment (UPLC-ESI-MS/MS). With this method, a complete list of the polyphenols from the extract was obtained. Finally, the antioxidant activity of the phenolic extracts and the isolated fractions was evaluated, showing great antioxidant capacities, between 3450 and 17900 μmol of Trolox equivalents/g of extract. With regard to the isolated fractions the most antioxidant were those that contained hydroxytyrosol and 3,4-DHPEA-EDA. The suitability of the solid residue extract obtained by the ASE procedure was demonstrated given the great range of phenolic compounds and the feasibility of production on an industrial scale.

KEYWORDS: Phenols; olive cake; DPPH; ORAC; ASE; antioxidants; semipreparative HPLC; UPLC-ESI-MS/MS

INTRODUCTION

Nowadays, there is a great interest in the use of natural products as a source of food ingredients with antioxidant activity. This increasing interest has been stepped up by the new advances in extraction procedures and chromatographic techniques that have made it possible to isolate and identify compounds from different plant sources. Furthermore, side effects have been reported for some synthetic antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), thus increasing the need to search for new natural sources of antioxidants (*1*). A range of byproducts from the food industry have been studied as alternatives to the synthetic antioxidants, with attention being focused on those that contain high levels of phenolic compounds.

Different byproducts from fruit and vegetable processing (peel, seeds, and stones) have been studied to find new sources of natural antioxidants (*2*). For example, the extraction of β-carotene and lycopene from tomato paste waste by supercritical fluid extraction has been proposed (*3*). Similarly, olive cake (also called wet pomace or *alperujo*) has been considered to be an interesting source of phenolic compounds. This semisolid byproduct is a harmful waste with negative impact on the environment and is the most important waste from olive oil extraction by the two-phase centrifugation system (which consumes little or no water), with approximately 4 million tonnes being generated annually in Spain (*4*).

Oleuropein and ligstroside are the major phenolic compounds in the olive fruit. During the virgin olive oil extraction process, crushing produces cellular destruction in the olive fruit and, after that, kneading leads to mixing of the cellular content. As a consequence of these two actions, a chain of reactions produces changes in the molecular structure of the oleuropein and ligstroside that lead to the formation of secoiridoid derivatives (oleuropein and ligstroside aglycones) (*5*). Finally, in the two-phase horizontal decanter, the phenolic compounds are partitioned into the different phases according to their affinity for water or oil and, as a result, almost all of the phenolic compounds present in the olive fruit are retained in the olive cake (only around 2% are transferred to the oil) (*6*). As a consequence, a wide range of phenolic compounds have been identified in the olive cake, the most important being phenolic alcohols, secoiridoid derivatives, phenolic acids, lignans, and flavonoids (*5*).

Different studies have shown the antioxidant activity of some of these individual and combined phenolic compounds using different test and lipid matrices that supported their contribution to the oxidative stability of virgin olive oil (*7*−*9*). The highest

* Corresponding author (telephone +34 973 702817; fax + 34 973 702596; e-mail motilva@tecal.udl.es).

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activity was found with phenolic compounds that possess 3,4-dihydroxyl and 3,4,5-trihydroxy structures linked to an aromatic ring (oleuropein and some of their derivatives, such as 3,4-DHPEA-EDA, and some flavones, such as luteolin and apigenin) that conferred a higher proton dislocation to the moiety, thus facilitating the scavenging activity.

Besides the antioxidant function in food systems, different studies have analyzed the potential effects of olive phenols on human health (*10*, *11*). The efficiency of the oleuropein derivatives and some flavonoids for increasing the resistance of LDL to oxidation in vitro system has been demonstrated (*12*, *13*). The results of the recent EUROLIVE study support the recommendation of olive oils with a high phenolic content as a source of fat to confer additional protection against cardiovascular risk factors (*14*). The main problem of the virgin olive oils with high phenolic content is the bitter taste, which makes them less easily accepted by nonhabitual consumers. Another problem related to the consumption of olive oil is the high caloric value, which imposes limits on its daily consumption. Considering the ingestion of a daily dose of virgin olive oil of 23 g recommended by the U.S. Food Drug Administration (FDA) (*15*), the daily ingestion of phenols is very low in relation to other food phenol sources. In that sense, the development of procedures focused on obtaining phenolic extracts containing the different components of the virgin olive oil phenolic fraction is supported by the current interest in natural antioxidants from plant sources for future applications in the enrichment of olive oils. This could be a convenient strategy for providing better health conditions for the consumer by increasing the phenol content and consequently the protective role of olive oil, without increasing calorie intake.

Previous works have focused on the development of methods to purify hydroxytyrosol from olive mill wastewaters due to its high antioxidant activity properties with regard to both nutrition and oil stability (*16*, *17*). In this way, Fernandez-Bolaños et al. (*16*) recovered high levels of hydroxytyrosol from olive cake by hydrothermal treatments that liberated it from other molecules such as oleuropein, demethyloleuropein, verbascoside, and hydroxytyrosol glucosides.

Considering the interest in olive cake as a source of antioxidants and previous works by other authors, the main objective of this work was to obtain an extract containing, besides hydroxytyrosol, the main components of the virgin olive oil phenolic fraction, including the oleuropein and ligtroside derivatives (secoiridoids), phenolic acids, flavonoids, and lignans. For this purpose, different procedures were evaluated to optimize the extraction from the two constituents of olive cake, vegetative waters and solid residue. The second point of the study was the fractionation of the extracts by semipreparative HPLC to evaluate the antioxidant capacity of the different phenolic fractions in relation to the whole phenolic extracts and to consider the interest of the purification step to increase the antioxidant capacity.

MATERIALS AND METHODS

Samples. The samples used in this study were byproduct taken from a commercial olive oil mill from the olive-growing area of Les Garrigues (Catalonia, Spain) during the olive harvest. A two-phase continuous system was used to extract the olive oil. In this system, after the olives are crushed, the paste is kneaded for 1 h at 28 °C and is then sent to a two-phase horizontal decanter, where the oil is separated from the olive oil cake. Finally, the oil goes to a vertical centrifuge to clean it of fine solids and water. Samples of olive cake were taken at the decanter outlet, and liquid nitrogen was immediately added to avoid oxidative damage. The samples were then stored at −40 °C until their processing.

To maximize the extraction of polyphenols from olive cake, the samples were centrifuged at 21600*g* at 15 °C for 10 min to separate the liquid fraction (containing vegetative water and residual oil) from the solid residue. In this way, the phenolic compounds were extracted from each of the two fractions, allowing an improvement in the recovery of all phenolic compounds retained in the olive cake.

Extraction of Phenolic Compounds from the Vegetative Water (VW). *Procedure A.* This method was based on the system developed by Visioli et al. (*18*) with some modifications. Vegetative water (100 mL) was transferred to a separatory funnel and cleaned three times with 15 mL of *n*-hexane to eliminate lipids, being shaken for 1 min each time. Then, the phenolic compounds were extracted three times with 25 mL of ethyl acetate and were concentrated in a rotary vacuum evaporator at 30 °C to dryness. Finally, the phenolic extract was redissolved in methanol and stored at −80 °C.

Procedure B: Alkaline Treatment. Before the initial centrifuging of the olive cake, the pH of the complete olive cake was increased to 9 using NaOH, and then it was left to rest for 16 h at room temperature. After that, the olive cake was centrifuged, and the extraction was continued with the vegetative water, similarly to procedure A. The pH of the sample was returned to the initial value (5.3), and then it was cleaned three times with 15 mL of *n*-hexane; the phenolic compounds were extracted with 25 mL of ethyl acetate (three times). After standing for 2 h to precipitate insoluble material, the extract was filtered, rotary evaporated to dryness at 30 °C, and finally redissolved in methanol and stored at −80 °C.

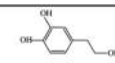
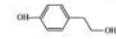
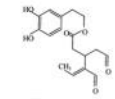
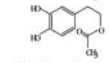
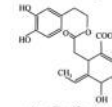
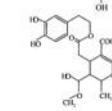
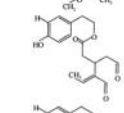
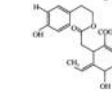
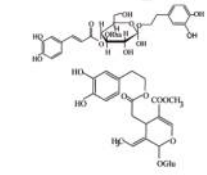
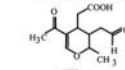
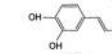
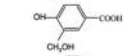
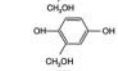
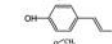
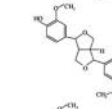
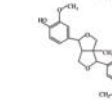
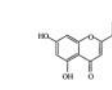
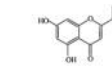
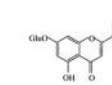
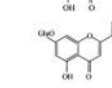
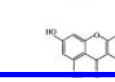
Extraction of Phenolic Compounds from the Solid Residue (SR). *Procedure C: Solid−Liquid Extraction at Atmospheric Pressure.* The SR of the centrifuged olive cake was soaked for 15 min in an ultrasonic bath with 150 mL of a solution of methanol/water (80:20, v/v). After that, the mixture was centrifuged at 21600*g* and 4 °C for 10 min. The methanolic fraction was then filtered through glass wool, and the pH was adjusted to 2 with 6 N HCl. It was immediately concentrated in a rotary vacuum evaporator at 30 °C until all of the methanol had evaporated, and then the phenolic compounds were extracted with 50 mL of ethyl acetate (three times). Finally, it was rotary evaporated to dryness at 30 °C and then freeze-dried (Telstar) and stored at −80 °C in N_2 atmosphere.

Procedure D: Solid−Liquid Extraction at High Pressure. To develop a simpler procedure, an accelerated solvent extractor ASE100 (Dionex) was used to extract the phenolic compounds from the olive cake. This extractor allows faster extractions using solvents at high temperatures and pressures. Ethanol/water (80:20) was used as an extraction solvent, and the selected temperature was 80 °C. To extract the phenolic compounds contained in 30 g of solid residue, an extraction cell with a volume of 100 mL was used, the flush volume being fixed at 60%. Two static cycles of 5 min were programmed in each extraction. After that, the sample was purged with nitrogen (≥99.99% purity, Aphagaz, Madrid, Spain). The resulting extract was rotary evaporated until all of the ethanol was eliminated, and then it was freeze-dried and stored at −80 °C in N_2 atmosphere.

Analysis of Phenolic Extracts. *HPLC-DAD.* HPLC analyses of the phenolic extracts from the VW and SR were performed according to the method described in Morello et al. (*19*). Chromatograms were obtained at 278 and 339 nm.

Semipreparative HPLC. The optimized phenolic extracts from the VW and SR were fractionated by semipreparative HPLC. The semipreparative system includes a Waters 1525EF binary HPLC pump, a Waters Flexinject, an Inertsil ODS-3 column (5 μm, 25 cm × 10 mm i.d., GL Sciences Inc.) equipped with a Spherisorb S5 ODS-2 (5 μm, 10 cm × 10 mm i.d., Technokroma, Barcelona, Spain) precolumn, a Waters 2487 dual λ absorbance detector (278 and 339 nm), and a Waters Fraction Collector II. The HPLC semipreparative system was operated using Brezze software. The extract (400 μL aliquot) was injected manually into the injector module (sample loop of 1 mL). The solvents employed during the chromatographic separation were water/acetic acid (100:0.2 v/v) as solvent A and methanol as solvent B, and the flow rate was 5 mL/min. Solvent A was initially 95% and was held

Table 1. SRM Conditions for the Analysis of Polyphenols by UPLC-ESI-MS/MS

Compound	Precursor ion (m/z)	Quantitation			Confirmation			Chemical structure
		Product ion	Cone voltage (V)	Collision energy (eV)	Product ion	Cone voltage (V)	Collision energy (eV)	
Hydroxytyrosol	153	123	35	10	95	35	25	
Tyrosol	137	106	40	15	119	40	15	
3,4-DHPEA-EDA	319	195	40	5	183	40	10	
3,4-DHPEA-AC	195	135	30	10	107	30	20	
3,4-DHPEA-EA	377	275	35	10	307	35	10	
ME 3,4-DHPEA-EA	409	377	30	5	275	30	10	
p-HPEA-EDA	303	285	30	5	179	30	5	
p-HPEA-EA	361	291	30	10	259	30	10	
Verbascoside	623	461	35	20	161	35	35	
Oleuropein	539	377	35	15	275	35	20	
Ligstroside derivative	335	199	40	10	155	40	5	Not availabe
Oleuropein derivative	365	229	35	10	185	35	15	Not available
Elenolic acid	241	139	30	15	165	30	10	
Caffeic acid	179	135	35	15	117	35	20	
Vanillic acid	167	123	30	10	152	30	15	
Vanillin	151	136	20	10	92	20	15	
p-cumaric acid	163	119	25	10	117	25	25	
Pinoresinol	357	151	40	10	136	40	25	
Acetoxy-pinoresinol	415	151	45	15	235	45	15	
Luteolin	285	133	55	25	151	55	25	
Apigenin	269	117	30	10	152	30	15	
Luteolin-7-glucoside	447	285	50	25	256	50	40	
Apigenin-7-glucoside	431	268	45	25	311	45	25	
Rutin	609	300	55	25	179	55	30	

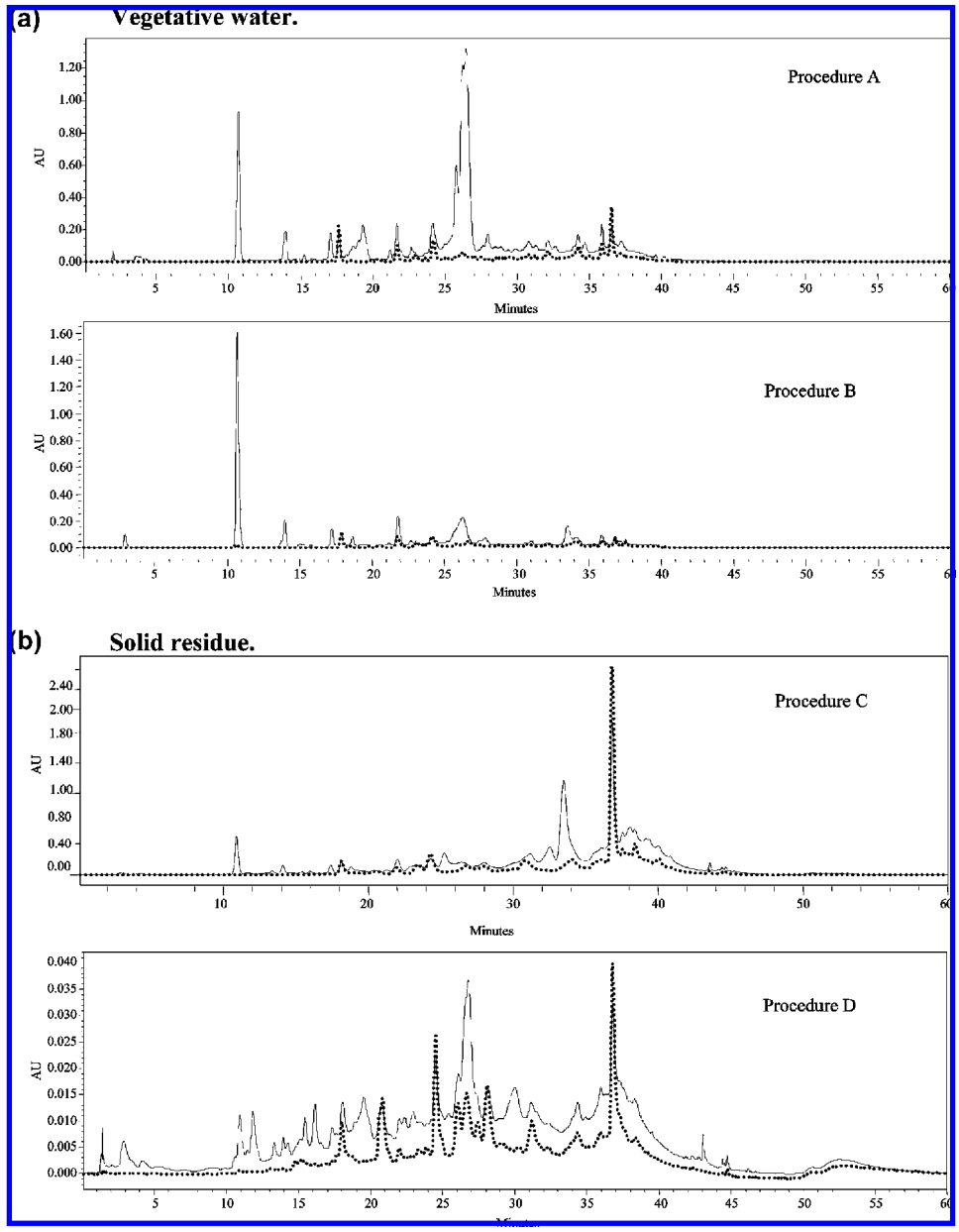


Figure 1. HPLC-DAD chromatograms of the vegetative water (**a**) and solid residue (**b**) extracts by the procedures under study. Procedure A was done using ethyl acetate as solvent of extraction; procedure B included an alkaline hydrolysis prior to the extraction with ethyl acetate. Procedure C was done by extraction at atmosphere pressure, and procedure D was done at high pressure using an ASE. Chromatograms were extracted at two wavelengths: 278 nm (—) and 339 nm (···).

isocratically for 2 min. Then it was reduced gradually to 75% at 10 min, to 60% at 20 min, and to 50% at 30 min. At 40 min, solvent A was 0% and remained isocratic for 5 min. Then, it was increased to 95% over 3 min and held there for up to 50 min. The fractions were collected manually according to their retention time and the output signal of the absorbance detector. After that, they were concentrated by rotary evaporation at reduced pressure to eliminate the methanol and were finally freeze-dried in a Lyobeta (Telstar, Spain) and stored at −80 °C in N_2 atmosphere.

UPLC-ESI-MS/MS. The composition of the complete and fractionated phenolic extracts from the VW and SR were characterized by UPLC tandem mass spectrometry equipment. The UPLC-MS/MS system consisted of an AcQuity UPLC equipped with a binary pump system Waters (Milford, MA) using an AcQuity UPLC BEH C18 column (1.7 μm, 100 mm × 2.1 mm i.d.) equipped with a VanGuard Pre-Column AcQuity UPLC BEH C18 (2.1 × 5 mm, 1.7 μm) also from Waters. The UPLC was coupled to a TQD mass spectrometer (Waters). The software used was MassLynx 4.1. During the analysis, the column was kept at 30 °C, and the flow rate was 0.4 mL/min. The solvent composition was solvent A, Milli-Q water/acetic acid (100:0.2 v/v), and solvent B, acetonitrile. Solvent B was initially 5% and was held isocratic during 2.1 min. Then it was increased gradually, reaching 10% at 7.5 min, 40% at 19 min, and 100% at 19.1 min. Then, solvent B was kept isocratic at 100% for 21 min. Finally, it was reduced to 5% A at 21.1 min and was held at this until 24 min to re-equilibrate the column at the initial conditions. The injection volume was 2.5 μL, and all of the freeze-dried samples were redissolved in methanol and filtered through 0.22 μm.

Ionization was done by electrospray (ESI) in the negative mode, and the data were collected in the selected reaction monitoring (SRM) mode. The ionization source parameters were as follows: capillary voltage, 3 kV; source temperature, 150 °C; desolvation gas temperature, 400 °C, with a flow rate of 800 L/h. Nitrogen (99.99% purity, N_2LCMS nitrogen generator, Claind, Lenno, Italy) and argon (≥99.99% purity, Aphagaz, Madrid, Spain) were used as the cone and collision gas, respectively. The SRM transitions and the individual cone voltage and collision energy for each phenolic compound were evaluated by infusing 10 mg/L of each compound to obtain the best instrumental conditions. The results of the SRM conditions are shown in **Table 1**.

Table 2. Phenolic Compounds Quantified by UPLC-ESI-MS/MS of the VW and SR Extracts[a]

compound	procedure A	procedure C	procedure D
tyrosol	1.92	0.32	0.09
hydroxytyrosol	98.6	2.79	2.54
total phenyl alcohols	100.5	3.11	2.63
vanillin	0.06	0.06	0.05
p-coumaric acid	0.18	0.20	0.02
vanillic acid	1.56	0.09	0.04
caffeic acid	0.00	0.43	0.09
total phenyl acids	1.80	0.78	0.20
3,4-DHPEA-AC	1.64	0.66	0.14
3,4-DHPEA-EDA	158.8	173.3	45.84
3,4-DHPEA-EA	104.1	28.21	10.09
methyl 3,4-DHPEA-EA	0.00	0.18	0.05
oleuropein derivative	0.10	0.19	0.08
oleuropein	0.02	0.45	0.32
elenolic acid	16.11	8.40	6.99
total oleuropein derivatives	280.8	211.4	63.5
verbascoside	2.50	2.18	0.56
p-HPEA-EA	3.76	1.64	0.26
p-HPEA-EDA	17.3	0.31	0.41
ligstroside derivative	1.63	3.05	1.59
total ligstroside derivatives	21.2	5.00	2.26
pinoresinol	0.25	0.33	0.10
acetoxypinoresinol	1.73	0.63	0.18
total lignans	1.98	0.96	0.28
rutin	0.20	1.24	1.21
apigenin	0.04	0.10	0.07
luteolin	1.24	5.07	2.84
apigenin-7-G	1.27	0.11	0.07
luteolin-7-G	0.71	3.38	2.91
total flavonoids	3.46	9.90	7.10

[a] The results are expressed as mg of phenol/g of lyophilized extract.

Individual phenols were quantified by a five-point regression curve on the basis of standards obtained from commercial suppliers or from semipreparative HPLC. Apigenin, apigenin 7-*O*-glucoside, luteolin, luteolin 7-*O*-glucoside, rutin, oleuropein, hydroxytyrosol, tyrosol, and vanillin were purchased from Extrasynthese (Genay, France). Caffeic, *p*-coumaric, and vanillic acids were purchased from Fluka Co. (Buchs, Switzerland), and (+)-pinoresinol was acquired from Arbo Nova (Turku, Finland). Secoiridoid derivatives, such as the dialdehydic form of elenolic acid linked to tyrosol (*p*-HPEA-EDA), the aldehydic form of elenolic acid linked to tyrosol (*p*-HPEA-EA), the dialdehydic form of elenolic acid linked to hydroxytyrosol (3,4-DHPEA-EDA), 4-(acetoxyethyl)-1,2-dihydroxybenzene (3,4-DHPEA-AC), oleuropein aglycone (3,4-DHPEA-EA) and its methylated form (methyl 3,4-DHPEA-EA), and the lignan acetoxypinoresinol were isolated from virgin olive oil by using the semipreparative HPLC method (*8*).

Antioxidant Activity. *DPPH Assay*. DPPH was used to evaluate the radical scavenging activity (RSA) of the phenolic extracts. An amount of 2.95 mL of a 0.1 mM methanolic DPPH solution was mixed in a cuvette with 50 μL of phenolic extract at different concentration levels. The RSA was determined by measuring the decrease in the absorption of the mixture at 517 nm at intervals of 15 s during 5 min (*7*). The results were expressed as percentage of inhibition of DPPH by the equation

$$\text{percentage of inhibition} = (A_0 - A_1)/A_0 \times 100 \qquad (1)$$

where A_0 is the initial absorbance of the mixture and A_1 is the absorbance of the mixture after 5 min. The assay was done in triplicate for each sample using pyrogallol as positive control. With the results obtained at different concentrations of the sample, the EC_{50} value (the concentration of substrate that causes a 50% drop in the DPPH activity) was found for each phenolic extract (*20*).

ORAC Assay. The ORAC assay was based on the methodology reported by Huang et al. (*21*) with some modifications. This method analyzes the peroxyl radical scavenging activity of the samples. The assay was carried out on a FLUORstar optima spectrofluorometric analyzer (BMG Labtechnologies GmbH, Offenburg, Germany) in 96-well microplates, using an excitation filter at 485 nm and an emission filter at 520 nm. The experiment was carried out at 37 °C using phosphate buffer at pH 7.4. The reaction mixed consisted of 150 μL of 68 nM fluorescein solution (substrate), 25 μL of 74 mM AAPH solution (initiator), made immediately before use in the assay buffer at 37 °C, and 25 μL of either phenolic extract or Trolox at different concentrations (from 0.415 to 4.15 μg/mL in the case of the phenolic extract and from 12.5 to 100 μM in the case of Trolox). Assay buffer was used as a blank. The ORAC values were calculated on the basis of area under curve (AUC) results with the data expressed as micromoles of Trolox equivalents per gram of phenolic extract using the Trolox and the sample calibration curves obtained in each analysis.

RESULTS AND DISCUSSION

Extraction Method Optimization. The selection of the optimum extraction procedure was based on the evaluation of the chromatographic areas obtained at 278 and 339 nm of the main peaks for each phenolic extract analyzed by HPLC-DAD.

Extraction of Phenolic Compounds from VW. **Figure 1a** shows the chromatographic profiles of the phenolic extracts obtained by procedures A and B, respectively. The resulting chromatogram of the extraction with ethyl acetate (procedure A) shows a great number of components. On the basis of the maximal absorption spectrum (with characteristic wavelength of 278 nm), almost all of the peaks appearing in the first part of the chromatogram could correspond to phenolic acids, phenolic alcohols, and secoiridoid derivates. Similarly to previous studies (*18*, *22*), ethyl acetate was considered to be a more appropriate solvent for extracting the phenolic compounds from the vegetative waters due to its high selectivity among other hydrosoluble compounds, mainly sugars, present in high concentration in this byproduct.

To increase the recovery of phenolic compunds, an alkaline pretreatment of the olive cake with NaOH, before centrifugation and separation of the vegetative water, was evaluated (procedure B). As can be observed in **Figure 1a**, the alkaline treatment produces an increase in the area of the peak with a retention time of 10 min that corresponds to hydroxytyrosol. In contrast, a reduction of the area of the peaks in the 20−30 min interval [corresponding to secoiridoid derivatives (*5*)] is observed. Considering that the main objective of this study was to obtain phenolic extracts with all of the components of the phenolic fraction of the virgin olive oil, besides hydroxytyrosol, we decided to avoid the alkaline pretreatment to obtain an extract with a balanced quantity of all phenolic compounds, including secoiridoids.

Extraction of Phenolic Compounds from SR. Initially, a solid−liquid extraction at atmospheric pressure was assayed to extract the phenolic compounds from the solid residue (procedure C). As can be seen in **Figure 1b**, the chromatographic profile of this extract is characterized by the presence of compounds with maximum wavelength at 339 nm (characteristic of flavonoids) in the second part of the chromatogram. These compounds eluted when the mobile phase had a high percentage of methanol, indicating the lower hydrophilic nature of the phenols retained in the solid residue after centrifugation of the olive cake compared with the main phenols from the vegetative water extract. The occurrence of these compounds might be explained by the extraction procedure. Thus, soaking the solid residue in the ultrasonic bath may have hydrolyzed the bonds between the flavonoids that were maintaining these compounds

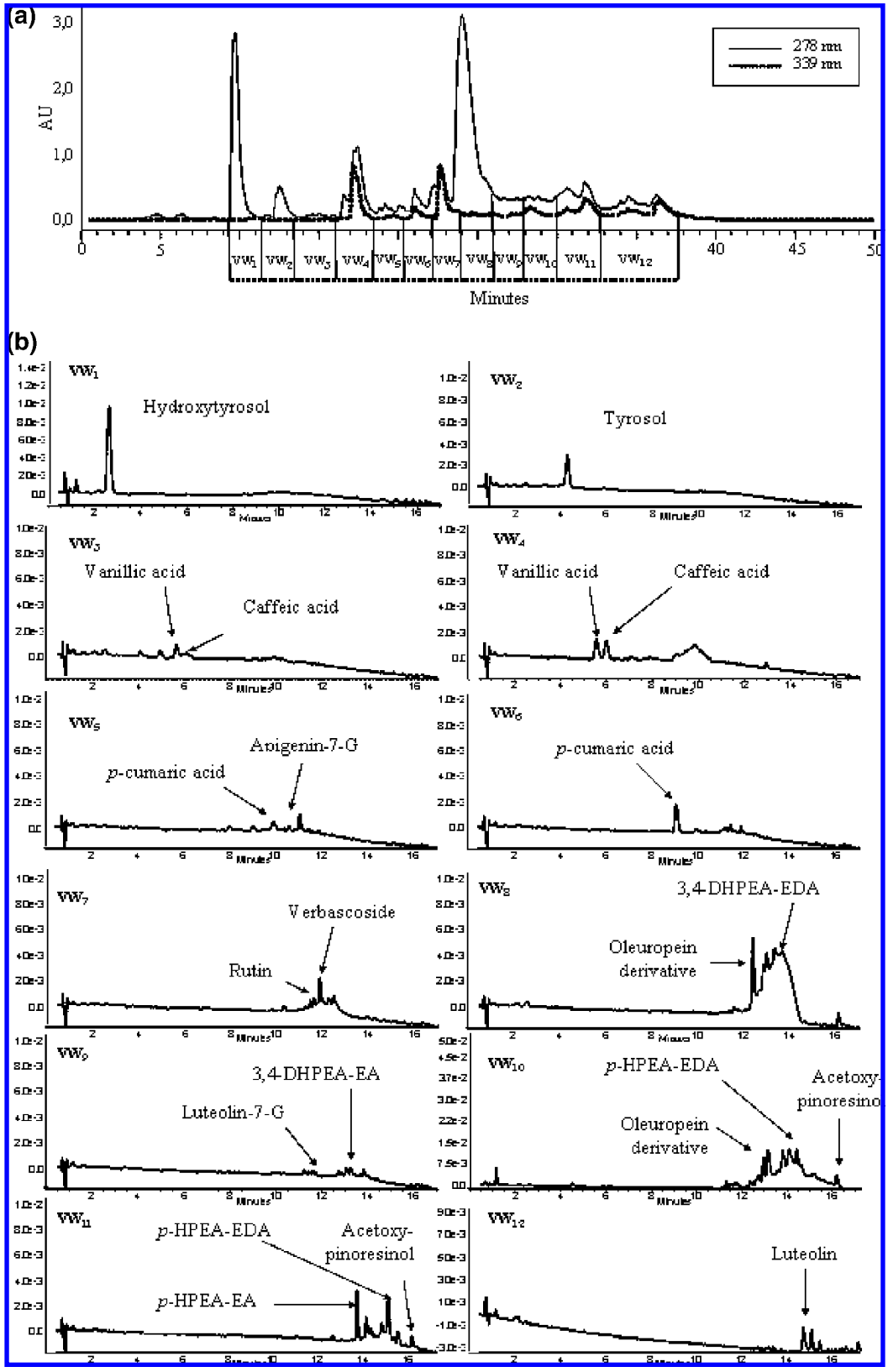


Figure 2. (**a**) Semipreparative HPLC chromatogram corresponding to the VW extract using procedure A showing the isolated fractions. The chromatogram was extracted at two wavelengths (278 and 339 nm). (**b**) UPLC-DAD chromatograms from the isolated fractions (VW_1-VW_{12}). The phenolic compounds in each fraction were identified by UPLC-ESI-MS/MS according to the SRM conditions that appear in **Table 1**.

in the solid matrix and allowed them to be extracted with methanol/water more easily. Furthermore, the use of aqueous methanol for solvent extraction has good selectivity for phenolic compounds in relation to other water-soluble compounds, mainly sugars, in the solid residue of the olive cake (*23*).

A second extraction method (procedure D) was evaluated using an ASE 100 to develop a faster extraction procedure and to achieve good recoveries of phenolic compounds from SR. This extractor allows faster extractions to be achieved using solvents at high temperatures and pressures. The chromato-

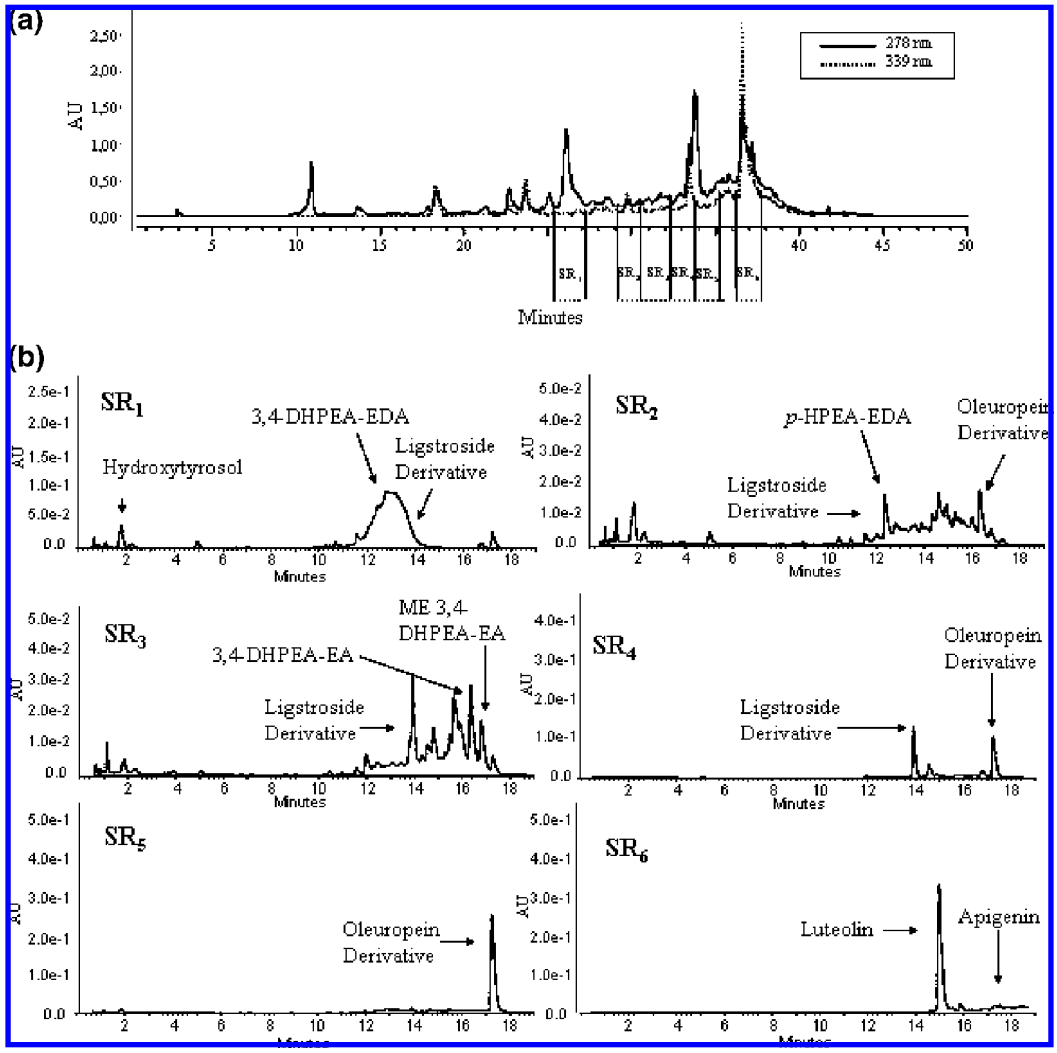


Figure 3. (**a**) Semipreparative HPLC chromatogram corresponding to the SR extract using procedure C showing the isolated fractions. The chromatogram was extracted at two wavelengths (278 and 339 nm). (**b**) UPLC-DAD chromatograms from the isolated fractions (SR_1−SR_6). The phenolic compounds in each fraction were identified by UPLC-ESI-MS/MS according to the SRM conditions that appear in **Table 1**.

graphic profile of the ASE extract (**Figure 1b**) was very similar to the one from procedure C, the same groups of phenolic compounds being obtained (secoiridoid derivatives and flavonoids). In addition to the simpler extraction procedure, the objective used authorized solvents included in European Regulation 88/344/CEE (later modified by directive 94/52/CEE) (*24*, *25*). Thus, methanol was replaced by ethanol for solvent extraction. Additionally, the purification step with ethyl acetate was omitted, so that the resulting extract was free of the organic smell that characterized the extract obtained by procedure C. In summary, the use of ethanol for solvent extraction, instead of methanol, and the elimination of the step purification with ethyl acetate increased the suitability of the extract obtained by procedure D for use as a food ingredient.

Characterization and Fractionation of the Phenolic Extracts. Due to the large number of components and the low chromatographic resolution of these phenols, DAD was not considered to be a suitable tool for the qualitative and quantitative analysis of the VW and SR phenolic extracts. As an alternative, the tandem mass spectrometry detector was chosen. Besides, the chromatographic analysis by UPLC in relation to HPLC achieved a better chromatographic separation of the compounds and reduced the analysis time noticeably (24 min against the 60 min needed in the HPLC method). Furthermore, high peak efficiency and a better signal-to-noise (S/N) ratio are obtained. As a consequence, ultraperformance liquid chromatography−tandem mass spectrometry becomes an interesting tool for developing more efficient methods in the chromatographic analysis of complex phenolic extracts.

For the phenolic quantification of the selected extracts, two product ions were studied in SRM: one was used to quantify the phenolic compound, and the other was used as a confirmation (**Table 1**). As can be seen in **Table 2**, the VW extract (procedure A) is especially rich in phenyl alcohols (mainly hydroxytyrosol) and secoiridoid derivatives (3,4-DHPEA-EDA). On the other hand, the SR extracts (procedures C and D), besides the secoiridoid derivatives, presented a higher concentration of flavonoids in relation to the extract from VW (procedure A). As can be observed in **Table 2**, the phenolic concentration of the SR by ASE extraction (procedure D) is lower than the extract obtained by procedure C. The differences can be explained by the purification step with ethyl acetate in procedure C that produces a more concentrated extract.

After the characterization, the complete phenolic extracts were fractionated by semipreparative HPLC to study the antioxidant capacity of the different fractions to identify the most interesting components of the extracts and to evaluate the interest of a second purification step. **Figures 2a** and **3a** show the semipreparative HPLC chromatograms corresponding to the VW (procedure A) and SR (procedure C) phenolic extracts, respec-

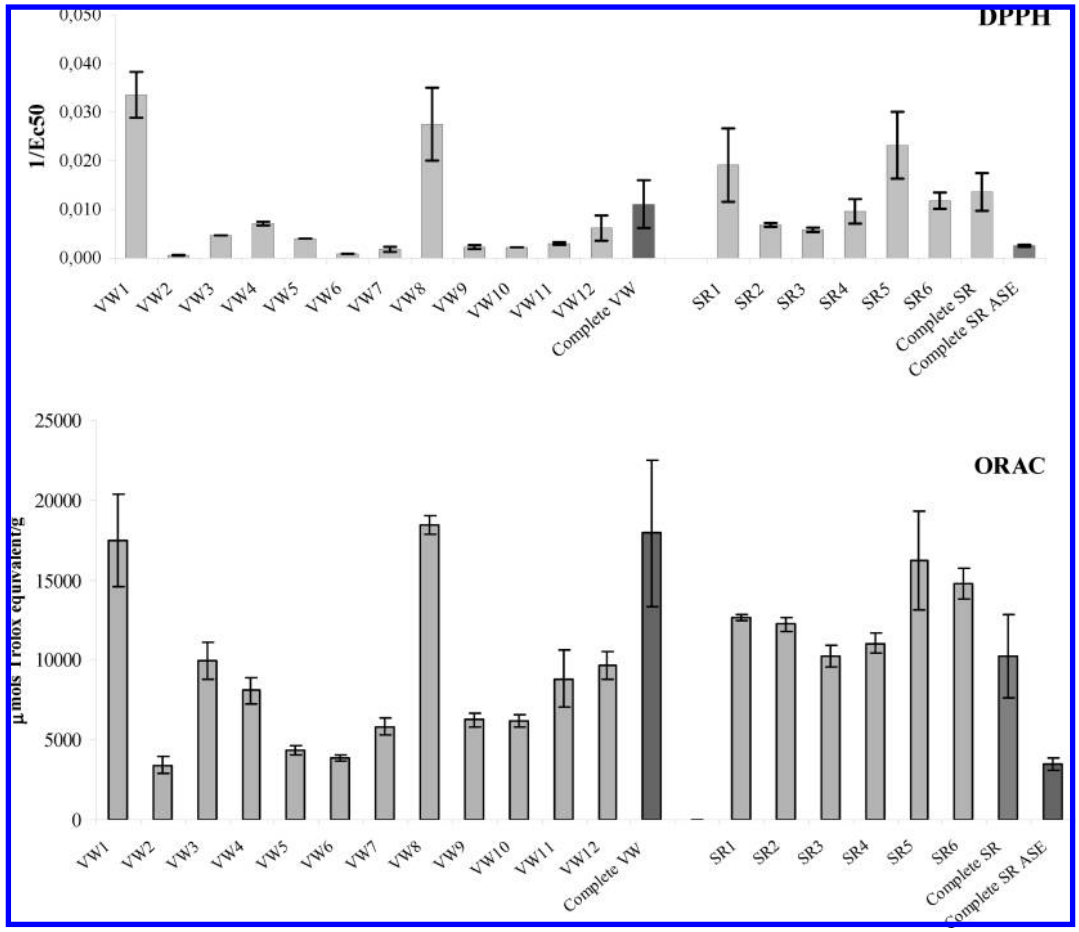


Figure 4. Antioxidant activity of the isolated fractions and the whole extracts by the DPPH method and the ORAC assay. The results are obtained from three repetitions, and standard deviation is included.

tively, and the various fractions that were collected. The VW extract was completely fractionated, obtaining 12 fractions (VW_1−VW_{12}) (**Figure 2a**). On the other hand, in the SR extract, the fractions of the second part of the chromatogram were selected (**Figure 3a**) containing the compounds with a minor hydrophilic nature. Once the phenolic extracts had been fractionated, the phenolic compounds present in each fraction were identified by UPLC-ESI-MS/MS (**Figures 2b** and **3b**).

Antioxidant Activity (DPPH and ORAC Assays). Antioxidant activity was evaluated by two methods to cover the great variability of assays that can be used. DPPH is a colorimetric assay that has been extensively used to evaluate antioxidant activity due to its speed. However, some authors have suggested that this method is not adequate because it uses stable radicals (which are foreign to biological systems) instead of short-lived radicals, such as the peroxyls (which act as intermediates in the real oxidation process) (*26*). On the other hand, the use of the ORAC assay has spread recently because it is especially useful for food samples that have complex reaction kinetics (*27*). The basis of this method is the use of peroxyl radicals to evaluate the loss of fluorescence in fluorescein.

As can be seen in **Figure 4**, both the complete phenolic extracts and the isolated fractions were active in the DPPH and ORAC assays. The DPPH results are expressed as $1/EC_{50}$ so that the more active the sample is in the ORAC assay, the higher the value will be. According to the results, the highest activity belonged to the fractions that have hydroxytyrosol, oleuropein derivatives, and flavonoids in their composition (**Figures 2** and **3**). The study of Obied et al. (*9*) concluded that the most antioxidant compounds from an olive cake extract were verbascoside and 3,4-DHPEA-EDA (which are compounds related to the secoiridoid derivatives). However, they also studied the activity of standard hydroxytyrosol, obtaining the best result of all. This agreed with our result in which the activity of VW_1, which contained hydroxytyrosol, was higher than that of VW_7 (containing verbascoside).

The resulting value for antioxidant activity can be explained by the chemical structure of the phenolic compounds found in each fraction (**Table 1**). Phenolic compounds are included in the group of chain-breaking antioxidants, which can lose a hydrogen radical to stop radical oxidation propagation in lipid oxidation. The power of the phenolic compounds to halt the oxidation reaction increases when the homolytic dissociation energy of the O−H bond decreases. Furthermore, the presence of an aromatic ring and some bulky groups allows the delocalization of the unpaired electron, thus restricting the reactivity of the radical formed (*28*).

With reference to the phenolic compounds present in the studied fractions, hydroxytyrosol, which is present in VW_1, has a 3,4-dihydroxy structure linked to an aromatic ring. This confers higher activity to hydroxytyrosol than to tyrosol (which has a similar structure but with only one hydroxyl group linked to an aromatic ring), allowing the moiety a higher proton dislocation. This was confirmed by our results, where VW_2, which mainly contained tyrosol, showed lower activity than VW_1.

3,4-DHPEA-EDA (present in VW_8 and SR_1) is one of the major secoiridoid derivatives in virgin olive oil and is of great interest because it is the most important source of hydroxytyrosol in human plasma after the intake of virgin olive oil (*29*). Its structure has two hydroxyl groups in the ortho position linked

to an aromatic ring, and this gives it its great radical scavenging activity. In addition, the double bonds of the elenolic structure in 3,4-DHPEA-EDA could facilitate the proton dislocation in the molecule and enhance its antioxidant activity. In contrast, the secoiridoid derivatives originated from ligstroside (*p*-HPEA-EDA and *p*-HPEA-EA) (VW_{11}) have lower antioxidant activity than those originated from oleuropein. This might be explained by the presence of a single hydroxyl group linked to the aromatic ring.

The recent identification of these oleuropein derivatives in postprandial human plasma after the ingestion of a single dose of 40 mL of virgin olive oil (*30*) could suggest their protective role against cardiovascular risk factors and the interest in including this phenolic group, besides hydroxytyrosol, in the phenolic extracts.

With regard to flavonoids, their characteristic structure with three aromatic rings confers good antioxidant activity specifically due to three structural groups (*31*): the catechol structure in the B ring (the radical target site), the 2,3 double bond in conjunction with the 4-oxo function (responsible for electron delocalization), and the presence of both 3 and 5 hydroxyl groups. Thus, luteolin and apigenin, two flavones present in SR_6, had good activity, being higher in the ORAC assay than in the DPPH method. In comparison, the activity of luteolin should be higher than that of apigenin due to the presence of two hydroxyl groups in the ortho position. However, this could not be confirmed by our results because both phenolic compounds appeared together in the same fraction.

One fact to be considered is that, in general, the fractions studied did not contain only one phenolic compound, but rather a mixture of a number of these. For this reason, the results obtained for antioxidant activity might contain synergistic effects between the activities of the compounds. In this case, the maximum synergistic effect was evaluated with the complete extracts. Some conclusions can be inferred from the composition of these extracts (**Table 2**) and the antioxidant results from the isolated fractions (**Figure 4**). Thus, the higher presence of hydroxytyrosol (majority compound in VW_1) in the VW (98.6 mg/g of extract) compared with the quantity in the SR (2.79 mg/g) could explain the higher antioxidant activity of the VW given that VW_1 was one of the most antioxidant fractions. Furthermore, there is a high concentration of oleuropein derivatives (3,4-DHPEA-EDA and 3,4-DHPEA-EA), mainly 3,4-DHPEA-EDA, which have also shown good activity (VW_8). On the other hand, the SR contains more flavonoids than the VW (9.9 mg/g compared with 3.46 mg/g), and these compounds have shown good antioxidant activity (SR_6). In addition, the quantity of the total oleuropein derivatives present in the SR extract is very important (203 mg/g). For these reasons, the SR might also have shown good activity, despite its low concentration of hydroxytyrosol.

The values of ORAC obtained with the complete extracts were higher than the values of a great variety of foods included in the database of the U.S. Department of Agriculture (*32*), demonstrating its great antioxidant activity. Compared to the ORAC values for different commercial dietary antioxidant supplements reported by Dávalos et al. (*33*), ranging from 79 to 3180 μmol of Trolox equivalent per gram of supplement, all extracts obtained in our study presented higher antioxidant activity, even the complete SR extract obtained by ASE (procedure D). Despite the lower ORAC, the main interest in this extract is based on the organoleptic characteristics when the extract was added to an oil matrix (data not show) in relation with the addition of the other complete extracts that transmitted a solvent aroma, even after the elimination of the ethyl acetate by rotary evaporation and freeze-drying.

Finally, the values from the DPPH method and the ORAC assay from each fraction were compared. The resulting coefficient of determination (R^2) between them was 0.7597, which indicated that some differences in the antioxidant result might be obtained using one or the other method. These differences must be the result of the different mechanisms involved: single electron transfer (SET) in the case of DPPH and hydrogen atom transfer (HAT) in the case of the ORAC assay. Some authors have suggested that an antioxidant compound can react quickly with peroxyl radicals and, in contrast, react slowly or not react at all with DPPH (*27*). On the contrary, in the ORAC assay AUC is used to join the antioxidant activity to the inhibition time, and this allows results closer to those of the biological systems to be achieved. In the present study, some differences were found in the quantitative results obtained by the two assays but, in general, the order of activity of the phenolic fractions studied was the same. The biggest difference between both methods was in the whole extracts. In this way, the ORAC could be considered as a more appropriate assay for studying the antioxidant activity in complex samples (such as natural extracts) that contain different compounds that could interact with each other (*27*).

On the basis of the results, the phenolic extract obtained by accelerated solvent extraction from the solid residue of olive cake could be proposed as a simple and rapid extraction procedure as an alternative to solid−liquid extraction at atmospheric pressure, which requires prior ultrasonic or thermal treatments to facilitate the solubilization of the phenols. This procedure permits a phenolic extract to be obtained containing the main components of the virgin olive oil phenolic fraction (oleuropein and ligtrosides derivatives, flavonoids, phenolic acids, and lignans) with a potent antioxidant activity (ORAC value higher than 5000) that supports interest in future applications as a natural antioxidant and as an ingredient in the development of supplemented olive oil.

ABBREVIATIONS USED

SR, solid residue; VW, vegetative water; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ORAC, oxygen radical absorbance capacity; UPLC, ultraperformance liquid chromatography; ESI, electrospray ionization; DAD, diode array detector; MS/MS, tandem mass spectrometry; SRM, selected reaction monitoring.

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