JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Evaluation of the Antioxidant Capacity of Individual Phenolic Compounds in Virgin Olive Oil

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Virgin olive oil has a high resistance to oxidative deterioration due to its tryacylglycerol composition low in polyunsaturated fatty acids and due to the presence of a group of phenolic antioxidants composed mainly of polyphenols and tocopherols. We isolated several phenolic compounds of extra virgin olive oil (phenyl-ethyl alcohols, lignans, and secoiridoids) by semipreparative high-performance liquid chromatography (HPLC) and identified them using ultraviolet, atmospheric pressure chemical ionization, and electrospray ionization MS detection. The purity of these extracts was confirmed by analytical HPLC using two different gradients. Finally, the antioxidant capacity of the isolated compounds was evaluated by measuring the radical scavenging effect on 1,1-diphenyl-2-picrylhydrazyl radical, by accelerated oxidation in a lipid model system (OSI, oxidative stability instrument), and by an electrochemical method.

KEYWORDS: Polyphenols; olive oil; HPLC; antioxidant activity; antioxidant power; DPPH; OSI

INTRODUCTION

Oxidative stability is a central parameter in the estimation of extra virgin olive oil (VOO) quality, as it gives a reliable evaluation of the susceptibility of the oil to oxidative degeneration, which is the main cause of its damage (*1*, *2*). Although inevitable, the oxidation process can be delayed by antioxidants that enhance the oxidative stability by preventing the propagation of lipid peroxidation or removing free radicals. Antioxidants are reported as molecules, which, when present even at low concentrations, significantly prevent oxidation (*3*).

Olives and olive-derived products are an important part of the Mediterranean diet and are recognized as a valuable source of natural phenolic antioxidants (*4*). VOO, one of the few oils consumed without any chemical treatment, has a high resistance to oxidative deterioration mainly due to two reasons: First, its fatty acid composition is characterized by a high monounsaturated-to-polyunsaturated fatty acid ratio, and second, it contains many minor compounds with powerful antioxidant activity among which polyphenols stand out (*5*, *6*). As a consequence of their fundamental chemical and antioxidant properties, the phenolic compounds continue to attract considerable research efforts.

A classification of the antioxidants based on their mechanism of action can be established as follows: primary antioxidants, synergistic, and secondary antioxidants (*7*). Polyphenols are one of the main groups of phenolic compounds acting as primary antioxidants (AH) to inhibit oxidation in VOO. They mainly act as chain breakers by donating a radical hydrogen to alkylperoxyl radicals ($ROO^{\bullet}$) formed during the initiation step of lipid oxidation and subsequently forming a stable radical ($A^{\bullet}$) through the reaction (*8*):

$$ROO^{\bullet} + AH \rightarrow ROOH + A^{\bullet}$$

Olive oil hydrophilic extracts contain a large number of phenolic compounds including simple phenols, lignans, and secoiridoids (*9*), which exhibit antioxidant properties. To evaluate the antioxidant contribution that these individual phenolic compounds have in olive oil, it is important to either synthesize or isolate individual polyphenols for their analysis. Synthesis is currently not practical, so isolation procedures must be used.

Much literature is available on the development of methods for the analysis, isolation, and identification of polyphenols in olives and olive oils (*4*, *9*−*20*). Typically, these methods have been applied to monitoring differences between olive varieties and changes during ripening and oil processing. The isolation of polyphenols has been performed to assess the properties of the two main parts of the polar fraction of VOO as antioxidants after separation by solid phase extraction (*21*), but only a few

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10.1021/jf0515680 CCC: $30.25
Published on Web 10/22/2005

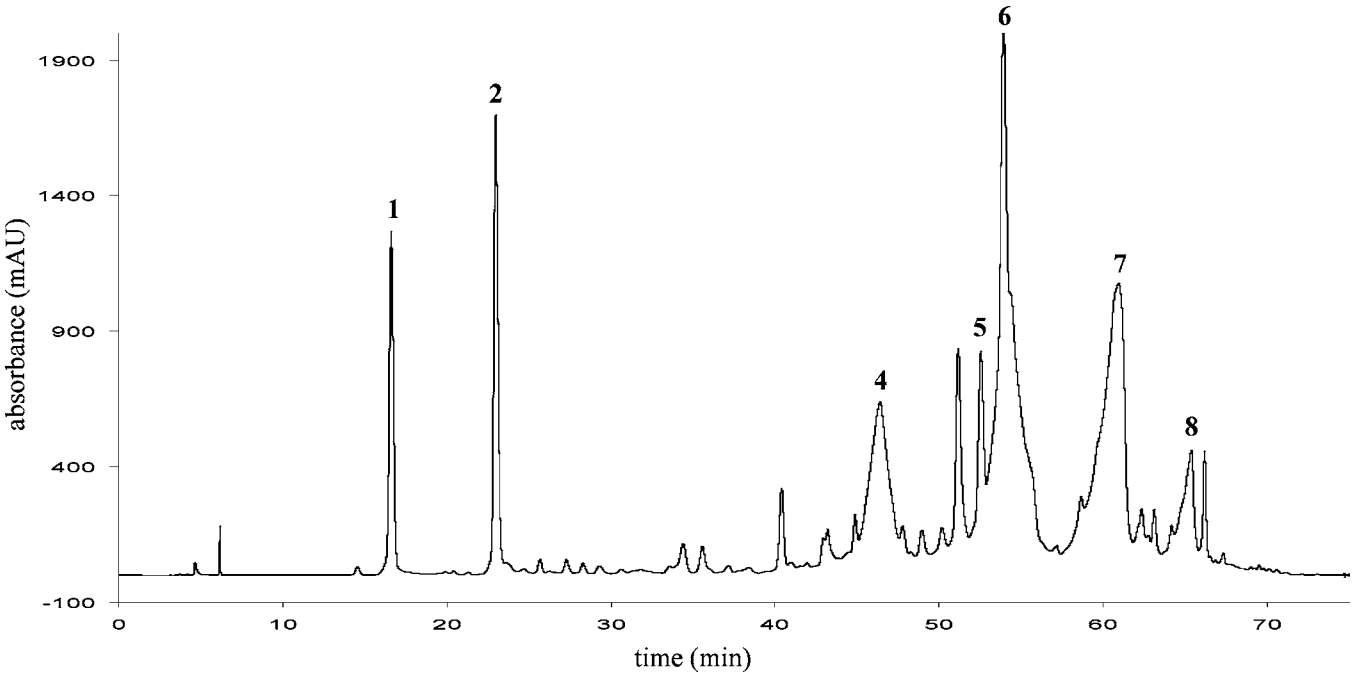

Figure 1. Chromatogram of extra VOO sample under optimized conditions using semipreparative HPLC. Detection was performed at 280 nm. Peak identification numbers: 1, hydroxytyrosol; 2, tyrosol; 3, elenolic acid (was detected at 240 nm); 4, deacetoxy oleuropein aglycon; 5, (+)-pinoresinol; 6, 1-(+)-acetoxypinoresinol; 7, oleuropein aglycon; 8, ligstroside aglycon.

data are available to the date to assess the individual antioxidant properties of these compounds directly isolated from extra VOO.

The aim of this work was to evaluate the antioxidant activity of different single phenolic compounds of VOO by chemical methods (1,1-diphenyl-2-picrylhydrazyl, DPPH), by accelerated oxidation in a lipid model system (OSI, oxidative stability instrument), and by an electrochemical method [flow injection analysis (FIA)−amperometry and cyclic voltammetry] and to compare the information to obtain insight of the chemical groups involved in the antioxidant mechanisms.

MATERIALS AND METHODS

Apparatus. High-performance liquid chromatography (HPLC) analyses were performed with a HP 1100 series (Agilent Technologies, Palo Alto, CA), equipped with a binary pump delivery system, degasser, autosampler, diode array UV−vis detector, and a mass spectrometer detector. The semipreparative HPLC column used was a Phenomenex Luna (C_{18}) column, 10 μm i.d., 25 cm × 10 mm, and the flow rate was 3 mL/min. The analytical HPLC column used was a C_{18} Luna column, 5 μm i.d., 25 cm × 3.0 mm (Phenomenex, Torrance, CA), with a C_{18} precolumn (Phenomenex) filter. The mobile phase flow rate was 0.5 mL/min.

The electrochemical behavior of the compounds studied was evaluated using hydrodynamic and cyclic voltammetry. The hydrodynamic voltammetry was performed in FIA, and cyclic voltammetry was performed using stop flow voltammetry. The apparatus consisted of a Minipuls II peristaltic pump (Gilson, France), a high-pressure injection valve model 7125 (Rheodyne, Rohnert Park, CA) equipped with a 20 μL loop, an electrochemical cell model UniJet (BAS, West Lafayette, IN) using a glassy carbon working electrode, and Autolab potentiostat (Ecochemie, Amsterdam, Netherlands).

Reagents, Stock Solutions, and Reference Compounds. Dopac (3,4-dihydroxyphenylacetic acid) was acquired from Sigma Aldrich (St. Louis, MO), and oleuropein (oleuropein glucoside) was obtained from Extrasynthèse (Genay, France). The stock solution containing these two analytes was prepared in methanol/water (50:50, v/v) and in methanol at a concentration of 500 μg/mL for each one. These compounds were used to do the calibration curves. DPPH and Trolox were acquired from Sigma Chemicals Co. (St. Louis, MO). Methanol and *n*-hexane (HPLC grade) were from Merck (Darmstadt, Germany). Doubly deionized water with a conductivity of 18.2 MΩ was obtained by using a Milli-Q system (Millipore, Bedford, MA).

Samples. Commercial extra VOO samples were used for this study (from Bertolli, Unilever Bestfoods Italia S.p.A., Inveruno-MI, Italy). Triolein was used as glyceridic matrix without phenolic compounds for OSI studies and was obtained from Fluka (Buchs, Switzerland).

Liquid−Liquid Extraction (LLE) of Phenolic Compounds from Olive Oils. A LLE system was used to extract the phenolic compounds present in VOO. According to Carrasco-Pancorbo et al. (*22*), 60 g of oil (±0.001 g) was dissolved in 60 mL of hexane, and the solution was extracted successively with four 20 mL portions of methanol/water (60:40, v/v) solution. The combined extracts of the hydrophilic layer were brought to dryness in a rotary evaporator under reduced pressure and a temperature of 40 °C. Finally, the residue was redissolved in 0.5 mL of methanol/water (50:50, v/v) and filtered through a 0.20 μm filter.

Isolation of Polyphenols Using Semipreparative HPLC. In the semipreparative HPLC analysis for the isolation of the single phenolic compounds, the mobile phases were water with acetic acid (0.5%) (phase A) and acetonitrile (phase B), and the solvent gradient changed according to the following conditions: from 0 to 30 min, 95% A:5% B to 80% A:20% B; from 30 to 40 min, 80% A:20% B to 70% A:30% B; from 40 to 50 min, 70% A:30% B to 65% A:35% B; from 50 to 60 min, 65% A:35% B to 50% A:50% B; from 60 to 70 min, 50% A:50% B to 5% A:95% B; from 70 to 75 min, 5% A:95% B to 95% A:5% B.

The injection volume for the isolation of the reference compounds was 100 μL of extracts obtained from extra VOO in methanol/water 50:50 (v/v) using the LLE system. The analyses were carried out at room temperature. The wavelengths were set at 240, 280, and 330 nm. In this way, we have used semipreparative reverse phase HPLC to isolate the following polyphenols from VOOs: hydroxytyrosol (CAS no. 10597-60-1), tyrosol (CAS no. 501-94-0), elenolic acid, deacetoxy oleuropein aglycon (named decarboxymethyl oleuropein aglycon also), (+)-pinoresinol (CAS no. 487-36-5), (+)-1-acetoxypinoresinol, oleuropein aglycon, and ligstroside aglycon. The analysis of these compounds was then done with an analytical column to check the purity of the isolated compounds and to confirm their identity, using the same gradient used in the semipreparative HPLC method plus another gradient. A chromatogram obtained using semipreparative HPLC is shown in **Figure 1**.

Analysis of the Isolated Compounds Using Analytical HPLC. After isolation, the analysis of these compounds was done with the

analytical HPLC column to check the purity of the isolated compounds and to confirm their identity, using the same gradient used in the semipreparative HPLC method. The injection volume was 10 μL. The wavelengths were set at 240, 280, and 330 nm. The detection was made using MS as well, and the analyses were carried out using an electrospray (ESI) interface operating in positive mode using the following conditions: drying gas flow, 9.0 L/min; nebulizer pressure, 35 psi; drying gas temperature, 350 °C; capillary voltage, 3000 V; and fragmentor voltage, 60 V; and also using atmospheric pressure chemical ionization (APCI) interface operating in negative mode using the following conditions: drying gas flow, 9.0 L/min; nebulizer pressure, 30 psi; drying gas temperature, 350 °C; vaporizer temperature, 450 °C; capillary voltage, 3000 V; and fragmentor voltage, 60 V. The polarity of ESI and APCI and all of the parameters of MS detector were optimized using the height of the MS signal for the phenolic compounds isolated previously from the methanol−water extracts of extra VOO as an analytical parameter.

Using the same mobile phases, another gradient was used to confirm the purity of the isolated compounds keeping in mind the fact that they only produced one peak in the chromatograms. The solvent gradient changed according to the following conditions: from 0 to 10 min, 90% A:10% B to 70% A:30% B; from 10 to 20 min, 70% A:30% B to 65% A:35% B; from 20 to 30 min, 65% A:35% B to 55% A:45% B; from 30 to 40 min, 55% A:45% B to 50% A:50% B; from 40 to 45 min, 50% A:50% B to 5% A:95% B; from 45 to 48 min, 5% A:95% B to 95% A:5% B.

Quantification of Isolated Compounds. Three standard calibration curves were done to quantify the isolated phenolic compounds using two commercial reference compounds, DOPAC (3,4-dihydroxyphenylacetic acid) and oleuropein (oleuropein glucoside). Tyrosol and hydroxytyrosol were quantified using the calibration curve of DOPAC at 280 nm; (+)-pinoresinol, (+)-1-acetoxypinoresinol, deacetoxy oleuropein aglycon, oleuropein aglycon, and ligstroside aglycon were quantified with the calibration curve of oleuropein obtained at 280 nm. However, the curve of oleuropein at 240 nm was used for the quantification of elenolic acid. The analyses were carried out using the analytical HPLC column and the gradient of 75 min.

Radical Scavenging Activity of Isolated Phenolic Compounds. The DPPH radical scavenging activity of the isolated compounds was evaluated following the Parejo et al. (*23*) and Brand-Williams et al. (*24*) analytical protocols, modified according to that of Rotondi et al. (*25*). Briefly, 2.9 mL of DPPH (100 μM) in methanol/water (80:20, v/v) solution was added to 0.1 mL of the solution of the isolated phenolic compound to be tested. After 30 min at 23 °C, absorbance was measured at 515 nm and compared to a control sample prepared without adding phenolic compounds [with 0.1 mL of methanol/water (50:50, v/v) solution]. The radical scavenging activity was expressed as TEAC (Trolox equivalent antioxidant capacity) based on a calibration curve ($r^2 = 0.9971$).

Oxidative Stability of Oil Samples. The oxidative stability of the phenolic compounds was also evaluated by the OSI, using an eight-channel OSI (Omnion, Decatur, IL). The instrumental conditions were set following the analytical protocol described by Jebe et al. (*26*); to obtain the OSI, a stream of purified air (120 mL min^{-1} air flow rate) was passed through the sample of oil-free phenolic compounds (with 50 or 218 mg/kg of each compound individually), and the effluent air for the oil sample was then bubbled through a vessel containing deionized water. The effluent air contained volatile organic acids swept from the oxidizing oil, which increased the conductivity of the water. The temperature to carry out this test was 110 °C. The OSI index (or OSI time) was expressed in hours ($n = 4$).

As we have commented before, triolein was used as a glyceridic matrix without phenolic compounds for OSI studies. We prepared triolein spiked with the individual phenolic compounds as follows: 0.25 or 1.09 mg of each isolated compound dissolved in methanol was added to 5 g of triolein for doing the experiments in the OSI. We added these quantities to check the results at two different concentrations of each analyte (50 or 218 mg/kg in each case). After homogenization by intensive shaking, the solvent was evaporated using nitrogen. We decided to add these quantities of the isolated compounds because the compounds under study were found in extra VOO samples at similar concentrations (or in the same order of magnitude).

Electrochemical Study of these Single Phenolic Compounds. The electrochemical behavior of the studied phenols was measured [in equimolar solution (10 μM; approximately between 1.38 and 4.16 ppm depending on the molecular weight of the compound)] using hydrodynamic voltammetry performed in FIA. Three replicate injections for each potential step were performed in the potential interval 0−300 mV vs Ag/AgCl; the increasing potential step was 25 mV, and the flow rate was 150 μL/min. The buffer was phosphate in a concentration of 50 mM at a pH of 7.4 with 50 mM KCl. Twenty microliters of sample was injected in the flowing buffer, and the current produced in the electrochemical oxidation was recorded.

The cyclic voltammograms of the single phenolic compounds were performed using a stop flow approach. This procedure allowed the use of smaller amounts of sample (a fact that is interesting because of the limited avaibility of HPLC fractions). The voltammetric scans were performed in the range −100 to +700 mV vs Ag/AgCl with a scan rate of 50 mV/s. The anodic peaks offered an insight into the electrochemistry of the single phenolic compounds.

A classification of the antioxidant power (AOP) of the evaluated phenols was proposed considering as a major feature the oxidation plateau regions of the hydrodynamic voltammograms; when a comparable oxidation potential was recorded, the higher current produced at that potential was considered as an index of energetically favored oxidation (higher AOP).

RESULTS AND DISCUSSION

Confirmation of the Purity of the Isolated Phenolic Compounds in VOO. With the use of two different gradients in the analytical scale column and performing the detection at three wavelengths and also using MS (ESI interface operating in positive mode and APCI interface operating in negative mode), we were able to confirm the purity of these isolated compounds. **Table 1** shows the retention time, absorption maxima, and the fragmentation patterns using the two interfaces for all of these described compounds, and in **Figure 2**, the chromatographic profiles of an extra VOO and individual isolated compounds obtained using an analytical column are shown. For the isolation of elenolic acid, we worked at 240 nm, but it is also added in the figure in order to show the analysis of all of the isolated compounds using the gradient of 75 min.

Calibration Curves. Standard calibration graphs were prepared for two reference compounds: DOPAC and oleuropein (oleuropein glucoside) at two wavelengths. It was impossible to use the standards (isolated phenolic compounds) obtained with semipreparative HPLC to investigate method linearity because the quantity obtained of each compound using HPLC was not enough to be weighed and to determine the exact weight of each one; this fact caused all of the compounds quantified in this work to be expressed in terms of two commercial standards (DOPAC and oleuropein glucoside).

The detection limit (DL), quantification limit (QL), and precision [as relative standard deviation (RSD) of the intermediate concentration value of the linear range] of this method were calculated for the studied analytes using the method proposed by Curie (*27*).

All calibration curves showed good linearity between different concentrations depending on the analytes studied. Each point of the calibration plot was repeated three times in the same way. The calibration plots indicate good correlation between peak areas and analyte concentrations; regression coefficients were higher than 0.990 for oleuropein and DOPAC at the two wavelengths. All of the features of the proposed method are summarized in **Table 2**.

Radical Scavenging Activity (DPPH Test). The H-transfer reactions are monitored by UV−vis spectroscopy recording the

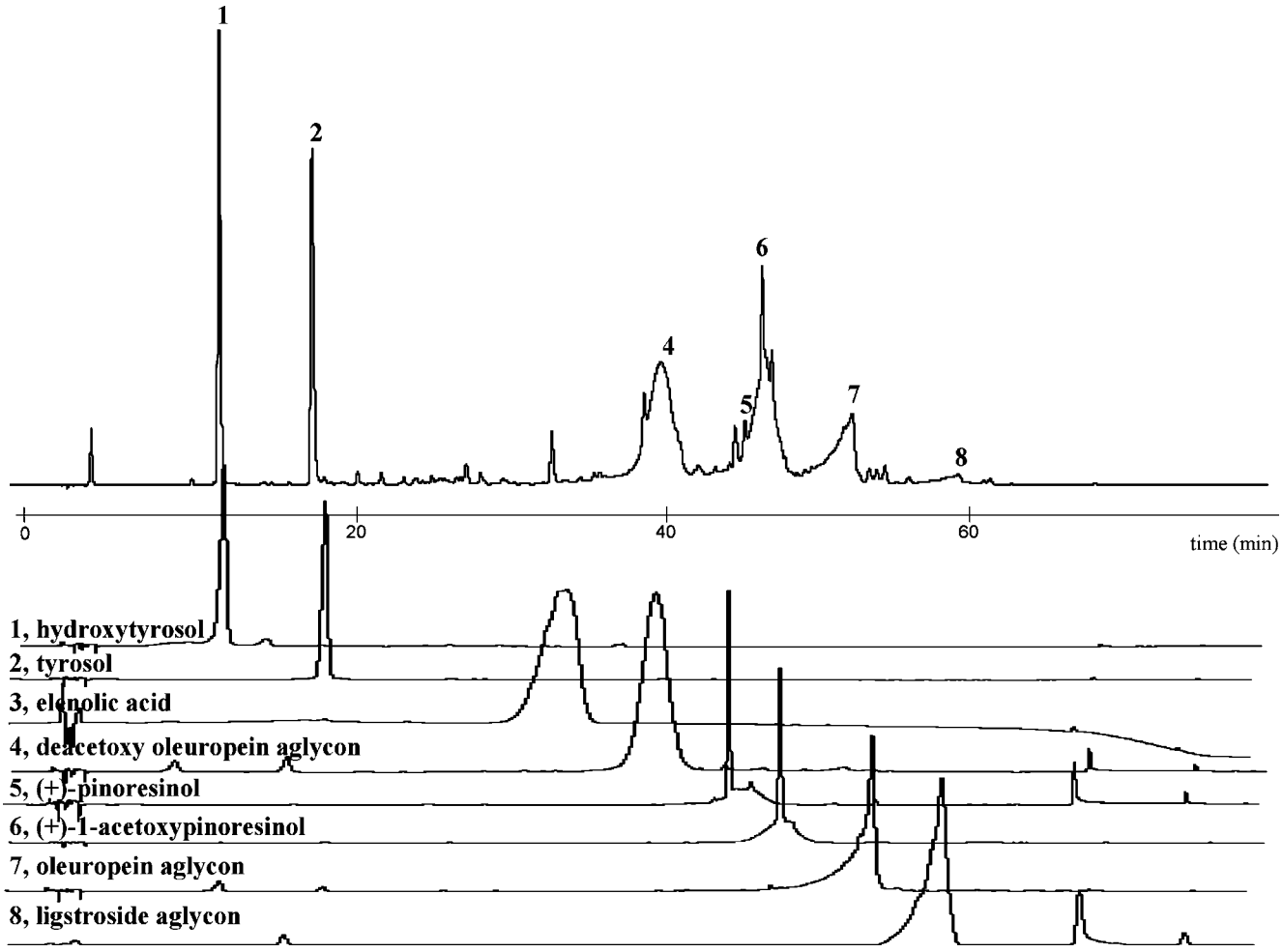

Figure 2. Chromatograms of extra VOO sample and the isolated phenolic compounds under optimized conditions using analytical HPLC. Detection was performed at 280 nm (only in the analysis of elenolic acid, number **3**, the detection was performed at 240 nm).

Table 1. Retention Times, Absorption Maxima, and the Fragmentation Patterns Using the Two Interfaces for All of the Compounds under Study[a]

				major fragments APCI negative				major fragments ESI positive					
analyte	t_r (min)	λ_{max} (nm)	molecular mass	$[M-H]^-$	$[2M-H]^-$	$[M-H_2O]^-$	other fragments	$[M+H]^+$	$[2M+H]^+$	$[M+Na]^+$	$[2M+Na]^+$	$[M-H_2O+H]^+$	other fragments
HYTY	11.64	232/280	154	153.1		137.1	225.1					137.1	
TY	17.74	230/276	138		nondetectable in APCI							121.1	183.1
EA	35.75	240	242	241.1				243.1	485.1	265.1	507.2	225.1	
DAOA	40.25	234/282	320	319.2	639.1	301	195			343.1			137.1
							163.1						
PIN	45.41	234/280	358	357.2				359.1		381.2	739.0	341.1	398.7
													403.1
ACPIN	46.57	234/280	416	415.1	831.1			417.1				398.4	385.1
													485
OA	53.11	236/282	378	377.1	755.1			379.1		401.1			417.1
													137.1
													225.1
LA	59.65	230/276	362	361.2		344		363.1		385.1			121.1
													225.1

[a] HYTY, hydroxytyrosol; TY, tyrosol; EA, elenolic acid; DAOA, deacetoxy oleuropein aglycon; PIN, (+)-pinoresinol; ACPIN, (+)-1-acetoxypinoresinol; OA, oleuropein aglycon; LA, ligstroside aglycon.

decay of the DPPH visible absorption band (λ_{max} = 515 nm in MeOH) that reflects the conversion of the DPPH radical into the corresponding colorless hydrazyne (DPPH-H) by the antioxidant. The antioxidant potential of the isolated phenolic compounds is shown in **Table 3**; the test was done at two different concentrations for each compound (50 and 218 ppm).

A number of structural features seem to be important in determining the antioxidant trend. The foremost consideration is the extent and nature of the hydroxylation pattern of the aromatic rings. We can establish a classification of these compounds according to their AOP. In decreasing order, with 218 ppm of each compound, we have hydroxytyrosol > deacetoxy oleuropein aglycon > oleuropein aglycon > (+)-pinoresinol > ligstroside aglycon > tyrosol > elenolic acid > (+)-1-acetoxypinoresinol. Doing the same test with 50 ppm of each compound, the order was practically the same: hydroxy-

Table 2. Analytical Parameters of the Proposed Method

analyte	RSD (%) (intermediate value)	DL (μg/mL)	QL (μg/mL)	calibration range (μg/mL)[a]	calibration equations	r^2
3,4-dihydroxyphenylacetic acid	3.65	0.22	0.73	2000	$y = 21.30x - 20.91$	0.9988
oleuropein glucoside, $\lambda = 280$ nm	3.37	0.99	3.30	4000	$y = 4.65x - 4.02$	0.9967
oleuropein glucoside, $\lambda = 240$ nm	4.93	0.34	1.11	4000	$y = 13.96x + 2464.60$	0.9901

[a] From QL to the value in the table.

Table 3. Values of DPPH Test for the Isolated Phenolic Compounds at Two Different Concentrations and Order of Classification in Terms of Antiradical Power [Mean Values ± SD ($n = 6$)][a]

	ARP (218 ppm)	classification	ARP (50 ppm)	classification
hydroxytyrosol	26.23 ± 0.56	**1**	5.03 ± 0.10	**1**
tyrosol	0.76 ± 0.05	**6**	0.18 ± 0.01	**8**
elenolic acid	0.69 ± 0.04	**7**	0.29 ± 0.01	**7**
deacetoxy oleuropein aglycon	7.61 ± 0.29	**2**	2.42 ± 0.14	**2**
(+)-pinoresinol	1.35 ± 0.08	**4**	1.08 ± 0.03	**4**
(+)-1-acetoxy-pinoresinol	0.60 ± 0.03	**8**	0.30 ± 0.01	**6**
oleuropein aglycon	6.11 ± 0.12	**3**	1.54 ± 0.03	**3**
ligstroside aglycon	0.79 ± 0.04	**5**	0.38 ± 0.03	**5**

[a] ARP, antiradical power (in μmol/g).

tyrosol > deacetoxy oleuropein aglycon > oleuropein aglycon > (+)-pinoresinol > ligstroside aglycon > (+)-1-acetoxy-pinoresinol > elenolic acid > tyrosol.

Phenolic compounds can be active as antioxidants for a number of potential factors. The most important is likely to be by free radical scavenging in which the phenol can break the free radical chain reaction. The presence of different substituents in the phenol backbone structures modulates their antioxidant properties, in particular their hydrogen-donating capacities.

We have proved that molecules with *o*-dihydroxyl functionalities are characterized by high antioxidant activity because of the formation of intramolecular hydrogen bonds during the reaction with the free radicals, but it is necessary to underline that the electronic and steric effects of substituents near the phenolic hydroxyl groups may be of importance in governing the hydrogen-donating capacity of monohydroxyl phenols. Electron-donating substituents in the ortho position tend to weaken the O−H bond of phenol and provide extra stability to the phenoxyl radical.

Hydroxytyrosol was the analyte that was found to exert the strongest antioxidant activity. Between deacetoxy oleuropein aglycon and oleuropein aglycon, it was logical that the first was more efficient as an antioxidant than oleuropein aglycon; the group $-COOCH_3$ in oleuropein aglycon caused a decrease in the antioxidant capacity because it is not an electron donor group. This fact could be observed when we studied the different antioxidant capacities of (+)-pinoresinol and (+)-1-acetoxy-pinoresinol where $-COOCH_3$ is also present. We also observed in these compounds that the *o*-methylation ($-OCH_3$) of the hydroxyl group of the benzoic rings caused a decrease in the antioxidant capacity, because this group was not able to form a hydrogen bond, in comparison with molecules having a catecholic group in their structure. Tyrosol and ligstroside aglycon are monosubstituted phenols and, as was expected, exhibited a very poor radical scavenging activity.

Elenolic acid is not a phenol, but undoubtedly, it is contained in the polar extract of VOO. Its presence in olive oil in a free form is due to the hydrolysis of oleuropein, ligstroside, and related compounds. At the two different concentrations that we studied, elenolic acid is one of the compounds that presents a weaker antioxidant activity; this result suggests that radical hydrogen donor ability is not easily attributable to compounds containing the closed ring of elenolic acid. Also, Briante et al. (*14*) showed in a previous paper that elenolic acid was a very poor antioxidant as compared to hydroxytyrosol.

OSI Results. For many years, *o*-diphenols are reported as being the highest contributors to oxidative stability in VOOs (*5*, *28*). Most of the papers studying the influence of different variables on changes in polyphenols also determined the stability measured by Rancimat (a method that works according to the same principle as OSI), and good correlation coefficients between the two parameters are found in all the studies (*1*, *28*−*30*). Studies of individual compounds added to refined oils or oils stripped of antioxidants demonstrate the important contribution of hydroxytyrosol in the effective inhibition of oxidation (*30*−*33*). Servili et al. (*33*) found that the oleosidic forms of hydroxytyrosol have the same antioxidant efficiency as hydroxytyrosol. However, in this study, the OSI test is applied to a glyceridic matrix-free phenolic compounds spiked with eight singular phenolic compounds.

To estimate the stability or susceptibily of a fat to oxidation, the sample can be subjected to an accelerated oxidation test under standardized conditions and a suitable end point is chosen to determine signs of oxidative deterioration. Several parameters (such as temperature, metal catalysts, oxygen pressure, and shaking) are manipulated to accelerate oxidation and the development of rancidity in oils and emulsions. The induction period (IP) is measured as the time required to reach an end point of oxidation corresponding to either a level of detectable rancidity or a sudden change in the rate of oxidation. Measurements of IP under standard conditions are generally used as an index of antioxidant effectiveness.

In our case, as commented before, to obtain the OSI time, we used as instrumental conditions a temperature of 110 °C and an air air flow rate of 120 mL min^{-1}. When the results of this test are observed (in **Table 4**), it can be seen that three of these compounds have antioxidant activity, and the others have pro-oxidant effects. The OSI value for the triolein without any phenolic compound was 9.08 h ($n = 8$); this time was longer when hydroxytyrosol, deacetoxy oleuropein aglycon, and oleuropein aglycon were added to the ≈5 g of triolein; however, the OSI time was shorter when triolein was spiked with elenolic acid, (+)-1-acetoxypinoresinol, ligstroside aglycon, tyrosol, and (+)-pinoresinol.

Among the isolated phenolic compounds with antioxidant activity, the classification in terms of AOP was, in decreasing order: hydroxytyrosol > deacetoxy oleuropein aglycon > oleuropein aglycon. The pro-oxidant compounds can be ranked according with the OSI index: (+)-pinoresinol > tyrosol > ligstroside aglycon > (+)-1-acetoxypinoresinol > elenolic acid. These classifications are for the compounds added in a

Table 4. OSI Results for the Eight Isolated Phenolic Compounds at Two Different Concentrations and Their Classification in Terms of Resistance Time to Oxidation [Mean Values ± SD ($n = 4$)]

	OSI (218 ppm)	classification	OSI (50 ppm)	classification
hydroxytyrosol	16.25 ± 0.61	**1**	11.40 ± 0.46	**1**
tyrosol	3.20 ± 0.12	**7**	5.15 ± 0.21	**7**
elenolic acid	7.98 ± 0.29	**4**	8.55 ± 0.28	**5**
deacetoxy oleuropein aglycon	11.25 ± 0.54	**2**	9.15 ± 0.23	**3**
(+)-pinoresinol	3.12 ± 0.09	**8**	4.86 ± 0.23	**8**
(+)-1-acetoxy-pinoresinol	7.58 ± 0.20	**5**	8.71 ± 0.43	**4**
oleuropein aglycon	9.76 ± 0.39	**3**	9.53 ± 0.41	**2**
ligstroside aglycon	3.50 ± 0.13	**6**	7.11 ± 0.21	**6**

concentration of 50 mg/kg. For 218 mg/kg, the order for the antioxidants was the same, but for the pro-oxidants, the order was (+)-pinoresinol > tyrosol > ligstroside aglycon > elenolic acid > (+)-1-acetoxypinoresinol.

Nevertheless, it is important to highlight that high temperature stability tests have several limitations. First, because reactions are very rapid and the concentration of hydroperoxide (ROOH) decreases rapidly with increasing temperature, it is obvious that phenolic antioxidants will become less effective with increasing temperature. Second, because ROOH are the products of the reaction of alkylperoxy radicals with the antioxidant, their immediate decomposition could even lead to chain propagation rather than chain breaking under these conditions. Finally, side reactions of oxidation or thermolabity of phenolic compounds also have to be considered. However, the result of the OSI test may help to understand how phenolic compounds act as antioxidants and how their chemical structure contributes.

Electrochemical Results for the Single Phenolic Compounds Studied. In a previous work, the correlation between the oxidation potential measured in cyclic voltammetry and the antioxidant activity was described (*34*). Our group proposed a FIA−amperometry method to evaluate the radical scavenging activity of phenolic extract of VOO (*2*). The electrochemical study of these phenolic compounds using a glassy carbon working electrode offers the possibility of investigating the functional (antioxidant) characteristics of the isolated phenols without the use of a reactive compound.

Both cyclic voltammetry and hydrodynamic voltammetry were performed on the single phenolic compounds obtained by semipreparative HPLC, and a classification of the AOP of the evaluated phenols was proposed on the basis of the electrochemical results.

Electron-donating substituents in the ortho position of phenolic molecules tend to weaken the O−H bond of phenol and provide extra stability to the phenoxyl radical. It is important to stress that the electrochemical oxidation of the phenolic moiety goes through the formation of a phenoxyl radical. Therefore, it can be assumed that the AOP, measured on the basis of electrochemical features (oxidation potential and current) of the phenolic molecules, may better describe the radical scavenging activity as previously hypothesized and experimentally shown (*2*, *35*).

Considering the results obtained in cyclic voltammetry experiments, it is logical to deduce that between deacetoxy oleuropein aglycon and oleuropein aglycon, the first has an higher AOP (lower oxidation potential) than the oleuropein aglycon. A similar electrochemical behavior was found when we studied the different AOPs of (+)-pinoresinol and (+)-1-acetoxypinoresinol where there is the $-COOCH_3$ too. We also

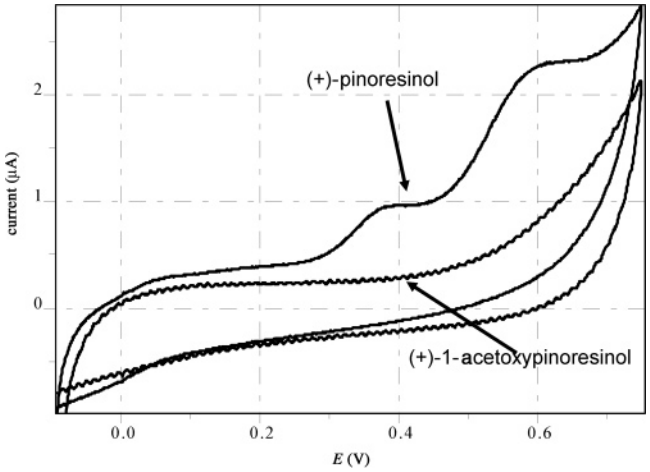

Figure 3. Cyclic voltammograms of (+)-pinoresinol and 1-(+)-acetoxy-pinoresinol.

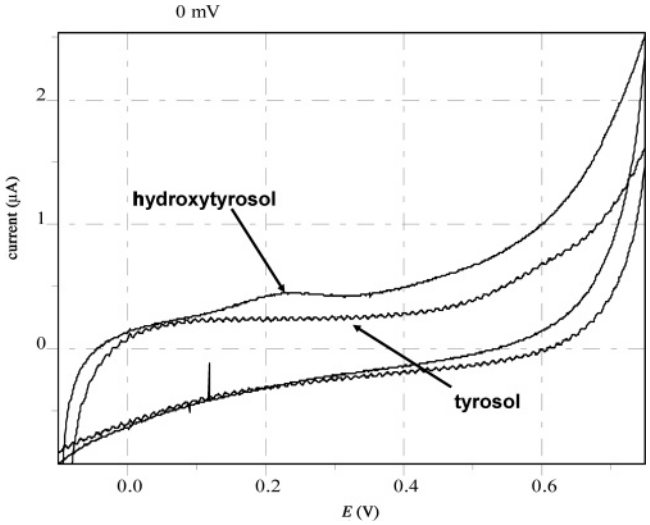

Figure 4. Cyclic voltammograms of hydroxytyrosol and tyrosol.

observed that the *o*-methylation (−OCH3) of the hydroxyl group of the benzoic rings caused a higher oxidation potential and a lower anodic current (see **Figure 3**). In these voltammograms, it is evident that the substitution of a hydrogen atom with an acetoxyl group completely changes the electrochemical behavior of the molecule eliminating the occurrence of anodic peaks. The importance of an electron donating group was also evidenced by the cyclic voltammograms of tyrosol and hydroxytyrosol where the presence of the −OH group determines the appearance of an anodic peak at +250 mV (**Figure 4**). Finally, ligstroside aglycon is a monosubstituted phenol and, as was expected, exhibited a very poor electrochemical activity, and elenolic acid, which is not a phenol, did not show electrochemical activity.

Considering the results obtained in hydrodynamic voltammetry experiments (data reported in **Table 5**), it is possible to see some plateau regions for each molecule. The plateau regions correspond to the oxidation potential of a molecule moiety, thus indicating the energy required for the oxidation; this value is inversely proportional to the AOP of the molecule (*2*, *35*). On the basis of the lower oxidation potential, we have proposed an AOP scale as follows: hydroxytyrosol > oleuropein aglycon > (+)-pinoresinol > deacetoxy oleuropein aglycon > (+)-1-acetoxypinoresinol > tyrosol> ligstroside aglycon = elenolic acid. This simple method, based on the electron-donating capability of each molecule to the electrode, may contribute to

Table 5. Plateau Potential Regions (E_{ox}) Evaluated by Hydrodynamic Voltammetry (HV)[a]

	E_{ox} (HV) mV vs Ag/AgCl	AOP classification
hydroxytyrosol	0.12–0.17	**1**
tyrosol	0.35	**6**
elenolic acid	inactive	**7**
deacetoxy oleuropein aglycon	0.15–0.30	**4**
(+)-pinoresinol	0.15–0.22–0.30	**3**
(+)-1-acetoxypinoresinol	0.22	**5**
oleuropein aglycon	0.15–0.20–0.30–0.40	**2**
ligstroside aglycon	inactive	**7**

[a] The AOP classification is based on the lower oxidation potential.

the understanding of the structure−function relationship of radical scavengers.

We should underline the differences among the results of the three tests. The reason for these discrepancies may be that while the DPPH test values the capacity of a group to block a radical (antiradical activity), the other test (OSI) allows the evaluation of the behavior of these groups also in the presence of oxygen (antioxigen and antiradical activity). To show the different behavior, we can use as an example the obtained values for lignans. The result of the chemical method (DPPH) showed that both lignans [(+)-pinoresinol and (+)-1-acetoxypinoresinol] are antioxidants and (+)-pinoresinol exerted a stronger antioxidant activity than (+)-1-acetoxypinoresinol; on the other hand, the result of the OSI test showed that both lignans have a pro-oxidant effect, but this pro-oxidant action is remarkable in the case of (+)-pinoresinol. To justify this result, it is important to consider their chemical structures, which contain one atom of oxigen in each central ring; this atom, in conditions of thermal stress, could cause the opening of the ring; therefore, the lignans could act as pro-oxidants. The $-COOCH_3$ group in the (+)-1-acetoxypinoresinol hinders the opening of the ring and causes a weaker pro-oxidant effect.

The electrochemical study of these phenolic compounds using a glassy carbon working electrode offers the possibility of investigating the functional (antioxidant) characteristics of the isolated phenols without the use of a reactive compound; their resulst are very similar to those obtained using the DPPH test.

In conclusion, in this work, the antioxidant capacity of simple phenols, lignans, and secoiridoids has been investigated. The antioxidant activity was evaluated by DPPH, OSI, and an electrochemical method and was closely related to the chemical structure of the isolated phenolic compounds.

The ability to act as a hydrogen donor and the inhibition of oxidation are enhanced by increasing the number of hydroxyl group in the phenols. We verified that, as is generally assumed, the presence of a single hydroxyl group confers a limited amount of antioxidant activity. On the other hand, the presence of an *o*-diphenol enhances the ability of the phenolic compounds to act as antioxidants.

The results obtained for the three tests show that hydroxytyrosol, deacetoxy oleuropein aglycon, and oleuropein aglycon are the strongest in the classification in terms of AOP. It is very interesting to observe the results obtained for the OSI test, where several of the compounds under study showed antioxidant activity, and the others showed pro-oxidant effects.

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Received for review July 1, 2005. Revised manuscript received September 8, 2005. Accepted September 14, 2005.

JF0515680