

**Phenolic Compounds:
Their Role During Olive Oil Extraction and in Flaxseed
– Transfer and Antioxidant Function**

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BITTER
INDEX**

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Objective 2.1

Objective 2.2

Objective 2.3

**Enrichment of Refined Olive Oil with Phenolic Compounds:
 Evaluation of Their Antioxidant Activity and Their Effect on the
 Bitter Index**

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The study of the antioxidant effects of biophenolic compounds is supported by the current interest in natural products and the ongoing replacement of synthetic antioxidants by natural antioxidants from plant sources. Olives and olive oil, especially extra virgin olive oil, contain a variety of bioactive compounds (phytochemicals) widely considered to be potentially beneficial for health. This research was focused on evaluating the antioxidant activity of the enriched refined olive oil to discover a possible functional food application. Different concentrations of individual and combined phenolic compounds were added to the refined olive oil as lipid matrix, and the antioxidant activity expressed as oxidative stability in hours was determined by using the Rancimat method. Additionally, the bitter index was evaluated to assess the effect of the enrichment in relation to the organoleptic quality. The results showed that the antioxidant activity depends on the concentration of the phenol used for the assay and the chemical structure. In general, the most positive effects were observed in 3,4-dihydroxy and 3,4,5-trihydroxy structures linked to an aromatic ring that conferred to the moiety a higher proton dislocation, thus facilitating the scavenging activity.

KEYWORDS: Phenolic compounds; enrichment; antioxidant activity; bitter index

INTRODUCTION

The study of the antioxidant effects of biophenolic compounds is supported by the current interest in natural products and the ongoing replacement of synthetic antioxidants by natural antioxidants from plant sources, as well as the screening of raw materials to identify new antioxidants (1–3). Synthetic antioxidants are widely used in the food industry. However, concerns about their safety have switched interest to natural antioxidants. Tocopherols, ascorbic acid, rosemary extracts, lycopene, and some flavonoids are now available for adding to foods as replacements for synthetic products.

The enrichment of processed food with polyphenols protects against oxidation and means better keeping quality because the formation of toxic oxidation products, such as cholesterol oxides, is prevented. Such enrichment also benefits human health. Both of these benefits, however, depend on the availability of the phenolic substances (4). In that sense, functional foods, including whole foods and fortified, enriched, or enhanced foods, have a potentially beneficial effect on health when consumed on a regular basis as part of a varied diet, at effective levels (5).

Foods preserved with added natural antioxidants could be considered as functional foods because these products can provide better health conditions to the consumer (6). The use

of plant material and plant extracts as food ingredients will go beyond antioxidative effects. The “green revolution” for the food ingredients industry is to modify plants to produce molecules that combine emulsifying and/or thickening effects with antioxidative and antimicrobial effects. Olives and olive oil, especially extra virgin olive oil, contain a variety of bioactive compounds (phytochemicals) widely considered to be potentially beneficial for health (7). The responsibility for such beneficial properties is assigned to both an adequate fatty acid profile and the presence of antioxidants such as the phenolic compounds. Evidence demonstrates that olive oil phenolic compounds are powerful antioxidants, both *in vitro* and *in vivo* (8). Although further studies are required, the water-soluble olive oil polyphenols might be good candidates as functional food ingredients in the future, potentially combating the development of cardiovascular disease, cancer, and inflammatory reactions.

There is scientific evidence for the availability of a qualified health claim for monounsaturated fat from olive oil and reduced risk of coronary heart disease (CHD). In 2004, the U.S. Food and Drug Administration (FDA) reported that there is limited but not conclusive scientific evidence to suggest that eating ≈23 g of olive oil daily may reduce the risk of coronary heart disease due to the monounsaturated fat in olive oil (9). To achieve this possible benefit, olive oil must replace a similar quantity of saturated fat without increasing the total daily calorie intake. However, virgin olive oil is not an important source of phenols

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compared with other plant sources. The phenol concentration in virgin olive oil is between 20 and a maximum of 800 mg/kg. That concentration does not suppose an important daily intake of phenols considering virgin olive oil as a source of dietetic phenols related to the limitation of this consumption and the supply of calories. As a consequence, the enrichment of olive oil with phenols could be an important development in functional food.

The antioxidant power of phenolic compounds occurring in olive fruit, olive oil, and olive mill wastewater has been studied recently, and different systems and methods have also been tested. These studies have focused on the evaluation of the antioxidant activities of phenol extracts or their fractions (3, 10–13). Farag et al. (14) found that phenolic extracts obtained from the olive plant (fruit, leaves, and pomace) showed remarkable antioxidant activity in retarding sunflower oil oxidative rancidity. Gordon et al. (15) compared the antioxidant activity of hydroxytyrosol acetate synthesized with that of other olive oil polyphenols in different lipid systems.

Adding antioxidants to food matrices leads to the evaluation of the real effect of these compounds and their possible interaction with other components occurring naturally in the product. Olive oil, the primary source of fat in the Mediterranean diet, is a good matrix for enrichment processes, not only as a product of direct consumption but also as an ingredient in new industrial foodstuffs.

A previous study carried out by our research group characterized the antioxidant activities of phenolic compounds that appear in olive pulp and olive oil using both radical scavenging and antioxidant activity tests (16). As an extension of these studies, this research was focused on evaluating the antioxidant activity of the enriched refined olive oil in order to discover a possible functional food application. Different concentrations of individual and combined phenolic compounds were added to the lipid matrix, and the antioxidant activity expressed as oxidative stability in hours was determined by using the Rancimat method. Additionally, the bitter index was evaluated to assess the effect of the enrichment in relation to the sensorial quality.

MATERIALS AND METHODS

Reference Compounds. α -Tocopherol was purchased from Sigma-Aldrich Chemical Co.. Phenolic standards from the following sources were used without further purification: 4',5,7-trihydroxyflavone (apigenin), apigenin 7-*O*-glucoside, *trans*-4-hydroxycinnamic acid (*p*-coumaric acid), 2-(3,4-dihydroxyphenyl) ethyl alcohol (hydroxytyrosol) (3,4-DHPEA), 3',4',5',7-tetrahydroxyflavone (luteolin), luteolin 7-*O*-glucoside, oleuropein, 2-(4-hydroxyphenyl) ethyl alcohol (tyrosol) (*p*-HPEA), and vanillin from Extrasynthese (Genay, France); 3,4-dihydroxycinnamic acid (caffeic acid), *trans*-2-hydroxycinnamic acid (*o*-coumaric acid), ferulic acid, 3,4,5-trihydroxybenzoic acid (gallic acid), and 4-hydroxy-3-methoxybenzoic acid (vanillic acid) from Fluka Co. (Buchs, Switzerland); and 3,4-dihydroxyphenylacetic acid (DOPAC), 4-hydroxybenzoic acid, 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid), and 3-phenyl-2-propenoic acid (*trans*-xinnamic acid) from Sigma-Aldrich Chemical Co.

Isolated and Acquired Compounds. Secoiridoid derivatives 4-(acetoxyethyl)-1,2-dihydroxybenzene (3,4-DHPEA-AC), 4-hexenoic acid, 4-formyl-3-(2-oxoethyl)-2-(3,4-dihydroxyphenyl) ethyl ester (3,4-DHPEA-EDA), 4-hexenoic acid, 4-formyl-3-(2-oxoethyl) 2-(4-hydroxyphenyl) ethyl ester (*p*-HPEA-EDA), 2*H*-pyran-4-acetic acid, 3-formyl-3,4-dihydro-5-(methoxycarbonyl)-2-methyl-2-(4-hydroxyphenyl) ethyl ester (3,4-DHPEA-EA), and 2*H*-pyran-4-acetic-, 3-formyl-3,4-dihydroxy-5-(methoxycarbonyl)-2-methyl-2-(4-hydroxyphenyl) ethyl ester (*p*-HPEA-EA) were isolated from virgin olive oil by using the semipreparative HPLC method.

Lipid Matrix. Refined olive oil (ROO) obtained from an industrial process was used as lipid matrix to perform the enrichment experiment

with the phenolic compounds and to carry out further studies on antioxidant activity and bitter index. The ROO was obtained by physical refining, a technology not commonly used for seed oils. The olive oil was bleached under vacuum with a mixture of synthetic silicas (aluminum silicate and magnesium silicate) and filtered. The bleached oil was deodorized in a continuous distillator operating at a vacuum (<2 mmHg) to remove the free fatty acids and volatiles. Then, the oil was alkali refined (sodium hydroxide) in a continuous system to eliminate the remaining free fatty acids. The ROO was stored at 5 °C in dark bottles with nitrogen in the headspace.

Lipid Matrix Characterization. Quality Parameters. The free fatty acid content, peroxide value, and ultraviolet absorption at 270 and 232 nm were determined according to the European Union Commission Regulation EEC/2568/91 (17). The results were expressed as a percentage of oleic acid, milliequivalents of active oxygen per kilogram of oil (mequiv of O₂/kg), and absorbance at 270 nm, respectively.

Oil Composition. α -Tocopherol was quantified by high-performance liquid chromatography (HPLC) with direct injection of an oil-in-hexane solution (18). Chromatograms were recorded at 295 nm, and α -tocopherol was quantified by using an external standard method. The results are expressed as milligrams per kilogram of oil.

The total phenol content was analyzed using the modified isolation method described by Vazquez-Roncero et al. (19). The total concentration of phenols was estimated with Folin–Ciocalteu reagent at 725 nm. The results are expressed as milligrams of caffeic acid per kilogram of oil. The phenolic profile was analyzed according to the method described in Romero et al. (20). Chromatograms were obtained at 280 and 339 nm.

The chlorophyll fraction at 670 nm and the carotenoid fraction at 470 nm were evaluated from the absorption spectrum of each olive oil sample dissolved in cyclohexane (21). The chlorophyll and carotenoid contents are expressed as milligrams of major pigment, pheophytin *a*, and lutein per kilogram of oil, respectively.

The fatty acid composition was determined by gas chromatography (GC) as fatty acid methyl esters (FAMES). FAMES were prepared by saponification/methylation with sodium methylate according to the European Union Commission modified Regulation EEC 2568/91 (17).

Isolation of Phenolic Compounds from Olive Oil by Semipreparative HPLC. Phenolic extracts were obtained from virgin olive oil according to the method of Romero et al. (20). The semipreparative system includes a Waters 1525EF binary HPLC pump, a Waters Flexinject, an Inertsil ODS-3 column (5 μ m, 25 cm \times 10 mm i.d., GL Sciences Inc.) equipped with a Sphersorb S5 ODS-2 (5 μ m, 10 cm \times 10 mm i.d., Technokroma, Barcelona, Spain) precolumn, a Waters 2487 dual λ absorbance detector (280 and 339 nm), and a Waters Fraction Collector II. The HPLC semipreparative system was operated using Brezze software.

Phenolic compounds were isolated from phenolic extract using the method reported by Morelló et al. (16) with modifications. Extract (500 μ L aliquot) was injected manually into the injector module (sample loop of 1 mL). The gradient elution program employed water/acetic acid (100:0.2 v/v) as solvent A and methanol as solvent B with a flow rate of 4 mL/min. Solvent A was decreased linearly from 90% at 0 min to 70% at 8 min with a 17-min isocratic time, then reduced to 60% at 35 min, followed by a further linear decrease to 50% at 40 min, held constant for 5 min, and then reduced to 40% at 49 min, followed by a further linear decrease to 0% at 51 min, held isocratically for 2 min, then ramped to 90% at 60 min, and held constant for 5 min. The fractions were collected manually by observing the detector output on the recorder and according to their retention time. The collected fractions were concentrated to dryness by rotary evaporation at reduced pressure, lyophilized, and stored at -40 °C and N₂ atmosphere before the enrichment of lipid matrices. Each selected (purified) fraction was confirmed by its retention time and UV spectra (diode array detector) in HPLC analytical conditions by comparison of HPLC chromatograms of phenolic extracts from virgin olive oil (20). Additionally, the mass spectra of selected (purified) fractions were performed on a ZMD mass spectrometer (Waters Inc., Milford, MA). The specific operating parameters for the electrospray mass spectrometry included the following: capillary voltage, 2.5 kV; cone voltage, 5 V; desolvation temperature, 400 °C; source temperature, 120 °C; ion mode, ESI⁺.

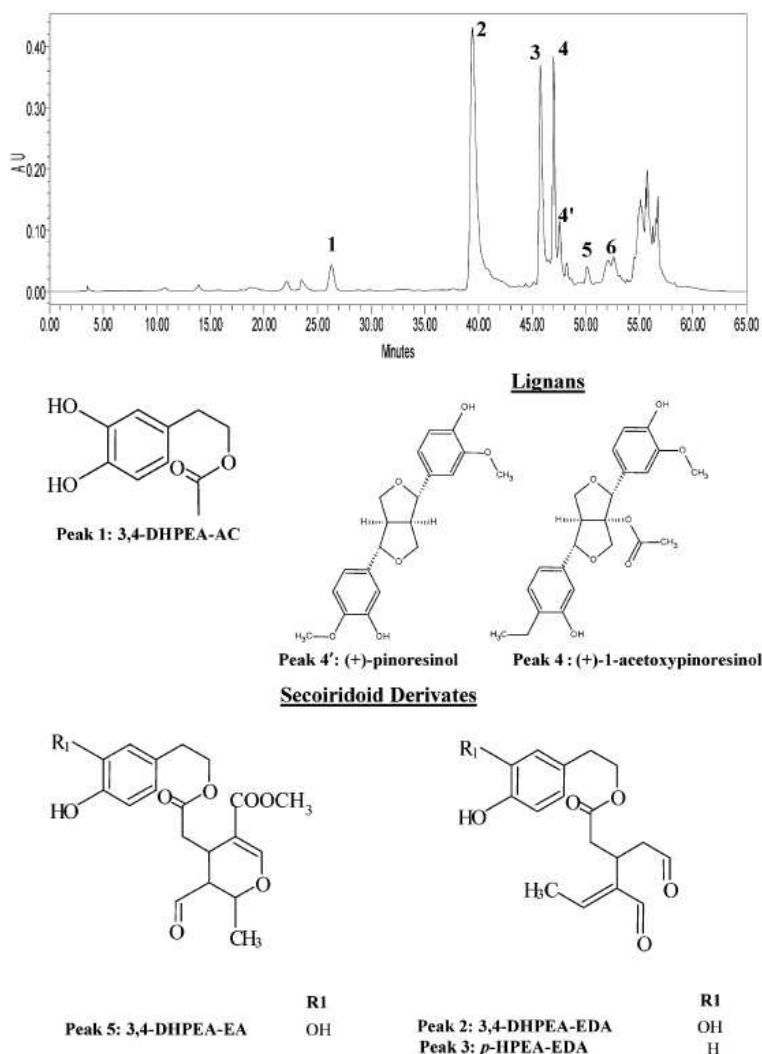


Figure 1. Semipreparative HPLC chromatogram (280 nm) of the phenolic extract from virgin olive oil and chemical structures of isolated phenolic compounds.

Figure 1 shows the semipreparative HPLC chromatogram of phenolic (280 nm) and chemical structures of the phenolic compounds isolated from virgin olive oil: 4-(acetoxylethyl)-1,2-dihydroxybenzene (3,4-DHPEA-AC), the dialdehydic form of elenolic acid linked to hydroxytyrosol (3,4-DHPEA-EDA), the dialdehydic form of elenolic acid linked to tyrosol (*p*-HPEA-EDA), lignans (1-acetoxypinoresinol + pinoresinol), oleuropein aglycon (3,4-DHPEA-EA), and the new compound (peak 6).

Figure 2 shows the chemical structures of the phenolic standards: hydroxytyrosol, tyrosol, apigenin; apigenin 7-*O*-glucoside, luteolin, luteolin 7-*O*-glucoside, vanillin, vanillic, *p*-coumaric, *o*-coumaric, caffeic, ferulic, gallic, 3,4-dihydroxyphenylacetic, 4-hydroxybenzoic, homovanillic, and *trans*-cinnamic acids, verbascoside, and oleuropein.

NMR. The NMR spectra of peak 6 were recorded on a Varian Mercury 400 spectrometer operating at 400 MHz for ^1H and at 100 for ^{13}C using TMS as external standard in methanol-*d*₄. About 20 mg of the sample was dissolved in 0.6 mL of deuterated methanol. ^1H , ^{13}C , 2D-HMBC, 2D-TOCSY, and 2D-HSQC were performed. All of the experiments were performed using standard Varian.

Phenolic Stock Solutions. Stock solutions of commercial standards were prepared by dissolving 1.0 mg of phenolic compound in 1 mL of Me-OH/H₂O (80:20 v/v). Stock solutions of phenolic compounds isolated from virgin olive oil were prepared by dissolving the dried collected fractions in MeOH/H₂O (80:20 v/v) to reach a final concentration depending on the quantity in which they occurred in virgin olive oil ranging from 0.5 to 1.0 mg/mL. Each solution was sonicated and stored at -40 °C before the enrichment of the lipid matrices.

Evaluation of Antioxidant Activity (AA). The AA was evaluated with Rancimat equipment model 679 (Metrohm, Ltda.). An air flow of 20 L/h and a temperature corresponding to 120 °C were the established parameters. Changes in conductivity caused by ionic volatile organic acids, mainly formic acids, were measured automatically and continuously. The peroxidation curve was recorded, and the flex point was selected as the induction time (IT, expressed in hours). The higher the IT value, the higher the oxidative stability of the sample.

Each phenolic stock solution was added to a reaction vessel containing 2.5 g of the lipid matrix (ROO) at the desired concentration, ranging from 40 to 320 mg/kg of oil. When the desired temperature

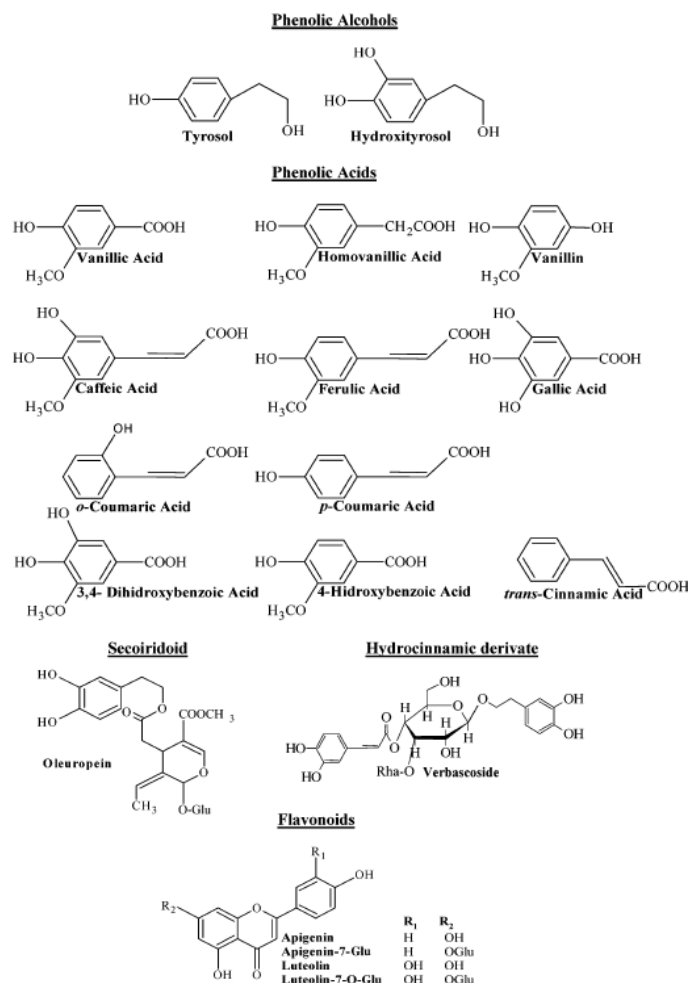


Figure 2. Chemical structures of standard phenolic compounds.

was reached, the analysis was started. Two replicates of two sets of experiments were done, and a control sample (lipid matrix without added phenolic) was evaluated in each experimental set. To normalize the effect of enrichment with different phenolic compounds at the evaluated concentrations, the antioxidant activity was expressed as the antioxidant activity index (AAI): $AAI = IT_e/IT_c$, where IT_e is the induction time of the enriched sample and IT_c is the induction time of the control sample.

The effect of antioxidant mixtures on the lipid matrix was evaluated through the Rancimat test as described above. 3,4-DHPEA-EDA, the most abundant compound in olive oil, was evaluated in combination with some standards and isolated phenolic compounds (hydroxytyrosol, caffeic acid, luteolin, apigenin, oleuropein, and lignans) at 200 and 400 mg/kg of oil concentrations. To measure the effect of phenolic compound mixtures, two sets of experiments were performed with two replicates in each case. ROO was used as a reference, and the induction time (hours) was recorded. The antioxidant activity was reported as the increase in the oxidative stability (hours) of the ROO containing phenolic compound mixtures with respect to the stability of the control sample (ROO).

Bitter Index (K_{225}) (BI). Phenol stock solution was added to 1.0 g of the lipid matrix (ROO) to the desired concentration ranging from 0

to 200 mg/kg of oil. The bitter index was measured as follows: a C18 column (Waters Sep-Pak cartridges) was activated with 6 mL of methanol and then washed with 6 mL of hexane; 1.00 ± 0.01 g of oil dissolved in 4 mL of hexane was passed through the column. After elution, 10 mL of hexane was passed to eliminate the fat, and the retained compounds were eluted with 25 mL of methanol/water (1:1). The absorbance was measured at 225 nm against methanol/water (1:1) in a 1-cm cuvette (22).

Statistical Analysis. Regression analysis was carried out with the 6.12 version SAS System package (SAS Institute Inc., Cary, NC) to evaluate the correlation between the induction time (hours) and the enrichment concentration of individual phenolic compounds.

RESULTS AND DISCUSSION

NMR Characterization of the Phenolic Compound (Peak 6). The peak number and retention time (RT) of the unknown compound are those quoted in the semipreparative HPLC chromatogram reported in Figure 1. The ^{13}C and 1H NMR data are reported in Tables 1 and 2, respectively. Peak 6 in methanol-*d* showed that resonances of the carbons 1'–8' relative

Table 1. ^{13}C NMR Data (Parts per Million) of Reference and New Phenolic Compound (Figure 1, Peak 6)^a

carbon	Montedoro et al. (23)	peak 6	carbon	Montedoro et al. (23)	peak 6
1	99.0	98.98	1'	66.5	66.57
2	156.2	156.28	2'	35.4	35.41
3	156.2	156.17	3'	130.8	130.77
4	108.0	108.05	4'	116.4	116.38
5	28.5	28.48	5'	144.9	146.24
6	39.8	39.83	6'	146.2	144.93
7	174.0	174.12	7'	117.0	117.0
8	47.7	47.52	8'	121.2	121.2
9	73.2	73.22	COOM	51.6	51.66
10	20.3	20.36	OMe	49.3	48.0
COOMe	169.5	169.47			

^aData obtained using metanol-*d*₄ as solvent.**Table 2.** ^1H NMR Data (Parts per Million) of Reference and the New Phenolic Compound (Figure 1, peak 6)^a

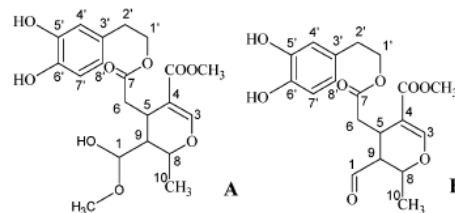
proton	Montedoro et al. (23)	peak 6	proton	Montedoro et al. (23)	peak 6
1	4.25 m		1'	4.13 m	4.21
2			2'	2.71 t	2.8
3	7.49 s	7.55	3'		
4			4'	6.80 d	6.68
5	3.8 m		5'		
6	3.20 m	2.17	6'		
7			7'	6.77 d	6.66
8	4.51 m	4.49	8'	6.63 dd	6.54
9			COOM		
10	1.25 d	1.95	OMe	3.29 s	
COOMe	3.60 s	3.67			

^aData obtained using metanol-*d*₄ as a solvent.

to 3,4-DHPEA-EA were present. The 3-4-5-6-7 fragment similar to that of 3,4-DHPEA-EA agreed with that reported by Montedoro et al. (23), excluding the resonances of C1, which give information about the presence of a -OCH₃ and a hydroxyl group linked to it.

Confirmation of the structure of this compound was done by comparing the results obtained by NMR with those assigned theoretically that correspond to the 3,4-DHPEA-EA described by Montedoro et al. (23). It was observed that the results obtained in the first part of the moiety were identical. Because hydroxytyrosol is a hydrocarbonate supplied by two constituent hydroxyls, carbon displacements were similar. On the other hand, part of the moiety formed by an elenolic ring with a high concentration of oxygen atoms in the structure interacts with the solvent used (methanol deuterated). This could explain why the theoretical displacements were not equal to theoreticals corresponding to the 3,4-DHPEA-EA. A second mass spectrometry was carried out, and the proposed structure is shown in Figure 3 with the denomination of the methylated form of oleuropein aglycone (ME 3,4-DHPEA-EA) and the structure of oleuropein aglycone (3,4-DHPEA-EA), the most similar secoiridoid derivative, previously identified by Montedoro et al. (23).

Lipid Matrix Characterization. The quality parameters, composition, oxidative stability, and bitter index of the lipid matrix used for phenolic compound enrichment are shown in Table 3. The olive oil refining process, especially the bleaching and the alkali refining, removes a considerable proportion of the antioxidant components, mainly phenols and pigments. The role of the individual phenolic compounds on the oxidative stability and the bitter index of olive oil could be evaluated

**Figure 3.** Methylated form of oleuropein aglycone (ME 3,4-DHPEA-EA) (A) (Figure 1, peak 6) and oleuropein aglycone (3,4-DHPEA-EA) (B).**Table 3.** Quality Parameters and Composition of the Refined Olive Oil (ROO) Used as a Base for Phenolic Enrichment^a

moisture (%)	0.04 ± 0.01
acidity (% oleic acid)	0.03 ± 0.01
peroxide value (mequiv of O ₂ /kg of oil)	2.9 ± 0.3
UV absorbance	
<i>K</i> ₂₇₀	0.54 ± 0.05
<i>K</i> ₂₃₂	2.2 ± 0.3
palmitic fatty acid (C16:0) (%)	12.5 ± 0.3
palmitoleic fatty acid (C16:1) (%)	1.3 ± 0.02
margaroleic fatty acid (C17:1) (%)	0.10 ± 0.01
stearic fatty acid (C18:1) (%)	3.3 ± 0.2
oleic fatty acid (C18:1) (%)	74.2 ± 0.5
linoleic fatty acid (C18:2) (%)	7.2 ± 0.4
linolenic fatty acid (C18:3) (%)	0.45 ± 0.02
arachidic fatty acid (C20:0) (%)	0.52 ± 0.03
gadoleic fatty acid (C20:1) (%)	0.46 ± 0.02
behenic fatty acid (C22:0) (%)	0.11 ± 0.02
α-tocopherol content (mg/kg of oil)	118.9 ± 1.9
pigment content (mg/kg of oil)	
chlorophylls	0.24 ± 0.03
carotenoids	0.16 ± 0.02
total phenol content (mg/kg of oil)	3.2 ± 0.4
oxidative stability (h)	2.7 ± 0.4
bitter index (<i>K</i> ₂₂₆)	0.014 ± 0.002

^aValues represent the mean of five analyses ± standard deviation.

because of the minor phenolic content. The values of these parameters in refined olive oil were considered as a control during the study.

Antioxidant Activity Index (AAI). Rancimat test limitations are known well because the phenolic compound activity could decrease considerably at high temperatures. The fast decomposition of the products of the alkyl-peroxy radical reaction with the antioxidant results in propagation rather than chain breaking under extreme conditions. However, this test has been widely used as a simple and useful measure of the oxidative stability of vegetal oils and animal fats. Moreover, a good correlation has been found between the concentration of total phenols (18) and the secoiridoid derivatives (3,4-DHPEA-EDA, *p*-HPEA-EDA, and 3,4-DHPEA-EA) (24) with the oxidative stability of the virgin olive oils measured by Rancimat test at 120 °C and 20 L/h air flow. A similar study by Carrasco-Pancorbo et al. (25) evaluated the antioxidant capacity of olive oil phenols in a lipid model system by accelerated oxidation using the oxidative stability instrument (OSI) working at 110 °C.

Table 4 shows the AAI of standard phenolic compounds and Table 5 the AAI of the phenolic compounds purified from virgin olive oil. This measure would be considered a reliable evaluation of the susceptibility to oxidative degeneration and can be used to specify the shelf life of olive oil containing different groups of phenolic compounds.

Phenolic Alcohols. The antioxidant activity expressed as the AAI of hydroxytyrosol and tyrosol was evaluated at concentrations from 0 to 300 mg/kg of oil. Hydroxytyrosol did have an

Table 4. Antioxidant Activity Index (AAI) of Standard Phenols Added to Refined Olive Oil^a

phenolic compound	phenolic compound addition				
	40 mg/ kg of oil	80 mg/ kg of oil	160 mg/ kg of oil	240 mg/ kg of oil	320 mg/ kg of oil
phenolic alcohols					
hydroxytyrosol	2.05	2.88	3.68	4.43	5.13
tyrosol	1.19	1.20	1.22	1.26	1.19
phenolic acids and derivatives					
vanillic	1.07	1.08	1.06	1.21	1.22
homovanillic	1.07	1.19	1.42	1.48	1.57
vanillin	1.00	1.02	1.00	1.14	1.09
caffeic	1.17	1.71	3.34	4.39	4.62
ferulic	1.12	1.23	1.29	1.32	1.44
gallic	3.00	4.34	7.89	10.4	10.5
o-coumaric	1.14	1.21	1.29	1.35	1.33
p-coumaric	1.09	1.11	1.11	1.21	1.23
3,4-dihydroxybenzoic	1.04	1.13	1.19	1.21	1.22
4-hydroxybenzoic	1.15	1.18	1.15	1.19	1.15
trans-cinnamic	1.00	0.90	0.77	0.64	0.67
hydrocinnamic derivative					
verbascoside	1.23	1.26	1.22	1.23	1.47
flavonoids					
luteolin	1.76	1.94	2.25	2.47	3.00
apigenin	1.23	1.34	1.50	1.62	1.71
luteolin 7-O-glucoside	1.38	1.35	1.70	1.76	1.78
apigenin 7-O-glucoside	1.09	1.28	1.31	1.49	1.67
secoiridoid					
oleuropein	1.02	1.13	1.13	1.19	1.76

^a AAI = induction time of enriched sample IT_e /induction time of control IT_c (AAI value of the control equal to 1.00 corresponds to a mean value of the induction time = 2.7 h). Values represent the mean of two replicates of two sets of experiments.

Table 5. Antioxidant Activity Index (AAI) of Phenolic Compounds Isolated from Virgin Olive Oil, Added to Refined Olive Oil^a

phenolic compound	phenolic compound addition				
	40 mg/ kg of oil	80 mg/ kg of oil	160 mg/ kg of oil	240 mg/ kg of oil	320 mg/ kg of oil
3,4-DHPEA-AC	1.11	1.08	1.13	1.19	0.92
3,4-DHPEA-EDA	1.14	1.16	1.28	2.06	2.11
phenolic compound	20 mg/ kg of oil	40 mg/ kg of oil	100 mg/ kg of oil	150 mg/ kg of oil	200 mg/ kg of oil
p-HPEA-EDA	0.94	0.92	0.77	0.64	0.60
3,4-DHPEA-EA	1.08	1.06	1.05	1.07	1.11
lignans	1.06	1.00	1.11	1.13	1.21
phenolic compound	5 mg/ kg of oil	10 mg/ kg of oil	20 mg/ kg of oil	30 mg/ kg of oil	40 mg/ kg of oil
ME 3,4-DHPEA-EA	1.05	0.97	1.13	1.24	1.31

^a AAI = induction time of enriched sample IT_e /induction time of control IT_c (AAI value of the control equal to 1.00 corresponds to a mean value of the induction time = 2.7 h). Values represent the mean of two replicates of two sets of experiments.

important effect on the concentration range with a positive trend, reaching an AAI value of 5.13 at 300 mg/kg of oil. Differences found between the oxidative stability of hydroxytyrosol and tyrosol were probably due to chemical structure, because hydroxytyrosol possesses a 3,4-dihydroxy structure linked to an aromatic ring that confers to the moiety a higher proton dislocation, facilitating a higher scavenging activity than observed with tyrosol, which possesses only a hydroxyl group linked to an aromatic ring (Figure 2).

Phenolic Acids. Gallic acid exhibited the most antioxidant effect with an important increment in the AAI, which reached

the value of 10.5 at 320 mg/kg of oil in the enrichment concentration. The antioxidant activity of gallic and caffeic acids could be explained by the presence in their structure of a 3,4-dihydroxyl structure linked to a phenolic ring as occurs in hydroxytyrosol. The higher antioxidant activity of gallic acid could be explained by the presence in its structure of a 3,4,5-trihydroxy structure linked to a phenolic ring that permits a higher proton dislocation than that observed in caffeic acid.

Flavonoids. The structural differences between apigenin and luteolin are characterized by the presence of a hydroxyl in the luteolin C ring, which appears in the ortho position. That structure could be responsible for the higher activity of luteolin versus apigenin. Luteolin and luteolin 7-glucoside fulfill all of the criteria for maximum radical scavenging activities as described by Morelló et al. (16). Luteolin and apigenin presented higher AAIs in the ROO matrix in relation to their 7-position glycosylated forms due to the presence of a sugar moiety in the flavonoid glycoside, which substitutes the hydroxyl group at C₇ linked to the phenolic ring. This agrees with the information reported in previous works (26–28).

Enrichment with *oleuropein* and *verbascoside*, the major phenolic compounds in olive fruit, showed an increase of the AAI from 1 to 1.47 and 1.76, respectively, at a concentration of 320 mg/kg of oil.

Secoiridoid and Derivatives. Secoiridoid derivatives of oleuropein were assayed at different enrichment concentrations related to their concentration in the virgin olive oil used as a source of the phenolic compounds isolated (Table 5). The secoiridoid derivative 3,4-DHPEA-EDA is a compound of especial interest because of its presence as one of the major secoiridoid antioxidant compounds in virgin olive oil (15), and it showed a positive result as an antioxidant with a maximum AAI index value of 2.11 at a concentration of 320 mg/kg of oil. However, the AAI index was not affected by enrichment with 3,4-DHPEA-AC. The results showed a slight antioxidant activity of 3,4-DHPEA-EA (oleuropein aglycone) with a maximum AAI index value of 1.11 in the range of evaluation (0–200 mg/kg of oil). This disagrees in part with the study that reported strong antioxidant activity by oleuropein aglycone in several lipid systems including oil emulsions (15) and triolein (25). It would thus appear that the lipid model system of the study could change the antioxidant activity of the phenolic structures. Moreover, enrichment with the methylated form of the oleuropein aglycone (ME 3,4-DHPEA-EA) showed an increase of the AAI to 1.31 at the concentration of 40 mg/kg compared with the AAI of 1.06 by the enrichment with the 3,4-DHPEA-EA at similar concentration (Table 5). The lack of a carboxymethyl group in the C₉-position of the oleuropein aglycone structure (methylated form of 3,4-DHPEA-EA) supposed an important effect on the antioxidant activity. Similarly, in a previous study, this phenolic structure (nominated as peak RT 36) was found to be one of the most active virgin olive oil antioxidants, showing an important radical scavenging activity (16).

The ligstroside derivative (p-HPEA-EDA), bearing one hydroxyl substituent, had a negative effect on antioxidant activity, decreasing to an AAI of 0.60 at a concentration of 200 mg/kg of oil with respect to the control sample.

Lignans. Lignans (acetoxypinoresinol and pinoresinol) provided a slight increase in the oxidative stability at the higher concentration studied (200 mg/kg of oil). This does not agree with the data reported by Carrasco et al. (25) because their study showed a pro-oxidant effect (OSI test) when triolein was enriched with (+)-1-acetoxypinoresinol and (+)-pinoresinol at

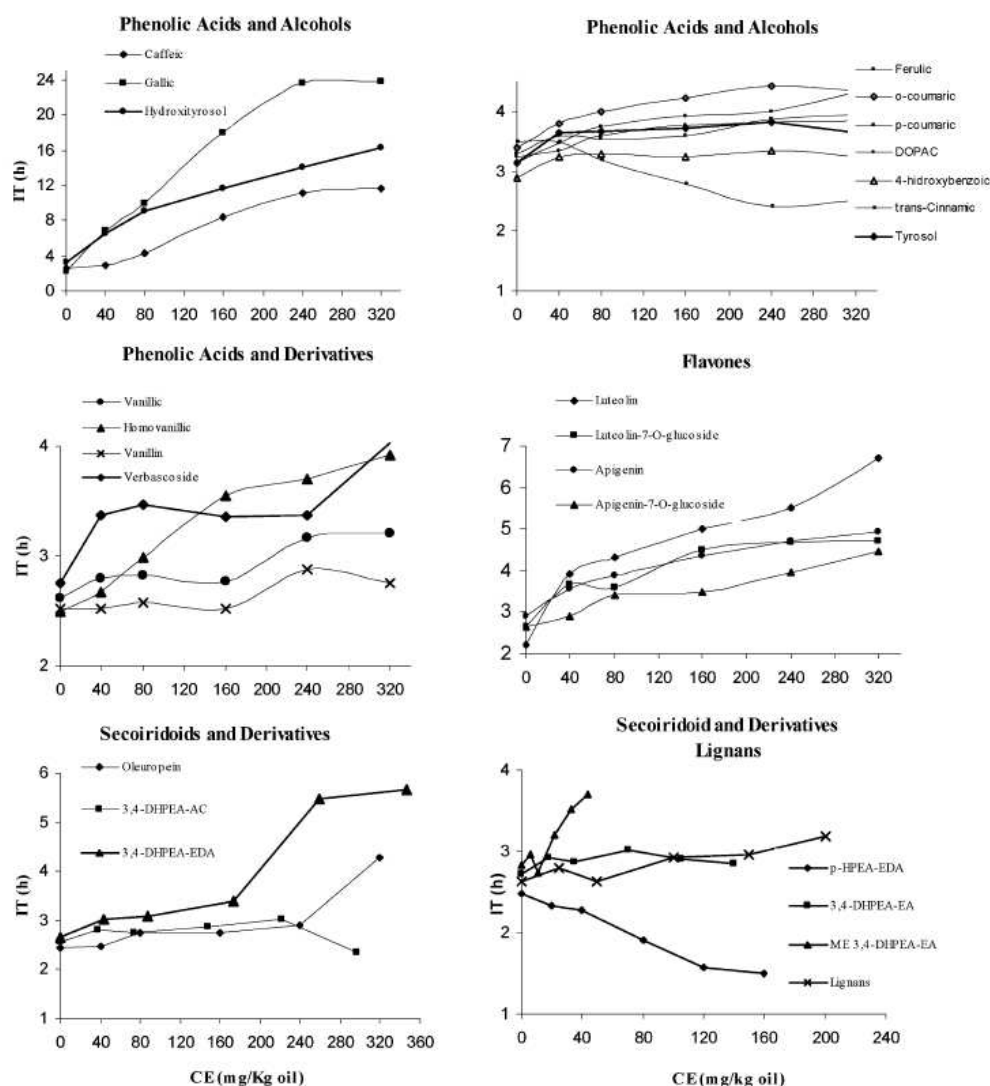


Figure 4. Relationship between phenol concentration of enrichment (CE, mg/kg of oil) of refined olive oil and oxidative stability expressed as induction time (IT) in hours. DOPAC, 3,4-dihydroxyphenylacetic. (Values represent the mean of two replicates of two sets of experiments.)

a concentration of 50 mg/kg. Differences observed could be attributed to the lipid matrix used in each study; the presence of α -tocopherol and the minor content of carotenoids (Table 3) could have a synergistic effect with lignans.

Relationship between Induction Time and Phenol Concentration of Enrichment. The antioxidant effects of phenolic compounds added to ROO, expressed as induction time (IT) by Rancimat, with different concentrations of enrichment are shown in Figure 4. As can be observed, gallic acid, hydroxytyrosol, and caffeic acid showed the most positive effect on the oxidative stability of ROO (control sample). The increase in the induction time to 21.5, 13.1, and 9.2 h, respectively, at the highest concentration (320 mg/kg of oil) showed a huge difference with respect to the other phenolic alcohols and acids evaluated. However, there was also an important increase in IT

values with the addition of homovanillic and ferulic acids, indicating the antioxidant activity of a wide range of phenolic compounds occurring in olive fruit and olive oil. This is in agreement with some studies that have demonstrated the antioxidant activity of the phenolic compounds tested using different antioxidant methods (7, 16, 27). Conversely, tyrosol had little effect; it would appear that the monohydroxy aromatic group without the secoiridoid moiety gives it a weak antioxidant activity.

The main flavones occurring in olive fruit and olive oil also had a strong effect on oxidative stability when they were added to a ROO control sample at concentrations ranging from 40 to 320 mg/kg of oil. An order of IT increase (hours) may be given: luteolin (4.47) > luteolin 7-O-glucoside (2.08) > apigenin (2.05) > apigenin 7-O-glucoside (1.78). Regarding

Table 6. Model To Describe the Relationship between Oxidative Stability (Induction Time) and Phenolic Concentration^a

phenolic compound	correlation parameters ^b			<i>r</i> ²
	<i>a</i>	<i>b</i>	<i>c</i>	
hydroxytyrosol	3.72	6.56×10^{-2}	-8.4×10^{-5}	0.989
caffeic acid	2.29	3.26×10^{-2}		0.956
gallic acid	1.73	1.33×10^{-1}	-1.94×10^{-4}	0.985
<i>o</i> -coumaric acid	2.95	7.70×10^{-3}	-1.54×10^{-5}	0.983
homovanillic acid	2.44	8.09×10^{-3}	-1.09×10^{-5}	0.988
luteolin	2.98	1.17×10^{-2}		0.907
luteolin 7- <i>O</i> -glucoside	2.81	1.57×10^{-2}	-2.68×10^{-5}	0.955
apigenin	3.23	5.93×10^{-3}		0.924
apigenin 7- <i>O</i> -glucoside	2.85	5.09×10^{-3}		0.936
oleuropein	2.26	4.80×10^{-3}		0.740
3,4-DHPEA-AC	2.77	-3.00×10^{-4}		0.019
3,4-DHPEA-EDA	2.46	9.30×10^{-3}		0.892
<i>p</i> -HPEA-EDA	2.47	-6.50×10^{-3}		0.973
3,4-DHPEA-EA	2.85	5.00×10^{-4}		0.061
ME 3,4 DHPEA-EA	2.75	2.17×10^{-2}		0.899
lignans	2.63	2.50×10^{-3}		0.856

^a Values represent the mean of two replicates of two sets of experiments.

^b Simple regression $y = a + bx$; polynomial regression $y = a + bx + cx^2$.

verbascoside, a hydrocinnamic derivative with *o*-dihydroxy structures, it was observed to have a positive result as an antioxidant with the increase of 1.3 h in the IT value at a concentration of 320 mg/kg of oil in relation to the control.

The aldehydic form of elenolic acid linked to hydroxytyrosol (3,4-DHPEA-EDA), at a concentration of 320 mg/kg of oil, was the most powerful antioxidant among the secoiridoids and derivatives, whereas oleuropein, containing an *o*-dihydroxy functional group esterified to a secoiridoid glycone, also produced a remarkable increase in induction time (1.84 h in relation to the control). On the contrary, 3,4-DHPEA-AC did not have an important effect on the oxidative stability of ROO even at concentrations higher than those of the other secoiridoid derivatives. Unlike the antioxidant effect of the phenolic compounds mentioned above, *trans*-cinnamic acid showed pro-oxidant activity. A decrease of 1 h with respect to the IT of the control sample was observed at 320 mg/kg, whereas *p*-HPEA-EDA also had similar behavior at lower concentrations. The

methylated form of 3,4-DHPEA-EDA demonstrated a considerable contribution to oxidative stability, taking into account the concentration range it was evaluated in.

The regression curve values of standard phenols with a good correlation coefficient (r^2) of >0.90 and the regression curve values of the isolated phenolic compounds are shown in Table 6. A positive linear trend between the IT and the concentration of antioxidant with a good correlation coefficient and higher slope could be established for some phenolic compounds. Gallic acid, hydroxytyrosol, caffeic acid, 3,4-DHPEA-EDA, and luteolin showed the highest slopes, indicating a significant increase in the IT at different concentrations. However, the regression coefficients obtained for hydroxytyrosol, gallic, *o*-coumaric, and homovanillic acids, and luteolin 7-*O*-glucoside were fitted to a second-order polynomial equation.

Effect of Mixing Antioxidants. Considering the results of the individual antioxidant activities, it was of interest to study the possible synergistic effect of some phenolic compounds (Figure 5). It was observed that 3,4-DHPEA-EDA produced increases of 0.8 and 2.6 h in relation to the control (average = 2.7 h, Table 3) at 200 and 400 mg/kg of oil, respectively. With respect to hydroxytyrosol, it was shown that this phenolic alcohol possessed the highest antioxidant power compared with the rest of the phenolic compounds evaluated. Moreover, it is of special interest to notice that a synergistic effect was not observed when hydroxytyrosol was added in combination with 3,4-DHPEA-EDA. The antioxidant effectiveness was similar when hydroxytyrosol was tested at 200 mg/kg of oil and when the phenolic compound mixture, 3,4-DHPEA-EDA plus hydroxytyrosol, was added at a total concentration of 400 mg/kg of oil. The same effect was observed for hydroxytyrosol at 400 mg/kg of oil and for the combined 3,4-DHPEA-EDA plus hydroxytyrosol solution at 800 mg/kg of oil. With regard to caffeic acid, the antioxidant activity was remarkable at the two concentrations (200 and 400 mg/kg of oil), and a positive synergistic effect was observed in combination with 3,4-DHPEA-EDA. The flavonoid luteolin in combination with 3,4-DHPEA-EDA showed a synergistic effect at a concentration of 400 mg/kg of oil, reaching a higher experimental increase in oxidative stability with the phenolic compound mixture than

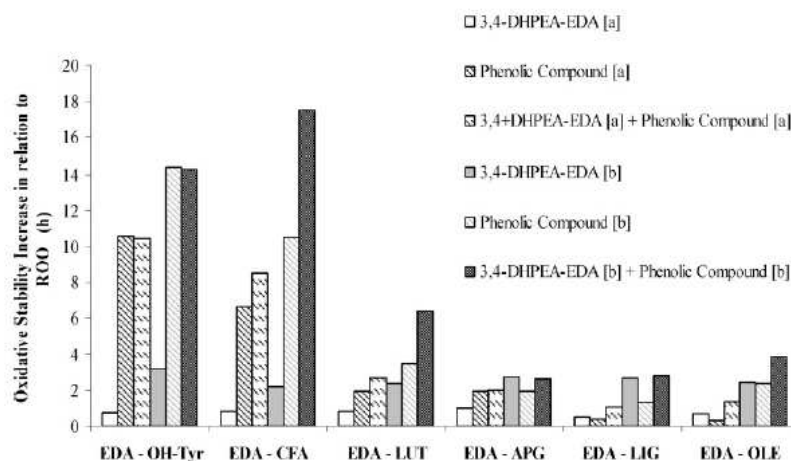


Figure 5. Effect of 3,4-DHPEA-EDA (EDA) and different phenolic compounds on the oxidative stability of refined olive oil matrix (ROO) at two concentrations. Values represent the mean of two replicates of two sets of experiments. OH-Tyr, hydroxytyrosol; CFA, caffeic acid; LUT, luteolin; APG, apigenin; LIG, lignans; OLE, oleuropein. Concentration [a] = 200 mg/kg of oil; concentration [b] = 400 mg/kg of oil.

Table 7. Effect on the Bitter Index (BI) of the Individual Phenols Added to Refined Olive Oil^a

phenolic compound	phenolic compound addition		
	50 mg/ kg of oil	100 mg/ kg of oil	200 mg/ kg of oil
hydroxytyrosol	1.76	2.25	4.24
tyrosol	1.72	3.46	5.77
vanillin	1.02	1.29	1.37
oleuropein	1.03	1.14	2.24
3,4-DHPEA-AC	0.95	1.16	1.37
3,4-DHPEA-EDA	1.11	2.17	2.35
p-HPEA-EDA	2.78	5.66	7.57
3,4-DHPEA-EA	1.37	1.42	3.54
ME 3,4-DHPEA-EA	2.29	3.44	8.57
lignans	1.14	1.60	2.10

^aBitter index of sample BI_s/bitter index of control BI_c (BI value of the control equal to 1.00 corresponds to the mean value of the bitter index = 0.014). Values represent the mean of two replicates of two sets of experiments.

that obtained with the theoretical sum of individual increases given by each phenolic compound. On the contrary, the resulting data for apigenin were not significant in relation to the oxidative stability of refined olive oil. Even at high concentrations, the antioxidant activity was not effective.

Lignans (1-acetoxypinoresinol and pinoresinol) and oleuropein provided a slight increase in the oxidative stability with respect to the control sample at concentrations of 200 and 400 mg/kg of oil. In addition, the phenolic compound mixtures with 3,4-DHPEA-EDA at the lowest concentrations had a synergistic effect. A higher increase in oxidative stability was observed than that resulting from the sum of the individual increments produced by lignans and oleuropein at 200 mg/kg of oil.

Bitter Index (K_{225}). The bitter index (K_{225}), an analytical determination to evaluate the bitter taste, is an important index to take into account because sensory quality plays a vital role in orientating the preference of consumers. To evaluate the effect of the enrichment on the sensory attributes of the lipid matrix, it was of interest to evaluate the bitter index of ROO with the addition of some selected phenolic compounds. The resulting data (Table 7) showed that the bitter index increased mainly with the enrichment with secoiridoid derivatives in the following order: the methylated form of 3,4-DHPEA-EA > p-HPEA-EDA > tyrosol > hydroxytyrosol > 3,4-DHPEA-EA. Although there is no clear or established limit, experience has shown that K_{225} values of the order of ≥ 0.360 correspond to quite bitter oils, which are rejected by many consumers (22). The values reached in the bitter index of the ROO with the phenolic enrichment in the concentrations used in this study were much lower than this value. In relation to the chemical structure of the phenols, the highest effect on the bitter index was found with ligstroside derivatives, such as p-HPEA-EDA, bearing only one hydroxyl substituent. The phenolic compounds that possess a 3,4-dihydroxyl structure linked to an aromatic ring showed a lower effect, excluding the methylated form of 3,4-DHPEA-EA, with a carboxymethyl group in the C₉-position of the oleuropein aglycone structure, which supposed an important effect on the bitter index.

As consequence of the results, higher antioxidant activities were found with phenolic compounds that possess 3,4-dihydroxyl and 3,4,5-trihydroxy structures linked to an aromatic ring (oleuropein, 3,4-DHPEA-EDA, and the methylated form of 3,4-DHPEA-EA) that conferred to the moiety a higher proton dislocation, thus facilitating the scavenging activity. The antioxidant activity among secoiridoid derivatives varies depending

on the elenolic acid structure, whether it is open (dialdehydic form) or closed (aldehydic form). Therefore, 3,4-DHPEA-EDA which bears a dialdehydic form of elenolic acid linked to hydroxytyrosol, exhibited a higher antioxidant activity than 3,4-DHPEA-EA (closed ring, aldehydic form) toward lipid matrix oxidation.

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Supporting Information Available: Elucidation of the chemical structure, TOCSY spectrum, and LC-¹H NMR spectra of peak 6. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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