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Olea europaea L. Leaf Extract and Derivatives: Antioxidant Properties

RAFFAELLA BRIANTE,[†] MAURIZIO PATUMI,[‡] STEFANO TERENZIANI,[‡] ETTORE BISMUTO,[§] FERDINANDO FEBBRAIO,[†] AND ROBERTO NUCCI*,[†]

Istituto di Biochimica delle Proteine ed Enzimologia del CNR, Via Marconi 10, 80125 Napoli, Italy; Istituto di Ricerche per la Olivicoltura del CNR, Via Madonna Alta 128, 60128 Perugia, Italy; and Facoltà di Medicina, Dipartimento di Biochimica e Biofisica, Seconda Università degli Studi di Napoli, Via Costantinopoli 16, 80131 Naples, Italy

This paper reports a very simple and fast method to collect eluates with high amounts of hydroxytyrosol, biotransforming *Olea europaea* L. leaf extract by a thermophilic β-glycosidase immobilized on chitosan. Some phenolic compounds in the leaf tissue and in the eluates obtained by biotransformation are identified. To propose the eluates as natural substances from a vegetal source, their antioxidant properties have been compared with those of the leaf extract from which they are originated. The eluates possess a higher concentration of simple phenols, characterized by a stronger antioxidant capacity, than those available in extra virgin olive oils and in many tablets of olive leaf extracts, commercially found as dietetic products and food integrators.

KEYWORDS: ***Olea europaea*** **L. leaf extract; olive oil polyphenols; antioxidant activity; biotransformation; LDL oxidation**

INTRODUCTION

Epidemiological evidence in the Mediterranean area of a lower incidence of coronary heart disease (*1*, *2*) and certain types of cancers (*3*, *4*) led to the hypothesis that a diet rich in grain, legumes, fresh fruits and vegetables, wine in moderate amounts, and olive oil has beneficial effects on human health. These effects have been attributed, in part, to the presence in the Mediterranean diet of antioxidant vitamins, flavonoids, and polyphenols that play an important role in disease prevention. The distinctive climate of the Mediterranean basin, characterized by warm weather and extended sunlight irradiation, has allowed the development of plants such as olive trees the fruits of which require a high proportion of antioxidant molecules (*5*). The synthesis of these compounds is activated by white light irradiation (*6*) and results in dark fruits that protect themselves from the noxious effects of extended exposure to sunlight.

In the Mediterranean diet, olive oil is the main source of polyphenols that constitute a complex mixture in both olive fruit and its derived product, oil. Some studies exist on the nature of these compounds (*7*−*10*), which influence the sensorial properties of olive fruits and oil (*11*) and are important markers for evaluating virgin olive oil quality (*12*). These molecules have pharmacological properties (*13*), are natural antioxidants (*14*, *15*), and inhibit the Gram-positive microorganisms involved in olive fruit fermentation (*8*, *16*).

The main phenols identified in *Olea europaea* L. tissues are shown in **Figure 1**. Oleuropein, a (3,4-dihydroxyphenyl)ethanol (hydroxytyrosol) ester with β-glucosylated elenolic acid, is the main phenol in olive fruits and leaves (*17*). The oleuropein aglycons are pharmacologically active molecules for their potential application as antimicrobial agents in some fairly common olive tree diseases (*18*). A third interesting compound present in extra virgin olive oil (*19*) is the lipid- and water-soluble simple phenol hydroxytyrosol. It was successfully tested for its free radical scavenging activity, strongly inhibiting superoxide production by either a cell-free system or activated human neutrophils (*20*), as well as for its protective role on oxidative stress in the human system (*21*).

Despite the reported importance of the hydroxytyrosol biological properties, very few data on its absorption and disposition have been published (*22*) due to the fact that this molecule is commercially unavailable. Chromatographic purification methods from olive mill waste waters (omww) (*23*), virgin olive oil (*24*), and olive leaves (*25*) as well as synthetic (*26*, *27*) procedures have been developed for hydroxytyrosol production. However, chromatographic purification methods give scarce yields as hydroxytyrosol undergoes both retention and chemical modification processes in the stationary phase during purification. On the other hand, the final purification step is usually carried out by a preparative TLC, which can be used only for laboratory applications. It is well-known that hydroxytyrosol recovery from omww, which could prove to be a useful process for their recycling, is more expensive than synthesis methods (*23*, *27*). Currently, enzymatic procedures for hydroxytyrosol production are not developed.

* Author to whom correspondence should be addressed (telephone/fax +39-081-7257300; e-mail nucci@dafne.ibpe.na.cnr.it).

[†] Istituto di Biochimica delle Proteine ed Enzimologia del CNR.

[‡] Istituto di Ricerche per la Olivicoltura del CNR.

[§] Seconda Università degli Studi di Napoli.

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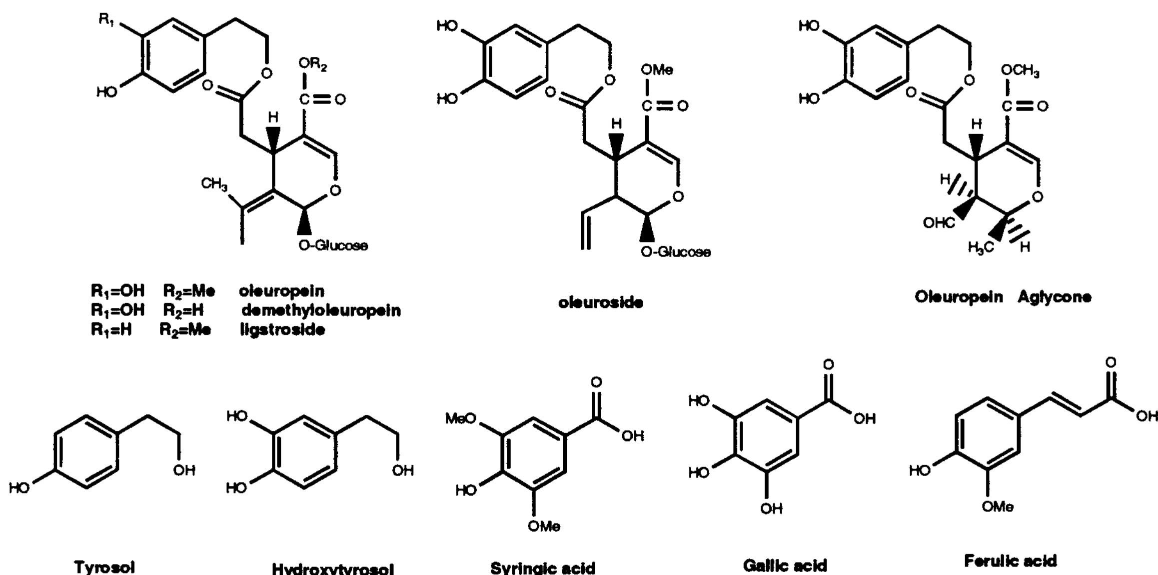

Figure 1. Chemical structures of phenolic compounds already identified in *O. europaea* L. leaf extract.

In previous works, we have described the hydrolysis of the oleuropein by a hyperthermophilic β-glycosidase immobilized on chitosan with the identification of the main reaction products (*28*, *29*). The enzyme quickly hydrolyzes oleuropein to its aglycon, which in its turn, at the temperature and pH conditions required for the enzyme activity, undergoes a fast chemical rearrangement leading to the monoaldehydic elenolate aglycon (**Figure 1**) that is hydrolyzed in hydroxytyrosol. Successively, the oleuropein present in *O. europaea* L. leaf extract was biotransformed in the presence of ethanol, thus improving the enzymatic activity, the extract solubility, and the reaction yield in hydroxytyrosol, which is also obtained from other phenols present in the extract as they are more soluble in organic solvent (*30*).

In this investigation, we have identified some phenols in *O. europaea* L. leaf extract and in the eluates by extract biotransformation. To propose the eluates as natural products from a vegetal source, we have compared their antioxidant properties with those of the leaf extract from which they are originated. The eluates have a higher content of simple phenols, characterized by a stronger antioxidant capacity, than many tablets of olive leaf extracts, containing hydroxytyrosol (0.7%) and tyrosol (0.7%), commercially available as dietetic products and food integrators.

MATERIALS AND METHODS

Chemicals. Low molecular weight chitosan ($M_r \sim 150000$), ferric chloride, copper(II) sulfate, and *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride (DMPD) were from Fluka Chimica (Milano, Italy); *p*-nitrophenyl β-D-glucopyranoside (PNPG), glutaraldehyde, bis(trimethylsilyl)trifluoroacetamide + 1% trimethylchlorosilane, 2-(*p*-hydroxyphenyl)ethanol (tyrosol), 4-hydroxy-3-methoxycinnamic acid (ferulic acid), 3,5-dimethoxy-4-hydroxybenzoic acid (syringic acid), 3,4,5-trihydroxybenzoic acid (gallic acid), and linoleic acid were from Sigma (St. Louis, MO). 2,2′-Azobis(2-amidinopropane) dihydrochloride (ABAP) was from Waco, oleuropein from Extrasynthèse (Genay, France), and bovine serum albumin (BSA) from Boeheringer Mannheim (Mannheim, Germany). Oleuropein aglycon and hydroxytyrosol were obtained according to the procedure reported in ref *29*. All chemicals used were of analytical grade.

Enzyme Purification and Immobilization. The partially purified β-glycosidase from the hyperthermophilic archaeon *Sulfolobus solfataricus* overexpressed in *Escherichia coli* (BL21de3 strain) was obtained by interrupting, at the thermoprecipitation step, the purification procedure described in ref *31*. The matrix preparation and the enzyme immobilization were carried out according to the method given in ref *30* using 1.3 g of low molecular weight chitosan, 11.9 mg of nonhomogeneous β-glycosidase, and 50.0 mg of BSA. The assay on PNPG as a substrate of the immobilized enzyme was carried out according to the procedure given in ref *28*. All spectroscopic assays were followed using a double-beam Cary 1E thermostated spectrophotometer (Varian, Victoria, Australia) equipped with Peltier temperature control.

Bioreactor Setup. In the one-step approach, a chromatographic column, connected to a thermostatic bath, was filled with the enzyme−chitosan matrix. In the two-step approach, a second chromatographic column, filled with 8.0 g of nontreated chitosan, was placed in series to the enzyme−chitosan column. The substrate solutions were downloaded onto the bioreactor by a peristaltic pump, and the eluates were recovered in a fraction collector. The bioreactor, in both approaches, was thermostated at 60 °C.

Extracts from Leaves of *Olea europaea* L. The leaves were randomly picked from the Moraiolo cultivar in the Umbria region of Italy and immediately freeze-dried in liquid N_2. The samples were blended into a fine powder and extracted with H_2O/EtOH (1:1, v/v). The suspension was shaken for 15 min at 4 °C and centrifuged before the supernatant was lyophilized.

GC and GC-MS Analyses. GC analyses were carried out using a Carlo Erba 8000^{TOP} (C. Erba, Milano, Italy) equipped with a splitless injection system and an FID detector on a Supelco fused silica capillary SE-54 (30 m length; 0.25 mm i.d.; 0.25 μm film thickness). The carrier gas was He at 150 kPa. The injector and detector temperatures were maintained at 290 °C. The oven temperature program was run at 90 °C, raised at 5 °C/min to 220 °C (not held) and then at 2 °C/min to 290 °C, and held for 20 min at this temperature. GC-MS analysis was performed by Varian Saturn 2000 MS interfaced to Varian 3800 GC with a Varian 1079 temperature programmable injector on a CP-Sil 8 CB fused silica column (60 m length; 0.25 mm i.d.; 0.25 μm film thickness) with a flow rate of 1 mL/min (He). The injector temperature was 290 °C. Electron ionization (EI) mass spectra were recorded at ionization control automatic, emission current = 10 μA, AGC target = 20000 counts, mass range = 50−650 *m*/*z*, and ion trap temperature = 210 °C. The oven temperature program was the same as used for GC determination. The samples, evaporated under N_2 flow, were dissolved in acetone and derivatized by bis(trimethylsilyl)trifluoroacetamide + 1% trimethylchlorosilane. The compounds were quantified on the basis of their GC peak areas by integration with Chrom-Card software for Windows (Carlo Erba, Rodano, Italy) using four point's calibration curves previously prepared by the corresponding standards.

The regression coefficients were in the 0.981−1.000 range. The experimental findings represent the average of three different measurements.

Biotransformation of *O. europaea* L. Leaf Extract. For the hydrolysis of the leaf extract by bioreactor, we applied two different approaches, one step and two step.

Two solutions in 10 mM sodium−phosphate buffer, pH 7.0, + 2.0 M EtOH containing 25.6 mg of lyophilized cv. Moraiolo leaf extract were prepared and loaded, in both approaches, onto the bioreactor thermostated at 60 °C with flow rate of 0.26 mL/min using the above buffer as eluent. In the two-step approach, the column filled with the enzyme−chitosan matrix was disconnected after 2 h, whereas the second column filled with nontreated chitosan continued to operate for 4 h. To remove the phosphate by precipitation, the bioreactor eluates were lyophilized and dissolved in a minimum volume of EtOH, filtered through filter paper in a Büchner funnel, and washed twice with EtOH. The obtained eluates were immediately used for testing their antioxidant activities in order to avoid autoxidation.

Colorimetric Evaluation of Total Phenols. Both leaf extract and the two eluates obtained by biotransformation (1 mL) (see previous section) were lyophilized and resuspended in MeOH. Total phenolic content was tested colorimetrically (*32*) and expressed as milligrams of tyrosol per milligram of lyophilized sample. A calibration curve was calculated using pure tyrosol concentrations ranging from 0.94 to 141.0 μg/mL with a regression coefficient of 0.9987.

Measurement of Antioxidant Activity by DMPD Method. The radical hydrogen-donating ability of leaf extract and eluates by biotransformation was measured colorimetrically at 505 nm using the DMPD method (*29*). The antioxidant efficiency (AE) was calculated for each sample by considering the absorbance at 505 nm as a percentage of uninhibited radical cation solution absorbance (blank), according to the equation

$$\text{AE}\ (\%) = [1 - (A_{\text{AO}}/A_0)] \times 100 \qquad (1)$$

where A_0 is the uninhibited radical cation absorbance and A_{AO} is the absorbance measured 15 min after the addition of antioxidant samples.

The efficient concentration (EC_{50}), which was the amount of antioxidants expressed as micrograms necessary to bleach the red radical cation by 50%, was calculated by the equation

$$1/\text{AE}\ (\%) = a + \text{slope}_{\text{AOi}} \times 1/\mu\text{g} \qquad (2)$$

where a and $\text{slope}_{\text{AOi}}$ were the intercept and the slope of the linear plot of 1/AE values versus 1/μg of antioxidants in the reaction mixture (1 mL final volume), respectively. The inverse antioxidant power (AP_i) of each sample was calculated as $AP_i = \text{slope}_{\text{AOi}}$, so when the EC_{50} and AP_i values were lower, the antioxidant capacity was greater.

Measurement of Antiperoxidative Activity by ABAP Method. The antiperoxidative efficiencies were tested as described in ref *29*, measuring the different rates of oxidation of linoleic acid to its conjugated diene hydroperoxide in the presence of the leaf extract and the eluates by biotransformation. The azo radical initiator (ABAP) was used to provide a constant rate of radical production (*33*). The antioxidant efficiency (AE) was defined as the ratio between the slope of the linear plot of peroxidation absorbance versus time (d*A*/d*t*) before and after the antioxidant solutions were added:

$$\text{AE}\ (\%) = [1 - (S_{\text{AO}}/S_{\text{ABAP}})] \times 100 \qquad (3)$$

S_{AO} and S_{ABAP} were the slopes before and after sample addition, respectively.

From the curve representing the AE (%) increase as a function of sample concentrations (μg/mL per mg of linoleic acid) (**Figure 3**) we have extracted two parameters in order to calculate the inhibition power of each sample: the slope of the initial linear phase of the curve (IP_S) and the amplitude corresponding to the intersection of the slope with the *Y* axis (IP_A). The higher they are, the stronger the antiperoxidative efficiencies are.

Low-Density Lipoprotein (LDL) Oxidation. The efficiency of leaf extract and eluates by biotransformation in delaying lipid peroxidation was evaluated by examining their inhibitory effects on the oxidative modification of LDL in vitro. Incubations were performed by 0.24 mg/mL LDL in PBF (10 mM, pH 7.4) supplemented with of each above samples. Cu^{2+}, at a final concentration of 5.0 μM, started the LDL oxidation. All incubations were performed at 37 °C for 120 min. The kinetics of oxidation, assessed by conjugated diene formation, were determined by continuously monitoring the absorbance at 234 nm.

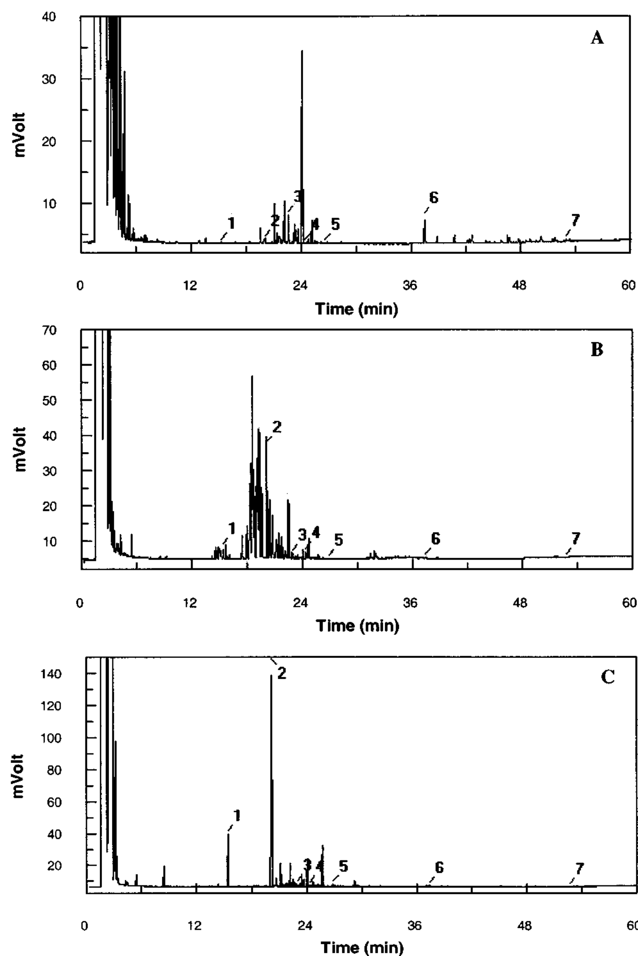

Figure 2. GC chromatographic profiles of cv. Moraiolo leaf extract (A) and bioreactor eluates obtained in one-step (B) and two-step (C) approaches. Peaks: 1, tyrosol; 2, hydroxytyrosol; 3, syringic acid; 4, gallic acid; 5, ferulic acid; 6, oleuropein; 7, oleuropein aglycon.

RESULT AND DISCUSSION

Biotransformation of *O. europaea* L. Leaf Extracts. Analyses of the GC spectrum of cv. Moraiolo leaf extract (**Figure 2A**) have shown the presence of tyrosol ($t_R = 15.4$), hydroxytyrosol ($t_R = 19.9$), syringic acid ($t_R = 22.8$), oleuropein ($t_R = 37.2$), and oleuropein aglycon ($t_R = 52.8$); furthermore, the presence in trace amounts of gallic ($t_R = 24.3$) and ferulic ($t_R = 26.6$) acid has been observed. These compounds were quantified (**Table 1**) with indication that cv. Moraiolo leaf extract is characterized by an elevated oleuropein concentration (∼38% in weight on lyophilized extract). The total phenols content makes reasonable the hypothesis that oleuropein is the main complex phenol in *O. europaea* L. leaves: expressing the total phenols also as milligrams of oleuropein (data not shown), they represent little more than 100% in weight of the extract.

In the GC profiles of the eluates produced by one- and two-step bioreactor approaches (**Figure 2B,C**), the same compounds characterized in leaf extract were identified and quantified (**Table 1**). The GC spectrum of the eluate by the two-step

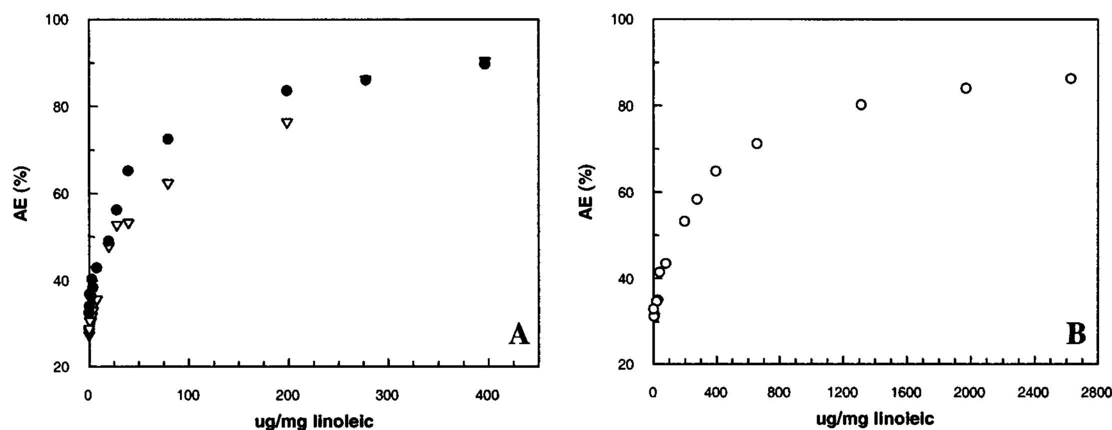

Figure 3. AE (percent) increase as a function of concentrations of (A) leaf extract (▽) and eluate from the two-step bioreactor (●) and (B) eluate from the one-step bioreactor (○).

Table 1. Phenolic Compounds in Cv. Moraiolo Leaf Extract and in Bioreactor Eluates Obtained in One- and Two-Step Approaches[a]

	Moraiolo leaf extract[b]	bioreactor eluate one-step approach[c]	bioreactor eluate two-step approach[c]
oleuropein	$0.377 \pm 4.6 \times 10^{-4}$	$0.0237 \pm 1.6 \times 10^{-3}$	$0.0625 \pm 1.15 \times 10^{-3}$
tyrosol	$8.89 \times 10^{-5} \pm 1.8 \times 10^{-5}$	$8.71 \times 10^{-4} \pm 8.57 \times 10^{-5}$	$1.15 \times 10^{-2} \pm 2.71 \times 10^{-4}$
hydroxytyrosol	$1.16 \times 10^{-3} \pm 6.9 \times 10^{-5}$	$2.29 \times 10^{-2} \pm 1.58 \times 10^{-3}$	0.208 ± 0.003
syringic acid	$1.74 \times 10^{-4} \pm 1.5 \times 10^{-5}$	$1.21 \times 10^{-3} \pm 1.35 \times 10^{-5}$	$1.59 \times 10^{-3} \pm 8.12 \times 10^{-5}$
oleuropein aglycon	$2.23 \times 10^{-3} \pm 7.6 \times 10^{-4}$	nd	nd
gallic acid	nd	$1.93 \times 10^{-3} \pm 2.71 \times 10^{-4}$	$1.12 \times 10^{-3} \pm 3.38 \times 10^{-5}$
ferulic acid	nd	nd	$1.94 \times 10^{-3} \pm 8.12 \times 10^{-5}$
total phenols	0.269 ± 0.006[d]	0.0546 ± 0.0004[e]	0.474 ± 0.002[e]

[a] Assays were run in triplicate, and data are expressed as the mean ($n = 3$) ± standard error. [b] Data are expressed as mg of compound/mg of lyophilized extract. [c] Data are expressed as mg of compound/mg of lyophilized eluate. [d] Expressed as mg of tyrosol/mg of lyophilized extract. [e] Expressed as mg of tyrosol/mg of lyophilized eluate.

Table 2. Biotransformation Data Relative to the One- and Two-Step Approaches on 25.6 mg of Cv. Moraiolo Leaf Extract[a]

	Moraiolo leaf extract (initial mg)	bioreactor eluate one-step approach (mg)	bioreactor eluate two-step approach (mg)
oleuropein	9.692 ± 0.053	2.621 ± 0.179	0.694 ± 0.013
tyrosol	$2.61 \times 10^{-3} \pm 9.9 \times 10^{-6}$	0.096 ± 0.009	0.127 ± 0.003
hydroxytyrosol	$2.99 \times 10^{-2} \pm 1.99 \times 10^{-4}$	2.533 ± 0.174	2.311 ± 0.038
syringic acid	$4.47 \times 10^{-3} \pm 2.76 \times 10^{-5}$	0.133 ± 0.001	0.018 ± 0.001
oleuropein aglycon	$5.74 \times 10^{-2} \pm 2.97 \times 10^{-4}$	nd	nd
gallic acid	nd	0.131 ± 0.003	$0.017 \pm 7.5 \times 10^{-5}$
ferulic acid	nd	nd	0.022 ± 0.001
total phenols[b]	6.554 ± 0.036	6.024 ± 0.024	5.257 ± 0.021

[a] Assays were run in triplicate and data are expressed as the mean ($n = 3$) ± standard error. [b] Data are expressed as mg of tyrosol.

bioreactor approach (**Figure 2C**) highlighted that hydroxytyrosol was the main product. In particular, the second column filled with nontreated chitosan was proved to be useful to purify preliminarily this compound: the GC profile of eluate by the onee-step bioreactor approach (**Figure 2B**) appears, in fact, more complex.

Data related to the one- and two-step biotransformation approaches are summarized in **Table 2**, showing that in a one-step configuration, we get, for oleuropein hydrolysis, a molar reaction yield in hydroxytyrosol >100%. This result can be explained by the presence, in the leaf extract, of nonglycosidic secoiridoids (i.e., the identified oleuropein aglycon) (*34*) but more probably of demethyloleuropein and oleuroside (*35*) (**Figure 1**), which, under the biotrasformation conditions, can be hydrolyzed in hydroxytyrosol. Also, the remarkable increase in tyrosol (**Table 2**) may be attributed to the chemical hydrolysis of ligstroside (**Figure 1**), which has been isolated from *O. europaea* L. leaf extract (*35*).

In the eluate from the two-step bioreactor, a slight decrease of the amount of hydroxytyrosol is observed (**Table 2**), whereas the amount of tyrosol does not show significant changes. We note, furthermore, a decrease of syringic and gallic acids as well as of total phenol content, having evidence that the chitosan in the second column interacts by its amino groups with phenols containing a carboxylic group. It is necessary to point out that, in the presence of the chitosan column, the mass of the recovered eluate (11.1 mg) considerably decreases: a decrease of 56.6% in weight, which cannot be attributed to only the decrease of total phenols. The chitosan matrix may react, by adsorption, with the carbohydrates available in the leaf extract as well as with those obtained by the hydrolysis catalyzed by the thermophilic β-glycosidase. Such enzyme, with a remarkable β-glucosidase activity and wide substrate specificity (36), may catalyze the hydrolysis of glycosidic bonds in several glucosidic oleosides, ligstrosides, and flavonoids isolated in leaf extract (*34*, *35*, *37*).

Table 3. Antioxidant Activities of Cv. Moraiolo Leaf Extract and Bioreactor Eluates by One- and Two-Step Approaches[a]

	DMPD method		ABAP method on linoleic acid	
	EC_{50} (μg/mL)	AP_i	IP_A	IP_S
Moraiolo leaf extract	97.57 ± 15.81	0.5419 ± 0.0023	29.88 ± 0.86	0.6893 ± 0.0512
bioreactor eluate one-step approach	496.91 ± 64.09	3.086 ± 0.056	34.27 ± 0.98	0.0823 ± 0.0058
bioreactor eluate two-step approach	49.97 ± 3.98	0.2562 ± 0.0067	35.26 ± 0.70	0.7554 ± 0.0414

[a] The EC_{50} and AP_i values determined by DMPD were obtained by eq 2 and the IP_A and IP_S for linoleic acid oxidation as described in the text. Assays were run in triplicate and data are expressed as the mean (n = 3) ± standard error.

To identify the complex phenols containing a hydroxytyrosol or tyrosol group, we have analyzed the GC-MS spectra of hydroxytyrosol, tyrosol, and oleuropein aglycon (**Figure 1**). According to the fragmentation mechanism of simple phenols (*38*), the mass spectrum of tyrosol showed a low abundance of molecular ion (M^+; m/z 282; 32%) and the main peak at m/z 179. Hydroxytyrosol was characterized by a similar trend with the molecular ion more abundant than tyrosol (M^+; m/z 370; 82%) and the higher peak at m/z 268. The GC-MS spectrum of the monoaldehydic elenolate oleuropein aglycon was more complicated because the fragmentation of these complex phenols has been so far not fully studied. We did not identify the significant peak at m/z 280, which was attributed to the hydroxytyrosol fragment formed through a McLafferty rearrangement of the open dialdehydic form of secoiridoid aglycons (*39*). This characteristic fragmentation of β-phenyl ethyl esters might be useful to establish if some complex phenols, in leaf extract and eluates, are attributable to hydroxytyrosol or tyrosol derivatives. In fact, in the GC-MS spectra of aglyconic phenols, the m/z 280 and 192 fragments have been related to β-phenyl ethanol derivatives (hydroxytyrosol and tyrosol, respectively) (*38*).

In the GC-MS spectrum of leaf extract, the m/z 192 fragment was present in three significant peaks of the spectrum (t_R = 28.4, 30.0, and 30.4) but a further four peaks with smaller intensities were also found. Their presence might justify the increase of tyrosol amount in the recovered eluates. The m/z 280 fragment was not identified in the GC-MS spectrum of leaf extract, whereas complex phenols containing the hydroxytyrosol group, related to this fragment, were identified in the GC-MS spectra of the eluates collected by biotransformation. These complex phenols probably derive from the hydrolysis, catalyzed by the immobilized β-glycosidase, of their corresponding glycosidic form, but the TMS derivatives of oleuropein and of other glycosidic phenols, which we hypothesized to be present in the leaf extract, are not detectable by our GC-MS analyses. In the GC-MS spectrum of eluate obtained by the one-step bioreactor, two peaks (t_R = 26.6 and 28.0) with the m/z 280 fragment were identified, whereas in the spectrum of the two-step bioreactor eluate, only one peak (t_R = 28.0) was present. As a matter of fact, the longer permanence of the latter eluate at the working temperature was proved to be useful to hydrolyze some aglyconic compounds in hydroxytyrosol.

Measurement of Antioxidant and Antiperoxidative Activities. At acidic pH in the presence of a suitable oxidant solution ($FeCl_3$), DMPD can make a stable red radical cation ($DMPD^{\bullet+}$). Antioxidant compounds, which transfer a hydrogen atom to $DMPD^{\bullet+}$, quench the red color and produce a bleaching of the solution proportional to their amounts (*40*). This reaction is fast, and its stable end point is taken as a measure of antioxidant activity; therefore, this assay reflects the radical hydrogen-donating ability to scavenge the single electron from $DMPD^{\bullet+}$.

The EC_{50} and AP_i values (**Table 3**) calculated for the three investigated samples have pointed out that the eluate obtained by the two-step bioreactor showed a higher antioxidant activity due to its higher simple phenols concentration (**Table 1**). The ability to donate radical hydrogen is, in fact, prevalently attributable to simple phenols and, to a lesser extent, to secoiridoids as oleuropein, whereas the elenolic acid derivatives show the lowest ability (*29*, *41*). Therefore, the antioxidant activity of leaf extract is due to its high oleuropein concentration (**Table 1**), whereas the lower simple phenols content (**Table 1**) and the presence of elenolic acid derivatives can explain the low antioxidant capacity of the eluate from the one-step bioreactor (**Table 3**). In the one-step bioreactor eluate, the hydrolysis of glycosidic and nonglycosidic secoiridoids originates elenolic acid derivatives, which might be retained on the chitosan column in the two-step bioreactor, thus explaining the higher activity of the eluate from this approach.

With regard to the antiperoxidative activities, those compounds that own a phenolic ring show additional activities, among the antioxidant properties, which may include chain breaking in radical propagation by trapping peroxyl radicals (*42*) and/or radical scavenging in lipidic peroxidation (*20*). Our previous data had displayed that the hydroxytyrosol exhibits a stronger radical-scavenging activity in the lipid peroxide production, and this capacity is attributable to the interaction of the phenolic ring, which is not bound to an elenolic structure, with a peroxyl radical. Oleuropein and its aglycon, in fact, produced a very slight effect on fatty acid peroxidation (*29*). The IP_A and IP_S for linoleic acid peroxidation by ABAP method (**Table 3**) point out that the eluate from the two-step bioreactor shows the higher antiperoxidative activity at concentrations ranging from 50 to 200 μg/mg linoleic acid (**Figure 3**), whereas its inhibition power is similar to that of leaf extract at lower concentration (1−35 μg/mg linoleic acid). The significative oleuropein content in the leaf extract, and the high hydroxytyrosol concentration in the eluate from two-step approach (**Table 1**), can explain their antiperoxidant activity. This property is reduced for the eluate from the one-step bioreactor (**Table 3**), which is characterized by the lower total phenols content (**Table 1**). In this eluate, the higher amount of elenolic acid derivatives, with lower antiperoxidative activity (*29*), contributes also to decrease its ability to inhibit the rate of conjugated diene hydroperoxide production. The LDL copper sulfate induced oxidation, in the investigated time (120 min), is totally inhibited by 28.1, 230.0, and 20.6 μg/mg LDL of leaf extract, eluates from the one- and two-step bioreactor approaches, respectively (**Table 4**). These results are understandable considering the concentrations of oleuropein and hydroxytyrosol (23.0 and 18.2 μg/mg of LDL, respectively) necessary to prevent the formation of conjugated dienes in the incubated LDL (**Table 4**). A significant degree of protection of LDL from oxidation was, also, observed for extracts from olive oil waste waters (20 ppm) with high hydroxytyrosol content (*43*). Our data show that the eluate from the two-step bioreactor (5 ppm) and the leaf extract (7 ppm) were more effective to inhibit LDL oxidation. On the other hand, the higher glucose concentration in the eluate from

Table 4. Amount of Cv. Moraiolo Leaf Extract, Bioreactor Eluates by One- and Two-Step Bioreactor Approaches, Oleuropein, and Hydroxytyrosol Necessary To Totally Inhibit the LDL Copper Sulfate Induced Oxidation, in the Investigated Time (120 min)

	μg/mg$_{LDL}$
Moraiolo leaf extract	28.1
bioreactor eluate, one-step approach	230.0
bioreactor eluate, two-step approach	20.6
oleuropein	23.0
hydroxytyrosol	18.2

the one-step bioreactor can explain its lower activity (55 ppm): a synergistic effect of transition metals and glucose can partially account for accelerated copper-mediated LDL oxidation (*44*).

Conclusion. We report a very simple and fast method to collect eluates with high amounts of hydroxytyrosol, using *O. europaea* L. leaf extract. In addition, the amount of the other simple phenols identified in these mixtures is higher than that in *O. europaea* L. leaf extract tablets as well as that in olive oils. In particular, in extra virgin olive oils are found (*12*) hydroxytyrosol (0.0−25.4 mg/kg), tyrosol (0.1−123.1 mg/kg), syringic acid (0.0−2.3 mg/kg), and ferulic acid (0.0−2.4 mg/kg).

Furthermore, using the bioreactor in a different approach we have collected a mixture of phenolic compounds with a significant antioxidant activity. Nevertheless, the possibility to bind by adsorption on the matrix in the second column carbohydrates such as glucose could make the obtained eluate utilizable as a dietetic integrator with a low carbohydrate content.

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