JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Isolation and Characterization of a New Hydroxytyrosol Derivative from Olive (*Olea europaea*) Leaves

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A new secoiridoid compound was isolated from the leaves of *Olea europaea*. This compound, not previously identified, is the bis methylacetal of oleuropein aglycone, the 3,4-dihydroxyphenylethyl [(2,6-dimethoxy-3-ethylidene)-tetrahydropyran-4-yl]acetate (3,4-DHPEA-DETA), and was found in different olive cultivar phenolic extracts as one of the major secoiridoid components. This compound was shown to be easily transformed in acidic aqueous media into 3,4-DHPEA-EDA, the major polyphenolic compound found in olive oil, and permitted us to increase the yield of 3,4-DHPEA-EDA isolation from the olive leaf extract. The antiradical activity of this new compound, evaluated by scavenging of 2,2-diphenyl-1-picrylhydrazyl radicals, was much higher than the one found for 3,4-DHPEA-EDA or α-tocopherol. Results also call to attention the need for a careful identification of compounds by HPLC-MS, usually performed in acidic conditions.

KEYWORDS: ***Olea europaea*; oleuropein aglycon; olive leaves; secoiridoids**

INTRODUCTION

The major polyphenolic constituent in olives and olive leaves (*Olea europaea* L.) is oleuropein glycoside, but this compound is almost completely absent from olive oil because of its low oil solubility and its extensive enzymatic degradation during olive oil production. Phenyl acids and phenyl alcohols, including 3,4-(dihydroxyphenyl)ethanol (hydroxytyrosol) or *p*-hydroxyphenylethanol (tyrosol), have been found in virgin olive oil, but the prevalent phenolic compounds are secoiridoid derivatives of oleuropein (**Figure 1**) and ligstrosid, such as the dialdehydic form of elenolic acid linked either to 3,4-(dihydroxyphenyl)ethanol (3,4-DHPEA-EDA) or to *p*- hydroxyphenylethanol (3-DHPEA-EDA or oleocanthal), and an isomer of oleuropein aglycon [3,4-(dihydroxyphenyl)ethanol elenolic acid ester, 3,4-DHPEA-EA] (*1–8*). These compounds are the most concentrated of those with a phenolic structure in virgin olive oil, where they can account for up to 55% of total phenolic fraction (*5–8*). Hydroxytyrosol derivatives are of particular significance because of their strong antioxidant/biological activity in several lipid systems including oil (*9–13*), emulsions (*14–16*), and low-density lipoprotein suspensions (*17–22*).

Animal and human studies suggest that the relatively high concentration of phenolic compounds in extra virgin olive oil contribute to the healthy nature of this oil (*20–28*). The oxidative stability of LDL isolated from animals and humans fed with virgin olive oil is increased, and this increased stability is attributable to the minor phenolic compounds in the oil (*19, 21, 27–30*). Direct evidence for the protective role of olive oil against cancer has also been recently published (*31*). Although many studies have investigated the antioxidant properties, the protective effects against cell injury, and the bioavailability of oleuropein and especially of hydroxytyrosol, relatively few studies have investigated the protective effects of oleuropein aglycones, mostly because of the difficulty in isolating these compounds.

Leaf extracts have been shown to be an important source of secoiridoids similar to the ones found in olive oil (*15*). This raw material is available throughout the year and is not under rapid enzymatic degradation as in other possible sources of oleuropein aglycones such as olive pomace. Therefore, their isolation from leaves in higher amounts than the ones obtained directly from olive oil has permitted some of the first studies on their biological and antioxidant activity. In particular, 3,4-DHPEA-EDA and 3,4-DHPEA-EA have shown important antioxidant activity in bulk oil, 5 to 7 times higher than that of α-tocopherol (*14, 16*).

The complexity of phenolic extracts is usually great, which is caused by the labile chemistry of the secoiridoid compounds that easily react with solvents, form nonconvalent adducts, and are easily converted in other compounds during extraction processes caused by the easy opening of the secoiridoid ring after hydrolyzation of the glucose moiety (**Figure 1**) (*2, 32–35*). Since the concentration of oleuropein and ligstroside derivatives rises largely during the mechanical extraction of olive oil, nuclear magnetic resonance (NMR) studies on enzymatic hydrolysis of oleuropein by β-glucosidase have been performed

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10.1021/jf800698y CCC: $40.75
Published on Web 06/27/2008

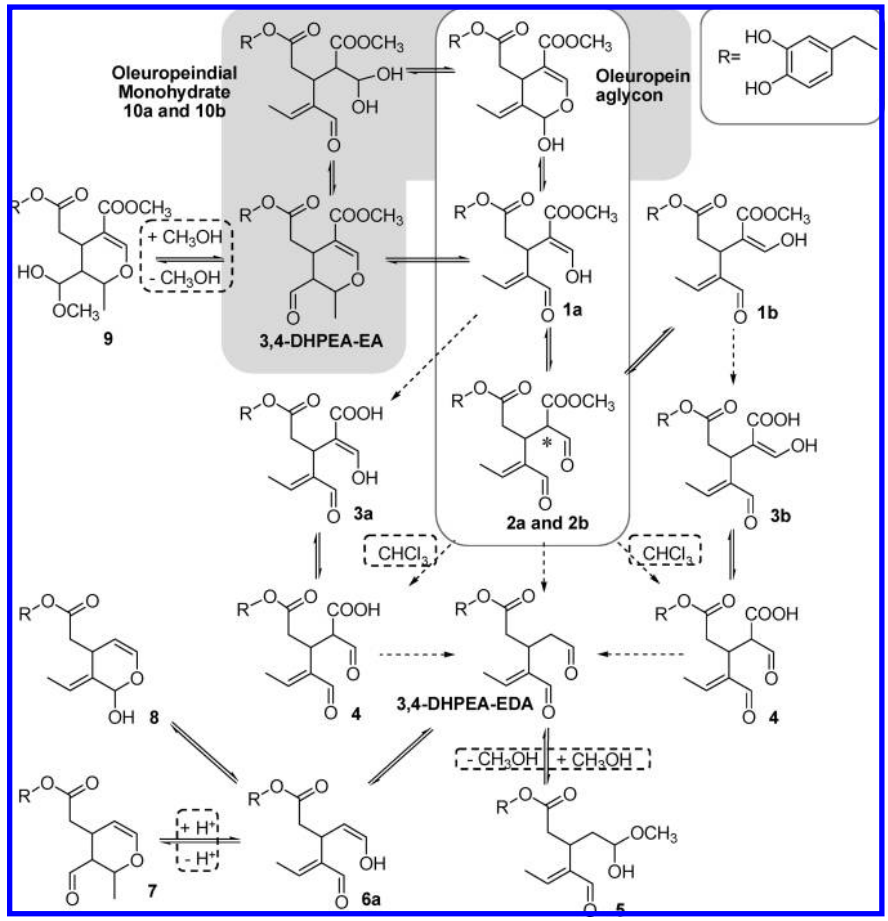


Figure 1. Structures of some olive oil phenolic compounds. Possible transformation pathways of oleuropein aglycone during olive oil extraction and in olive leaves. Adapted from refs *2, 15, and 32–36*. Transformations observed in *d*-chloroform/*d*-water (1:1) (gray box), in *d*-water (white box) and in methanol, chloroform and acidic solutions (solvents in doted boxes). * = epimeric center.

in chloroform/water (1:1) and in water in order to understand the mechanism of synthesis/biosynthesis of these derivatives (*32–34*). In fact, oleuropein aglycones may exist in a number of keto−enolic tautomeric equilibria involving the opening of the heterocyclic ring, yielding compounds of different structures. **Figure 1** summarizes some of these transformations.

This article reports on the isolation of a new hydroxytyrosol derivative, which is one of the major secoiridoids found in olive leaf extracts, and that can be easily transformed into one of the most abundant secoiridoids found in olive oil, the 3,4-DHPEA-EDA. In some olive oils, the compound 3,4-DHPEA-EDA may represent 50% of the phenolic fraction of this oil (*7, 8*).

MATERIALS AND METHODS

Reference Compounds. Oleuropein was purchased from Extrasynthese (Genay, France) or extracted from olive leaves according to the procedure of Gariboldi et al. (*32*). The oleuropein aglycon 3,4-DHPEA-EA was obtained from oleuropein by enzymatic reaction using β-glycosidase (Fluka, Buchs, Switzerland) according to the procedure of Limirioli et al. (*33*). The dialdehydic form of elenolic acid linked to hydroxytyrosol (3,4-DHPEA-EDA) was obtained from olive leaves according to the procedure of Paiva-Martins and Gordon (*15*).

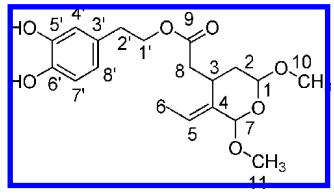
Isolation of Polyphenols from Leaves. Leaf polyphenolic extracts were obtained from Carrasca, Ripa, Negruche, Cordovil, Verdeal, Madural, and Bical cultivars. Leaves from each cultivar (150 g), enclosed in a plastic bag without air, were stored at 38 °C for 18 h. This procedure was necessary in order to obtain 3,4-DHPEA-EDA according to the procedure of Paiva-Martins and Gordon (*15*). Then leaves were macerated in 250 mL of ethanol for 5 days in the dark at room temperature. The extract was separated by filtration, and the solvent was evaporated under vacuum. The residue was taken up in 50 mL of acetone/water (1:1) (v/v). The aqueous mixture was successively extracted with *n*-hexane followed by chloroform (Merck, Lisbon, Portugal). After solvent evaporation, the extract was dissolved in 10 mL of methanol and analyzed by TLC and HPLC.

Isolation of 3,4-Dihydroxyphenylethyl [(2,6-dimethoxy-3-ethylidene)-tetrahydropyran-4-yl]acetate (3,4-DHPEA-DETA) and 3,4-DHPEA-EDA. After solvent evaporation, the Carrasca extract (0.42 g) was purified by column chromatography using silica gel 60 (Merck, 230−400 mesh ASTM, 140 g) and eluted with diethyl ether/methanol (35:2) (Merck, Lisbon, Portugal). Combined fractions gave 0.0435 g of pure 3,4-DHPEA-EDA (identified by NMR and MS) and 0.0984 g of pure 3,4-DHPEA-DETA identified by NMR and MS as 3,4-dihydroxyphenylethyl [(2,6-dimethoxy-3-ethylidene)-tetrahydropyran-4-yl]acetate (3,4-DHPEA-DETA) (**Table 1** and **Figure 3**).

NMR. ^{1}H, ^{13}C, and ^{13}C DEPT-135 (Distortionless Enhancement by Polarization Transfer) spectra were recorded using a Brucker AMX 300. The COSY (Homonuclear Correlated Spectroscopy) spectrum, HETCOR (Heteronuclear Correlation Spectroscopy) spectrum, and another ^{1}H NMR spectrum, used in the calculation of the coupling constants, were recorded using a Varian Inova 750 spectrometer. Compounds were dissolved in acetone-d_6.

HPLC. The HPLC system comprised a Merck-Hitachi chromatograph with a Merck Hitachi L-6200 Intelligent Pump and a 250 mm × 4.6 mm Waters Spherisorb ODS2 5 μm column (Supelco Inc.), coupled to a Merck Hitachi L-4200 UV−vis detector, and components were detected at 280 nm with elution at room temperature. The composition of samples was determined by HPLC using a flow rate of 1 mL·min^{-1} and, as a mobile phase, a mixture of 0.1% formic acid (pH 3.1) in water (A) and methanol (B) with a total analysis time of 70 min, according to the method previously described (*15*). Another run was performed in the same conditions but changing solvent A for distilled water.

Table 1. ^{1}H and ^{13}C Nuclear Magnetic Resonance Data in Acetone-d^a



position	$\delta^{1}H$	$\delta^{13}C$
1	4.88 dd J1,2a = 2 J1,2b = 2	96.5
2a	1.85 m	41.0
2b	1.68 m	41.0
3	3.23 m	35.9
4		137.4
5	5.52 q J5,4 = 7	125.5
6	1.63 d J6,5 = 7	13.7
7	4.84 s	106.0
8a	2.58 dd J8a,8b = 16 J8a,3 = 5	49.8
8b	2.50 dd J8a,8b = 16 J8b,3 = 10	49.5
9		173.5
10	3.34 s	55.9
11	3.38 s	57.1
1′	4.18 m	66.7
2′	2.77 t J2′,1′ = 7	36.2
3′		132.5
4′	6.74 dd J4′,8′ = 2	122.0
5′		146.8
6′		145.5
7′	6.74 d J7′,8′ = 8	117.7
8′	6.58 dd J8′,4′ = 2 J8′,7′ = 8	117.0

[a] Chemical shifts are in ppm and coupling constants in Hz.

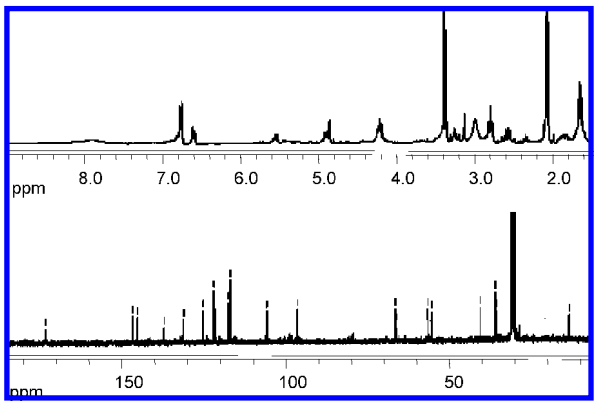


Figure 2. ^{1}H and ^{13}C spectra of the new compound 3,4-DHPEA-DETA, recorded using a Brucker AMX 300.

Table 2. 1,1-Diphenyl-2-picrylhydrazyl Radical Scavenging Effects of 3,4-DHPEA-DETA, 3,4-DHPEA-EDA, and α-Tocopherol after 5 and 15 min

	time 5 min[a]		time 15 min[a]	
compound	EC_{50}[b]	no. of reduced radicals	EC_{50}[b]	no. of reduced radicals
3,4-DHPEA-DETA	0.091 a (±0.01)	5.5	0.053 a (±0.01)	9.4
3,4-DHPEA-EDA	0.30 b (±0.01)	1.7	0.22 b (±0.01)	2.3
α-tocopherol	0.29 b (±0.01)	1.7	0.25 c (±0.01)	2.0

[a] Mean (standard deviation in parentheses) of eight determinations. Superscripts within a column indicate samples that were significantly different ($p < 0.05$). [b] EC_{50} expressed as mol of antioxidant/mol of 1,1-diphenyl-2-picrylhydrazyl radical.

For the LC-MS analysis, the flow rate used was 0.5 mL. min^{-1}; the mobile phase used was also a mixture of 0.1% formic acid (pH 3.1) in water (A) and methanol (B) with a total analysis time of 100 min, and the gradient changed as follows: 95% A/5% B for 15 min, 80% A/20% B in 15 min, 70% A/30% B in 15 min, 70% A/30% B for 10 min, 50% A/50% B in 10 min, 40% A/60% B in 10 min, 30% A/70% B in 10 min, 30% A/70% B for 10 min, 100% B in 1 min, and maintained until the end of the analysis. Samples were analyzed using 20 μL of each solution dissolved in methanol. Solvents were HPLC grade. Peak identification and quantification was performed by comparison of retention times using a standard solution containing reference polyphenols.

Thin Layer Chromatography (TLC). Phenolic extracts and reference compounds were analyzed by TLC. Two microliters of extract from each olive leaf cultivar was applied on a Silica gel GF_{254} plate (Merck); spots were visualized by iodine. Eluent, diethyl ether/methanol (35:2); R_f (3,4-DHPEA-DETA) = 0.75.

ESI/MS Analysis. Mass spectrometry analysis was performed using a Finnigan LCQ DECA XP MAX equipped with an API source, using an electrospray ionization (ESI) probe. Compound was injected after the HPLC separation in the conditions previously described or directly in the MS spectrometer with a pump at a flow rate of 3 μL/min. The capillary temperature and voltage used were 180 °C and 3 V, respectively, and spectra were obtained in negative ion mode. When the molecular ion of the phenolic compound was detected, its MS^2 spectrum was obtained using a relative energy collision of 27. MS^3 of the main fragment ion in the MS^2 spectrum was also obtained using a relative energy collision of 26.

Synthesis of 3,4-DHPEA-EDA. 3,4-DHPEA-DETA (0.015 g) was dissolved in a mixture of acetone/water/HCOOH (10:89:1) and stirred for 2 h at room temperature. The aqueous mixture was extracted with chloroform. After solvent evaporation, the extract (0.0134 g) was analyzed by NMR. The compound identified in the extract showed the same ^{1}H and ^{13}C spectral data as that reported by Montedoro et al. and Paiva-Martins and Gordon for 3,4-DHPEA-EDA (*2, 15*).

The same procedure was performed in distilled water (pH 6.95) in order to evaluate the stability of 3,4-DHPEA-DETA in a neutral aqueous solvent. This time, a mixture of compounds was obtained, observed by TLC analysis.

Determination of Radical Scavenging Activity. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was used as a stable radical (*36*). Several concentrations of 3,4-DHPEA-DETA were tested. 3,4-DHPEA-EDA and α-tocopherol were also tested in the same conditions. Phenolic compound solution (0.1 mL) was added to 3.5 mL of a 0.06 mM methanolic DPPH radical solution. The decrease in absorbance was determined at 515 nm at 0, 5, and 15 min. The exact initial DPPH radical concentration in the reaction was calculated from a calibration curve determined by linear regression. The change in absorbance with time was plotted, and from this graph, the percentage of DPPH radical remaining at several times was determined. The values were transferred onto another graph showing the percentage of residual DPPH radical as a function of the molar ratio of phenolic compound to DPPH radical. Antiradical activity was defined as the relative concentration of phenolic compound required to lower the initial DPPH radical concentration by 50% [EC50 (mol/L 5 per unit DPPH radical concentration)]. The test was performed twice in quadruplicate. SPSS 15.0 software was used for statistical analysis by one-way analysis of variance (ANOVA) with the level of significance set at $P < 0.05$.

RESULTS AND DISCUSSION

The structure of the new isolated compound, a derivative of oleuropein aglycone, was determined on the basis of proton and carbon magnetic resonance experiments. The ^{1}H and ^{13}C spectra showed four interesting characteristics important for the identification of this compound: the presence of typical resonances for the hydroxytyrosol moiety, with the aromatic proton signals in the range of 6.58−6.74 ppm and the methylene part as triplet at 2.77 ppm, and multiplet at 4.18 ppm; the lack of aldeidic protons; the presence of two resonances (3.34 and 3.38 ppm as singlets) corresponding to CH_3 protons linked to an oxygen; and the presence of only one carbonylic carbon (173.5 ppm) (**Figure 2**). These observations led us to believe that we were

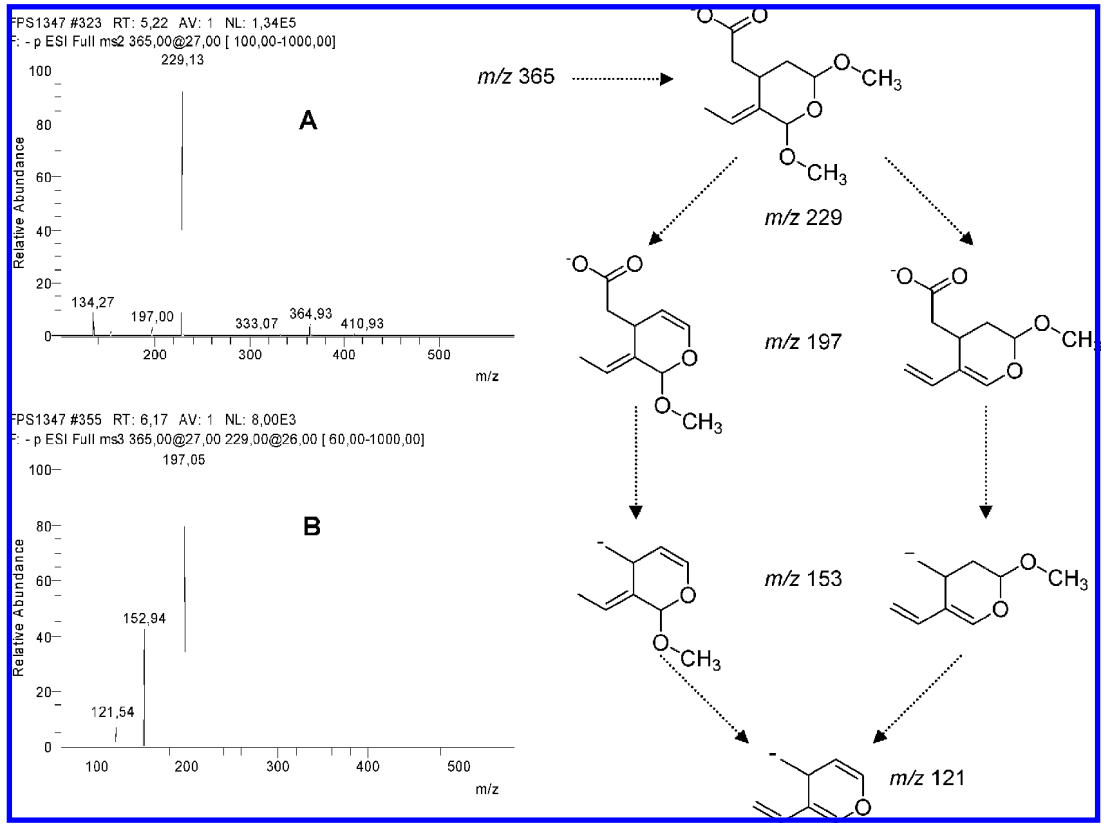


Figure 3. Mass spectra in product ion scan mode of *m/z* 365 (**A**, MS^2) and *m/z* 229 (**B**, MS^3) and proposed negative electrospray ionization fragmentation scheme for 3,4-DHPEA-DETA.

in the presence of an oleuropein derivative molecule with two methoxy groups. Moreover, the singlet at 4.84 and the doublet of doublets at 4.88 ppm indicate two acetal protons at C7 and C1. The two-dimensional H1 (COSY) NMR spectrum reveals coupling between H1 and both H2, coupling between both H2 and H3, and coupling between H3 and both H8, suggesting a molecular structure similar to that of 3,4-DHPEA-DETA. This spectrum also showed coupling between H5 and H6 and the absence of coupling for both CH_3 signals, as expected for the proposed structure. The HETCOR and ^{13}C DEPT-135 spectral data confirmed the proposed structure.

The deprotoneted molecule $[M - H]^-$ ($m/z = 365$), the noncovalent dimer $[2M - H]^-$ ($m/z = 731$), the noncovalent adduct $[M + 197\text{-}H]^-$ ($m/z = 561$), the noncovalent adduct $[M - HCOOH\text{-}H]^-$ ($m/z = 411$), resulting from some residual formic acid in the system from a previous solvent, and the fragment $m/z = 229$ were identified in full-scan mode as the main picks. Mass spectra in production scan mode of *m/z* 365, together with the mass spectra of the ion mode of *m/z* 229, proved useful in confirming the proposed fragmentation scheme (**Figure 3**) for this phenolic compound. In contrast, the positive spectrum derived from this compound did not yield the $[M + H]^+$, being the strongest fragment arising from cleavage of one methoxy group $[M - 31+H]^+$ ($m/z = 335$).

Analysis of several leaf extracts by TLC with iodine detection showed that the spot corresponding to this compound appeared as one of the major compounds in the TLC chromatogram of all leaf extracts obtained from several cultivars (**Figure 4**). The determination of this compound was therefore attempted using HPLC. However, the signal of this compound showed an unexpected behavior. In fact, the retention time of this compound was similar to that obtained for the dialdeidic form of the oleuropein aglycone, 3,4-DHPEA-EDA, in several eluent HPLC programs, and no separation was achieved. When analyzed by

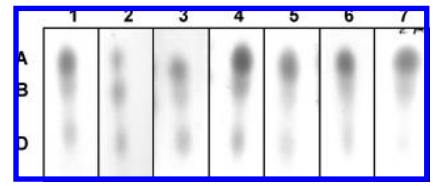


Figure 4. TLC of several olive leaf extracts. Spot identification: A, 3,4-DHPEA-DETA; B, mixture of 3,4-DHPEA-EA and hemiacetalic derivative of 3,4-DHPEA-EDA; C, mixture of 3,4-DHPEA-EDA with other secoiridoids. Cultivar identification: 1, Bical; 2, Cordovil; 3, Ripa; 4, Negruche; 5, Madural; 6, Verdial; 7, Carrasca.

LC-MS, the spectra obtained was also similar to the spectra obtained for 3,4-DHPEA-EDA. Apparently, this new compound was converted into 3,4-DHPEA-EDA in aqueous acidic solutions, such as the eluent used (0,1% HCOOH aqueous solution) (**Figure 5**). This was also supported by the MS spectra in positive mode (**Figure 6**) by the presence of signals at ion *m/z* 335 and ion *m/z* 199 (corresponding to the loss of the hydroxytyrosol moiety of fragment *m/z* 335) and the lack of the $[M + H]^+$ ion, probably caused by the instability of this compound in acidic media. This behavior has already been described for other secoiridoids (*35, 37*) with protic solvents with pK_a values lower than that of water.

Therefore, a small amount of 3,4-DHPEA-DETA was dissolved in a mixture of acetone/water/HCOOH (10:89:1) and stirred for 2 h at room temperature. The aqueous mixture was then extracted with chloroform. After solvent evaporation, 3,4-DHPEA-DETA showed a complete transformation in 3,4-DHPEA-EDA, as proved by its ^1H and ^{13}C NMR spectra.

The same procedure was performed without formic acid in order to evaluate the stability of 3,4-DHPEA-DETA in a neutral aqueous solvent. When assessed by TLC, a mixture of compounds, including 3,4-DHPEA-EDA, was obtained, but the spot

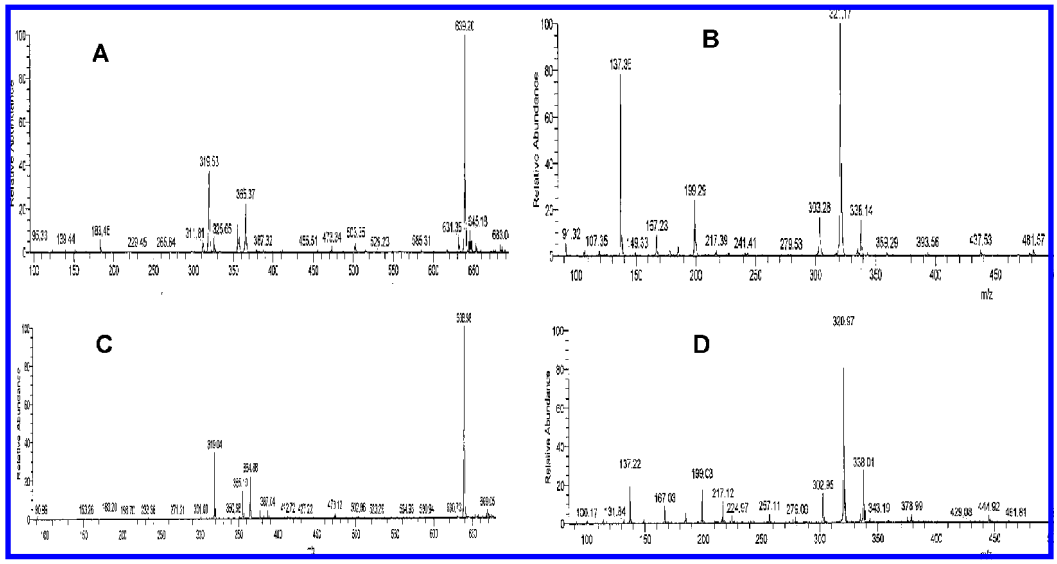


Figure 5. Mass spectra in full scan mode of compounds 3,4-DHPEA-DETA (**A** and **B**) and 3,4-DHPEA-EDA (**C** and **D**) after elution with acidic eluent in the HPLC column. **A** and **C**, negative mode; **B** and **D**, positive mode.

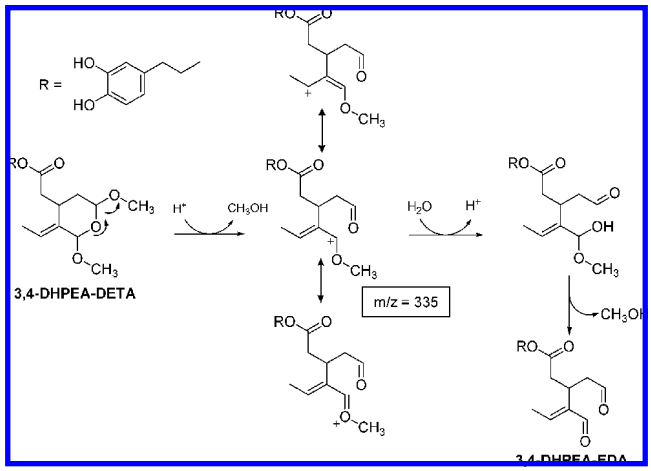


Figure 6. Proposed behavior of compound in aqueous acidic solution. Proposed positive electrospray ionization fragment for *m/z* 335.

corresponding to 3,4-DHPEA-DETA was still important (data not shown). A tentative quantification/identification was then performed by HPLC using distilled water as eluent A instead of a water/HCOOH mixture, and several overlapped picks (main pick at $RT = 40.1$ min) were obtained with a different retention time of 3,4-DHPEA-EDA ($RT_{3,4\text{-DHPEA-EDA}} = 33.6$ min) (**Figure 7**).

In the aprotic solvent acetone-d_6, 3,4-DHPEA-DETA was found to be stable for several months at −20 °C as confirmed by several ^{1}H NMR spectra and TLC chromatograms performed during that time.

3,4-DHPEA-DETA was examined for its radical scavenging activity toward the stable DPPH free radical. The compound reacted rapidly with the DPPH radical in the first 15 min of reaction and showed a significant high antiradical scavenger activity when compared with that of α-tocopherol and 3,4-DHPEA-EDA (**Table 2**). This high activity is probably caused by the labile acid elenolic derivative ring of the molecule that may also react with radicals and therefore contribute to the radical scavenging activity assessed by this method.

This is the first report of this compound in olive leaves extracts, and, to our knowledge, its identification has never been performed before. However, a MS spectrum similar to the one found for 3,4-DHPEA-DETA was reported by De la Torre-Carbot et al. (*38*) for an unidentified secoiridoid derivative detected in olive oil extracts. Therefore, if this compound is present in olive oil because of its high radical scavenging activity, it will have an important role in the oxidative stability of the oil.

It could be unclear if this compound was only formed during the extraction process with methanol. Nevertheless, this compound has never been detected in 3,4-DHPEA-EDA methanolic stock solutions, even after several months of storage at −20 °C. Moreover, this compound is also detected by TLC in phenolic extracts when ethanol is used as extraction solvent.

Since 3,4-DHPEA-DETA can be easily transformed in 3,4-DHPEA-EDA, the major compound found in olive oil, these

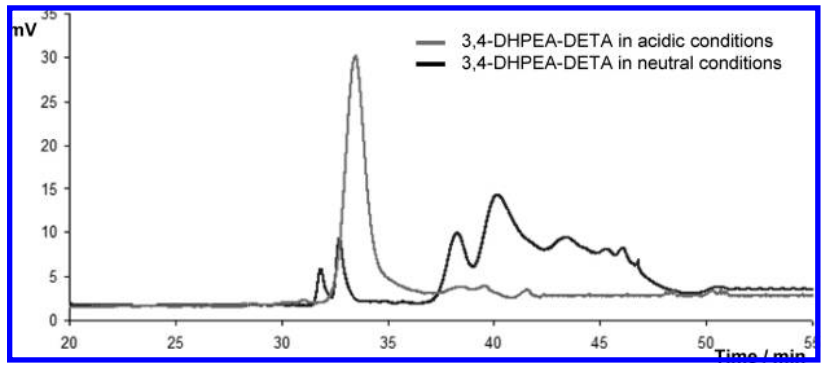


Figure 7. HPLC chromatogram obtained after eluition of 3,4-DHPEA-DETA with an acidic eluent (—) and with a more neutral eluent (—).

findings may open a new way of obtaining this phenolic compound from olive leaves with higher recovery. Moreover, both compounds can be separated with the same chromatographic column separation procedure. One of the major difficulties in separating 3,4-DHPEA-EDA is that this compound can easily elute with other secoiridoids with the same R_f value (*15*) in all eluent systems used so far. However, as discussed in the Introduction section, the complexity of phenolic extracts is usually great and is caused by the labile chemistry of these compounds that easily react with solvents, form nonconvalent adducts, and are easily converted into other compounds during extraction processes. 3,4-DHPEA-DETA is easier to separate from the whole extract because of its different R_f value and because apparently no other compounds coeluted. This procedure has permitted us to triple the yield of 3,4-DHPEA-EDA isolation from the olive leaf extract. However, like any other natural product, 3,4-DHPEA-DETA concentration in leaves and therefore the yield of the whole procedure will depend on the cultivar, time of the year, and weather conditions. As can be observed in **Figure 4**, the Cordovil cultivar showed on the TLC a less intense 3,4-DHPEA-DETA spot when compared with the 3,4-DHPEA-EDA spot, and in fact, less quantity of phenols was recovered from this cultivar (0.0203 g of 3,4-DHPEA-EDA plus 0.0134 g of 3,4-DHPEA-DETA).

This compound can also be detected in olive leaf extracts obtained from fresh leaves. Apparently, the treatment of leaves at 37 °C in a plastic bag without air decreases somewhat the concentration of this compound in the extract (assessed by TLC). However, the concentration of 3,4-DHPEA-DETA plus 3,4-DHPEA-EDA, assessed by HPLC, is lower in leaves without this treatment (*15*). Moreover, the separation by column chromatography of both compounds is more difficult since more compounds are present in the extract (*15*).

These results also bring to attention the need for a careful identification of compounds by LC-MS, usually performed in acidic conditions. Also, compounds with this behavior will never be separated as pure by preparative HPLC with acidic solvents.

ACKNOWLEDGMENT

We thank Zélia Azevedo for technical assistance.

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Received for review November 20, 2007. Revised manuscript received April 24, 2008. Accepted May 8, 2008.

JF800698Y