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Separation and Identification of Phenolic Compounds in Olive Oil by Coupling High-Performance Liquid Chromatography with Postcolumn Solid-Phase Extraction to Nuclear Magnetic Resonance Spectroscopy (LC-SPE-NMR)

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This study reports the first application of the hyphenated LC-SPE-NMR technique using postcolumn solid-phase extraction to the direct analysis of phenolic compounds in the polar part of olive oil. Apart from the identification and structure elucidation of simple phenols (hydroxytyrosol, tyrosol, vanillic acid, vanillin, *p*-coumaric acid, hydroxytyrosol, and tyrosol acetates), lignans (pinoresinol and 1-acetoxypinoresinol), flavonoids (apigenin and luteolin), and a large number of secoiridoid derivatives, this technique enables the identification of several new phenolic components, which had not been reported previously as constituents in the polar part of olive oil.

KEYWORDS: LC-SPE-NMR; phenolic compounds; olive oil

INTRODUCTION

Among other minor constituents, olive oil contains phenolic compounds usually referred to as phenolic acids or simply polyphenols. The major phenolic compounds identified and quantified in olive oil belong to four different classes: simple phenols, (hydroxytyrosol, tyrosol, *p*-coumaric acid, vanillic acid, etc.), secoiridoids (oleuropein, ligstroside, and, in particular, their hydrolysis derivatives), lignans [(+)-pinoresinol and (+)-1-acetoxypinoresinol)], and the flavonoids (apigenin and luteolin). Members of these four classes have potent antioxidant activity and contribute significantly to the extraordinary stability of olive oil against oxidation (*1*, *2*). Several epidemiological studies (*3*, *4*) have shown that phenolic compounds afford considerable protection against cancer (skin, breast, and colon), coronary heart disease, and aging by inhibiting oxidative stress. Also, phenolic compounds are related to the sensory and nutritional qualities of virgin olive oil (*2*). Because of these properties, cold-pressed extra virgin olive oil is considered to be the major component of the so-called Mediterranean diet.

There exists an extensive literature concerning the detection and quantification of phenolic compounds in olive oil. Starting from the early days, nonspecific analytical methods, such as paper, thin-layer, and column chromatography, as well as UV spectroscopy were applied to polyphenols analysis with limited success. Significant progress was achieved by employing specific analytical methods, such as high-resolution gas chromatography (GC) and, in particular, high-performance liquid chromatography (HPLC) (*2*, *5*). Despite the fact that these analytical techniques are characterized by very low detection limits, a careful assessment indicates that chromatographic data are not homogeneous. In several cases contradictory results are obtained when different mobile phases are used for elution, in particular, with unknown compounds with the same retention times as the polyphenols present. It is worth mentioning that several peaks in the chromatograms, especially at long retention times, remain unknown.

During the past decade proton nuclear magnetic resonance spectroscopy has been successfully employed in olive oil analysis (*6*, *7*). Although the usefulness of ^{1}H NMR spectroscopy has been increasingly recognized for its noninvasiveness, rapidity, and sensitivity to a wide range of compounds in a single measurement, difficulties arise in relation to the information obtained from spectra of multicomponent mixtures such as olive oil. It is worth mentioning that no attempt has been made in the past to detect phenolic compounds in olive oil by employing one-dimensional ^{1}H NMR spectroscopy. Strong signal overlap, dynamic range problems, diversity of intensities due to various concentrations of the food constituents, and inherent lack of scalar coupling information between different moieties lead to ambiguous or incomplete assignments, thus hindering detection even with the use of multidimensional NMR.

A possible approach to these problems could involve the combination of the advantages of NMR spectroscopy with those of chromatography. Several attempts have been made in the

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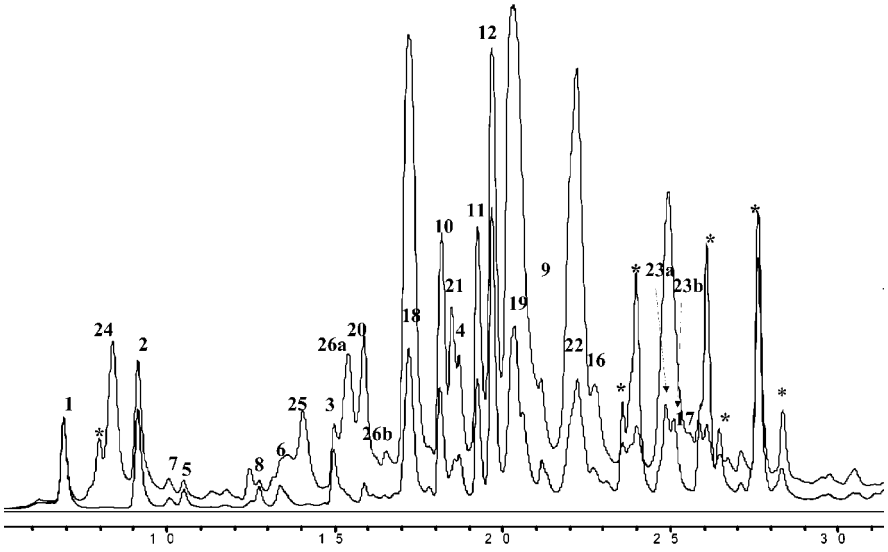

Figure 1. UV chromatogram obtained at 230 nm (upper trace) or 280 nm (lower trace) for the phenolic fraction of a virgin olive oil sample from Ilia.

past by using preparative or semipreparative thin-layer chromatography, liquid chromatography, and column chromatography to isolate individual phenols, the structures of which are determined subsequently by NMR off-line (*8*−*13*). The isolation procedure is time-consuming, although it has the advantage that the isolated phenols can be used as standards in subsequent experimental work. It would be advantageous to be able to speed up this part of the work by performing the separation and structure elucidation on-line. Such an approach requires the hyphenation of the most powerful separation technique of liquid chromatography (LC) with the most information-rich spectroscopic technique (NMR) for structure elucidation. Some practical aspects of this special coupling technique are given elsewhere (*14*, *15*). LC-NMR has been used in recent years in several instances, such as the identification of hop bitter acids (*16*), tocotrienol isomers in a palm oil extract (*17*), and quercetin and phloretin glucosides in an apple peel extract (*18*). Further attempts to explore the applicability of the method include drug metabolite identification (*19*), the identification of natural products (*20*), and the characterization of the aromatic composition of some liquid foods, such as beer, grape juice, and wine phenolic extracts (*21*).

Further improvement to the LC-NMR sensitivity could be obtained by adding a postcolumn solid-phase extraction (SPE) system to replace loop collection. S/N improvements of up to a factor of 4 could be demonstrated with this new technology (*22*−*24*). This study is the first application of LC-SPE-NMR to the identification and confirmation of phenolic compounds in the polar part of virgin olive oil.

MATERIALS AND METHODS

Samples. Two samples of virgin olive oils from Ilia (harvesting period 2001−2002), one sample from Sitia (2002−2003), and one sample from Lesvos (2002−2003) were used in the present analysis. The samples from Sitia and Ilia were extracted from the same olive variety, Koroneiki, whereas the sample from Lesvos originated from a different variety (Kolovi).

Sample Preparation. The virgin olive oil phenolic extract was prepared by phase partitioning following the procedure described by Montedoro et al. (*25*). Fifty grams of oil was dissolved in hexane (1:1, w/v) and extracted using 3 × 30 mL of methanol/water (80:20, v/v). The mixture was stirred for 2 min in a vortex apparatus and centrifuged at 3000 rpm for 15 min. The extracts were combined and washed with 3 × 30 mL of *n*-hexane. The *n*-hexane was discarded, and the methanolic solution was evaporated under vacuum giving a residue (35−40 mg) that was dissolved in 0.4 mL of methanol-d_4 and analyzed directly by the hyphenated method. Aliquots (20 μL) of the final solutions were injected into the HPLC system.

The reference materials hydroxytyrosol, tyrosol, vanillic acid, vanillin, homovanillyl alcohol, and *p*-coumaric acid were obtained from Sigma-Aldrich (Athens, Greece). Apigenin and luteolin were purchased from Extrasynthese (Genay France), and (+) pinoresinol was obtained from Separation Research (Turku, Finland). These materials were used without further purification.

LC-SPE-NMR System. For the LC-NMR analysis, the HPLC system consisted of an Agilent 1100 delivery pump with a vacuum degasser (Agilent, Waldbronn, Germany), a model 7725i manual injector (Rheodyne, Cotati, CA), equipped with a 1 mL sample loop, and a diode array detector from Bruker-Biospin (Rheinstetten, Germany). An LC-SPE interface coupled to a Bruker Avance-600 spectrometer, the latter being equipped with a ^{1}H−^{13}C inverse detection flow probe (cell of 3 mm with an active volume of 30 μL) from Bruker-Biospin was used. HPLC fractions were transferred to a Prospekt II peak-trapping unit (Bruker-Biospin) equipped with 10 × 2 mm Hysphere trap cartridges (Spark Holland) after UV detection and water addition for temporary storage, dried with nitrogen gas, and transferred to the NMR with deuterated acetonitrile. The Bruker Daltonik software HyStar 2.3 (Bremen, Germany) controlled the whole process of LC-SPE-NMR under fully automated conditions.

Chromatographic Separation of Phenolic Compounds. Separation of the phenolic compounds in olive oil extracts was carried out at 35 °C, using a 250 mm × 4.6 mm i.d., 5.0 μm, Phenomenex C-18 reverse phase column (Darmstadt, Germany) with diode array detection set at 280 and 230 nm. Elution was performed at a flow rate of 0.8 mL/min using as mobile phase a mixture of water (H_2O) acidified with 0.1% of trifluoroacetic acid (TFA-d) (solvent A) and acetonitrile (CH_3CN) acidified with 0.1% TFA-d (solvent B), utilizing linear gradient elution over a total run time of 40−45 min.

NMR Experiments. One-dimensional (1D) and two-dimensional (2D) NMR spectra were recorded on a Bruker Avance-600 spectrometer operating at 600.13 MHz for the proton nucleus and using both the stop flow and loop sampling. One-dimensional ^{1}H NMR spectra of olive oil extracts were acquired with 80−1024 scans depending on the peak height in the chromatogram, applying 24 dummy scans, 16384 data points, 12019.23 Hz spectral width, acquisition time of 0.68 s, and relaxation delay of 3 s. Line broadening of 1 Hz and zero-filling to 32K were applied prior to Fourier transform. The residual signals from the transfer solvent (CD_2H−CN, traces of H_2O) were suppressed using a 1D NOESY pulse sequence for double-solvent suppression

Table 1. Chromatographic and NMR Data for Phenolic Compounds Identified in Olive Oil by LC-SPE-NMR

LC peak/ structure	RT (min)	compound	NMR signals δ (ppm) (multiplicity, J in Hz, assignment)
1	7.0	hydroxytyrosol	3.62 (t, 6.8, H1′), 2.62 (t, 6.8, H2′), 6.68 (d, 1.8, H4′), 6.70 (d, 8.1, H7′), 6.56 (dd, 1.8, 8.1, H8′)
24	8.4	dialdehydic form of elenolic acid lacking a carboxymethyl group	9.25 (d, 1.5, H1), 9.57 (s br, H3), 2.80 (ddd, 1.5, 8.3, 17.8, H4a), 2.73 (ddd, 1.0, 5.3, 17.8, H4b), 3.60 (m, H5), 2.62 (dd, 8.4, 16.0, H6a), 2.57 (ddd, 1.5, 6.2, 16.0, H6b), 6.72 (q, 7.0, H8), 2.03 (d, 7.0, H10)
2	9.2	tyrosol	3.62 (t, 6.8, H1′), 2.67 (t, 6.8, H2′), 7.04 (d, 8.4, H4′, H8′), 6.71 (d, 8.4, H5′, H7′)
7	10.1	homovanillyl alcohol	3.65 (t, 7.0, H8), 2.68 (t, 7.0, H7), 6.81 (d, 1.5, H2), 6.71 (d, 8.2, H5), 6.67 (dd, 1.5, 8.2, H6), 3.82 (s, OCH_3)
5	10.5	vanillic acid	6.87 (d, 8.1, H5), 7.52 (d, 1.8, H2), 7.53 (dd, 1.8, 8.1, H6), 3.88 (s, OCH_3)
8	12.7	*p*-coumaric acid	7.48 (d, 8.6, H2, H6), 6.83 (d, 8.6, H3, H5), 7.58 (d, 16.0, H7), 6.28 (d, 16.0, H8)
6	13.6	vanillin	6.98 (d, 8.4, H2), 7.42 (d, 1.5, H5), 7.43 (dd, 1.5, 8.4, H6), 9.79 (s, H7), 3.91 (s, OCH_3)
25	14.1	dialdehydic form of the methyl ester of elenolic acid lacking a carboxymethyl group	9.24 (d, 1.8, H1), 9.57 (s br, H-3), 2.81 (ddd, 1.5, 8.0, 17.8, H4a), 2.75 (ddd, 1.0, 5.3, 17.8, H4b), 3.64 (m, H5), 2.65 (dd, 9.2, 15.8, H6a), 2.58 (ddd, 1.5, 6.2, 15.8, H6b), 6.72 (q, 7.1, H8), 2.03 (d, 7.1, H10), 3.55 (s, $COOCH_3$)
3	15.0	hydroxytyrosol acetate	4.16 (t, 7.0, H1′), 2.75 (t, 7.0, H2′), 6.69 (d, 1.8, H4′), 6.71 (d, 8.1, H7′), 6.58 (dd, 1.8, 8.1, H8′)
26a	15.4	elenolic acid (5*S*, 8*R*, 9*S*)	9.56 (d, 1.8, H1), 7.53 (s, H3), 3.32 (m, H5), 2.53 (dd, 9.2, 16.5, H6b), 2.77 (dd, 3.3, 16.5, H6a), 4.55 (dq, 5.0, 6.6, H8), 2.73 (dd, 5.0, 2.2, H9), 1.37 (dd, 2.2, 5.0, H10), 3.66 (s, $COOCH_3$)
20	16.0	hemiacetal of the dialdehydic form of oleuropein lacking a carbo-methoxygroup	4.17 (dt, 6.9, 10.9, H1a′), 4.08 (dt, 6.6, 10.9, H1b′), 2.71 (dd, 6.9, 6.6, H2′), 6.65 (d, 1.8, H4′), 6.70 (d, 8.1, H7′), 6.55 (dd, 1.8, 8.1, H8′), 9.20 (d, 1.8, H1), 4.17 (m, H3), 2.61 (dd, 8.8, 16.0, H4a), 2.56 (dd, 7.1, 16.0, H4b), 3.46 (m, H5), 2.63 (dd, 9.4, 15.4, H6a), 2.55 (dd, 15.4, H6b), 6.66 (q, 7.0, H8),
26b	16.5	elenolic acid (5*S*, 8*S*, 9*S*)	9.66 (d, 1.2, H1), 7.58 (s, H3), 3.24 (m, H5), 2.74 (dd, 2.9, 16.9, H6a), 2.29 (dd, 11.0, 16.9, H6b), 4.27 (dq, 2.4, 6.6, H8), 2.70 (br, H9), 1.54 (d, 6.6, H10), 3.66 (s, $COOCH_3$)
18	17.2	dialdehydic form of oleuropein lacking a carboxymethyl group	4.17 (dt, 7.0, 11.0, H1a′), 4.09 (dt, 6.7, 11.0, H1b′), 2.71 (dd, 7.0, 6.7, H2′), 6.66 (d, 1.8, H4′), 6.71 (d, 8.1, H7′), 6.55 (dd, 1.8, 8.1, H8′), 9.19 (d, 1.8, H1), 9.54 (s, br, H3), 2.78 (ddd, 1.3, 8.2, 17.8, H4a), 2.69 (ddd, 1.0, 6.1, 17.8, H4b), 3.57 (m, H5), 2.61 (dd, 9.0, 15.5, H6a), 2.52 (dd, 6.2, 15.5, H6b), 6.65 (q, 7.0, H8), 1.97 (d, 7.0, H10)
10	18.2	luteolin	7.43 (d, 2.2, H2′), 6.96 (d, 8.8, H5′), 7.42 (dd, 2.2, 8.8, H6′), 6.55 (s, H3), 6.22 (d, 1.8, H6), 6.47 (d, 1.8, H8)
10/13	18.2	syringaresinol	6.63 (s, H2′, H6′, H2“, H6“), 3.08 (m, 4.0, 7.0, H1, H5), 4.65 (d, br, 4.0, H2, H6), 4.20 (dd, 9.0, 7.0, H4a, H8a), 3.81 (br, 9.0, 3.7, H4b, H8b), 3.81 (s, OCH_3)
21	18.55	hemiacetal of the dialdehydic form of ligstroside lacking a carboxymethyl group	4.17 (dt, 7.0, 11.0, H1a′), 4.09 (dt, 6.8, 11.0, H1b′), 2.76 (t, 6.9, H2′), 7.03 (d, 8.4, H4′, H8′), 6.71 (d, 8.4, H5′, H7′), 9.21 (d, 1.8, H1), 4.17 (m, H3), 2.61 (dd, 8.8, 16.0, H4a), 2.56 (dd, 6.4, 16.0, H4b), 3.46 (m, H5), 2.62 (dd, 9.3, 15.6, H6a), 2.55 (dd, 5.8, 15.6 H6b), 6.67 (q, 7.0, H8),
4	18.8	tyrosol acetate	4.17 (t, 8.2, H1′), 2.80 (t, 8.2, H2′), 7.06 (d, 8.4, H4′, H8′), 6.72 (d, 8.4, H5′, H7′)
11	19.2	(+)-pinoresinol	3.07 (m,4.0, H1, H5), 4.66 (d, 4.0, H2, H6), 4.17 (dd, 9.1, 6.8, H4a, H8a), 3.78 (dd, 3.6, 9.1, H4b, H8b), 6.94 (d, 1.8, H2′, H2“), 6.77 (d, 8.1, H5′, H5“), 6.81 (dd, 1.8, 8.1, H6′, H-6“), 3.84 (s, OCH_3)
12	19.9	(+)-1-acetoxypinoresinol	4.95 (s, br H2), 4.38 (dd, 9.2, 8.1, H4a), 3.70 (dd, 9.2, 5.1, H4b), 3.32 (ddd, 8.1, 4.8, 5.1, H5), 4.74 (d, 4.8, H6), 4.29 (d, 10.6, H8a), 4.19 (d, 10.6, H8b), 6.91 (d, 1.8, H2′), 7.00 (d, 1.8, H2“), 6.75 (d, 8.1, H5′), 6.81 (d, 8.1, H5“), 6.82 (dd, 1.8, 8.1, H6′), 6.87 (dd, 1.8, 8.1, H-6“), 3.82 (s, 3′-OCH_3), 3.85 (s, 3“-OCH_3), 1.65 (s, $COOCH_3$)
19	20.4	dialdehydic form of ligstroside lacking a carboxymethyl group	4.18 (dt, 6.9, 10.9, H1a′), 4.10 (dt, 6.8, 10.9, H1b′), 2.76 (t, 6.9, H2′), 7.03 (d, 8.4, H4′, H8′), 6.72 (d, 8.4, H5′, H7′), 9.20 (d, 1.8, H1), 9.54 (s br, H3), 2.77 (ddd, 1.7, 8.5, 17.8, H4a), 2.69 (ddd, 1.0, 5.7, 17.8, H4b), 3.57 (m, H5), 2.61 (dd, 9.2, 15.4, H6a), 2.53 (dd, 6.1, 15.4, H6b), 6.65 (q, 7.0, H8), 1.97 (d, 7.0, H10)
9	21.1	apigenin	7.87 (d, 8.8, H2′, H6′), 6.96 (d, 8.8, H3′, H-5′), 6.59 (s, H3), 6.22 (d, 1.8, H6), 6.48 (d, 1.8, H8)
22	22.25	aldehydic form of oleuropein (5*S*, 8*S*, 9*S*)	4.24 (dt, 6.7, 11.0, H1a′), 4.21 (dt, 6.6, 11.0, H1b′), 2.77 (dd, 6.6, 6.7, H2′), 6.70 (d, 1.8, H4′), 6.72 (d, 8.1, H7′), 6.58 (dd, 1.8, 8.1, H8′), 9.61 (d, 1.2, H1), 7.56 (s, H3), 3.23 (dd, 2.9, 11.0, H5), 2.72 (dd, 2.9, 16.3, H6a), 2.26 (dd, 11.0, 16.2, H6b), 4.24 (dq, 6.59, 6.61, H8), 2.56 (br, H9), 1.52 (d, 6.6, H10), 3.66 (s, OCH_3)
22	22.25	aldehydic form of oleuropein (5*S*, 8*R*, 9*S*)	4.22 (dt, 6.7, 10.7, H1a′), 4.18 (dt, 6.6, 10.7, H1b′), 2.76 (dd, 6.6, 6.7, H2′), 6.69 (d, 1.8, H4′), 6.72 (d, 8.1, H7′), 6.58 (dd, 1.8, 8.1, H8′), 9.45 (d, 1.5, H1), 7.50 (s, H3), 3.29 (m, 3.9, 5.0, 9.7, H5), 2.75 (dd. 3.9, 15.8, H6a), 2.51 (dd, 9.7, 15.8, H6b), 4.52 (dq, 5.1, 6.6, H8), 2.55 (ddd, 1.2, 5.0, 5.1, H9), 1.34 (d, 6.6, H10), 3.65 (s, OCH_3)

Table 1. (Continued)

LC peak/ structure	RT (min)	compound	NMR signals δ (ppm) (multiplicity, J in Hz, assignment)
16	22.9	oleuropein aglycon	4.15 (dt, 6.9, 1057, H1a′), 4.10 (dt, 6.6, 10.5, H1b′), 2.70 (t, 6.7, H2′), 6.65 (d, 1.8, H4′), 6.70 (d, 8.1, H7′), 6.53 (dd, 1.8, 8.1, H8′), 4.20 (d, 1.1, H1), 7.51 (s, H3), 3.94 (m, H5), 2.73 (dd, 4.8, 16.5, H6a), 2.58 (dd, 4.8, 16.5, H6b), 7.04 (dq, 1.1, 7.3, H8), 1.85 (d, 7.3, H10)
23a	24.9	aldehydic form of ligstroside (5*S*, 8*R*, 9*S*)	4.22 (dt, 6.7, 10.9, H1a′), 4.18 (dt, 6.9, 10.9, H1b′), 2.81 (t, 6.8, H2′), 7.06 (d, 8.4, H4′, H8′), 6.72 (d, 8.4, H5′, H7′), 9.46 (d, 1.5, H1), 7.50 (s, H3), 3.29 (m, 3.9, 5.0, 9.5, H5), 2.75 (dd, 3.9, 15.8, H6a), 2.51 (dd, 9.5, 15.8, H6b), 4.51 (dq, 5.1, 6.6, H8), 2.56 (ddd, 1.5, 5.0, 5.1, H9), 1.34 (d, 6.6, H10), 3.65 (s, OCH_3)
23b	25.1	aldehydic form of ligstroside (5*S*, 8*S*, 9*S*)	4.22 (dt, 6.7, 10.9, H1a′), 4.18 (dt, 6.9, 10.9, H1b′), 2.81 (t, 6.8, H2′), 7.06 (d, 8.4, H4′, H8′), 6.72 (d, 8.4, H5′, H7′), 9.61 (d, 1.2, H1), 7.56 (s, H3), 3.23 (dd, 2.9, 11.0, H5), 2.71 (dd, 2.9, 16.4, H6a), 2.26 (dd, 11.0, 16.4, H6b), 4.24 (m, H8), 2.56 (br, H9), 1.52 (d, 6.8, H10), 3.66 (s, OCH_3)
17	26.0	ligstroside aglycon	4.16 (dt, 6.9, 10.8, H1a′), 4.18 (dt, 6.9, 10.9, H1b′), 2.75 (t, 6.9, H2′), 7.01 (d, 8.4, H4′, H8′), 6.71 (d, 8.4, H5′, H7′), 4.21 (d, 1.3, H1), 7.51 (s, H3), 3.95 (m, H5), 2.81 (dd, 4.8, 16.5, H6a), 2.59 (dd, 4.6, 16.5, H6b), 7.06 (dq, 1.3, 7.5, H8), 1.87 (d, 7.5, H10)

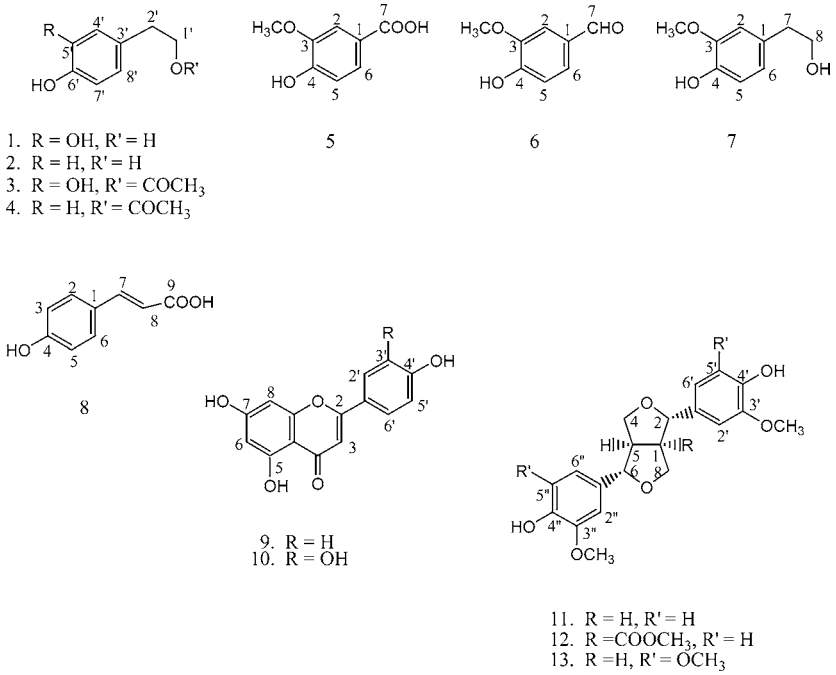

Figure 2. Chemical structures and numbering system of simple phenols, lignans, and flavonoids.

(pulse program "lc1pncwps" from Bruker pulse program library). The total correlation spectroscopy (TOCSY) spectra were acquired in the phase sensitive mode with TPPI, using the MLEV17 pulse sequence for the spin lock, and a multiple WET solvent suppression of water and acetonitrile resonances (pulse program "mlevdcphwt" from Bruker pulse program library). Typically, 32 dummy scans and then 16 scans were collected for each of 256 increments with a spectral width of 7183.91 Hz in both dimensions, 2048 data points, mixing time of 60 ms, and a relaxation delay of 2.4 s. The spectra were zero-filled to a final size of 2K × 2K prior to Fourier transform.

RESULTS AND DISCUSSION

The UV chromatogram resulting from HPLC elution of the polar part of a virgin olive oil sample from Ilia at two wavelengths of 280 and 230 nm is shown in **Figure 1**. The choice of the two wavelengths was dictated by the fact that a wide range of phenolic compounds shows strong absorption at these wavelengths (*5*). Similar chromatograms were obtained for the remaining three olive oil samples, although variation in polyphenols concentration was observed. This interoil variation is due to a complex interaction among several factors including geographical origin, climatic conditions, olive variety and ripeness, agricultural practices, and olive oil production techniques, as pointed out by several authors (*2*, *5*, *26*, *27*).

A large number of phenolic acids were detected by HPLC under the conditions described under Materials and Methods and verified by ^{1}H NMR spectroscopy. These compounds are summarized in **Table 1** along with the corresponding peak number in the chromatogram, retention times, and assignments of the various signals in their LC-SPE-^{1}H NMR spectra. **Figure 2** depicts the chemical structures and numbering systems of simple phenols, lignans, and flavonoids, whereas **Figure 3** illustrates the secoiridoid derivatives identified in the present analysis.

Detection of Simple Phenolic Compounds, Lignans, and Flavonoids. Major phenolic compounds observed in several

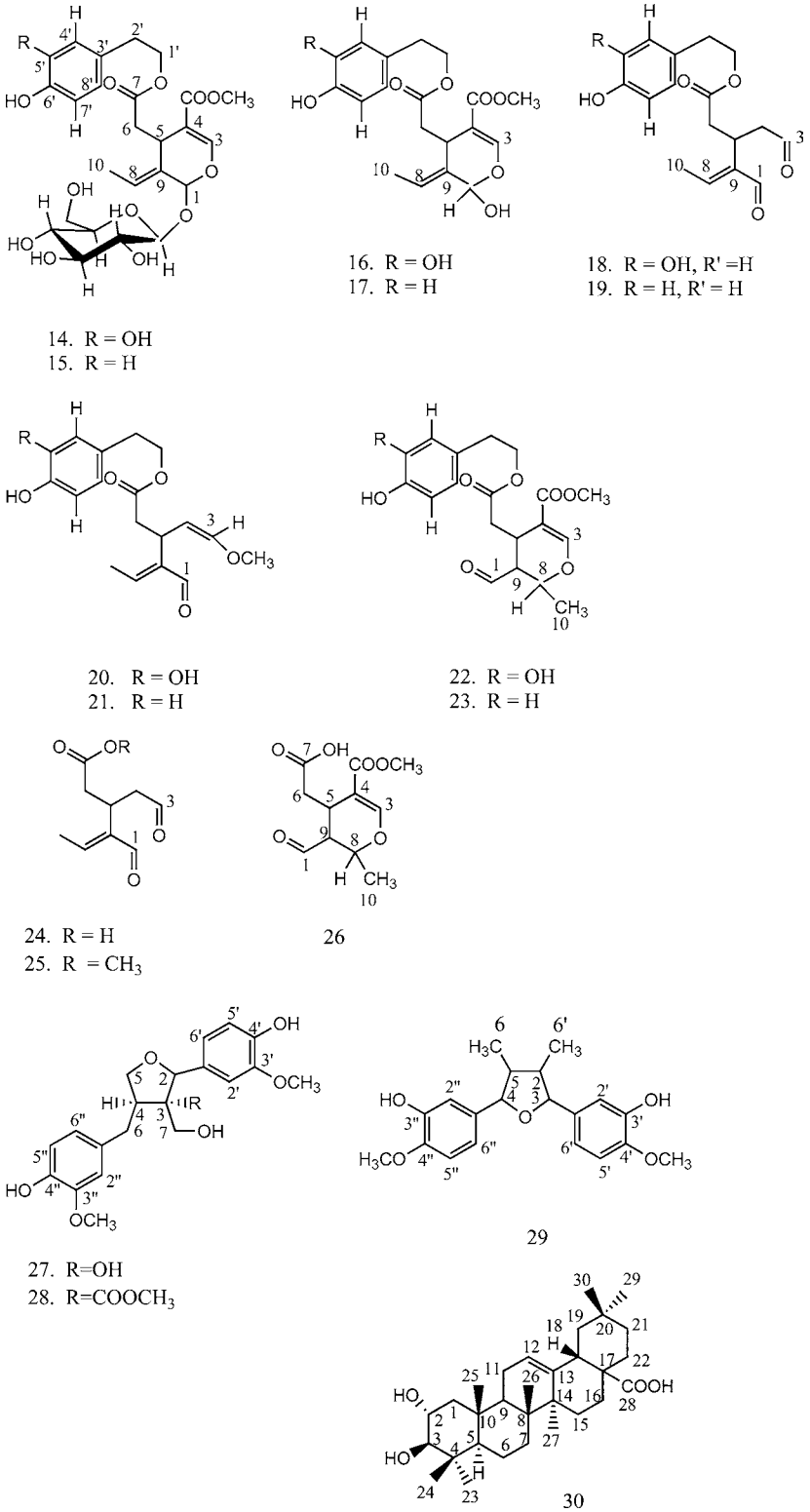

Figure 3. Chemical structures and numbering system of the secoiridoids oleuropein and ligstroside and their derivatives.

studies (*4*, *8*, *9*, *28*−*32*) are detected as well in the present chromatograms. These are hydroxytyrosol (**1**), tyrosol (**2**), vanillic acid (**5**), *p*-coumaric acid (**8**), vanillin (**6**), hydroxytyrosol acetate (**3**), and tyrosol acetate (**4**), the latter compound being described recently in olive oil (*28*), and the flavonoids apigenin (**9**) and luteolin (**10**). Their presence was confirmed from the respective LC-SPE-^{1}H NMR spectra, which were assigned on the basis of existing ^{1}H NMR databases (*9*, *33*, *34*), and verified, when necessary, with TOCSY experiments and/or upon comparison of their spectra with those of available model compounds. Acetylation of the aliphatic hydroxyl proton in hydroxytyrosol and tyrosol acetates produced downfield shifts for the side-chain aliphatic protons relative to those of the free phenols. The ^{1}H NMR spectroscopic data of these phenolic compounds are summarized in **Table 1**.

Also, we were able to detect and confirm the presence of lignans (+)-pinoresinol (**11**) and (+)-1-acetoxypinoresinol (**12**). The spectroscopic data for the aliphatic protons for both compounds agree closely with those reported by Owen et al. (*8*) at 500 MHz in chloroform-*d* solutions. However, differences

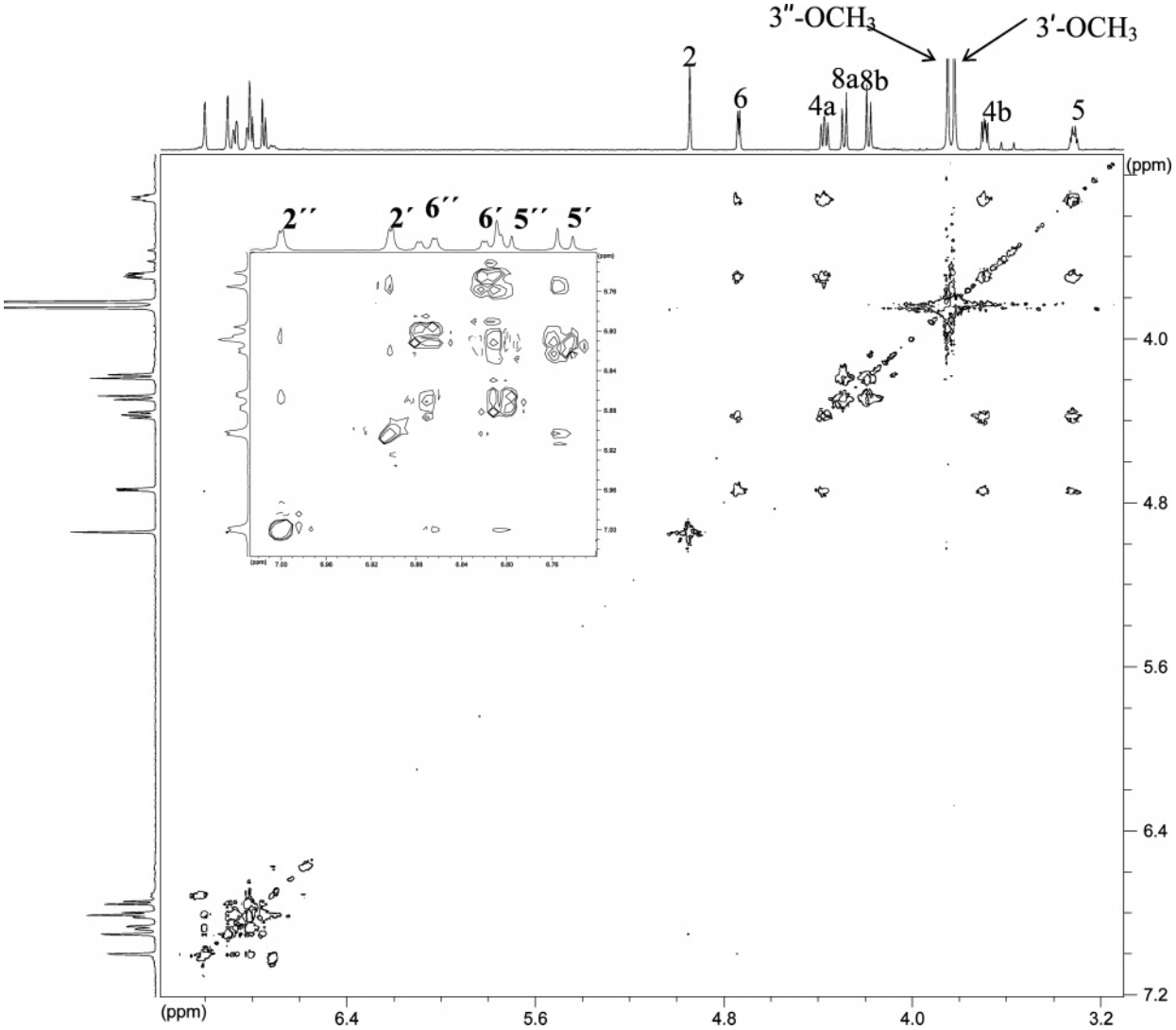

Figure 4. 600 MHz TOCSY spectrum of (+)-1-acetoxypinoresinol (**12**) detected by LC-SPE-NMR.

were observed in the assignment of the aromatic protons, due to higher resolution achieved at 600 MHz, and the use of a different solvent system (water/acetonitrile), which facilitated considerably the assignment of the aromatic protons of these compounds. This is shown in **Figure 4**, which depicts the TOCSY spectrum of peak 12 in the chromatogram. The aromatic protons of compound **12**, shown in the inset of **Figure 4**, give almost first-order spectra. Data acquisition has been done in the stop-flow mode. Following the cross-peak pattern connecting the various aromatic protons, we were able to assign unambiguously the chemical shifts and coupling constants of the prime and double-prime protons. The signals of H2′ and H2″ protons of the two aryl rings appear as broad doublets at high frequencies, whereas those of H5′ and H5″ protons appear at low frequencies. The well-resolved resonances of the aromatic and aliphatic protons of the other lignan, **11**, were easily assigned with a TOCSY experiment (not shown). The ^{1}H NMR spectroscopic data of these compounds are summarized in **Table 1**.

Upon examining the TOCSY spectrum of luteolin (**10**) (**Figure 5**), we discovered that apart from the signals of luteolin (indicated by L), it contains several additional signals indicating the presence of an unknown phenolic compound coeluted with luteolin. The singlet at δ 6.63 could be attributed to either an isolated aromatic proton or to two equivalent aromatic protons with no coupling with any other proton in the molecule. The chemical shifts and coupling constant pattern of the signals at high magnetic fields were similar to those observed for the protons of the 3,7-dioxabicyclo[3.3.0]octane skeleton of lignans **11** and **12** bearing two aryl groups at C2 and C6 carbons (**Table 1**). Moreover, the signal intensity of the singlet at δ 3.81 corresponded to 12 protons, and thus it was assigned to four equivalent methoxy groups. On the basis of the above data, the structure of this compound appears to be the lignan syringaresinol with two methoxy groups flanking one hydroxy group in each of the two-aryl rings. The assignment of the ^{1}H NMR spectrum and the derived structure of this compound are in accord with those of syringaresinol (**13**) isolated from the plant *Leptademia arborea* (*35*). Pinoresinol and syringaresinol are members of the group of plant lignans that occur in roots, leaves, seeds, fruits, and woody parts of several plants (*36*). Most of the plant lignans, including pinoresinol and, to a lesser extent, syringaresinol are metabolized in humans to mammalian lignans enterolactone and enterodiols (*37*). Plant lignans or phytoestrogens as they are usually named, are known to affect the production of sex hormones binding globulin, and they inhibit the growth of tumor cells (*38*, *39*). Moreover, high serum enterolactone values are associated with decreased risk of acute coronary event (*40*) and decreased risk of breast cancer (*41*).

The peak in the chromatogram with a retention time of 10.1 min observed at both wavelengths has not been detected previously. Apart from a singlet at δ 3.82, which is ascribed to

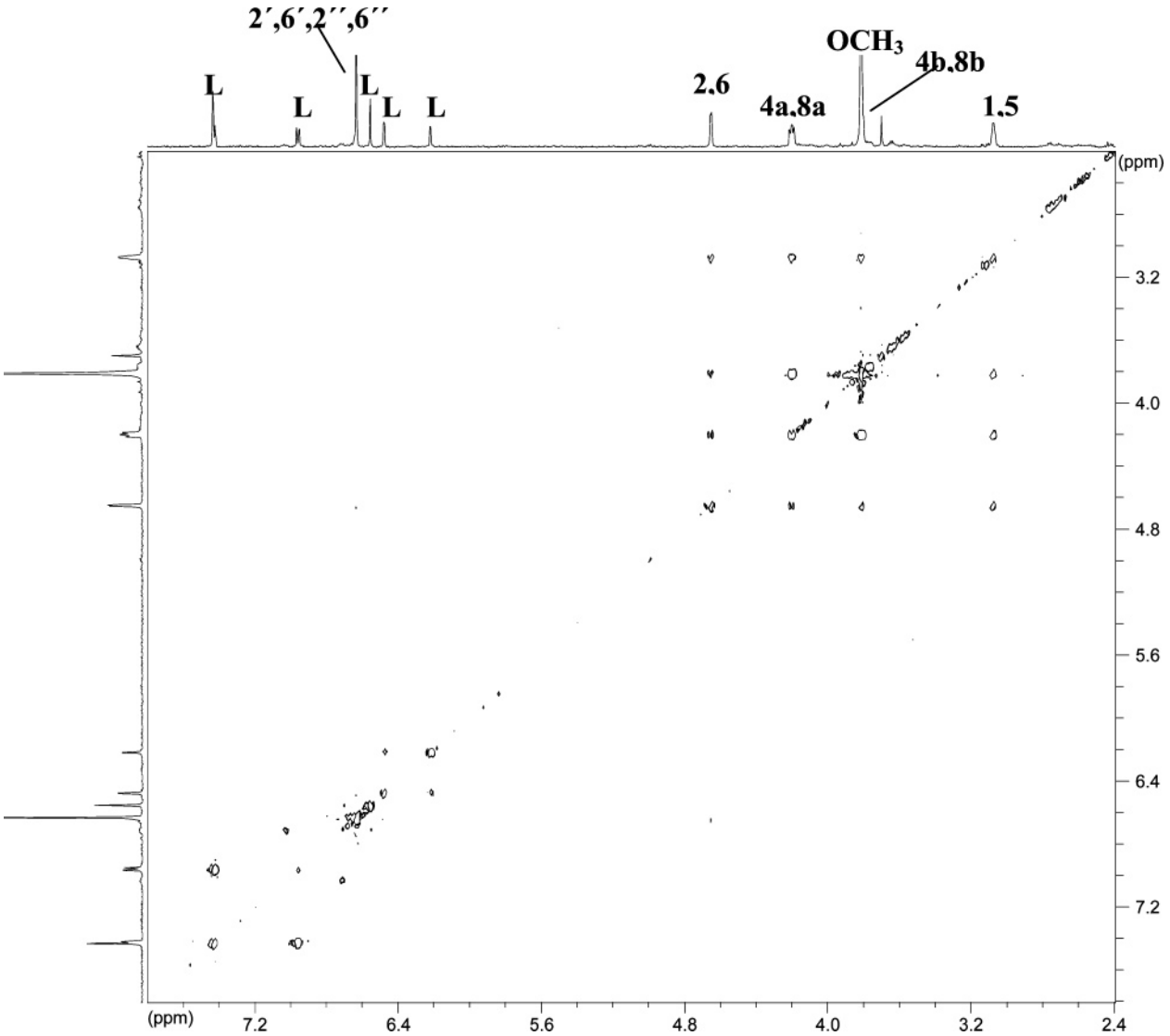

Figure 5. 600 MHz TOCSY spectrum of peak 10 in the chromatogram indicating the presence of the lignan syringaresinol. The signals denoted by L belong to luteolin.

a methoxy group on the basis of its relative intensity, the LC-SPE-^{1}H NMR spectrum of this compound depicted in **Figure 6** is similar to that of hydroxytyrosol with two triplets at δ 3.65 and 2.68, which must belong to two vicinal aliphatic protons, two doublets at δ 6.81 and 6.71, and one doublet of doublets at δ 6.67, which are attributed to three aromatic protons. This pattern is consistent with the structure of homovanillyl alcohol (**7**) and matched the spectrum of an authentic sample of homovanillyl alcohol run in a mixture of D_2O and acetonitrile-d_3. The spectroscopic parameters of this compound are summarized in **Table 1**. Homovanillyl alcohol was found in the phenolic fractions of all virgin olive oil samples analyzed in the present study, although its concentration varied from sample to sample. It is considered to be a common component of the lignin fraction. Moreover, it has been detected in human excretion as a major metabolite of hydroxytyrosol after administration of extra virgin olive oil. Preliminary studies in our laboratory have shown that this compound is also a component of the phenolic fraction obtained from brined olive drupes. The question of how this phenolic compound becomes a constituent of the phenolic fraction of olive fruits and olive oil has yet to be answered.

Detection of Secoiridoids. Our next step was to verify the chromatographic peaks originating from the hydrolysis products of the secoiridoids. Several past studies (*8*, *10*−*13*, *42*, *43*) support the conclusion that intact secoiridoids, such as oleuropein glucoside (**14**) and ligstroside glucoside (**15**), are almost completely absent in olive oil because of their high solubility in water. They undergo enzymatic hydrolysis during olive oil extraction and olive oil storage, producing first oleuropein aglycon (**16**) and ligstroside aglycon (**17**) upon removal of the attached glucose moiety and then a number of secoiridoid derivatives upon further molecular transformations via ring opening and rearranged reclosure. It has been suggested (*44*, *45*) that these metabolites show strong bioactive properties and offer protection against microbe and insect attack via a multichemical defense mechanism, not shown directly by the secoiridoid glucosides. Several of these individual compounds have been isolated from olive oil or olive fruits by preparative or semipreparative thin-layer chromatography and HPLC, and their structures were elucidated by ^{1}H and ^{13}C NMR spectroscopy (*8*, *10*−*13*, *42*, *43*). **Figure 7** illustrates selections of LC-SPE-^{1}H NMR spectra, indicating the presence of four different metabolites of oleuropein. The first ^{1}H NMR spectrum (**Figure 7A**) is consistent with the dialdehydic form of oleuropein lacking a carboxymethyl group (**18**). This is reflected on the chemical shift and coupling constant pattern of the aliphatic H4a and H4b protons at δ 2.77 and 2.69, respectively, the quartet at δ 6.65,

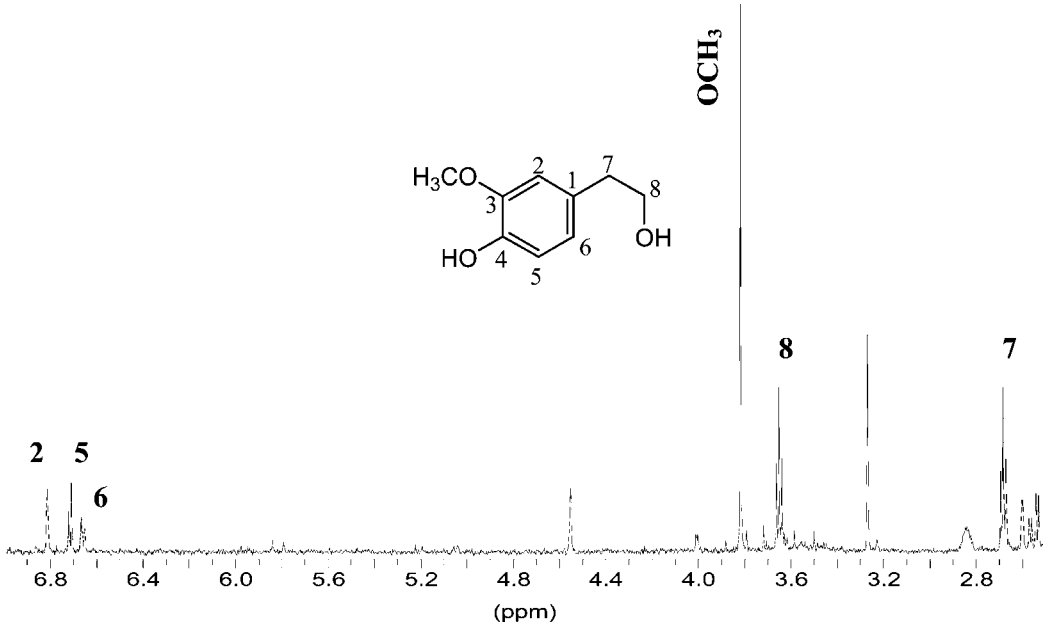

Figure 6. 600 MHz LC-SPE ^{1}H NMR spectrum of peak 7 in the chromatogram, indicating the presence of homovanillyl alcohol.

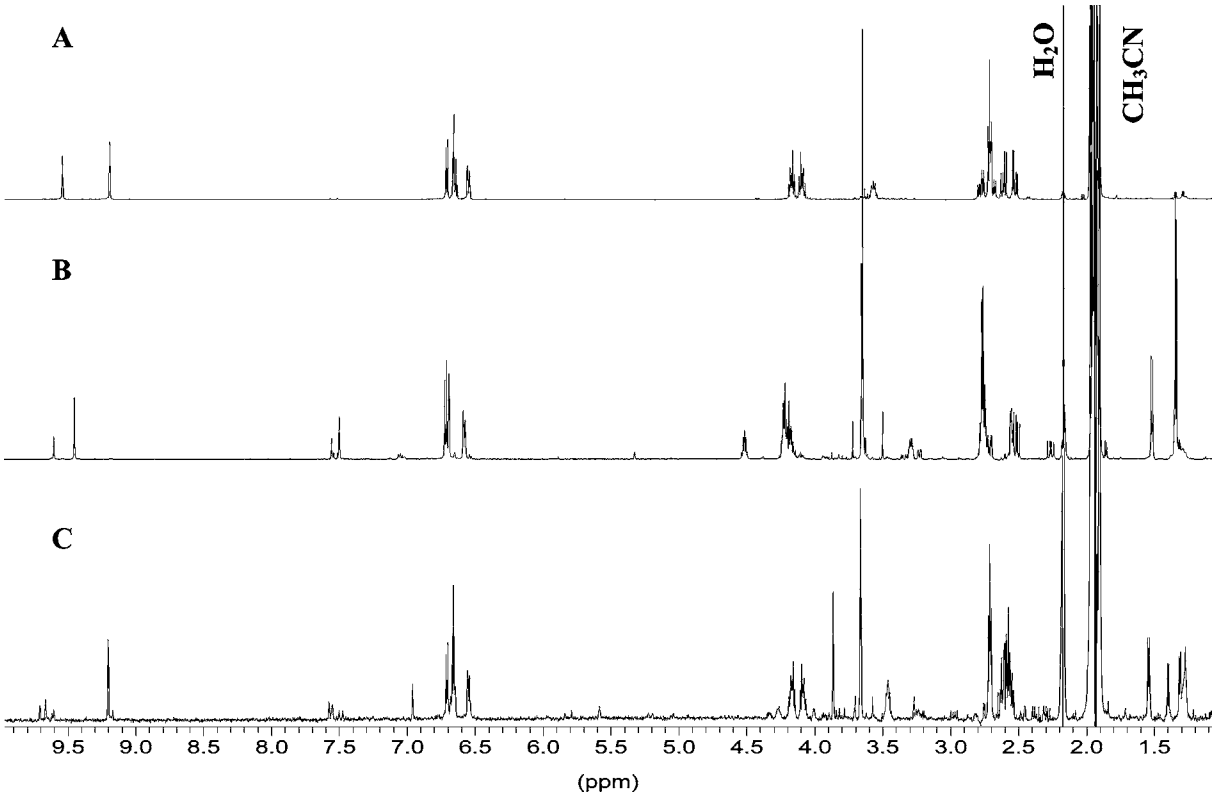

Figure 7. 600 MHz LC-SPE ^{1}H NMR spectra of oleuropein derivatives: (A) dialdehydic form of oleuropein lacking a carboxymethyl group (peak 18 in the chromatogram); (**B**) two coeluted isomers of the aldehydic form of oleuropein (peak 22 in the chromatogram); (**C**) hemiacetal of the dialdehydic form of oleuropein (peak 20 in the chromatogram). The suppressed signals of H_2O and CH_3CN solvents give spikes at δ ~1.95 and ~2.18.

and the multiplet at δ 3.57 showing the presence of H-8 and H-5 protons, respectively, the appearance of a doublet at δ 9.19 and a broad singlet at δ 9.54, indicative of the aldehydic H1 and H3 protons, respectively, the doublet of the methyl protons at δ 1.97, and the lack of the carboxymethyl protons signal. Finally, all proton resonances of the hydroxytyrosyl moiety were detected in the spectrum. This assignment agrees with that obtained in the literature (*11*, *13*). The spectrum in **Figure 7B** is more interesting, because it reveals the existence of two coeluted isomers of the aldehydic form of oleuropein (**22**). The strong and weak sets of signals with the same multiplicity belong to two different isomers, namely, 5*S*, 8*R*, 9*S* and 5*S*, 8*S*, 9*S* at C8 (the ring of elenolic acid linked to the hydroxytyrosyl moiety contains three chiral centers at C5, C8, and C9). These two isomers were detected in olive leaves in a ratio of 1:7 (*12*), but not in olive oil, in which only the isomer 5*S*, 8*R*, 9*S* has been identified (*11*). The isomer 5*S*, 8*S*, 9*S* escaped detection presumably because of its low concentration. Moreover, compound **22** is considered to be the final stable biomolecule of the oleuropein transformation (*11*, *12*, *42*). The spectrum in

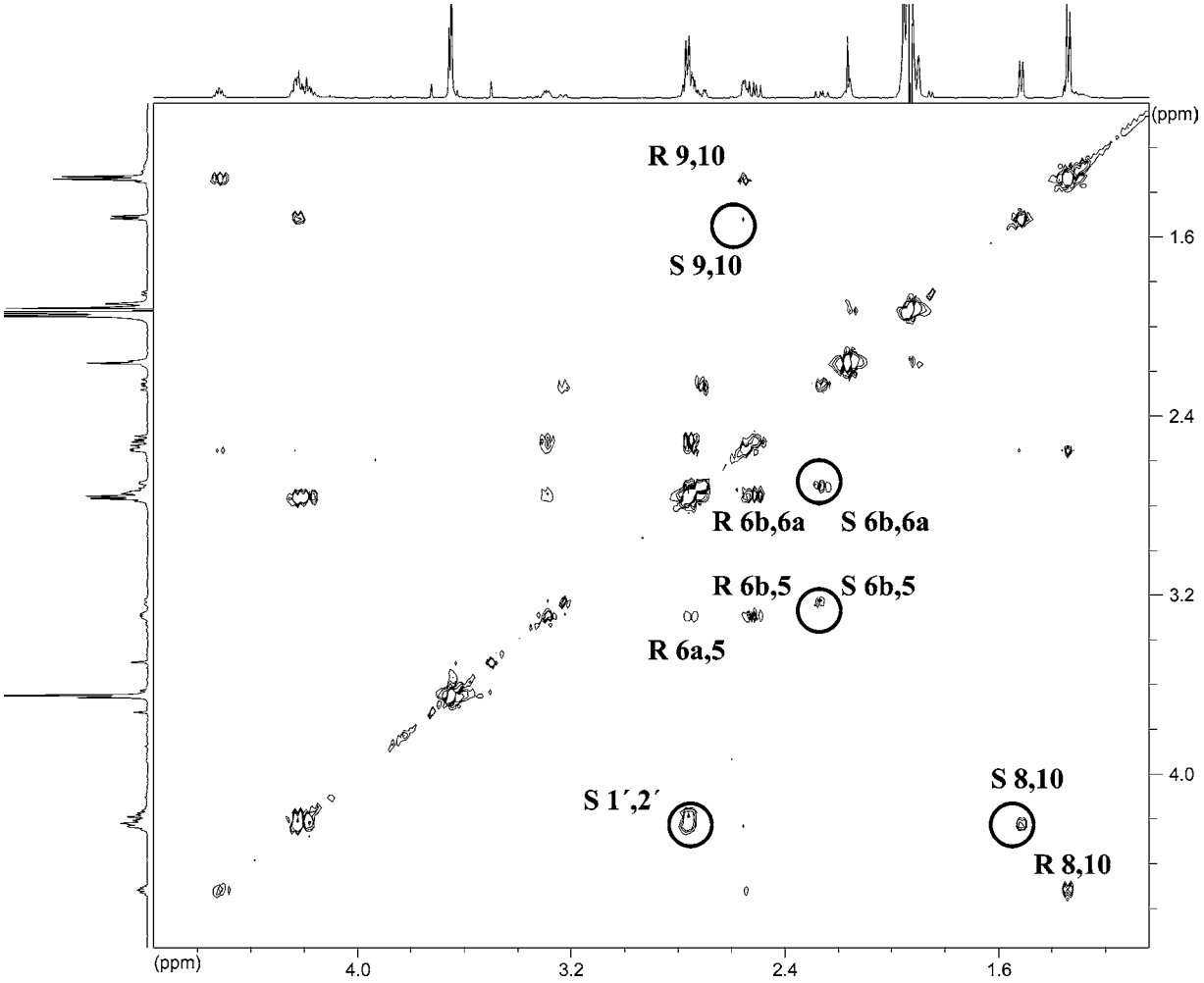

Figure 8. 600 MHz TOCSY spectrum of peak 22 in the chromatogram indicating the presence of the two isomers of the aldehydic form of oleuropein. S and R denote the cross-peaks correlating the signals of isomers 5*S*, 8*S*, 9*S* and 5*S*, 8*R*, 9*S*, respectively.

Figure 7B has been assigned by TOCSY depicted in **Figure 8**. S denotes the cross-peaks correlating the signals of the isomer 5*S*, 8*S*, 9*S*, whereas R is used to discriminate the cross-peaks connecting the isomer 5*S*, 8*R*, 9*S*. The spectroscopic results for these two phenolic isomers summarized in **Table 1** agree very closely with those reported in the literature (*11*, *12*). **Figure 7C** illustrates the spectrum of the hemiacetal at C-3 of the dialdehydic form of oleuropein lacking a carbomethoxy group (**20**) detected by Montedoro et al. (*11*) in olive oil. The chemical shifts and coupling constants of this compound agree closely with those reported in the literature (*11*). However, it is important to note that compound **20** is not a direct product of the oleuropein transformation. It is formed when the phenolic fraction of olive oil is dissolved in methanol just before the HPLC analysis (*11*). Previous studies (*10*, *42*) have shown that enzymatic hydrolysis of oleuropein carried out in water leads to hydration of the aldehydic group at position 3. This conclusion is further supported by the fact that the signal of the OCH_3 group was not observed in the spectrum of **20**, because the polar part of the olive oil used in the present analysis was dissolved in deuterated methanol. The spectroscopic data of oleuropein metabolites detected by LC-SPE-NMR is given in **Table 1**.

On the basis of the NMR data of the oleuropein derivatives, we were able to detect and verify the presence of the dialdehydic form of ligstroside lacking a carboxymethyl group (**19**), the two isomers 5*S*, 8*R*, 9*S* and 5*S*, 8*S*, 9*S* of the aldehydic form of ligstroside (**23**) characterized by different retention times of 24.8 min (**23a**) and 25.1 min (**23b**), respectively, and the hemiacetal at C3 of the dialdehydic form of ligstroside (**21**). The chemical shifts and coupling constants of these compounds, which are reported in **Table 1**, differ from those of the oleuropein derivatives only in the spectroscopic pattern of the aromatic protons.

At 230 nm, and at early retention times (7−10 min), a peak at 8.4 min is eluted between the well-established peaks representing hydroxytyrosol and tyrosol (**Figure 1**). This peak is very small at 280 nm and apparently escaped scrutiny in other studies. The LC-SPE-^{1}H NMR spectrum of this peak does not show the signals of the hydroxytyrosyl or tyrosyl moieties, but all remaining spectroscopic patterns are similar to those of the pairs of compounds **18** and **19**, respectively. The spectrum in **Figure 9A** is consistent with the dialdehydic form of elenolic acid lacking a carboxymethyl group (**24**), respectively. It appears that this compound, which is detected for the first time in olive oil, is the product of a more extended degradation of secoiridoids. The spectroscopic parameters of compound **24** are summarized in **Table 1**. Also, **Table 1** lists the chemical shifts and coupling constants for two phenolic metabolites, which are frequently isolated from olive oil (*11*, *12*). These are the two isomers 5*S*, 8*R*, 9*S* (**26a**, 15.4 min) and 5*S*, 8*S*, 9*S* (**26b**, 16.5 min) of elenolic acid and the methyl ester of the dialdehydic form of elenolic acid lacking a carboxymethyl group at C4 (**25**). The LC-SPE-^{1}H NMR spectra for the two isomers of elenolic acid are shown in spectra **B** and **C** of **Figure 9**, respectively, whereas that of compound **25** is shown in **Figure 9D**. The assignment of ^{1}H NMR spectra in **Figure 9** was facilitated by

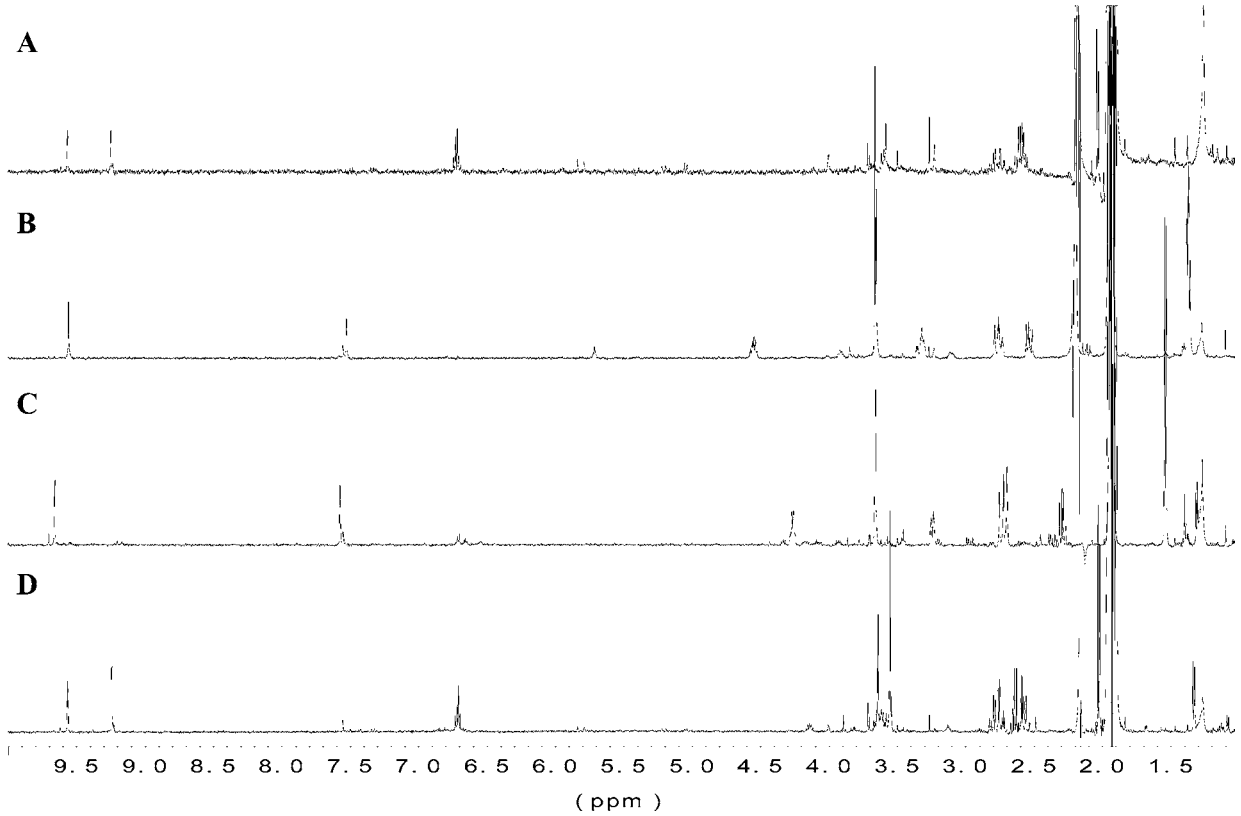

Figure 9. 600 MHz LC-SPE-^{1}H NMR spectra of elenolic acid derivatives: (**A**) dialdehydic form of elenolic acid lacking a carboxymethyl group (**24**); (**B**) 5*S*, 8*R*, 9*S* isomer of the aldehydic form of elenolic acid (**26a**, 15.4 min); (**C**) 5*S*, 8*S* 9*S* isomer of elenolic acid (**26b**, 16.5 min); (**D**) dialdehydic form of the methyl ester of elenolic acid lacking a carboxymethyl group (**25**). The suppressed signals of H_2O and CH_3CN solvents give spikes at $\delta \sim 1.95$ and ~ 2.18.

the fact that the chemical shifts and coupling constants for the protons of the free and esterified elenolic ring (linked to hydroxytyrosol and tyrosol fragments in oleuropein and ligstroside derivatives) did not differ significantly from each other, except perhaps for those of the geminal protons of carbon C-6; for instance, compare the data in **Table 1** for compounds **18** and **24** or for compounds **22** and **26**.

Other important intermediate derivatives from oleuropein and ligstroside glucosides that have been detected in considerable amounts in olive oil (*4*, *13*) are oleuropein aglycon (**16**) and ligstroside aglycon (**17**). The structure of oleuropein aglycon obtained by biomimetic experiments with β-endogenous glucosidase has been resolved by employing ^{1}H and ^{13}C NMR spectroscopy (*13*, *42*). Both metabolites have been detected in the present analysis (peaks at 22.9 and 26.0 min in the chromatogram of **Figure 1**). The assignment of the LC-SPE-^{1}H NMR of compounds **16** and **17** was facilitated by TOCSY experiments. **Figure 10** shows the TOCSY spectrum of compound **16**, where the correlation of the various protons is clearly seen. The spectroscopic parameters of both compounds are depicted in **Table 1**. Our spectroscopic data for **16** are comparable with those reported by Bianco et al. (*13*, *42*) except for the resonance of the H8 proton, which appears at lower fields. Spectroscopic information for compound **17** is reported for the first time.

The chromatogram in **Figure 1** shows a number of peaks (denoted by asterisks) that are either unknown or cannot be identified unambiguously with ^{1}H NMR alone. The peak eluting at 8.0 min, which is very close to peak 24, is ascribed tentatively to the hemiacetalic form of elenolic acid lacking a carboxymethyl group. Shift predictions were based on the LC-SPE-^{1}H NMR spectra of the corresponding hemiacetals of oleuropein (**20**) and ligstroside (**21**) (**Table 1**). Nevertheless, the presence of the H3 proton expected at δ 4.1−4.2 was not confirmed in the spectrum. Four peaks with retention times of 25.9, 26.2, 26.6, and 27.2 min detected at 380 nm appear to correspond to a family of specific lignans characterized by a substituted diaryl epoxy skeleton. These compounds, which are distributed widely in the plant kingdom (*46*−*51*), differ by the substitution pattern of the furanoid ring. The first two compounds, which were coeluted at 230 nm (**Figure 1**), exhibited the same spectroscopic pattern of a 2,3,3,4-tetrasubsituted furanoid ring, bearing the two aryl rings at positions C2 and C4. These compounds are tentatively related to the lariciresinol-type epoxylignans, the first bearing a hydroxyl group at C3, the so-called berchemol (**27**, 26.2 min), and the second probably a carboxymethyl group at C3 (**28**, 25.9 min) (*46*−*48*). It has been suggested (*52*, *53*) that lariciresinol-type epoxylignans are formed by enantiospecific reduction of (+)-pinoresinol and perhaps of (+)-1-acetoxypinoresinol. The LC-SPE-^{1}H NMR spectra of the remaining two lignans were consistent with a 2,3,4,5-tetrasubstituted tetrahydrofuran, bearing the two aryl rings at C2 and C5 carbon sites, which is a common feature of the so-called neolignans (*49*−*51*). On the basis of the LC-SPE-^{1}H NMR spectroscopic data the lignan eluted at 27.2 min is tentatively ascribed to the 2*S*, 3*R*, 4*S*, 6*S* isomer, the so-called verucosin (**29**) (*49*), whereas that eluted at 26.6 min may be defined as the 2*R*, 3*R*, 4*S*, 5*S* isomer of *meso*-3′,3″-dihydroxy-4′,4″-dimethoxy-2,5-epoxyliganan (**29**) (*51*).

Detection of Maslinic Acid. Upon examining our LC-SPE-^{1}H NMR spectra for peaks at long retention times in the HPLC chromatogram at 280 nm, we discovered a peak eluting at 37.1 min, which gave a spectrum (not shown) with the characteristics of maslinic acid (**30**) (*54*, *55*): seven singlets at high fields (δ

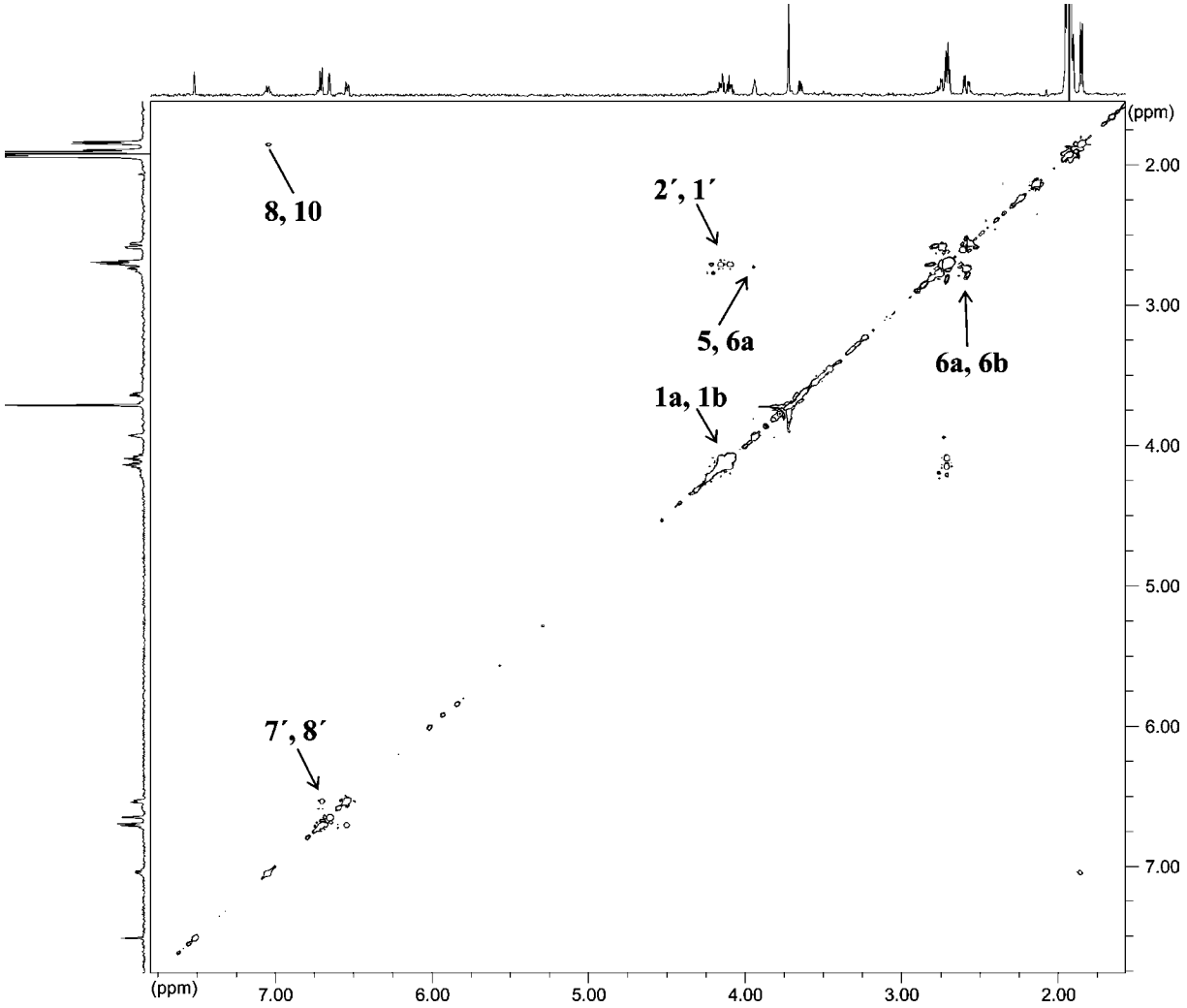

Figure 10. 600 MHz TOCSY spectrum of peak 16 in the chromatogram indicating the presence of the oleuropein aglycon.

0.70−1.15) attributed to an equal number of methyl groups; a doublet at δ 2.83; and a multiplet at δ 3.53 assigned to H3α and H2β protons ($J_{3\alpha,2\beta}$ = 9.5 Hz), respectively. Moreover, a doublet of doublets at δ 2.78 was ascribed to the H18 proton attached to a tertiary carbon and a triplet at δ 5.22 for the olefinic proton H12. To the best of our knowledge, a complete assignment of the ^{1}H spectrum of maslinic acid has not been reported yet. Nevertheless, the high-resolution 600 MHz LC-SPE-^{1}H NMR spectrum of this compound allowed the assignment of a few additional signals of the triterpenic skeleton assisted by TOCSY (not shown). Following the cross-peaks correlating the known signals of H3α, H2β, H12, and H18 protons in TOCSY, the geminal protons H1α (δ 1.83, dd, $J_{1\alpha,1\beta}$ = 12.5 Hz, $J_{1\alpha,2}$ = 4.6 Hz), H1β (δ 0.86, dd, $J_{1\beta,2}$ = 11.4 Hz), H9 (δ 1.62, dd, $J_{9,11\alpha}$ = 7.0 Hz, $J_{9,11\beta}$ = 11.0 Hz), H11α (δ 1.91, m, $J_{12,11\alpha}$ = 3.7 Hz, $J_{12,11\beta}$ = 3.7 Hz), H19α (δ 1.68, $J_{18,19\alpha}$ = 13.9 Hz), and H19β (δ 1.08, dd, $J_{18,19\beta}$ = 4.8 Hz) were easily assigned. It should be noted that maslinic acid was absent in the chromatogram at 230 nm.

In summary, the results presented in this study have demonstrated the tremendous potential of LC-SPE-NMR as a method of analysis of multicomponent mixtures. Using this technique, we were able to assign 27 constituents of the phenolic fraction of olive oil, which were difficult or impossible to identify by NMR alone, mainly because of their low concentration and severe overlap with other signals in the same region. The main achievements of this study include the unambiguous identification and confirmation of a large number of phenolic compounds reported previously and, more importantly, the detection and structure elucidation of new phenolic compounds, such as syringaresinol, homovanillyl alcohol, the 5*S*, 8*S*, 9*S* isomer of the aldehydic form of oleuropein, the two isomers of the aldehydic form of ligstroside, the ligstroside aglycon, the dialdehydic form of elenolic acid lacking a carboxymethyl group, and, finally, maslinic acid, not detected in the past.

Supporting Information Available: LC-SPE ^{1}H NMR spectra of peaks 1−15. This material is available free of charge via the Internet at http://pubs.acs.org.

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