# High-Performance Liquid Chromatography Evaluation of Phenols in Olive Fruit, Virgin Olive Oil, Vegetation Waters, and Pomace and 1D- and 2D-Nuclear Magnetic Resonance Characterization

**Maurizio Servili[a,1], Maura Baldioli[a], Roberto Selvaggini[a], Enrico Miniati[a], Alceo Macchioni[b], and Gianfrancesco Montedoro[a,*]**

[a]Istituto di Industrie Agrarie, University of Perugia, 06126 Perugia, Italy, and [b]Dipartimento di Chimica, University of Perugia, 06123 Perugia, Italy

**ABSTRACT:** Phenolic compounds are the most important antioxidants of virgin olive oil. This paper reports on the application of solid phase extraction (SPE) in the separation of phenolic compounds from olive fruit, olive oil, and by-products of the mechanical extraction of the oil and the complete spectroscopic characterization by nuclear magnetic resonance of demethyloleuropein and verbascoside extracted from olive fruit. SPE led to a higher recovery of phenolic compounds from olives than did liquid/liquid extraction. SPE also was used to separate phenolic compounds from pomaces and vegetation waters. Phenyl-acid and phenyl-alcohol concentrations in extracts obtained from SPE and liquid/liquid extraction were not significantly different ($P < 0.05$). The recovery of the dialdehydic form of elenolic acid linked to 3,4-(dihydroxyphenyl)ethanol and an isomer of oleuropein aglycon, however, was low.

Paper no. J8898 in *JAOCS 76,* 873–882 (July 1999).

**KEY WORDS:** HPLC analysis, NMR, phenols, secoiridoids, solid phase extraction, virgin olive oil.

Phenolic compounds of olive fruit and virgin olive oil have been found to correlate with the pungent and bitter taste of the oil (1); they also inhibit blood platelet aggregation, are involved in the synthesis of thromboxane in human cells (2), and inhibit phospholipid oxidation (3). The most important classes of phenolic compounds of olive fruit include phenyl acids, phenyl alcohols, flavonoids, and secoiridoids (4). The main phenyl alcohols of olive are 3,4-(dihydroxyphenyl)ethanol (3,4-DHPEA) and *p*-(hydroxyphenyl)ethanol (*p*-HPEA) (5,6). The flavonoids include the flavonol glycosides, luteolin-7-glucoside, and rutin, and the anthocyanins, cyanidin, and delphinidin glycosides (7,8). Oleuropein and demethyloleuropein are the predominant secoiridoids of olive fruit (9–11) which also contains verbascoside (4,12).

Phenyl acids and phenyl alcohols have also been found in virgin olive oil (13,14) but the prevalent phenolic compounds are secoiridoid derivatives, such as the dialdehydic form of elenolic acid linked to 3,4-DHPEA, or *p*-HPEA (3,4-DHPEA-EDA or *p*-HPEA-EDA, where EDA is elenolic acid dialdehyde) and an isomer of oleuropein aglycon (3,4-DHPEA-EA, where EA is elenolic acid) (15). These compounds are the most concentrated phenolic antioxidants of virgin olive oil (16). Secoiridoid derivatives have also been found in the by-products of oil extracted mechanically such as from vegetation waters and pomaces (17–19).

Various analytical methods have been studied to evaluate phenolic compounds in olive fruit and olive oil (5,6,13,14, 20–26). A few chromatographic methods have been used to study the phenolic compounds of olive fruit (27,28). This paper reports the separation of phenolic compounds from olive fruit, virgin olive oil, wastewaters separated during the oil mechanical extraction process (vegetation waters), and pomaces using solid phase extraction (SPE) and nuclear magnetic resonance (NMR) characterization of demethyloleuropein and verbascoside extracted from olive fruits.

## MATERIALS AND METHODS

*Sample preparation*. Olive fruits (*Olea europaea* L.) from frantoio and coratina cultivars were used. To extract phenolic compounds from olive fruit, 500 g of olives were destoned. To study phenolic compounds in oil, vegetation waters, and pomaces, 3 kg of olives were crushed with a hammer mill and slowly mixed (malaxed) at 30°C for 60 min. The oil was extracted using a laboratory hydraulic press (maximum pressure 220 bar). Destoned fruit, stones, vegetation waters, and pomaces were immediately frozen in liquid nitrogen to inhibit enzymatic activity, freeze-dried, and stored at −30°C before analysis.

*Reference compounds.* The 3,4-DHPEA was synthesized in the laboratory according to the procedure of Baraldi *et al.* (29). The 3,4-DHPEA-EDA and 3,4-DHPEA-EA were extracted from virgin olive oil and the chemical structures were

[1]Present address: University of Molise, Via De Sanctis, 86100 Campobasso, Italy.

*To whom correspondence should be addressed at Istituto di Industrie Agrarie, University of Perugia, Via S. Costanzo, 06126 Perugia, Italy. E-mail: alceo@unipg.it

verified by NMR according to Montedoro *et al.* (15). Oleuropein glucoside was obtained from Extrasynthèse Co. (Genay, France). The *p*-HPEA was obtained from Janssen Chemical Co. (Beerse, Belgium); *p*-hydroxybenzoic acid, vanillic acid, and caffeic acid were obtained from Fluka Co. (Buchs, Switzerland); luteolin-7-glycoside was obtained from Roth Co. (Karlsruhe, Germany); and rutin was obtained from BDH Co. (Poole, England). Demethyloleuropein and verbascoside were extracted and characterized by NMR according to the procedure reported below.

*Phenolic compounds from olive fruit and pomaces.* The extraction, purification, and separation procedure of phenolic compounds was optimized using freeze-dried olives from the frantoio cultivar, and optimal conditions were applied to the extraction and separation of phenolic compounds from the pomaces (Fig. 1).

Freeze-dried stones were crushed and added to the destoned olives in the same ratio as the original olive. Whole fruits (10 g) were mixed at −25°C with 50 mL of 80% methanol added containing 20 mg/L sodium diethyldithiocarbamate (DIECA) (30), to inhibit polyphenoloxidase and lipoxygenase activities. The mixture was homogenized in an Omni-mixer (Sorvall) for 30 s at 1050 × *g* and filtered using a Buchner funnel apparatus. The extraction was repeated six times. The extracts were collected and the methanol was evaporated in vacuum under nitrogen flow at 35°C. The remaining aqueous extract is referred to as the water extract. Phenolic compounds may be oxidized during methanol evaporation and successive separation of the water extract in the $C_{18}$ cartridge. For this reason, different combinations of antioxidants, such as $SO_2$ (90 mg/g), $SO_2$ (25 mg/g) + ascorbic acid (2 mg/g), and ascorbic acid (2 mg/g) were added to the extract before methanol evaporation.

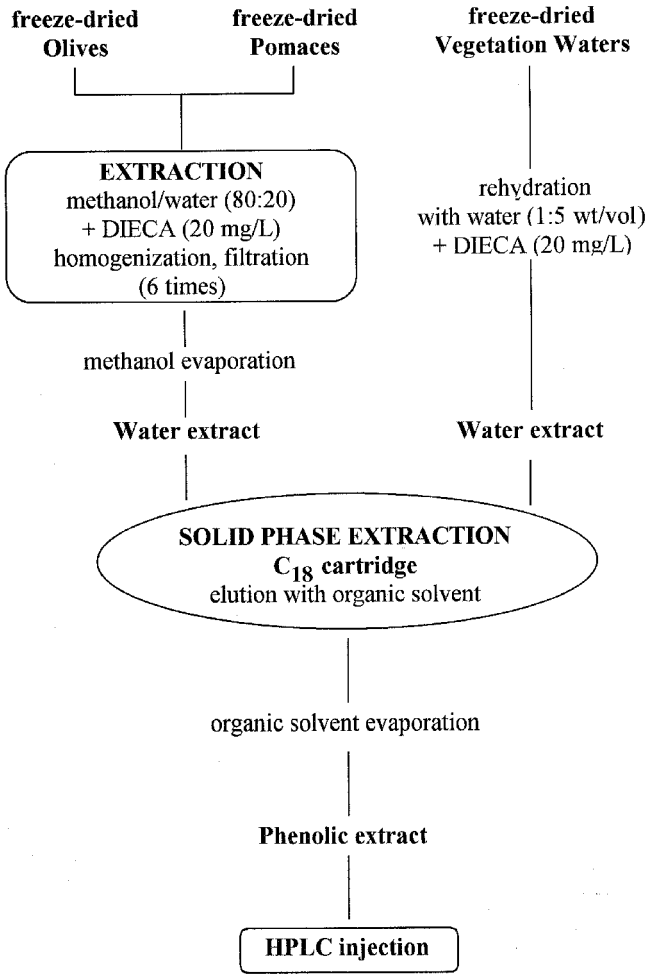


**FIG. 1.** Flow sheet of the extraction and separation of phenolic compounds from olives, pomaces, and vegetation waters. HPLC, high-performance liquid chromatography; DIECA, diethyldithiocarbamate.

SPE was used to separate the phenolic compounds present in the water extract. Two milliliters of water extract was added to a 5 g/20 mL Extract-Clean highload $C_{18}$ cartridge (Alltech Italia Srl, Milan, Italy) and the following two sequences of organic solvents were used to recover phenolic compounds: ethyl ether (300 mL), ethyl acetate (100 mL) and methanol (200 mL) or methanol (600 mL). To reduce phenol oxidation, elution from the $C_{18}$ cartridge under a flow of nitrogen also was tested. The eluate was collected and the organic solvent evaporated in vacuum under nitrogen flow at 35°C. The residue was dissolved in 1 mL of methanol and injected into the high-performance liquid chromatograph (HPLC).

The results obtained from the olive fruit by SPE were compared with those from the liquid/liquid extraction performed according to Amiot *et al.* (27).

*Phenolic compounds from vegetation waters.* Freeze-dried vegetation waters were rehydrated with water containing DIECA (20 mg/L), and 2 mL was loaded on a 5 g/20 mL Extract-Clean highload $C_{18}$ cartridge (Alltech Italia Srl). To recover and fractionate phenolic compounds the following three sequences of organic solvents were studied: ethyl acetate (500 mL) and methanol (100 mL), or ethyl ether (100 mL) and ethyl acetate (500 mL), or ethyl ether (600 mL). The eluate concentration and HPLC injection were carried out as above (Fig. 1).

The HPLC system was composed of a Varian 9010 solvent delivery system (Varian Associates, Inc., Walnut Creek, CA) with a 150 × 4.6 mm i.d. Inertsil ODS-3 column (Alltech Italia Srl) coupled with a Varian Polychrom 9065 ultraviolet (UV) diode array detector, operating in the UV region. The samples were dissolved in methanol, and a sample loop of 20 μL capacity was used. The mobile phase was a mixture of solution A (0.2% acetic acid, pH 3.1) and methanol (B), and the flow rate was 1.5 mL/m. The total run time was 55 min and the gradient changed as follows: 95% A/5% B for 2 min; 75% A/25% B for 8 min; 60% A/40% B for 10 min; 50% A/50% B for 10 min; 0% A/100% B for 10 min; the mixture was maintained for 5 min, then returned to 95% A/5% B for 10 min.

*Phenolic compounds from virgin olive oil.* Phenols were extracted from virgin olive oil using a 5 g/20 mL Extract-Clean highload $C_{18}$ cartridge (Alltech Italia Srl). Twenty milliliters of either hexane or hexane/ethyl ether (98:2 vol/vol) was used to condition the cartridge, 5 g of oil was introduced,

and the sample was washed with 100 mL of the particular conditioning solvent to remove the nonpolar fraction. To elute phenolic compounds, 80 mL of the following solvents were tested: methanol, methanol/water (80:20 vol/vol), and acetonitrile. The solvents from the eluent were evaporated to dryness in vacuum under nitrogen flow at 35°C. The dried residue was dissolved in 1 mL of methanol and analyzed by HPLC using the same chromatographic conditions reported in a previous paper (23). Results were compared with the liquid/liquid extraction performed according to Montedoro *et al*. (23). To study the effect of nonionic surfactants in phenol extraction, Tween® 20, Tween® 80 (BDH Co.) and Triton® X-100 (LKB Bromma, Sweden) were tested. The nonionic surfactants (2% wt/vol) were added to the extraction (methanol/water 80:20, vol/vol) and the elution (methanol) solvents for liquid/liquid extraction and SPE, respectively.

*Separation and NMR characterization of verbascoside and demethyloleuropein.* Verbascoside and demethyloleuropein were extracted from the freeze-dried destoned olives (coratina cultivar), using the extraction procedure reported above. The separation and purification of these compounds were achieved using preparative HPLC. A Varian liquid chromatograph Model 5000 equipped with a 500 × 9.4 mm i.d. Whatman Partisil 10 ODS-2 semipreparative column (Alltech Italia Srl), coupled with a Varian Polychrom 9065 diode array detector was used. The phenolic extract was injected in the column using a sample loop of 1 mL, and compounds were detected at 278 nm. The peaks corresponding to the demethyloleuropein and verbascoside were recovered using a Gilson Model 201 fraction collector (Gilson Medical Electronics, Inc., Middleton, WI). The mobile phase was a mixture of solution A (0.2% acetic acid, pH 3.1) and methanol (B) (flow rate 5.6 mL/m). The total running time of the analysis was 65 min and the gradient was changed as follows: 80% A/20% B at time 0 min, 60% A/40% B realized in 20 min; the mixture was maintained for 20 min, 0% A/100% B for 5 min, and the mixture was maintained for 10 min, and returned to 80% A/20% B for 5 min.

Each phenolic extract injection corresponded to about 10 mg of total phenols, expressed as 3,4-DHPEA equivalent, as determined with the Folin-Ciocalteau reagent (23). The collected compounds were recovered according to the procedure described in a previous paper (15).

The NMR spectra were recorded on Bruker AC 200 and Bruker DRX 500 spectrometers (Bruker, Karlsruhe, Germany) (operating at 200.13 and 500.13 MHz for $^1H$ and 50.13 and 125.77 MHz for $^{13}C$) using TMS as the external standard. About 20 mg of sample was dissolved in 0.6 mL in methanol-$d_4$. $^1H$, $^{13}C\{^1H\}$, $^{13}C$-$^1HJ$ Modulated, $^1H$-COSY (with gradients), $^1H$-NOESY phase-sensitive, $^1H$-$\{^{13}C\}$-correlation (with gradients), and $^1H$-$\{^{13}C\}$-long range correlation experiments (with gradients) were performed (31).

The $^1H$-NOESY phase-sensitive spectrum was obtained with a mixing time of 800 ms. The H-$\{^{13}C\}$-correlation and $^1H$-$\{^{13}C\}$-long range correlation experiments were obtained using the Bruker pulse programs *inv4gs* and *inv4gslnd*, respectively, setting the delays for evolution of couplings at 3.3 ms and 50 ms, respectively. The $^1H$-COSY experiment was obtained with the *cosygs* Bruker pulse program.

Structural assignments (Table 1) were achieved by first recording the $^{13}C\{^1H\}$ NMR spectrum which gives the total number of carbons in the molecule. Successively, from the $^{13}C$-$^1HJ$ Modulated spectrum it was possible to distinguish quaternary and $CH_2$ from CH and $CH_3$ groups. All the resonances were assigned crossing together the information derived from the analysis of $^1H$, $^1H$-COSY, H-$\{^{13}C\}$-correlation and $^1H$-$\{^{13}C\}$-long range correlation experiments. Results from this last experiment also gave final information needed to assign the final structures. Indications of the conformation were obtained by the $^1H$-NOESY phase-sensitive experiment as well as a confirmation of the total assignment.

*Statistical analysis.* All chemical and instrumental measurements were replicated three times. Means ± standard deviations are reported in the tables. To evaluate the significance of differences between mean values among three or more different experimental groups the one-way analysis of variance using the Tukey test was performed. To compare two groups of values the paired *t*-test was employed. Statgraphics Version 6.1 (Statistical Graphics Corp., 1992, Manugistics, Inc., Rockville, MD) was used to perform all statistical analyses.

## RESULTS AND DISCUSSION

*Phenolic compounds from oive fruit and pomace*. The effect of antioxidants on the recovery of phenols was studied. As shown in Table 2, the phenolic concentration decreased when ascorbic acid was added to the extract before methanol evaporation. Significant differences ($P < 0.05$) were observed in all classes of phenolic compounds including secoiridoids. The great decrease in 3,4-DHPEA and oleuropein suggested a prooxidant activity of ascorbic acid (32); however, no significant differences were found in the concentration of other compounds such as demethyloleuropein and verbascoside, which also contain 3,4-DHPEA.

$SO_2$ strongly reduced the concentration of 3,4-DHPEA-EDA, probably due to the nucleophilic addition of $HSO_3^-$ to the dialdehydic groups of 3,4-DHPEA-EDA causing its precipitation from the aqueous solution (33). The elution under nitrogen significantly improved only the recovery of *p*-hydroxybenzoic, caffeic, and vanillic acids ($P < 0.05$).

Phenyl alcohols, phenyl acids, flavonoids and secoiridoids are characterized by different affinities with different organic solvents (4). For this reason the elution of phenolic compounds from SPE was carried out using a sequence of ethyl ether, ethyl acetate, and methanol to study the selective recovery of phenolic compounds from the water extract. Results reported in Table 3 show that there was no selective effect of the organic solvent in the elution of phenolic compounds. In fact, secoiridoids such as oleuropein and demethyloleuropein were found in all the fractions analyzed and recovery was highest when methanol was used alone (Table 3). The comparison between SPE and liquid/liquid extraction (27), reported in Table

**TABLE 1**
**$^1$H and $^{13}$C Nuclear Magnetic Resonance Data for Demethyloleuropein (1) and Verbascoside (2) in Methanol-$d_4$**

| Atom no. | Demethyloleuropein (**1**) | | Verbascoside (**2**) | | Atom no. | Demethyloleuropein (**1**) | | Verbascoside (**2**) | |
|---|---|---|---|---|---|---|---|---|---|
| | $^{13}$C in ppm (*J* in Hz) | $^1$H in ppm (*J* in Hz) | $^{13}$C in ppm (*J* in Hz) | $^1$H in ppm (*J* in Hz) | | $^{13}$C in ppm (*J* in Hz) | $^1$H in ppm (*J* in Hz) | $^{13}$C in ppm (*J* in Hz) | $^1$H in ppm (*J* in Hz) |
| 1 | 95.1 | 5.73 *m* | 127.9 | | Glucose | | | | |
| 2 | — | — | 115.5 | 7.14 *d* ($^4J_{2,6}$ = 2.0) | 1′ | 100.9 | 4.66 *d* ($^3J_{1',2'}$ = 7.5) | 104.5 | 4.47 *d* ($^3J_{1',2'}$ = 7.9) |
| | | | | | 2′ | 74.7 | 3.20 *m* buried under $CHD_2OD$ | 76.5 | 3.48 *dd* ($^3J_{2',3'}$ = 9.1; $^3J_{2',1'}$ = 8.0) |
| 3 | 154.8 | 7.34 *s* | 147.1 | — | | | | | |
| 4 | 110.2 | | 150.1 | | | | | | |
| 5 | 31.9 | 3.83 *m* | 116.8 | 6.87 *d* ($^3J_{5,6}$ = 8.2) | 3′ | 77.9 | 3.85 *m* | 81.9 | 3.91 *t* [($^3J_{3',4\tilde{O}}$ + $^3J_{3',2'}$)/2 = 9.2] |
| 6 | 41.2 | 2.60 *dd* buried under 2′ | 123.5 | 7.05 *dd* ($^3J_{6,5}$ = 8.3; $^4J_{6,2}$ = 1.9) | 4′ | 71.4 | 4.78 buried under $CD_3OH$ | 70.9 | 5.00 *t* [($^3J_{4',3'}$ + $^3J_{4',5'}$)/2 = 9.5] |
| | | 2.25 *dd* ($^2J_{6a,6b}$ = 13.4; $^3J_{5,6b}$ = 9.3) | | | 5′ | 78.3 | 3.50 *m* | 76.3 | 3.78 *m* |
| | | | | | 6′ | 62.7 | 3.75 *d* ($^2J_{6a',6b'}$ = 11.0) | 62.7 | 3.71 *d* ($^2J_{6a',6b'}$ = 10.0) |
| | | | | | | | 3.54 *d* | | 3.62 *m* |
| 7 | 173.3 | — | 148.3 | 7.68 *d* ($^3J_{7,8}$ = 15.8) | Rhamnose | | | | |
| 8 | 124.6 | 5.91 *qd* ($^3J_{8,10}$ = 7.1; $^4J_{8,1}$ = 1.4) | 115.0 | 6.37 *d* ($^3J_{8,7}$ = 15.9) | 1′ | | | 103.3 | 5.28 *d* ($^3J_{1\tilde{O},2\tilde{O}}$ = 1.4) |
| | | | | | 2′ | | | 72.7 | 4.01 *dd* ($^3J_{2\tilde{O},3\tilde{O}}$ = 3.1; $^3J_{2\tilde{O},1\tilde{O}}$ = 1.3) |
| 9 | 130.7 | | 168.6 | | | | | | |
| 10 | 13.5 | 1.50 *dd* ($^5J_{10,1}$ = 1.3) | | | 3′ | | | 72.3 | 3.66 *dd* ($^3J_{3\tilde{O},4\tilde{O}}$ = 9.5; $^3J_{3\tilde{O},2\tilde{O}}$ = 3.3) |
| *C*OOH | 171.0 | | | | | | | | |
| Aglycone | | | | | 4′ | | | 74.1 | 3.38 *t* [($^3J_{4\tilde{O},3\tilde{O}}$ + $^3J_{4\tilde{O},5\tilde{O}}$)/2 = 9.5] |
| 1′ | 66.9 | 3.99 *m* | 72.6 | 4.14 *m* | | | | | |
| | | | | 3.83 *m* | 5′ | | | 70.7 | 3.62 *m* |
| 2′ | 35.3 | 2.60 *t* ($^3J_{2',1'}$ = 7.0) | 36.9 | 2.89 *m* | 6′ | | | 18.8 | 1.18 *d* ($^3J_{6\tilde{O},5\tilde{O}}$ = 6.2) |
| 3′ | 130.8 | | 131.7 | | | | | | |
| 4′ | 116.5 | 6.53 *d* | 117.4 | 6.78 *d* | | | | | |
| 5′ | 144.8 | | 145.0 | | | | | | |
| 6′ | 146.1 | | 146.4 | | | | | | |
| 7′ | 117.1 | 6.53 *d* | 116.6 | 6.76 *d* ($^3J_{5\tilde{O},6\tilde{O}}$ = 8.0) | | | | | |
| 8′ | 121.3 | 6.39 *dd* ($^3J_{7',8'}$ = 8.0; $^4J_{4',8'}$ = 2.0) | 121.5 | 6.66 *dd* ($^3J_{7\tilde{O},8\tilde{O}}$ = 8.0; $^4J_{4\tilde{O},8\tilde{O}}$ = 2.0) | | | | | |

4, shows that SPE significantly improved the recovery of phenolic compounds from the water extract ($P < 0.05$).

The analytical procedure studied for the olive fruit also was applied to the pomaces. The HPLC chromatogram reported in Figure 2 shows a phenolic composition of pomaces similar to that from the olive fruit. In fact, secoiridoid aglycons, such as 3,4-DHPEA-EDA, which originate by hydrolysis of oleuropein and demethyloleuropein during the oil mechanical extraction process, were not predominant in the HPLC chromatogram (17,19,30).

*Phenolic compounds from vegetation waters.* To separate phenolic compounds from vegetation waters using SPE, a sequence of organic solvents was tested. The highest phenolic recovery was obtained using ethyl ether (Table 5).

Methanol was also tested but did not provide good results (data not shown) due to the high background noise in the UV detector during HPLC analysis. It is likely that the high concentrations of quinones and melanoidins occurring in the vegetation waters (33), and soluble in methanol, may interfere with the detector response.

Vegetation waters showed a phenolic composition very different from that of olive fruit. In fact, secoiridoid glucosides, such as oleuropein and demethyloleuropein, were greatly concentrated in olives, whereas the vegetation waters had high concentrations of secoiridoid derivatives, such as 3,4-DHPEA and 3,4-DHPEA-EDA.

*Phenolic compounds from virgin olive oil.* As reported in previous papers (15,16) virgin olive oil showed low amounts of phenyl acids and phenyl alcohols and high concentrations of secoiridoid derivatives such as 3,4-DHPEA-EDA, 3,4-DHPEA-EA, and *p*-HPEA-EDA, which originate from oleuropein, demethyloleuropein, and ligstroside during the oil me-

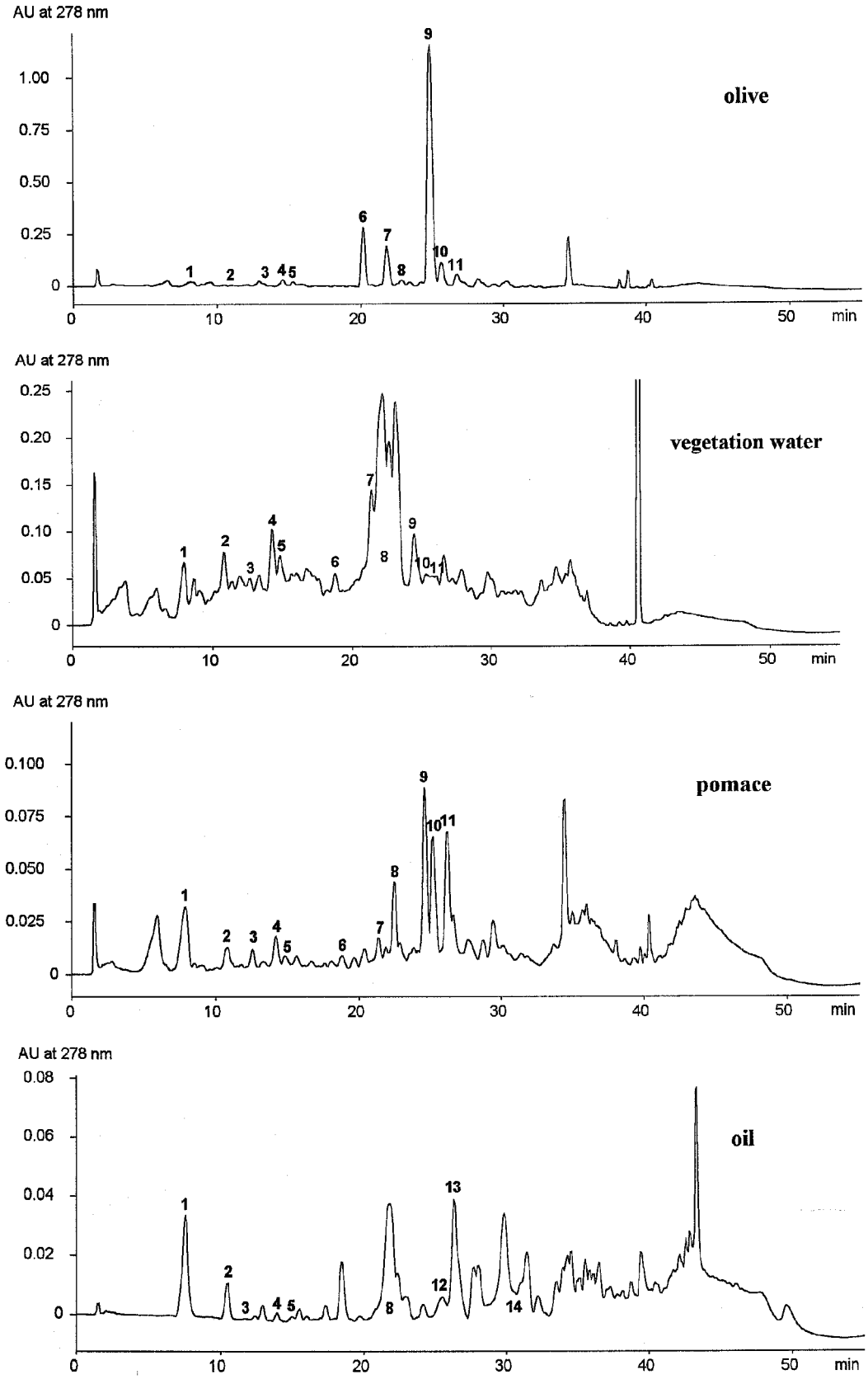


**FIG. 2.** HPLC chromatograms of olive, vegetation water, pomace and virgin olive oil. Peak numbers are identified as: (1) 3,4-(dihydroxyphenyl)ethanol (3,4-DHPEA); (2) *p*-(hydroxyphenyl)ethanol (*p*-HPEA); (3) *p*-hydroxybenzoic acid; (4) vanillic acid; (5) caffeic acid; (6) demethyloleuropein; (7) verbascoside; (8) 3,4-DHPEA-EDA (where EDA is elenolic acid dialdehyde); (9) oleuropein; (10) luteolin-7-glycoside; (11) rutin; (12) *p*-HPEA-EDA; (13) *p*-HPEA derivative; (14) 3,4-DHPEA-EA (where EA is elenolic acid). AU, absorbancy units. See Figure 1 for other abbreviation.

**TABLE 2**
**Use of Nitrogen Flow, $SO_2$ and Ascorbic Acid on the Solid Phase Extraction (SPE) Recovery of Olive Phenolic Compounds**

| Phenolic compound | Control | Recovery under $N_2$ flow | Ascorbic acid[a] (2 mg $g^{-1}$ d.w) | $SO_2$ + ascorbic acid[a] (25 mg $g^{-1}$ + 2 mg $g^{-1}$ d.w.) | $SO_2$[a] (90 mg $g^{-1}$ d.w.) |
|---|---|---|---|---|---|
| (1) 3,4-DHPEA[b,c,d] | 49.7 ± 3.5$^{f,h}$ | 55.6 ± 3.8$^{f}$ | 37.2 ± 2.5$^{g}$ | 44.8 ± 2.7$^{g,h}$ | 47.7 ± 3.6$^{f,h}$ |
| (2) *p*-HPEA[b,c,d] | 10.1 ± 0.7$^{f,h}$ | 11.5 ± 0.9$^{f}$ | 12.3 ± 0.9$^{f}$ | 14.8 ± 1.1$^{g}$ | 9.1 ± 0.8$^{h}$ |
| (3) *p*-Hydroxybenzoic acid[b,c,d] | 18.4 ± 1.4$^{f}$ | 25.5 ± 1.7$^{g}$ | 19.3 ± 1.5$^{f}$ | 16.7 ± 1.2$^{f}$ | 17.9 ± 1.4$^{f}$ |
| (4) Vanillic acid[b,c,d] | 17.0 ± 1.2$^{f,h}$ | 23.0 ± 1.4$^{g,i}$ | 14.2 ± 1.2$^{f}$ | 19.3 ± 1.5$^{h,i}$ | 21.1 ± 1.6$^{i}$ |
| (5) Caffeic acid[b,c,d] | 9.3 ± 0.7$^{f}$ | 14.5 ± 1.1$^{g}$ | 9.1 ± 0.6$^{f}$ | 6.9 ± 0.3$^{i}$ | 8.8 ± 0.6$^{f,i}$ |
| (6) Demethyloleuropein[b,c,d] | 2014.6 ± 143.6$^{f}$ | 2159.4 ± 150.2$^{f}$ | 1900.4 ± 142.6$^{f}$ | 2191.4 ± 174.2$^{f}$ | 2120.3 ± 179.6$^{f}$ |
| (7) Verbascoside[b,c,d] | 250.7 ± 10.4$^{f}$ | 251.7 ± 12.4$^{f}$ | 230.2 ± 9.8$^{f}$ | 131.5 ± 6.3$^{g}$ | 120.6 ± 1.6$^{g}$ |
| (8) 3,4-DHPEA-EDA[b,c,d] | 188.5 ± 14.9$^{f}$ | 198.3 ± 16.1$^{f}$ | 174.1 ± 10.4$^{f}$ | 92.0 ± 5.2$^{g}$ | 69.8 ± 4.3$^{h}$ |
| (9) Oleuropein[b,c,d] | 2750.4 ± 113.6$^{f,g}$ | 2822.5 ± 120.6$^{f}$ | 2395.4 ± 155.6$^{g,h}$ | 2690.3 ± 194.6$^{f,h}$ | 2325.2 ± 128.1$^{h}$ |
| (10) Luteolin-7-glucoside[b,c,d] | 52.5 ± 2.8$^{f}$ | 54.1 ± 3.2$^{f,h}$ | 61.3 ± 2.9$^{g,h}$ | 54.6 ± 2.5$^{f,g}$ | 62.6 ± 3.5$^{g}$ |
| (11) Rutin[b,c,d] | 115.9 ± 6.0$^{f,g}$ | 119.6 ± 6.4$^{f}$ | 103.6 ± 5.9$^{g,h}$ | 96.5 ± 5.7$^{h}$ | 92.5 ± 3.7$^{h}$ |
| Total phenolic compounds[b,e] | 2800.2 ± 89.6$^{f,h}$ | 3330.6 ± 105.4$^{h}$ | 2580.4 ± 78.5$^{f}$ | 2990.3 ± 164.2$^{h}$ | 2625.5 ± 140.2$^{f}$ |

[a]Antioxidants were added before methanol evaporation.
[b]Results are mean values of three independent determinations ± standard deviation. Values in each row bearing the same superscripts (f–i) are not significantly ($P < 0.05$) different from one another.
[c]The number in parentheses is the peak number shown in the high-performance liquid chromatography (HPLC) chromatogram of Figure 2.
[d]Values are expressed as mg $L^{-1}$ of phenolic extract injected in HPLC.
[e]Total phenolic compounds expressed as mg $L^{-1}$ of 3,4-(dihydroxyphenyl)ethanol (3,4-DHPEA) (Ref. 23). HPEA, (hydroxyphenyl)ethanol; EDA, elenolic acid dialdehyde.

chanical extraction process. SPE also was tested in the extraction of phenols from virgin olive oil and different organic solvents were studied to remove the nonpolar fraction and to elute phenolic compounds. Methanol led to the highest recovery in total phenols when the cartridge was conditioned with hexane (data not shown). An HPLC chromatogram of phenolic extract obtained by SPE is reported in Figure 2. A comparison of HPLC results obtained by SPE and liquid/liquid extraction is reported in Table 6. No significant differences in the recovery of phenyl alcohols and phenyl acids were found, but the liquid/liquid extraction improved the recovery of 3,4-DHPEA-EDA, 3,4-DHPEA-EA and *p*-HPEA-EDA ($P < 0.05$). As reported in Table 6 the use of nonionic surfactants tested in both methods significantly decreased the recovery of 3,4-DHPEA-EDA and 3,4-DHPEA-EA, particularly when used in liquid/liquid extraction ($P < 0.05$).

*NMR characterization of demethyloleuropein and verbascoside.* The $^{1}H$ and $^{13}C$ NMR data for demethyloleuropein (**1**) and verbascoside (**2**) are reported in Table 1. The numeration of the different carbons and protons is shown in Figure 3.

Despite the fact that demethyloleuropein has been known for more that two decades (11) we believe its NMR characterization has not been reported. The assignment of the carbon and proton resonances was achieved by comparing proton and carbon data with those reported for oleuropein (15). The NMR characterization of verbascoside has been reported (12), but we revised the assignment using the results of several two-dimensional experiments.

**TABLE 3**
**Use of Some Organic Solvents on the SPE Recovery of Phenolic Compounds from Olive**

| | Progressive elution with: | | | | Elution with: |
|---|---|---|---|---|---|
| Phenolic compound | Ethyl ether (300 mL) | Ethyl acetate (100 mL) | Methanol (200 mL) | Total | Methanol (600 mL) |
| (1) 3,4-DHPEA[a,b,c] | 6.4 ± 0.5 | 4.4 ± 0.5 | 28.5 ± 2.3 | 39.2 ± 2.4$^{e}$ | 44.3 ± 2.1$^{f}$ |
| (2) *p*-HPEA[a,b,c] | 3.7 ± 0.4 | 6.7 ± 0.5 | 5.8 ± 0.5 | 16.2 ± 0.8$^{e}$ | 10.8 ± 0.9$^{f}$ |
| (3) *p*-Hydroxybenzoic acid[a,b,c] | 1.9 ± 0.1 | n.d.[a] | 8.6 ± 0.6 | 10.5 ± 0.5$^{e}$ | 16.9 ± 1.0$^{f}$ |
| (4) Vanillic acid[a,b,c] | 4.1 ± 0.3 | n.d. | 20.0 ± 0.8 | 24.1 ± 0.9$^{e}$ | 18.1 ± 1.1$^{f}$ |
| (5) Caffeic acid[a,b,c] | 1.0 ± 0.0 | n.d. | 9.0 ± 0.5 | 10.0 ± 0.6$^{e}$ | 9.9 ± 0.8$^{e}$ |
| (6) Demethyloleuropein[a,b,c] | 281.2 ± 24.0 | 886.3 ± 66.5 | 1095.2 ± 82.0 | 2262.7 ± 108.2$^{e}$ | 2200.6 ± 143.1$^{e}$ |
| (7) Verbascoside[a,b,c] | n.d. | n.d. | 218.9 ± 10.5 | 218.9 ± 10.5$^{e}$ | 271.3 ± 10.2$^{f}$ |
| (8) 3,4-DHPEA-EDA[a,b,c] | 43.0 ± 3.4 | 36.6 ± 2.9 | 30.5 ± 2.1 | 110.0 ± 4.9$^{e}$ | 198.1 ± 13.8$^{f}$ |
| (9) Oleuropein[a,b,c] | 515.4 ± 38.5 | 555.2 ± 36.5 | 575.6 ± 43.0 | 1646.2 ± 67.7$^{e}$ | 2808.2 ± 118.3$^{f}$ |
| (10) Luteolin-7-glucoside[a,b,c] | 4.1 ± 0.2 | 21.0 ± 1.5 | 21.8 ± 1.6 | 46.8 ± 1.8$^{e}$ | 52.3 ± 2.7$^{f}$ |
| (11) Rutin[a,b,c] | 10.3 ± 1.1 | 10.1 ± 0.8 | 76.5 ± 4.8 | 96.9 ± 5.0$^{e}$ | 107.5 ± 5.6$^{f}$ |
| Total phenolic compounds[a,d] | 1408.5 ± 62.8 | 2170.2 ± 83.5 | 2208.6 ± 88.5 | 5787.3 ± 135.9$^{e}$ | 7400.5 ± 161.1$^{f}$ |

[a]Results are mean values of three independent determinations ± standard deviation. Values in each row bearing the same superscripts (e,f) are not significantly ($P \leq 0.05$) different from one another. n.d. = not detected.
[b]Values are expressed as mg $L^{-1}$ of phenolic extract injected in HPLC.
[c]The number in parentheses is the peak number shown in the HPLC chromatogram of Figure 2.
[d]Total phenolic compounds expressed as mg $L^{-1}$ of 3,4-DHPEA (Ref. 23). See Table 2 for abbreviations.

**TABLE 4**
**Recovery of Olive Phenolic Compounds by Liquid/Liquid and Solid Phase Extraction**

| Phenolic compound | Liquid/liquid separation | SPE[a] |
|---|---|---|
| (1) 3,4-DHPEA[b,c,d] | 8.5 ± 0.7[g] | 53.2 ± 3.6[h] |
| (2) *p*-HPEA[b,c,d] | 4.1 ± 0.3[g] | 12.1 ± 0.8[h] |
| (3) *p*-Hydroxybenzoic acid[b,c,d] | 7.3 ± 0.4[g] | 24.4 ± 1.4[h] |
| (4) Vanillic acid[b,c,d] | 5.8 ± 0.4[g] | 24.1 ± 1.3[h] |
| (5) Caffeic acid[b,c,d] | 3.1 ± 0.2[g] | 13.4 ± 0.9[h] |
| (6) Demethyloleuropein[b,c,d] | 516.9 ± 41.7[g] | 2036.4 ± 143.6[h] |
| (7) Verbascoside[b,c,d] | 118.4 ± 6.6[g] | 261.9 ± 12.1[h] |
| (8) 3,4-DHPEA-EDA[b,c,d] | 75.4 ± 4.9[g] | 210.5 ± 16.9[h] |
| (9) Oleuropein[b,c,d] | 1732.8 ± 103.2[g] | 2805.1 ± 206.1[h] |
| (10) Luteolin-7-glucoside[b,c,d] | 39.6 ± 2.7[g] | 53.1 ± 2.5[h] |
| (11) Rutin[b,c,d] | 77.6 ± 3.8[g] | 117.9 ± 6.8[h] |
| Total phenolic compounds[b,e] | 1510.9 ± 50.4[g] | 3260.6 ± 100.7[h] |
| Total recovery (%)[f] | 60.2 ± 3.5[g] | 94.4 ± 4.1[h] |

[a]Methanol (600 mL) was used as liquid phase under $N_2$ flow.
[b]Results are mean values of three independent determinations ± standard deviation. Values in each row bearing the same superscripts (g,h) are not significantly ($P < 0.05$) different from one another.
[c]The number in parentheses is the peak number shown in the HPLC chromatogram of Figure 2.
[d]Values are expressed as mg $L^{-1}$ of phenolic extract injected in HPLC.
[e]Total phenolic compounds expressed as mg $L^{-1}$ of 3,4-DHPEA (Ref. 23).
[f]Data are expressed in percentage in relation to the phenol concentration of water extracts (100%).
See Table 2 for abbreviations.

*$^1H$-COSY*. Having first identified in the $^1H$ NMR spectra the resonances of H-1 protons belonging to the glucose and rhamnose moieties and H-6 protons of caffeic acid and aglycon moieties, it is possible to assign all the other resonances besides every moiety simply by following the $^3J_{HH}$ "network."

*$^1H\{^{13}C\}$-correlation*. Based on the proton assignment it was easy to assign all the nonquaternary carbons detecting the cross peaks with "their" protons. An example concerning a section of the $^1H\{^{13}C\}$-correlation NMR spectrum (for verbascoside in methanol-$d_4$) relative to the olefinic and aromatic proton and carbon is reported in Figure 4. By knowing the proton resonances it is possible to assign the carbon resonances from the position of the cross peaks in the carbon dimension.

*$^1H\{^{13}C\}$-long range correlation.* This experiment was performed in order to unambiguously assign the quaternary carbons and to connect the different fragments of the molecules. C-3, C-4, C-1 aromatic quaternary carbons of caffeic acid and aglycon correlated with their H-5, H-2 and H-6, H-5 protons,

**TABLE 5**
**Use of Some Organic Solvents on the SPE Recovery of Phenolic Compounds from Vegetation Waters**

| | Progressive elution with: | | | Progressive elution with: | | | Elution with: |
|---|---|---|---|---|---|---|---|
| Phenolic compound | Ethyl acetate (500 mL) | Methanol (100 mL) | Total | Ethyl ether (100 mL) | Ethyl acetate (500 mL) | Total | Ethyl ether (600 mL) |
| (1) 3,4-DHPEA[a,b,c] | 85.6 ± 4.1 | 24.4 ± 1.8 | 110.0 ± 4.7[e] | 55.1 ± 1.4 | 11.0 ± 0.4 | 66.1 ± 1.5[f] | 121.4 ± 4.9[g] |
| (2) *p*-HPEA[a,b,c] | 167.2 ± 12.4 | 156.1 ± 13.4 | 323.3 ± 18.2[e] | 158.2 ± 11.6 | 30.2 ± 2.4 | 188.4 ± 11.8[f] | 320.4 ± 20.8[e] |
| (3) *p*-Hydroxybenzoic acid[a,b,c] | 34.5 ± 3.1 | n.d. | 34.5 ± 3.1[e] | 40.3 ± 3.8 | 4.0 ± 0.6 | 44.3 ± 3.9[f] | 44.5 ± 2.6[f] |
| (4) Vanillic acid[a,b,c] | 76.3 ± 4.0 | 64.2 ± 4.1 | 140.5 ± 5.7[e] | 62.0 ± 5.1 | 12.2 ± 0.5 | 74.2 ± 5.2[f] | 164.3 ± 9.8[g] |
| (5) Caffeic acid[a,b,c] | 24.0 ± 1.8 | 30.0 ± 2.6 | 54.0 ± 3.1[e] | 30.3 ± 3.8 | 3.2 ± 0.6 | 33.5 ± 3.8[f] | 52.5 ± 3.8[e] |
| (6) Demethyloleuropein[a,b,c] | 644.2 ± 49.1 | n.d. | 644.2 ± 49.1[e] | 314.8 ± 21.0 | 52.8 ± 3.1 | 367.6 ± 20.2[f] | 506.4 ± 49.3[g] |
| (7) Verbascoside[a,b,c] | 98.4 ± 5.1 | n.d. | 98.4 ± 5.1[e] | 78.2 ± 4.2 | 36.8 ± 2.1 | 115.0 ± 5.8[f] | 132.9 ± 7.5[g] |
| (8) 3,4-DHPEA-EDA[a,b,c] | 17860.3 ± 910.2 | n.d. | 17860.3 ± 910.2[e] | 17369.5 ± 872.8 | 1533.7 ± 90.1 | 18902.0 ± 876.6[e] | 18390.4 ± 927.6[e] |
| (9) Oleuropein[a,b,c] | 1200.8 ± 60.0 | n.d. | 1200.8 ± 60.0[e] | 570.3 ± 31.8 | 54.2 ± 1.4 | 624.5 ± 31.9[f] | 1268.3 ± 65.5[e] |
| (10) Luteolin-7-glucoside[a,b,c] | 22.0 ± 1.3 | n.d. | 22.0 ± 1.3[e] | 7.2 ± 0.5 | 1.4 ± 0.1 | 8.6 ± 0.6[f] | 17.0 ± 0.8[g] |
| (11) Rutin[a,b,c] | 36.1 ± 2.6 | n.d. | 36.1 ± 2.6[e] | 15.5 ± 1.2 | 1.4 ± 0.2 | 16.9 ± 1.1[f] | 32.9 ± 1.8[e] |
| Total phenolic compounds[a,d] | 16400.5 ± 820 | 1200.5 ± 68.6 | 17601.0 ± 823.2[e] | 15200.5 ± 836.5 | 1800.4 ± 102.5 | 16037.0 ± 842.2[e] | 17300.6 ± 825.4[e] |

[a]Results are mean values of three independent determinations ± standard deviation. Values in each row bearing the same superscripts (e–g) are not significantly ($P < 0.05$) different from one another.
[b]Values are expressed as mg $L^{-1}$ of phenolic extract injected in HPLC.
[c]The number in parentheses is the peak number shown in the HPLC chromatogram of Figure 2.
[d]Total phenolic compounds expressed as mg $L^{-1}$ of 3,4-DHPEA (Ref. 23). See Tables 2 and 3 for abbreviations.

**TABLE 6**
**Comparison Between SPE and Liquid/Liquid Extractions on the Recovery of Phenolic Compounds from Virgin Olive Oil**

| | SPE | | | | Liquid/liquid extraction | | | |
|---|---|---|---|---|---|---|---|---|
| Phenolic compound | Methanol | Methanol/ Tween 20 98:2 vol/wt | Methanol/ Tween 80 98:2 vol/wt | Methanol/ Triton X-100 98:2 vol/wt | Methanol/ water 80:20 vol/vol | Methanol/ water 80:20 vol/vol Tween 20 98:2 vol/wt | Methanol/ water 80:20 vol/vol Tween 80 98:2 vol/wt | Methanol/ water 80:20 vol/vol Triton X-100 98:2 vol/wt |
| (1) 3,4-DHPEA[a,b,c] | 14.7 ± 1.32$^{A,e}$ | 15.2 ± 1.10$^{e}$ | 9.0 ± 0.60$^{f}$ | 12.8 ± 0.65$^{e}$ | 15.6 ± 1.44$^{B,e}$ | 14.6 ± 0.91$^{e}$ | 13.1 ± 0.70$^{e}$ | 14.7 ± 0.88$^{e}$ |
| (2) *p*-HPEA[a,b,c] | 4.5 ± 0.57$^{A,e,g}$ | 5.6 ± 0.75$^{e}$ | 2.4 ± 0.22$^{f,h}$ | 3.6 ± 0.32$^{g,h}$ | 4.9 ± 0.051$^{A,e}$ | 3.8 ± 0.32$^{f}$ | 3.4 ± 0.22$^{f}$ | 3.8 ± 0.31$^{f}$ |
| (3) *p*-Hydroxybenzoic acid[a,b,c] | 0.6 ± 0.07$^{A,e}$ | 0.7 ± 0.08$^{e}$ | 0.7 ± 0.06$^{e}$ | n.d. | 0.6 ± 0.030$^{A,e,f}$ | 0.6 ± 0.04$^{d}$ | 0.5 ± 0.03$^{f}$ | 0.2 ± 0.01$^{g}$ |
| (4) Vanillic acid[a,b,c] | 0.6 ± 0.03$^{A,e}$ | 0.7 ± 0.06$^{e}$ | 0.7 ± 0.03$^{e}$ | n.d | 0.6 ± 0.060$^{A,e}$ | 0.6 ± 0.02$^{e}$ | 0.7 ± 0.05$^{e}$ | 0.4 ± 0.03$^{f}$ |
| (5) Caffeic acid[a,b,c] | 0.5 ± 0.02$^{A,e}$ | 0.9 ± 0.09$^{f}$ | 0.5 ± 0.02$^{e}$ | n.d. | 0.6 ± 0.031$^{B,e,f}$ | 0.6 ± 0.02$^{f}$ | 0.5 ± 0.03$^{e,g}$ | 0.5 ± 0.04$^{g}$ |
| (8) 3,4-DHPEA-EDA[a,b,c] | 289.4 ± 20.1$^{A,e,g}$ | 192.4 ± 11.7$^{f}$ | 261.2 ± 18.4$^{e}$ | 311.7 ± 23.5$^{g}$ | 461.8 ± 33.7$^{B,e}$ | 370.5 ± 20.2$^{f}$ | 342.4 ± 20.6$^{f}$ | 363.0 ± 20.1$^{f}$ |
| (12) *p*-HPEA-EDA[a,b,c] | 29.7 ± 2.15$^{A,e}$ | 29.9 ± 1.80$^{e}$ | 10.6 ± 0.65$^{f}$ | 18.5 ± 1.21$^{g}$ | 31.2 ± 2.22$^{B,e}$ | 23.0 ± 1.42$^{f}$ | 21.7 ± 1.81$^{f}$ | 14.0 ± 0.88$^{g}$ |
| (13) *p*-HPEA derivative[a,b,c,d] | 19.3 ± 1.70$^{A,e}$ | 17.1 ± 1.35$^{e}$ | 18.3 ± 0.80$^{e}$ | 7.9 ± 0.44$^{f}$ | 16.1 ± 1.41$^{B,e}$ | 10.2 ± 0.54$^{f}$ | 8.9 ± 0.66$^{f}$ | 23.7 ± 1.44$^{g}$ |
| (14) 3,4-DHPEA-EA[a,b,c] | 221.8 ± 12.4$^{A,e}$ | 231.4 ± 11.8$^{e}$ | 104.4 ± 5.8$^{f}$ | 114.2 ± 4.8$^{f}$ | 290.5 ± 14.8$^{B,e}$ | 227.4 ± 5.3$^{f}$ | 152.3 ± 6.0$^{g}$ | 183.3 ± 6.2$^{h}$ |

[a]Results are mean values of three independent determinations ± standard deviation. Values in each row bearing the same superscripts are not significantly ($P < 0.05$) different from one another. Superscript capital letters (A,B) refer to the comparison between SPE and liquid/liquid extraction, whereas the superscript lowercase letters (e–h) report the comparison among the values with in the same extraction method.
[b]Values are expressed as mg $kg^{-1}$ of virgin olive oil.
[c]The number in parentheses is the peak number shown in the HPLC chromatogram of Figure 2.
[d]Reference 23. See Tables 2 and 3 for abbreviations.

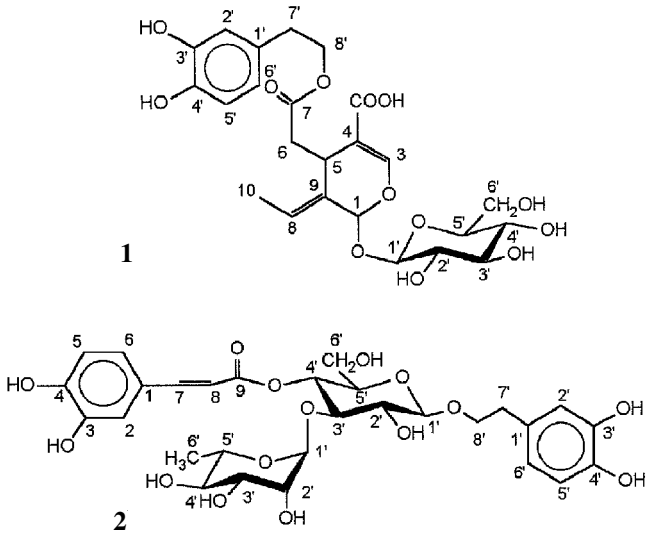


**FIG. 3.** Chemical structure of demethyloleuropein (**1**) and verbascoside (**2**).

respectively. C-9 correlated with H-8 and H-4 of the glucose, thus, the caffeic moiety is connected to glucose. C-3 of glucose correlated with H-1 of rhamnose and C-1 of rhamnose with H-3 of glucose, indicating that the two moieties are connected in these positions. A strong cross peak between C-8 of aglycon and H-1 of glucose ensures that the fragments are connected. The $^{1}$H-NOESY experiment confirmed the assignment discussed above.

## ACKNOWLEDGMENTS

This research work was supported by the European Economic Community, under the project AIR3–CT–94 1355. The authors wish to thank Roberto Santibacci for his technical assistance.

## REFERENCES

1. Gutiérrez Rosales, F., S. Perdiguero, R. Gutiérrez, and J.M. Olias, Evaluation of the Bitter Taste in Virgin Olive Oil, *J. Am. Oil Chem. Soc. 69*:394–395 (1992).
2. Petroni, A., M. Blasevich, M. Salami, N. Papini, G.F. Montedoro, and C. Galli, Inhibition of Platelet Aggregation and Eicosanoid Production by Phenolic Component of Olive Oil, *Thrombosis Res. 78*:151–160 (1995).
3. Visioli, F., G. Bellomo, G.F. Montedoro, and C. Galli, Low Density Lipoprotein Oxidation Is Inhibited *in vitro* by Olive Oil Constituents, *Atherosclerosis 117*:25–32 (1995).
4. Macheix, J.J., A. Fleuriet, and J. Billot, *Fruit Phenolics*, CRC Press, Boca Raton, 1990, pp. 1–126.
5. Vazquez Roncero, A., C. Janer Del Valle, and L. Janer Del Valle, Determination of Total Polyphenols in Olive Oils, *Grasas Aceites 24*:350–357 (1973).
6. Vazquez Roncero, A., C. Janer Del Valle, and L. Janer Del Valle, Phenolic Compounds in Olives. III. Polyphenols in Olive Oil, *Grasas Aceites 27*:185–191 (1976).
7. Brenes-Balbuena, M., P. Garcia-Garcia, and A. Garrido-Fernandez, Phenolic Compounds Related to the Black Color Formed During the Processing of Ripe Olive, *J. Agric. Food Chem. 40*:1192–1196 (1992).
8. Mazza, G., and E. Miniati, *Anthocyanins in Fruits, Vegetables and Grains,* CRC Press, Boca Raton, 1993, pp. 64–67.
9. Bourquelot, E., and J. Vintilesco, *C.R. Hebd. Seances Acad. Sci. 147*:533–535 (1908).
10. Panizzi, L., M.L. Scarpati, and G. Oriente, Chemical Structure of Oleuropein, Bitter Glucoside of Olive with Hypotensive Activity, *Gazz. Chim. Ital. 90*:1449–1485 (1960).
11. Ragazzi, E., G. Veronese, and A. Guitto, The Demethyloleuropein, a New Glucoside Extracted from Ripe Olives, *Ann. Chim. 63*:13–20 (1973).
12. Andary, C., R. Wylde, C. Laffite, G. Privat, and F. Winternitz, Structure of Verbascoside and Orobancoside, Caffeic Acid Sugar Esters from *Orobanche rapum-genistae*, *Phytochemistry 21*:1123–1127 (1982).
13. Montedoro, G.F., Phenolic Constituents of Virgin Olive Oils. I.

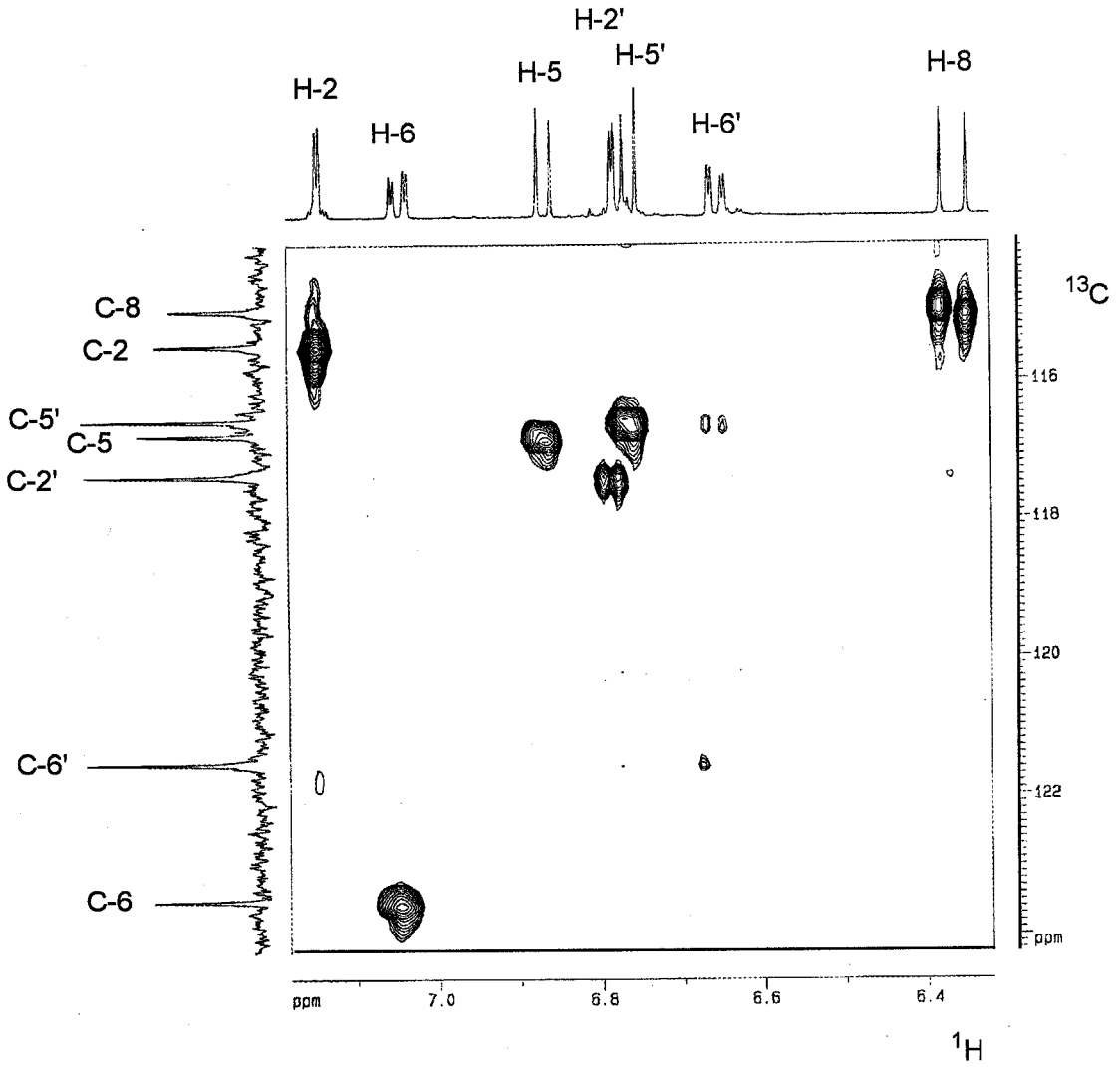


**FIG. 4.** Section of $^{1}H\{^{13}C\}$-correlation nuclear magnetic resonance spectrum for compound **2** (verbascoside), recorded at 500.13 MHz in methanol-$d_4$, showing the cross peaks between all the aromatic protons and carbons and between H-8 and C-8.

Identification of Some Phenolic Acids and Their Antioxidant Capacity, *Sci. Tecnol. Alimenti*. *2*:177–186 (1972).
14. Vazquez-Roncero, A., A Study of the Polar Compounds in Olive Oil by Gas Chromatography, *Grasas Aceites 31*:309–316 (1980).
15. Montedoro, G.F., M. Servili, M. Baldioli, R. Selvaggini, E. Miniati, and A. Macchioni, Simple and Hydrolyzable Compounds in Virgin Olive Oil. 3. Spectroscopic Characterization of the Secoiridoids Derivatives, *J. Agric. Food Chem. 41*: 2228–2234 (1993).
16. Baldioli, M., M. Servili, G. Perretti, and G.F. Montedoro, Antioxidant Activity of Tocopherols and Phenolic Compounds of Virgin Olive Oil, *J. Am. Oil Chem. Soc. 73*:1589–1593 (1996).
17. Lo Scalzo, R., and M.L. Scarpati, A New Secoiridoid from Olive Waste Waters, *J. Nat. Products 56*:621–623 (1993).
18. Limiroli, R., R. Consonni, A. Ranalli, G. Bianchi, and L. Zetta, $^{1}$H NMR Study of Phenolics in the Vegetation Waters of Three Cultivars of *Olea europaea*: Similarities and Differences, *J. Agric. Food Chem. 44*:2040–2048 (1996).
19. Servili, M., M. Baldioli, F. Mariotti, and G.F. Montedoro, Secoiridoids of Virgin Olive Oil: Modification During Mechanical Extraction Oil, *Proceedings of the World Conference on Oilseed and Edible Oils Processing*, Vol. II, edited by S.S. Koseoglu, K.C. Rhee, and R.F. Wilson, AOCS Press, Champaign, 1998, pp. 289–295.
20. Montedoro, G.F., and C. Cantarelli, Investigation on the Phenolic Compounds of Virgin Olive Oils, *Riv. Ital. Sostanze Grasse 46*:115–124 (1969).
21. Solinas, M., and A. Cichelli, Determination of Phenolic Substances in Olive Oil by GLC and HPLC; Possible Role of Tyrosol in Determination of the Quantity of Virgin Oil in Blends with Refined Oil, *Riv. Soc. Ital. Sci. Aliment. 11*:223–230 (1982).
22. Cortesi, N., and E. Fedeli, Polar Compounds of Virgin Olive Oil. Note 1, *Riv. Ital. Sostanze Grasse 60*:341–351 (1983).
23. Montedoro, G.F., M. Servili, M. Baldioli, and E. Miniati, Simple and Hydrolyzable Compounds in Virgin Olive Oil. 1. Their Extraction, Separation and Quantitative and Semiquantitative Evaluation by HPLC, *J. Agric. Food Chem. 40*:1571–1576 (1992).
24. Mannino, S., M.S. Cosio, and M. Bertuccioli, High Performance Liquid Chromatography of Phenolic Compounds in Virgin Olive Oils Using Amperometric Detection, *Ital. J. Food Sci. 2*: 150–157 (1993).

25. Angerosa, F., N. D'Alessandro, P. Konstantinou, L. Di Giacinto, and N. D'Alessandro, GC–MS Evaluation of Phenolic Compounds in Virgin Olive Oil, *J. Agric. Food Chem. 43*: 1802–1807 (1995).
26. Tsimidou, M., M. Lytridou, D. Boskou, A. Pappalouisi, F. Kotsifaki, and C. Petrakis, On the Determination of Minor Phenolic Acids of Virgin Olive Oil by RP-HPLC, *Grasas Aceites 47*: 151–157 (1996).
27. Amiot, M.J., A. Fleuriet, and J.J. Macheix, Importance and Evolution of Phenolic Compounds in Olive During Growth and Maturation, *J. Agric. Food Chem. 34*:823–826 (1986).
28. Vlahov, G., Flavonoids in Three Olive (*Olea europaea*) Fruit Varieties During Maturation, *J. Sci. Food Agric. 58*:157–159 (1992).
29. Baraldi, P.G., D. Simoni, F. Manfredini, and E. Menziani, Preparation of 3,4-Dihydroxy-1-benzeneethanol: A Reinvestigation, *Liebigs Ann. Chem.,* 684–686 (1983).
30. Servili, M., M. Baldioli, F. Mariotti, and G.F. Montedoro, Phenolic Composition of Olive Fruit and Virgin Olive Oil: Distribution in the Constitutive Parts of Fruit and Evolution During the Oil Mechanical Extraction Process, *Third International Symposium on "Olive Growing,"* Crete, 19–24 September 1997 (in press).
31. Sanders, J.K.M., and B.K. Hunter, *Modern NMR Spectroscopy,* Oxford University Press, New York, 1987, p. 235.
32. Wedzicha, B.L., *Chemistry of Sulphur Dioxide in Foods*, Elsevier Applied Science Publishers, London, 1984, pp. 183–229.
33. Vogel, A.I., *Practical Organic Chemistry,* Longmans, Green and Co. Press, London, 1959, pp. 318–333.
33. Ragazzi, E., and G. Veronese, Investigation on the Hydrosoluble Compounds of Olives. Note 1: Sugars and Phenols, *Ann. Chim. 57*:1386–1397 (1967).

[Received June 8, 1998; accepted February 18, 1999]