Determination of Phenols, Flavones, and Lignans in Virgin Olive Oils by Solid-Phase Extraction and High-Performance Liquid Chromatography with Diode Array Ultraviolet Detection

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A simple analytical method for the quantitative determination of phenols, flavones, and lignans in virgin olive oils was developed. The polar fraction was isolated from small amounts of oil sample (2.5 g) by solid-phase extraction (SPE) using diol-phase cartridges, and the extract was analyzed by reversed-phase HPLC coupled with diode array UV detection. Chromatographic separation of pinoresinol, cinnamic acid, and 1-acetoxypinoresinol was achieved. Repeatability (RSD < 6.5%), recovery (> 90%), and response factors for each identified component were determined. SPE on amino-phase cartridges was used for isolating acidic phenols and as an aid for phenol identification. For the first time, 2-(4-hydroxyphenyl)ethyl acetate was detected in olive oils. The aldehydic structure of the ligstroside aglycon was confirmed by NMR spectroscopy. The colorimetric determination of total *o*-diphenolic compounds by reaction with molybdate was consistent with their HPLC determination. Differences between results obtained by liquid−liquid extraction and SPE were not statistically significant.

Keywords: *Phenols; quantitative determination; solid-phase extraction; virgin olive oil*

INTRODUCTION

The main phenolic compounds in olive fruit are oleuropein and ligstroside, two glucosides the aglycons of which are esters of elenolic acid with 2-(3,4-dihydroxyphenyl)ethanol (hydroxytyrosol) and 2-(4-hydroxyphenyl)ethanol (tyrosol), respectively. In virgin olive oil, the respective aglycons and compounds arising from them by loss of the carboxymethyl moiety are the main phenolic constituents (*1*). These aglycons may exist in a number of keto−enolic tautomeric equilibria involving opening of the heterocyclic ring, yielding compounds of different structures (*2*). Some of these have been identified by LC-MS (*3*) and by GC-MS of their trimethylsilyl derivatives (*2*), and solvent effects on the equilibrium have been reported (*4*). Nuclear magnetic resonance (NMR) studies (*5*) on isolated phenolic compounds demonstrated that the aldehydic form of oleuropein aglycon (**1**) and the dialdehydic forms of decarboxymethyl aglycons (**3** and **4)** (Figure 1) predominate over other isomers in deuteriochloroform, whereas reversible equilibria toward their hemiacetal derivatives occurred in methanol-d_4. By analogy with **1**, the aldehydic form **2** has been suggested (*2*) for the ligstroside aglycon, but NMR studies have not yet been done. Variable amounts of hydroxytyrosol (**5**) and tyrosol (**6**) have also been found (*1*). Minor amounts of vanillin, cinnamic acid, and phenolic derivatives of benzoic, phenylacetic, cinnamic, and phenylpropionic acids have been reported (*1*), but only some of these compounds have been repeatedly found (*6*−*8*). Recently, 2-(3,4-dihydroxyphenyl)ethyl acetate (hydroxytyrosyl acetate) (**7**) (*8*, *9*) and two lignans, pinoresinol (**8**) and 1-acetoxypinoresinol (**9**) (*10*, *11*), have been identified as components of the phenolic fraction in olive oils. The flavones luteolin and apigenin were detected many years ago (*12*).

Traditionally, the phenolic fraction of olive oils has been isolated by extraction of an oil solution in hexane with several portions of water/methanol, followed by solvent evaporation of the aqueous extract and a cleanup of the residue by solvent partition (*7*, *10*). Extraction with tetrahydrofuran/water followed by centrifugation has also been assayed (*13*). These extraction procedures are very laborious, and alteration of phenolic compounds may occur. Consequently, attempts have been made to isolate the phenolic fraction by solid-phase extraction (SPE) using C_{18} (*14*, *15*) and C_8 (*4*, *16*) cartridges, but incomplete extraction of the phenolic fraction (*13*) and partial oil separation (*16*) have been reported. Anionic exchange cartridges have been used to isolate the phenolic fraction from various seed oils, but recoveries were low (53−62%) for some components (*17*). Recently, the use of SPE cartridges packed with 5 g of high-load C_{18} phase showed low recoveries for the secoiridoid derivatives (*18*).

Currently, analysis of the phenolic fraction is performed by reversed-phase high-performance liquid chromatography (RP-HPLC) with UV detection operated at 225, 240, or 280 nm, using gradient elution with two solvents, one of them being a water/acid admixture, and the other acetonitrile (*4*), methanol (*6*, *7*, *10*, *11*), or methanol/acetonitrile (*13*, *16*, *19*). However, a better separation between some chromatographic peaks is

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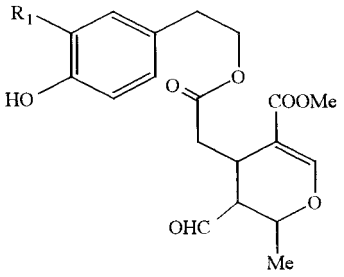
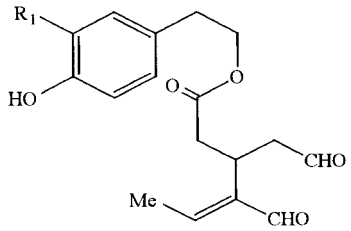
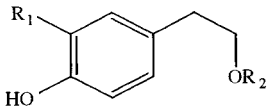


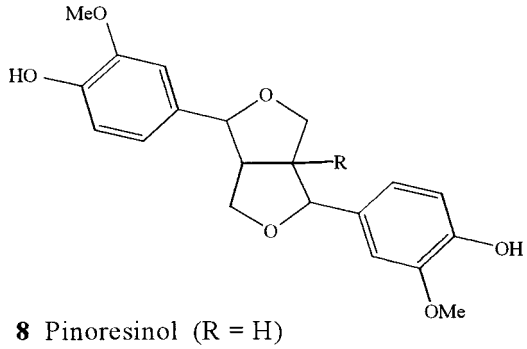
1 Aldehydic form of oleuropein aglycone (R_1= OH)
2 Aldehydic form of ligstroside aglycone (R_1= H)



3 Dialdehydic form of decarboxymethyl oleuropein aglycone (R_1= OH)
4 Dialdehydic form of decarboxymethyl ligstroside aglycone (R_1= H)



5 2-(3,4-Dihydroxyphenyl)ethanol (Hydroxytyrosol) (R_1= OH, R_2= H)
6 2-(4-Hydroxyphenyl)ethanol (Tyrosol) (R_1= H, R_2= H)
7 2-(3,4-Dihydroxyphenyl)ethyl acetate (Hydroxytyrosyl acetate) (R_1= OH, R_2= CH_3CO)



8 Pinoresinol (R = H)
9 1-Acetoxypinoresinol (R = CH_3COO)

Figure 1. Main phenolic compounds reported in olive oils.

desirable. Flavone analysis by HPLC with UV detection at 340 nm has been reported (*20*).

In this work, a simple and precise analytical method for qualitative and quantitative determination of phenolic compounds and flavones in virgin olive oils is presented. The isolation of the phenolic compounds using SPE on diol cartridges is investigated. For peak identification, SPE on amino phase and detection by HPLC at several UV wavelengths are used. To characterize the phenolic compounds and calculate their response factors, some compounds were synthesized or isolated from olive oils and from a "crude phenolic extract". The HPLC operating conditions are investigated to improve the chromatographic resolution. Precision and recovery data are calculated. The results are compared with those obtained by extraction with methanol/water and by colorimetric determination of *o*-diphenols.

EXPERIMENTAL PROCEDURES

Samples. Virgin olive oils were obtained from industrial oil mills and filtered thorough filter paper. Refined sunflower oils were used as glyceridic matrix for precision and recovery studies. A solution of sunflower oil in hexane was passed through a glass chromatographic column packed with neutral alumina grade I. The eluate was evaporated at room temperature under vacuum.

Reference Compounds. The following commercial products were used: caffeic, *o*-, *m*-, and *p*-coumaric, vanillic, sinapinic, protocatechuic, syringic, gentisic, homoveratric, salicylic, *p*-hydroxyphenylacetic, 3,4-dihydroxyphenylacetic, *m*- and *p*-hydroxybenzoic, 2,3-dihydroxybenzoic, 3,4-dimethoxybenzoic, and 3-(*p*-hydroxyphenyl)propionic acids and homovanillyl alcohol, vanillin, guaiacol, luteolin, apigenin, (±)-catechin, morin, and quercetin were obtained from Sigma Chemical Co. (St. Louis, MO); gallic, ferulic, and *trans*-cinnamic acids and 3-hydroxybenzaldehyde, 4-hydroxy-3-methoxybenzyl alcohol, *o*-vanillin, and baicalein were from Aldrich (Steinheim, Germany); homovanillic acid was from Fluka AG (Buchs, Switzerland); 2-(4-hydroxyphenyl)ethanol (tyrosol) (**6**) was from Janssen Chemical Co. (Beerse, Belgium); and oleuropein ws from Extrasynthese (Z.I. Lyon-Nord, Genay, France).

The following compounds were isolated or synthesized:

A "crude phenolic extract" was obtained from fresh residual vegetation water originated in the olive oil extraction process. The wastewater was evaporated under vacuum, and the residue was washed several times with hexane. The residue was extracted several times with methanol/water (1:1), and the combined extracts were evaporated under vacuum.

2-(3,4-Dihydroxyphenyl)ethanol (hydroxytyrosol) (**5**) was synthesized from 3,4-dihydroxyphenylacetic acid by reduction with $LiAlH_4$ (*21*).

2-(3,4-Dihydroxyphenyl)ethyl acetate (hydroxytyrosyl acetate) (**7**) was isolated from the crude phenolic extract by fractionating on a silica gel column and eluting with hexane/ethyl acetate/methanol (2:6:1). The ^1H and ^{13}C NMR data were in agreement with those of the compound isolated from olive oil (*8*).

By reaction of tyrosol with acetic anhydride at room temperature during 48 h, a mixture (40:60) of 2-(4-hydroxyphenyl)-ethyl acetate (tyrosyl acetate) (**10**) and tyrosyl diacetate was obtained. Tyrosyl acetate was isolated by fractioning on a silica gel column with hexane/diethyl ether (2:1). ^{13}C NMR data were in agreement with those earlier reported (*22*).

The aldehydic form of oleuropein aglycon (**1**) was obtained by enzymatic hydrolysis of oleuropein with β-glucosidase (from almonds, Sigma Chemical Co.) (*23*). This compound was purified by fractioning on a silica gel column using dichloromethane/acetone/hexane (3:2:5) as mobile phase. NMR data were in accordance with those reported by Montedoro et al. (*5*).

The aldehydic form of ligstroside aglycon (**2**) and the dialdehydic forms (**3** and **4**) were isolated from 100 g of virgin olive oil. The oil, dissolved in hexane, was poured onto a preparative silica gel chromatographic column and then eluted consecutively with hexane, hexane/ethyl acetate (90:10), and methanol. The methanolic fraction was evaporated and the residue extracted with methanol/water (1:1). The extract was evaporated and the residue fractionated by thin-layer chromatography (TLC) on silica gel plates, eluting with hexane/ethyl acetate/methanol (9:9:2). Compound **2** was isolated as a single compound, whereas **3** and **4** contained minor amounts

of hydroxytyrosol and tyrosol, respectively. RMN data of **3** and **4** were consistent with those reported by Montedoro et al. (*5*).

Pinoresinol (**8**) and 1-acetoxypinoresinol (**9**) were isolated by preparative HPLC from phenolic extracts obtained by SPE on amino phase from Cornicabra and Arbequina olive oils, respectively. The MS data were in agreement with those earlier described (*10*). The MS spectra of their TMSi derivatives indicated the presence of two hydroxyl groups in both compounds (*11*).

Elenolic acid was obtained from oleuropein by hydrolysis with 1 N sulfuric acid at 55 °C (*24*).

Analytical Materials and Reagents. All reagents were of analytical reagent grade. Acetonitrile (far-UV), acetic acid, and methanol were of HPLC grade (Romil Ltd., Cambridge, U.K.). SPE cartridges (3 mL), packed with diol- and amino-bonded phase, were from Supelco (Bellefonte, PA). Silica gel 60 for column chromatography and silica gel plates for TLC were from Merck, KgaA (Darmstadt, Germany). Sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$) was also from Merck.

Analytical Procedure. *Internal Standard and Sample Preparation*. A solution of *p*-hydroxyphenylacetic (4.64×10^{-2} mg/mL) and *o*-coumaric acids (9.6×10^{-3} mg/mL) in methanol was used as internal standard.

A sample of filtered virgin olive oil (2.5 ± 0.001 g) was weighed, and 0.5 mL of standard solution was added. The solvent was evaporated in a rotary evaporator at 40 °C under vacuum, and the oily residue was dissolved in 6 mL of hexane.

SPE. A diol-bonded phase cartridge was placed in a vacuum elution apparatus and conditioned by the consecutive passing of 6 mL of methanol and 6 mL of hexane. The vacuum was then released to prevent drying of the column. The oil solution was applied to the column, and the solvent was pulled through, leaving the sample and the standard on the solid phase. The sample container was washed with two 3-mL portions of hexane, which were run out of the cartridge. The sample container was washed again with 4 mL of hexane/ethyl acetate (90:10, v/v), which were run out of the cartridge and discarded. Finally, the column was eluted with 10 mL of methanol, and the solvent was evaporated in a rotary evaporator at room temperature under vacuum until dryness. The residue was extracted with 500 μL of methanol/water (1:1, v/v) at 40 °C. An aliquot (20 μL) of the final colorless solution was injected into the HPLC system.

For extractions using amino-phase cartridges, the procedure was similar, but the elution with methanol was followed by subsequent elution with methanol/concentrated hydrochloric acid (98:2 v/v).

HPLC. HPLC was performed in a Hewlett-Packard series 1100 liquid chromatographic system equipped with a diode array UV detector and a Rheodyne injection valve (20-μL loop). A Lichrospher 100RP-18 column (4.0 mm i.d. × 250 mm; particle size = 5 μm) (Merck), maintained at 30 °C, was used. Elution was performed at a flow rate of 1.0 mL/min, using as mobile phase a mixture of water/acetic acid (97:3, v/v) (solvent A) and methanol/acetonitrile (50:50 v/v) (solvent B). The solvent gradient changed according to the following conditions: from 95% (A)−5% (B) to 70% (A)−30% (B) in 25 min; 65% (A)−35% (B) in 10 min; 60% (A)−40% (B) in 5 min; 30% (A)−70% (B) in 10 min; and 100% (B) in 5 min, followed by 5 min of maintenance. Chromatograms were acquired at 240, 280, and 335 nm.

Quantitative Determination by HPLC. Quantification of phenols (except ferulic acid), cinnamic acid, and lignans was carried out at 280 nm using *p*-hydroxyphenylacetic acid as internal standard. Quantification of flavones and ferulic acid was done at 335 nm using *o*-coumaric acid as internal standard.

Response factors and recoveries were calculated by analyzing, four times, refined sunflower oil spiked with reference compounds at concentrations similar to those found in virgin olive oils. Because the available amounts of compounds **3**, **4**, **8**, and **9** were too small to be accurately weighed, response factors were indirectly determined. To do it, a solution of **3** and solutions of **4**, **8**, and **9** were spiked with hydroxytyrosol and tyrosol, respectively, and then analyzed by ^{1}H NMR. Molar ratios were calculated by comparing the signals due to aliphatic or heterocyclic protons. Finally, concentrations of hydroxytysosol or tyrosol in the four solutions were determined by HPLC analysis at 280 nm, and the concentrations of compounds **3**, **4**, **8**, and **9** were calculated using the molar ratios. Linear response of the main compounds was determined by spiking refined sunflower oil with different amounts of a pool of olive oil extracts.

GP-HPLC Analysis of Phenolic Extract. The methanolic extract obtained by SPE on diol phase was evaporated, the residue was dissolved in tetrahydrofuran and analyzed by HPLC using a refractive index detector, and two 100- and 500-Å PLGel columns (30 cm × 0.75 cm i.d.) were connected in series. Tetrahydrofuran was used as mobile phase at flow rate of 1 mL/min (*25*).

Colorimetric Determination of o-Diphenols. The methanolic extract obtained from olive oil by SPE on diol phase was evaporated, the residue dissolved in 10 mL of methanol/water (1:1), and the solution filtered. A mixture of 4 mL of the solution with 1 mL of a 5% solution of sodium molybdate dihydrate in ethanol/water (1:1) was shaken vigorously. After 15 min, the absorbance at 370 nm was measured. A blank was obtained by measuring a mixture of 4 mL of phenolic solution with 1 mL of ethanol/water (1:1) (*26*).

Extraction with Methanol/Water. In a separator, olive oil (10 ± 0.001 g) was extracted with 10 mL of methanol/water (80:20, v/v) containing the internal standards. The upper layer was again extracted by using 3 × 10 mL of methanol/water (80:20, v/v). The combined methanolic extracts were evaporated in a rotary evaporator at 40 °C under vacuum. The residue was taken up to 10 mL with acetonitrile and the solution washed with 3 × 13 mL of hexane. The acetonitrile solution was evaporated at 40 °C under vacuum, and the residue was dissolved in 2 mL of methanol/water (1:1) (*10*).

NMR. NMR spectra were recorded at 303 K on a Bruker AMX 500 spectrometer operating at 500.13 MHz (^{1}H) and 125.75 MHz (^{13}C). Samples were examined as solutions in 99.8% DMSO-d_6. Chemical shifts are given in parts per million, using the DMSO-d_5 signals (2.49 and 39.5 ppm for ^{1}H and ^{13}C, respectively) as references. To confirm the assignments of NMR signals, extensive 2D homo- and heteronuclear correlation experiments were carried out.

Gas Chromatography—Mass Spectrometry (GC-MS). A MAT 95-S mass spectrometer (Finnigan, Manchester, U.K.) was coupled directly to an HP-5890 gas chromatograph (Hewlett-Packard, Wilmington, DE) fitted with a fused silica column (0.25 mm × 30 m) coated with DB-5 phase. Electron impact ionization at 70 eV and a resolution of 2500 were used. The GC conditions included an initial temperature of 90 °C, which was held for 2 min and then programmed at 4 °C/min to 300 °C.

RESULTS AND DISCUSSION

RP-HPLC Analysis. A pool of phenolic extracts obtained from Picual olive oil was used to optimize solvent composition and elution gradient. Using a methanol/acetonitrile (50:50) admixture as second solvent and the elution gradient indicated under Experimental Procedures, separations between peaks 11, 12, and 13 (Figure 2, at 280 nm) were better than those recently reported (*10*, *11*), making possible the determination of pinoresinol, cinnamic acid, and 1-acetoxypinoresinol.

SPE Isolation of the Phenolic Fraction. Because nonpolar phases are not appropriate for the extraction of polar fractions from nonpolar matrices, only polar phases were taken in account. Diol phase was chosen because of its negligible activity on labile esters (*27*). Passing of the hexane solution of the olive oil through a diol cartridge retained the polar compounds on the solid phase. Hexane washing eliminated hydrocarbons, waxes, tocopherols, and triacylglycerols. Subsequent

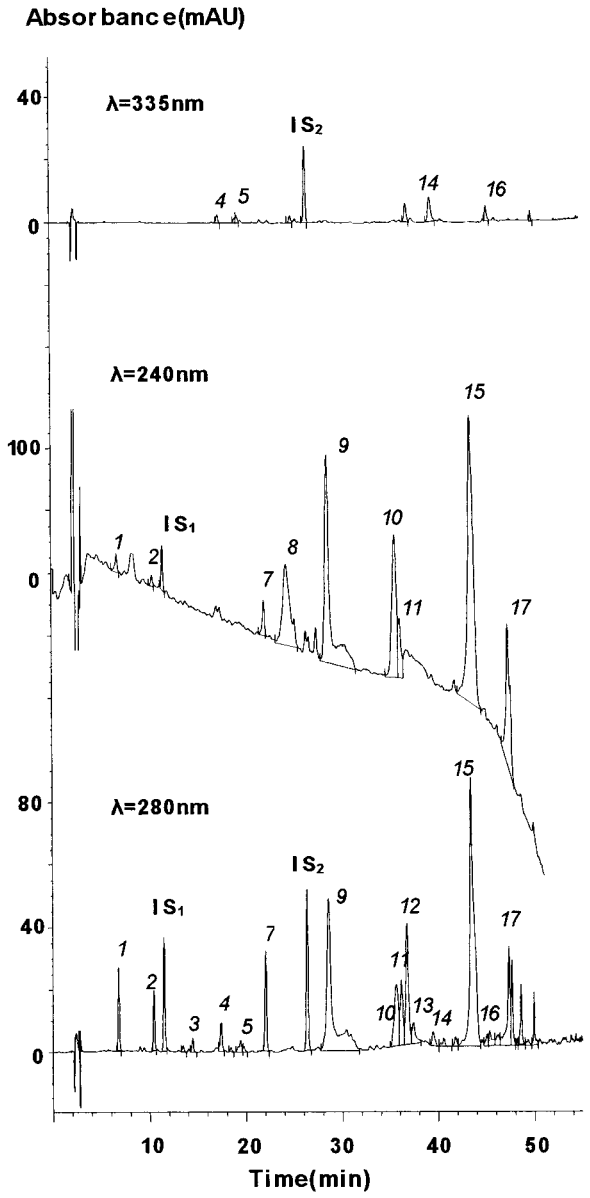


Figure 2. HPLC chromatogram of phenolic compounds isolated from Picual virgin olive oil by SPE on diol phase (detection at $\lambda = 280$, 240, and 335 nm). Peaks: (1) hydroxytyrosol; (2) tyrosol; (IS_1) *p*-hydroxyphenylacetic acid; (3) vanillic acid; (4) vanillin; (5) *p*-coumaric acid; (7) hydroxytyrosyl acetate; (8) elenolic acid; (IS_2) *o*-coumaric acid; (9) dialdehydic form of decarboxymethyl oleuropein aglycon; (10) dialdehydic form of decarboxymethyl ligstroside aglycon; (11) pinoresinol; (12) cinnamic acid; (13) 1-acetoxypinoresinol; (14) luteolin; (15) aldehydic form of oleuropein aglycon; (16) apigenin; (17) aldehydic form of ligstroside aglycon.

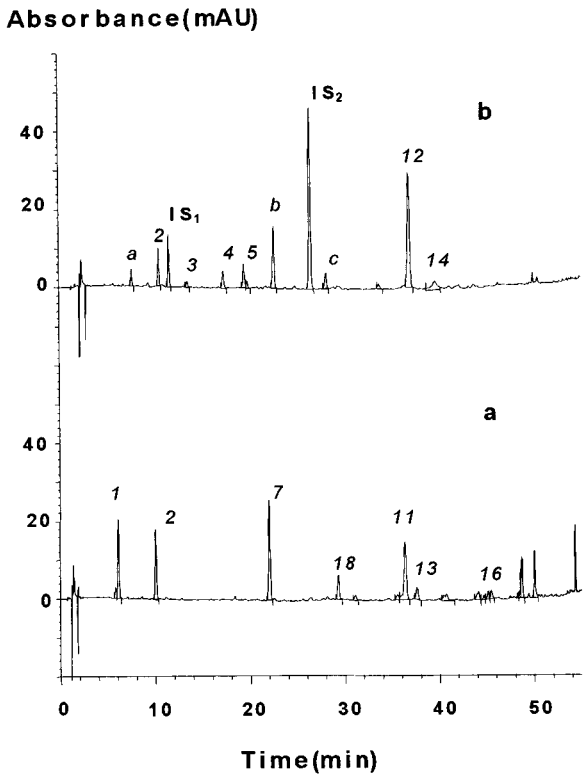


Figure 3. HPLC chromatograms of phenolic compounds isolated from Picual virgin olive oil by SPE on amino phase using consecutively several eluting solvents (detection at $\lambda = 280$ nm): (a) with methanol [peaks: (1) hydroxytyrosol; (2) tyrosol; (7) hydroxytyrosyl acetate; (18) tyrosyl acetate; (11) pinoresinol; (13) 1-acetoxypinoresinol; (16) apigenin]; (b) with methanol/HCl (98:2, v/v) [peaks: (a) artifact; (2) tyrosol; (IS_1) *p*-hydroxyphenylacetic acid; (3) vanillic acid; (4) vanillin; (5) *p*-coumaric acid; (b) artifact; (IS_2) *o*-coumaric acid; (c) artifact; (12) cinnamic acid; (14) luteolin].

washing with hexane/ethyl acetate (90:10, v/v) removed the major part of the oxidized triacylglycerols, sterols, and diacylglycerols. The very polar fraction was eluted with 10 mL of methanol, and then the solution was evaporated, yielding a yellow solid residue. Analysis of this residue by gel permeation liquid chromatography showed the presence of minor amounts of di- and monoacylglycerols and free fatty acids. TLC analysis on silica gel plates gave spots corresponding to triterpenic acids and pigments. To eliminate undesired compounds, the extraction of the residue was assayed using the admixtures water/methanol, water/acetonitrile, and water/acetonitrile/methanol at different ratios. Extraction with 0.5 mL of water/methanol (1:1, v/v) at 40 °C for 1 min yielded a colorless solution and an oily residue. The water/methanol admixture contained all of the phenolic compounds because the HPLC analysis of the oily residue dissolved in methanol did not show phenolic peaks. Elution of the cartridge with water/methanol instead of methanol did not improve the desorption efficiency, making solvent evaporation difficult. The analysis of sunflower oil spiked with the phenolic extract obtained from Picual olive oil, eluting with methanol yielded the same HPLC profile as the initial extract (Figure 2). This indicates negligible alteration during extraction on the diol phase.

In contrast, the use of amino-phase cartridges caused drastic changes in phenol recovery (compare Figure 2 at 280 nm with Figure 3a). Thus, in the profiles obtained by elution with methanol, peaks 3−5, 9, 10, 12, 14, 15, and 17 were absent, and a new peak (number 18) could be observed. Subsequent elution with methanol/HCl (Figure 3b) yielded peaks 3−5, 12, and 14, additional amounts of peaks 1 and 2, and three new peaks (marked a−c), but peaks 9, 10, 15, and 17 were not recovered. To investigate the new peaks, the phenols retained on a diol-phase cartridge were eluted with methanol/HCl. Peaks a−c did not appear, suggesting that they were artifacts originated by the combined action of the HCl and the amino phase. These facts indicated that nonacidic compounds were eluted from the amino phase with methanol, acidic compounds were eluted with methanol/HCl, and some components interact with amino groups. Therefore, the amino phase is not appropriate for isolating the total phenol fraction from olive oils, but it

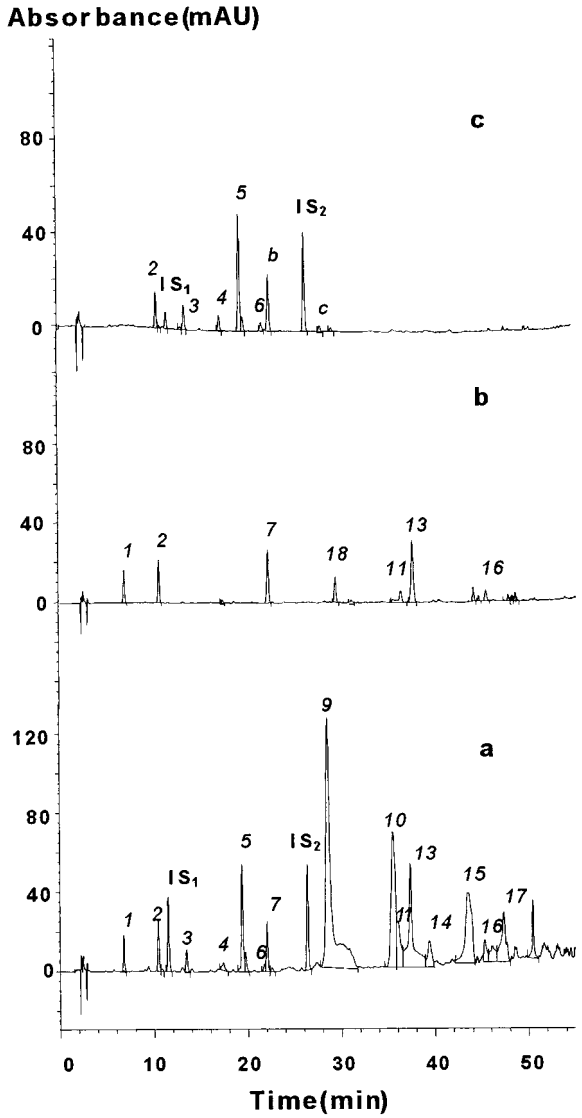


Figure 4. HPLC chromatograms of phenolic compounds isolated from Manzanilla virgin olive oil by SPE (detection at $\lambda = 280$ nm): (a) diol phase eluted with methanol [peaks: (1) hydroxytyrosol; (2) tyrosol; (IS_1) *p*-hydroxyphenylacetic acid; (3) vanillic acid; (4) vanillin; (5) *p*-coumaric acid; (6) ferulic acid; (7) hydroxytyrosyl acetate; (IS_2) *o*-coumaric acid; (9) dialdehydic form of decarboxymethyl oleuropein aglycon; (10) dialdehydic form of decarboxymethyl ligstroside aglycon; (11) pinoresinol; (13) 1-acetoxypinoresinol; (14) luteolin; (15) aldehydic form of oleuropein aglycon; (16) apigenin; (17) aldehydic form of ligstroside aglycon]; (b) amino phase eluted with methanol [peaks: (1) hydroxytyrosol; (2) tyrosol; (7) hydroxytyrosyl acetate; (11) pinoresinol; (13) 1-acetoxypinoresinol; (16) apigenin; (18) tyrosyl acetate]; (c) amino phase eluted with methanol/HCl (98:2, v/v) subsequent to methanol elution [peaks: (2) tyrosol; (IS_1) *p*-hydroxyphenylacetic acid; (3) vanillic acid; (4) vanillin; (5) *p*-coumaric acid; (6) ferulic acid; (b) artifact; (IS_2) *o*-coumaric acid; (c) artifact].

can be useful to isolate specific components and as an aid for peak identification.

Identification of Phenolic Compounds in Olive Oil Extract. The HPLC analyses of phenolic extracts obtained from several olive oil varieties by SPE on diol-phase cartridges yielded HPLC profiles containing the same chromatographic peaks but in variable proportions (Figures 2 and 4a). Identification of peaks was from the UV spectrum, retention time, and elution behavior on amino cartridges (Figures 3 and 4b,c), in comparison with authentic samples. Peak 1 was assigned to hydroxytyrosol because the diminution at 240 nm, the absence of a UV maximum at 300 nm, and the complete elution with methanol eliminated the presence of protocatechuic acid. The lack of absorption at 335 nm of peak 2 indicated the presence of tyrosol and the absence of gentisic acid.

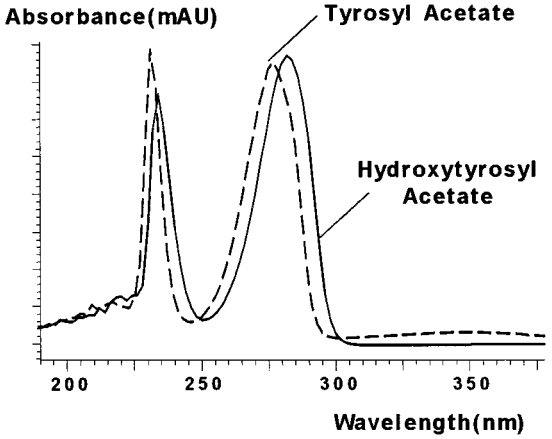


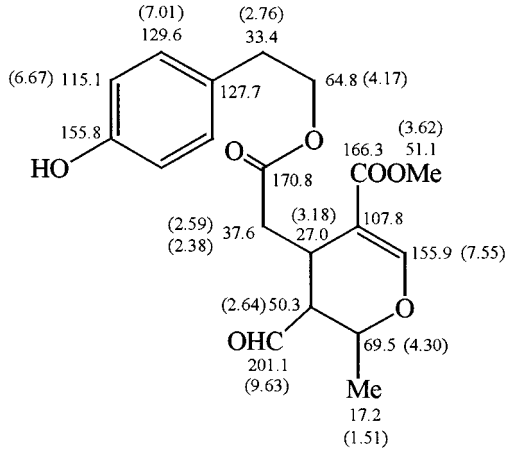
Figure 5. UV spectra obtained during HPLC analysis.

In the extract obtained through diol phase from Manzanilla oil, two peaks were observed at ~22.10 min (Figure 4a). Using the amino-phase cartridge, peak 7 was eluted with methanol (Figure 4b), and the subsequent elution with methanol/HCl (Figure 4c) showed a small peak due to ferulic acid (peak 6). The elution of peak 7 with methanol through the amino phase suggested a phenolic compound without carboxylic or aldehydic groups. The retention time and UV spectrum (Figure 5) of this peak were identical to those of hydroxytyrosyl acetate (**7**) isolated from the crude phenolic extract obtained from wastewater. This compound cannot be considered an artifact originating from reaction with acetic acid during the HPLC analysis because it appeared in the HPLC analysis using 0.5% phosphoric acid in water as mobile phase A. There is no evidence of the formation of **7** by transformation from other hydroxytyrosol derivatives during the SPE procedure because it was isolated from the crude phenolic extract and from olive oils by extraction with methanol/water (*8*).

Peak 8 (Figure 2, at 240 nm), which did not show absorption at 280 nm, was due to elenolic acid. This compound cannot be attributed to hydrolysis of **1** or **2** during the analysis, as this peak did not appear in the analysis of sunflower oil spiked with these compounds.

Peaks 9 and 10 were the dialdehydic forms of decarboxymethyl oleuropein and ligstroside aglycons (**3** and **4**, respectively) and peaks 15 and 17 the aldehydic forms of oleuropein and ligstroside aglycons (**1** and **2**, respectively). These four peaks were not eluted with methanol from amino-phase cartridges (Figures 3a and 4b), due to the presence of aldehydic groups, which react with the amino groups. These four peaks were broader than the other ones in the chromatogram, probably due to equilibria toward hemiacetal forms during the HPLC analysis. The aldehydic structure suggested by Montedoro et al. (*5*) for the tyrosol derivative **2** was confirmed by its NMR data (Figure 6), which were in accordance with those reported for the homologous hydroxytyrosol derivative **1** (*28*).

In the elution with methanol from the amino-phase cartridge (Figures 3a and 4b) a minor peak (number 18) appeared at a retention time similar to that of compound



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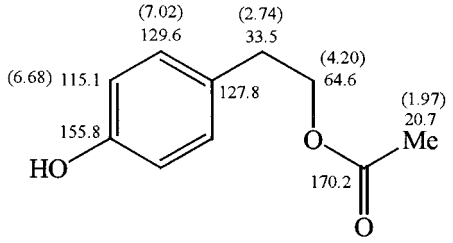

10

Figure 6. NMR data of aldehydic form of ligstroside aglycon (**2**) and tyrosyl acetate (**10**) in dimethyl sulfoxide-d_6. Figures on carbon atoms indicate chemical shift of ^{13}C atoms. Figures in parentheses indicate chemical shifts of protons.

3 (peak 9), but its UV absorption did not increase at 240 nm. Its retention time, UV spectrum (Figure 5), and ^1H and ^{13}C NMR spectra (Figure 6) were identical to those of the synthesized tyrosyl acetate (**10**). The mass spectrum, *m*/*z* (relative intensity), was M^+ 180 (2), 121 (10), $[M - AcOH]^+$ 120 (100), 119 (5), 108 (4), $[M - (AcO - CH_2)]^+$ 107 (44), 93 (2), 91 (7), 78 (4), 77 (13). When the extract obtained from the diol-phase cartridge was analyzed by HPLC, using as solvent A 0.5% of phosphoric acid in water instead of water/acetic acid, a complete separation between peaks 9 and 18 was achieved. This fact indicates that the tyrosyl acetate (**10**) is not an artifact originating from the SPE extraction on the amino-phase or HPLC analysis. Using the HPLC operating conditions indicated under Analytical Procedure, a phenolic extract spiked with tyrosyl acetate showed a shoulder on the tail of peak 9. In all oil samples analyzed, this shoulder was absent, indicating that tyrosyl acetate is a minor component.

Peaks 11 and 13 (Figures 2 and 4) were pinoresinol (**8**) and 1-acetoxypinoresinol (**9**), respectively, as demonstrated by their mass spectra. Cinnamic acid (peak 12) was identified by retention time, UV spectrum, and behavior on amino-phase cartridge. This compound was very abundant in Picual (Figure 2 at 280 nm and Figure 3b), absent in Manzanilla (Figure 4a,c), and scarce in Arbequina, and it has been also reported by Montedoro et al. (*7*).

Quantitative Analysis. For quantitative analysis of phenols, except ferulic acid, and lignans, UV detection was carried out at 280 nm using *p*-hydroxyphenylacetic acid as internal standard. All phenolic compounds absorb at this wavelength, and the standard does not overlap any peak present in olive oil extract. At lower wavelengths (240 nm), the UV absorption of some hydroxytyrosol derivatives was greater (Figure 2), but the absorption of the mobile phase interfered. For flavones and ferulic acid, detection at 335 nm enhances the response of these compounds and diminishes that of most phenolic compounds. The *o*-coumaric acid was chosen as internal standard because other commercial flavonoids (catechin, morin, quercetin, and baicalein) overlap peaks of olive oils. The calculated response factors are indicated in Table 1. Hydroxytyrosol and its derivatives showed similar response factors expressed in millimoles per kilogram ($\sim 4.1 \times 10^{-3}$), and the same occurred for tyrosol and its derivatives ($\sim 6.0 \times 10^{-3}$ mmol/kg). For the main compounds (**1**−**4**, **7**, and **9**), the responses showed a linear relationship with concentration in the range of 0.01−0.08 mmol/kg. The results of four determinations in spiked sunflower oils indicated

Table 1. Retention Time, UV Absorption Maxima, Recovery, Response Factor, and Precision of Phenols, Flavones, and Lignans Isolated by SPE and Analyzed by RP-HPLC

HPLC peak	compound	RT (min)	λ (nm) $max_1/max_2/max_3$	recovery[a] (%)	response factor[b]	RSD[c] (%)
1	hydroxytyrosol (**5**)	6.78	230:280	98.9	0.646[d]	1.82
2	tyrosol (**6**)	10.45	230:280	99.8	0.829[d]	2.41
IS_1	*p*-hydroxyphenylacetic acid	11.52	240:280	99.9	1.000	
3	vanillic acid	14.44	228:265:295	99.2	0.206[d]	6.47
4	vanillin	17.39	240:282:310	98.7	0.126[d]	4.56
5	*p*-coumaric acid	19.41	230:310	99.2	0.106[d]	0.38
6	ferulic acid	22.03	240:295:325	90.7	0.542[e]	0.42
7	hydroxytyrosyl acetate (**7**)	22.06	232:285	98.2	0.788[d]	1.38
8	elenolic acid	24.30	240			
IS^2	*o*-coumaric acid	26.36	235:280:325	98.9	1.000	
9	dialdehydic form decarboxymethyl oleuropein aglycon (**3**)	28.58	235:285	98.2	1.303[d]	3.24
18	tyrosyl acetate (**10**)	29.29	230:280	98.3	1.093[d]	3.42
10	dialdehydic form decarboxymethyl ligstroside aglycon (**4**)	35.62	235:280	98.6	1.843[d]	2.97
11	pinoresinol (**8**)	36.16	232:280	98.4	0.197[d]	1.55
12	cinnamic acid	36.74	280	95.3	0.057[d]	2.45
13	1-acetoxypinoresinol (**9**)	37.40	240:282	99.4	0.584[d]	3.25
14	luteolin	39.43	255:350	94.7	0.836[e]	3.85
15	aldehydic form oleuropein aglycon (**1**)	43.45	235:285	99.4	1.587[d]	1.37
16	apigenin	45.31	230:270:340	94.4	0.833[e]	4.84
17	aldehydic form ligtroside aglycon (**2**)	47.33	235:280	96.2	2.121[d]	2.98

[a] Mean value ($n = 4$). [b] Mean value of response factor relative to internal standard ($n = 4$). [c] Relative standard deviation of response factor ($n = 4$). [d] Relative to *p*-hydroxyphenylacetic acid. [e] Relative to *o*-coumaric acid.

good repeatability of the complete method. The recoveries relative to the standard solutions were excellent (Table 1).

The HPLC method was compared with the colorimetric quantification of *o*-diphenols by reaction with molybdate. For this, the colorimetric method was applied to several hydroxytyrosol solutions, and the following calibration line was obtained:

$$C\ (\text{mmol/mL}) = 1.36 \times 10^{-3} \times \text{absorbance} + 1.2 \times 10^{-5}$$

The method was then applied to standard solutions of phenolic compounds possessing different functional groups. As expected, vanillin, tyrosol, and α-tocopherol did not react with molybdate, whereas the molar responses of hydroxytyrosol, hydroxytyrosyl acetate, the aldehydic form **1**, and luteolin were similar. Application of the colorimetric method to methanol extracts obtained by SPE on diol phase yielded results similar to those obtained with the HPLC method, when only compounds having *o*-diphenol structure (hydroxytyrosol, hydroxytyrosol derivatives, and luteolin) were considered. The results for Picual olive oil were 0.52 and 0.52, for Manzanilla olive oil, 1.01 and 1.04, and for Arbequina olive oil, 0.16 and 0.17 mmol/kg, respectively.

For comparing SPE on diol-phase and liquid−liquid extraction, phenolic extracts were obtained from a Picual virgin olive oil using both analytical methods, each one in duplicate. The four extracts were analyzed by HPLC using the described method. The concentrations of each phenolic compound obtained by both extraction methods did not show differences at 95% of statistical significance level. In summary, the SPE on diol phase followed by HPLC with UV detection is a simple and precise analytical method for determining phenolic compounds, lignans, flavones, and cinnamic acid in olive oils.

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