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Characterization and Quantification of Phenolic Compounds in Olive Oils by Solid-Phase Extraction, HPLC-DAD, and HPLC-MS/MS

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A simple and reproducible method for qualitative and quantitative analysis of phenolic compounds in virgin olive oils by solid-phase extraction (SPE), high performance liquid chromatography with diode array detector (HPLC-DAD), and HPLC-mass spectrometry (MS) in tandem mode was developed. The polar fraction was obtained from samples of three different virgin olive oils. Detection and quantification were performed at 280, 240, and 320 nm. For identification purposes, HPLC-MS/MS was equipped with turbo ion spray source in the negative-ion mode. Twenty compounds of twenty-three detected and quantified were characterized. The method showed satisfactory linearity ($r > 0.99$), good recovery, satisfactory precision, and appropriate limits of detection (LOD) and quantification (LOQ).

KEYWORDS: Phenolic compounds; olive oil; secoiridoids; liquid chromatography; mass spectrometry

INTRODUCTION

Phenolic compounds are a complex but important group of naturally occurring compounds in plants (*1*). Although the main phenolic compounds in olive fruit are secoiridoid derivatives, olives also contain phenyl acids, phenyl alcohols, and flavonoids (*2*). The secoiridoids is a very specific group that are abundant in *Oleaceas* and many other plants that are produced from the secondary metabolism of terpenes as precursors of various indole alkaloids, and are usually derived from the oleoside type of glucoside oleosides, which are characterized by a combination of elenolic acid and a glucosidic residue. It could be stated that these compounds proceed from the acetate/mevalonate pathway (*3*, *4*).

Because virgin olive oil is a natural product, its chemical composition varies. Thus, the phenolic content of virgin olive oil is affected by the variety, location, environmental conditions, degree of ripeness, and the type of oil extraction (*2*−*6*). However, phenolic compounds are removed when the oil is refined.

Phenolic compounds present in olive oil have received considerable attention in recent years because they are essential to its quality and nutritional properties. They affect its shelf life because they retard oxidation (*4*, *7*, *8*) and its sensorial properties: color, astringency, bitterness, and flavor (*4*, *9*−*12*). In addition, some studies have been performed on the protective effects of olive oil phenolic compounds on health, including the protection on risk factors for cardiovascular disease (*13*−*17*).

To identify and quantify these compounds in olive oil, several extraction procedures and analytical methods have been developed. Traditionally, the phenolic fraction of olive oil has been isolated by liquid−liquid extraction (*5*, *18*−*20*). However, as extraction with these methods is very laborious, more time and higher quantities of dissolvent are required. Some attempts to isolate these compounds by solid-phase extraction have been made (*21*−*24*), but good recovery is not regularly achieved (*25*).

Total phenolic compounds in oil are quantified mainly by the Folin-Ciocalteau method, based on the reduction properties of phenols in alkaline medium. However, this is a nonspecific colorimetric method. Thus, some authors have tried to separate and quantify specific phenols by gas chromatography (*21*, *26*), by HPLC-DAD (*22*−*24*, *27*, *28*), or by HPLC-MS (*29*−*31*), but the phenolic concentrations reported in the literature are often not comparable. The formality of expression and the spectrophotometric features of the reference phenol dramatically affect the calculation of phenolic concentration in the same oil (*24*).

However, among the methods used for the determination of phenols, the coupling of HPLC-MS with atmospheric pressure

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ionization techniques, that is, electrospray ionization (ESI) (*32*) or atmospheric pressure chemical ionization (APCI) (*29*), is a powerful tool for identification of natural products in crude plant extracts because of their soft ionization. Its main advantage over the gas chromatography coupled to MS methods described in the literature is that no derivatization is needed.

Many studies have provided good information, and some of them have helped to clarify the structures of some phenolic compounds in oil. Nevertheless, because of the complexity of the wide group of secoiridoids, many of these phenolic compounds in olive oils remain unidentified (*27*). It is difficult to compare data within the literature, because of the lack of consistency: information is not only incomplete but sometimes contradictory as well.

The aims of this study were to develop a simple and reproducible method for the qualitative and quantitative analysis of phenolic compounds in virgin olive oils by SPE, HPLC-DAD, and HPLC-MS, and to summarize the information compiled on olive oil secoiridoids information.

MATERIAL AND METHODS

Chemicals. Phenolic standards: tyrosol, oleuropein, luteolin, methoxyluteolin, and apigenin were purchased from Extrasynthèse (Genay, France), *p*-coumaric acid and vanillic acid were purchased from Fluka (Buchs, Switzerland), and vanillin was purchased from Panreac (Barcelona, Spain). All solvents (methanol, acetonitrile, and *n*-hexane) were of HPLC grade and were purchased from SDS (Peypin, France). Formic acid was purchased from Sigma Chemical Co. (St. Louis, MO). Ultrapure water generated by the MilliQ system (Millipore, Bedford, MA) was used. MilliQ water:methanol (90:10 v:v) was used as the most suitable solvent for the standards.

Samples. The analyses were run on three virgin olive oils: oil A, from Arbequina olives (Catalonia Spain); oil B, from Picual olives (Andalucía Spain); and oil C, a commercial virgin olive oil. Samples were stored in dark-brown glass bottles at 4 °C until analysis. The oil was extracted from high-quality olives and met the standards set by the European Commission (*33*) for extra-virgin quality.

Instruments. Samples were extracted by an SPE-Vacuum Manifold from Tecknokroma (Barcelona, Spain).

HPLC-DAD Instrument. The phenolic compounds were analyzed in a Hewlett-Packard-1050 Series liquid chromatograph with an automatic injector and DAD 1050 series instrument and with a HP Chemstation (Waldbronn, Germany). A 5-μm particle size C_{18} Luna column, 15 cm × 2 mm i.d., was used (Phenomenex, UK).

HPLC-MS/MS Instrument. An Agilent 1100 HPLC (Waldbronn, Germany) equipped with an autosampler and coupled to an API3000 triple-quadrupole mass spectrometer (PE Sciex, Concord, Ontario, Canada) with a turbo ion spray source was used with the same column.

Extraction of Phenolic Compounds. To clean and concentrate the sample, the polar fraction was obtained from 3 g of oil sample using an SPE diol cartridge (Vac RC 500 mg, Waters, Milford, MA). The cartridge was activated with 6 mL of *n*-hexane, 6 mL of methanol:water (80:20), and 3 mL of acetonitrile. The oil was dissolved in 6 mL of *n*-hexane and percolated into the cartridge. To remove the nonpolar fraction, the oil was washed under vacuum with 10 mL of *n*-hexane. Afterward, phenolic compounds were eluted with 8 mL of methanol:water and 4 mL of acetonitrile. During the entire process, the vacuum was maintained at less than 30 kPa. The eluent was evaporated to 2 mL by a rotary evaporator, and the temperature was always controlled (<40 °C), to avoid the deterioration of phenols. The samples were then filtered through a 13-mm PTFE 0.45 μm membrane filter from Waters. According to our previous experience (*34*), filters were checked after various retention assays of the phenolic compounds with olive oil extracted samples. After this, 20 μL was injected into the liquid chromatograph. The entire process was performed in conditions of darkness and with brown glass material.

HPLC-DAD Conditions and Quantification. The column was kept at 40 °C. The mobile phase consisted of a binary solvent system using water acidified with 0.1% formic acid (solvent A) and 100% acetonitrile (solvent B), kept at a flow rate of 0.5 mL/min. The gradient program started with 90% eluent A and 10% eluent B, which ramped linearly to 25% in 12 min. This percentage was maintained for 7 min, and eluent B was ramped again linearly to 40% at 30 min and to 60% at 40 min. Initial conditions were reached in 5 min, so the total run time was 45 min. Detection and quantification were performed at 280, 240, and 320 nm.

Each phenolic compound was expressed with its standard when it was available. Due to the absence of standard for all of the compounds detected, both phenolic alcohols 3,4-dihydroxyphenyl and 4-hydroxyphenyl were expressed as tyrosol, secoiridoids and elenolic acid were expressed as oleuropein, and unknown flavonoids were expressed as luteolin.

HPLC-MS Conditions. The HPLC conditions were as described for the HPLC-DAD system. All of the analyses used the turbo ionspray source in negative mode with the following settings: capillary voltage −3500 V, nebulizer gas (N_2) 10 (arbitrary units), curtain gas (N_2) 12 (arbitrary units), collision gas (N_2) 4 (arbitrary units), declustering potential (DP) −30 V, focusing potential −250 V, entrance potential 10 V, and collision energy (CE) −30 V. Drying gas was heated to 300 °C and introduced at a flow-rate of 5000 $cm^3\ min^{-1}$. Full-scan data were acquired by scanning from *m*/*z* 100 to 800 in profile mode using a cycle time of 2 s with a step size of 0.1 u and a pause between each scan of 2 ms. In product ion scan experiments, MS/MS product ions were produced by collision-activated dissociation (CAD) of selected precursor ions in the collision cell of the triple quadrupole mass spectrometer and mass-analyzed with the second analyzer of the instrument.

Log α. All peaks in the three chromatograms were compared and identified using logarithms of selectivity values (log α). The reference peaks were selected because they appear in the three studied samples and present a good stability. Log α was calculated on the basis of the retention time of each phenolic compound, relative to peak 23 (see **Table 1**), in the case of compounds observed at 280 nm, and peak 11 (luteolin), in the case of compounds observed at 320 nm, considering in both cases, the peak corresponding to the first eluted peak as t_0.

RESULTS AND DISCUSSION

Sample Preparation. Traditionally, the phenolic fraction of olive oils is laboriously isolated by extraction of an oil solution in hexane with several portions of water:methanol, followed by solvent evaporation of the hydro alcoholic extract. SPE extraction is quicker than liquid−liquid extraction and reduces the amount of solvents used. However, previous research using C_{18} (*21*, *35*), C_8 (*23*), and diol cartridges has shown low recoveries (*24*, *25*). In our research, diol cartridge was selected because of its negligible activity on labile esters (*22*) in comparison with reversed phases. Washing and elution conditions were studied to improve the recovery of the extraction. Initially, a mixture of *n*-hexane and ethyl acetate was tried as washing solvent. However, ethyl acetate also elutes some phenolic compounds, because it is a solvent used to extract phenolic compounds, except anthocianins (*36*). In fact, some investigators use it to elute phenolic compounds of non-oily plant extract (*37*, *38*), then it was decided to use *n*-hexane alone. Afterward, elution solvents were also studied: assays were done with methanol, but some polar phenols did not completely elute, so water had to be added to the elution solvents. Nevertheless, when acetonitrile was also added, better recovery was observed. An average increment of 36% was observed when ethyl acetate was eliminated from the washing process and the two solvents were used for the elution: methanol:water (80:20) and acetonitrile. Moreover, the product was concentrated up to 2 mL, instead of evaporated to dryness prior to its final extraction with solvent: methanol, water, acetonitrile, or a combination of these. This evaporation to dryness causes a decrease in recovery of

Table 1. Phenolic Compounds Found in Olive Oils

peak	RT[a]	log α[b]	compound	*m*/*z*[c]	MS/MS ions	standard
1	2.23	−1.4765	hydroxytyrosol	153	123(10)	no
2	3.48	−1.1110	tyrosol	137		yes
3	4.50	−0.9481	vanillic acid	167		yes
4	7.00	−0.4373	vanillin	151	123(60)	yes
5	8.11	−0.3567	*p*-coumaric acid	163		yes
6	9.63	−0.5327	ligstroside derivative	553	137(100), 257(98), 181(70), 109(38), 341(32)	no
7	12.89	−0.3895	ligstroside derivative	335	199(100), 111(40), 155(27)	no
8	13.38	−0.3712	oleuropein derivative	377	307(100), 275(90), 139(16), 111(7)	no
9	14.20	−0.3428	oleuropein derivative	377	275(100), 307(90), 139(18), 111(5)	no
10	15.77	−0.2925	oleuropein derivative	319	199(100), 111(37), 153(5)	no
11	15.79	0.0000	luteolin	285	133(35), 199(13), 107(10), 217(10), 175(8)	yes
12	16.39	−0.2750	ligstroside derivative	361	291(100), 259(30), 101(25), 127(7), 138(8)	no
			oleuropein derivative	365	229(100), 153(25), 138(18)	no
13	16.92	0.0332	apigenin	269	117(30), 107(35), 151(15)	yes
14	17.28	−0.2500	ligstroside derivative	361	291(100), 101(27), 259(25), 127(5), 139(3)	no
			ligstroside derivative	393	317(30), 257(15), 349(5)	no
15	18.07	−0.2291	ligstroside derivative	393	317(55), 349(10), 257(6)	no
16	18.74	−0.2122	ligstroside derivative	393	317(97), 349(15), 257(8)	no
17	19.92	−0.1964	oleuropein derivative	377	275(100), 307(90), 139(18), 111(5)	no
18	20.66	0.1262	methoxyluteolin	299	199(25), 191(20), 227(10)	yes
19	22.60	−0.1254	oleuropein derivative	377	275(100), 307(80), 333(20), 301(7), 181(5)	no
			ligstroside derivative	361	291(100), 101(25), 259(23), 127(7), 139(3)	no
20	25.03	−0.0254	unknown			no
21	27.23	0.2145	ligstroside derivative	361	291(100), 101(27), 259(25), 127(8), 139(3)	no
22	28.97	0.2529	unknown			no
23	29.74	0.000	ligstroside derivative	361	291(100), 101(35), 259(30), 127(8), 139(3)	no

[a] Retention time (min). [b] Logarithms of selectivity values. [c] Mass charge value.

phenolic compounds. Thus, recovery of phenolic compounds was 20% higher on average than when the product was evaporated to dryness.

In summary, olive oil was washed with *n*-hexane to eliminate apolar compounds. Subsequently, the polar fraction was eluted with 8 mL of methanol:water (80:20) and 4 mL of acetonitrile and was later concentrated to 2 mL, prior to its HPLC injection.

HPLC Conditions. The composition of the HPLC mobile phase was optimized to achieve a good resolution between peaks. Different gradients between formic acid (0.1%) in water and acetonitrile, methanol, or a mixture acetonitrile:methanol (1:1) were assayed. In the case of the use of methanol as phase B, the appearance of the final chromatogram was better, but there was more coelution. The best resolution and peak shapes were obtained of a gradient formic acid 0.1% as phase A and acetonitrile as phase B. Detection was performed at 280, 320, and 240 nm. **Figure 1** shows the LC-DAD chromatograms of the phenolic compounds present in the virgin olive oils studied.

Identification of Phenolic Compounds in Olive Oil Samples. MS/MS Experiments. The deprotonated molecule $[M - H]^-$ in full-scan mode and the main product ion of each compound are reported in **Table 1**. HPLC-DAD gives rise to complex profiles of the phenolic fraction due to overlapping of various compounds (peaks 7, 8, and 9). MS offers the possibility of excluding the presence of interference, mainly when high complex matrixes such as olive oil polar compounds are analyzed. MS modes (such as full scan) and MS/MS modes (such as product ion scan) verify structural information of the compounds present in a virgin olive oil extract. The combined use of HPLC-MS/MS systems identifies olive oil phenols further.

The examination of the chromatograms in full-scan mode revealed the presence of several compounds that were positively identified by means of comparison with available standards. When these standards were not available and thus this comparison was not possible (the case of secoiridoids), MS/MS experiments had to be used.

In full-scan mode, several compounds with the same *m*/*z* were observed. To identify differences between them, samples were injected in the product ion scan mode of 393 and 361, both of which were ligstroside derivatives, and 377 an oleuropein derivative. These MS/MS experiments split the derivatives into a number of fragments according to strict pathways, which may enable their differences to be identified.

Olive oil extracts were injected in product ion scan mode of *m*/*z* 241, 285, 269, and 299 (corresponding to elenolic acid, luteolin, apigenin, and metoxyluteolin), to confirm the presence of these compounds.

The product ion scan modes of *m*/*z* 335 and 319 (for ligstroside and oleuropein derivatives) were evaluated to clarify where the different models came from.

Nine basic models of ligstroside and oleuropein aglycons were found in the bibliography. Each model shares the same elenolic acid derivative ring structure. In **Table 2**, these possible forms of derivatives of aglycons of oleuropein and ligstroside in virgin olive oil are shown.

Phenolic Alcohols. Hydroxytyrosol (peak 1) was identified by examination of the chromatogram belonging to the different sub-fractions in full-scan mode. The spectra generated for this compound in negative ion mode gave the deprotonated molecule $[M - H]^-$ at *m*/*z* 153. The ion fragment at *m*/*z* 123 is due to the loss of the CH_2OH group.

Tyrosol (peak 2) was identified by comparison with standard even when its mass spectrum was hidden by background.

Phenolic Acids. Peaks 3 and 5 are minor constituents (vanillic acid and *p*-coumaric acid) and were confirmed by using standard, spectral data, and log α. The mass spectrum of vanillic acid was hidden by background, but the deprotonated molecule $[M - H]^-$ of *p*-coumaric (163) was clearly visible.

Vanillin. The spectra generated for peak 4, vanillin, in negative ion mode gave the deprotonated molecule $[M - H]^-$ at *m*/*z* 151. The ion fragment at *m*/*z* 123 present is due to the loss of the CHO. This compound was confirmed by using standard, spectral data, and log α.

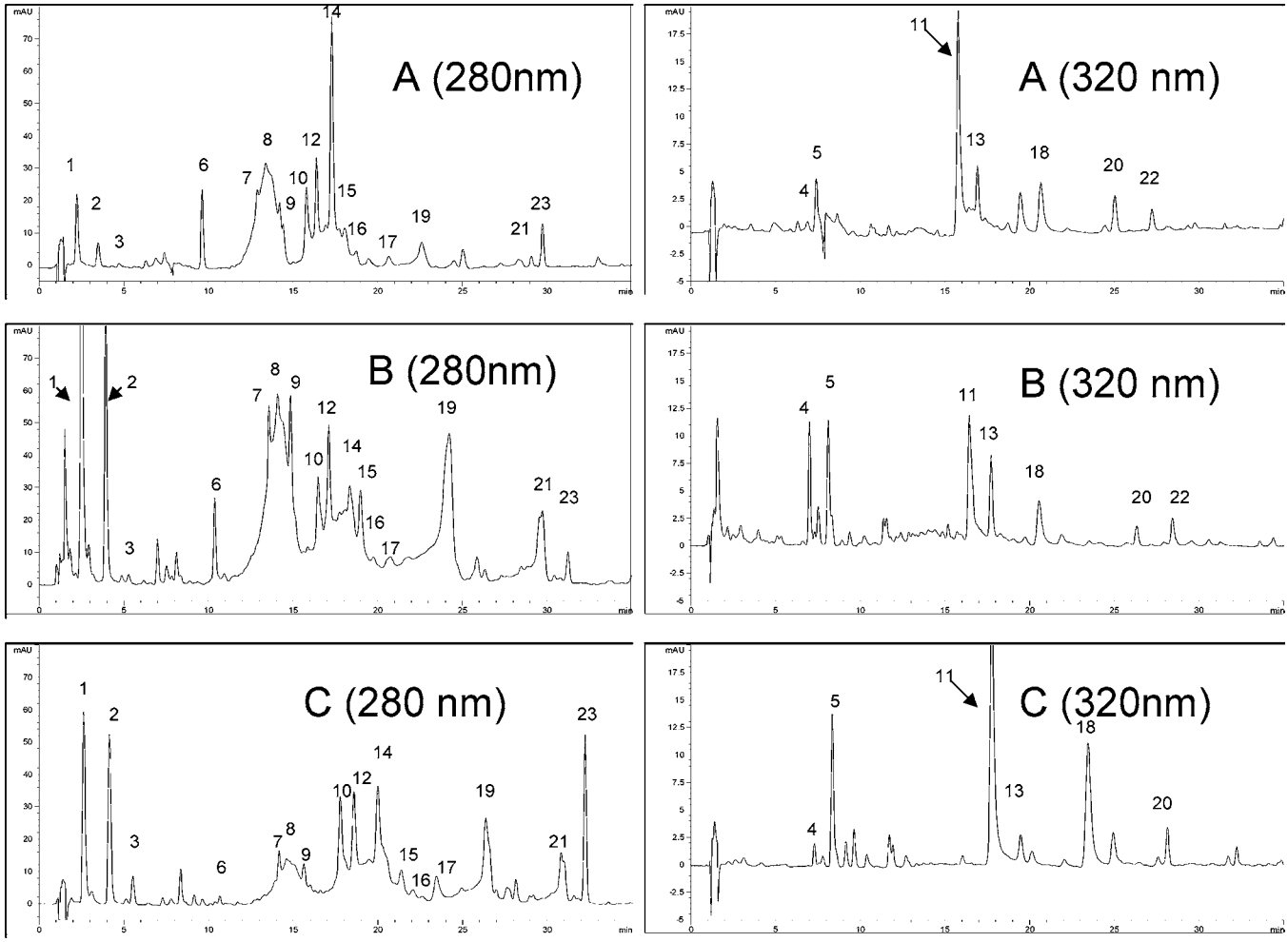


Figure 1. Phenolic compounds in virgin olive oil studied. Oil A, from Arbequina olives, Catalonia, Spain; oil B, from Picual olives, Jaén, Andalucía Spain; and oil C, a commercial virgin olive oil. Phenolic compounds: (1) hydroxytyrosol, (2) tyrosol, (3) vanillic acid, (4) vanillin, (5) *p*-coumaric acid, (6) ligstroside derivative *m*/*z* 553, (7) ligstroside derivative *m*/*z* 335, (8) oleuropein derivative *m*/*z* 377, (9) oleuropein derivative *m*/*z* 377, (10) oleuropein derivative *m*/*z* 319, (11) luteolin, (12) ligstroside derivative *m*/*z* 361, oleuropein derivative *m*/*z* 365, (13) apigenin, (14) ligstroside derivative *m*/*z* 361, ligstroside derivative *m*/*z* 393, (15) ligstroside derivative *m*/*z* 393, (16) ligstroside derivative *m*/*z* 393, (17) oleuropein derivative *m*/*z* 377, (18) methoxyluteolin, (19) oleuropein derivative *m*/*z* 377, ligstroside derivative *m*/*z* 361, (20) unknown, (21) ligstroside derivative *m*/*z* 361, (22) unknown, (23) ligstroside derivative *m*/*z* 361. HPLC-DAD conditions are as described in text.

Ligstroside Derivatives. Peak 7 had a deprotonated molecule at *m*/*z* 335. The product ion scan spectrum showed the *m*/*z* 199 ion (loss of tyrosol unit). The *m*/*z* 111 ion can be explained by the loss of 104 mass units of molecular mass of the elenolic derivative fragment (*m*/*z* 215) formed by an aldehyde and the COOH group loss. The *m*/*z* 155 ion can be explained by the loss of the $CH_2OH-OCH_3$ group of the elenolic derivative fragment too. This compound was assigned to a ligstroside derivative, which for the purpose of these tests corresponded to model 8 present in **Table 2**.

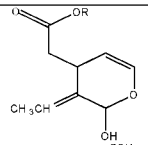
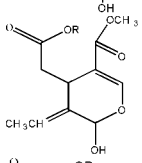
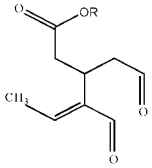
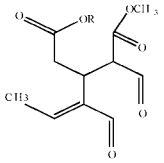
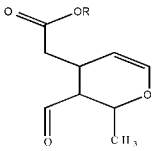
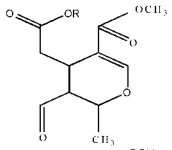
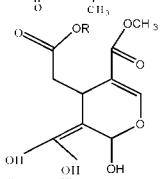
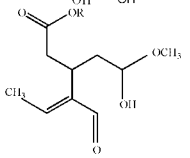
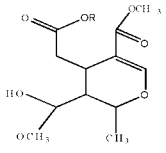
Peak 12 showed *m*/*z* 361 in full-scan mode. Trace chromatogram of this *m*/*z* ratio gave four more peaks (14, 19, 21, and 23). According to the literature, these *m*/*z* 361 compounds may correspond to models 2, 4, and 6 (*12*, *23*, *24*, *26*, *29*, *30*, *39*−*44*). Injection of the extract in product ion scan mode of *m*/*z* 361 gave no differences for the five peaks even when they were injected at different collision energies (up to −50 V) (see **Figure 2**). For all of the compounds, *m*/*z* 291 had a relative abundance of 100% in product ion spectra. This ion at *m*/*z* 291 is probably derived from the C_4H_6O loss fragment, in the case of models 2 and 6 as described by Caruso et al. (*29*). In the case of model 4, it could be justified by the two dialdehydes and a methyl group loss. The 259 and 101 ions may be due to rearrangement fragments. The ion at *m*/*z* 127 is justified by the loss of the upper C_7H_7O fragment of the molecule, the same C_4H_6O group mentioned above, and carboxylic group loss in the case of models 2 and 6 (see **Figure 3**). In model 4, it could be justified by a loss of 114 units of molecular mass of the elenolic fragment (*m*/*z* 241) caused by the liberation of the COOH, both aldehyde and a methyl group, or for the two dialdehyde groups and the CH_2-COOH loss of the same elenolic fragment. In models 2 and 6, it can be justified by the loss of the COOH group and the C_4H_6O group loss of the elenolic fragment. The loss of 103 units of mass of the elenolic fragment caused by carboxylic liberation and the COOH group gave a molecule at *m*/*z* 139 justified in the three models.

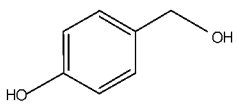
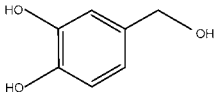
Peak 14 showed a deprotonated molecule at *m*/*z* 393 that could be attributed to a ligstroside derivative because its product ion scan spectra gave the ion for a loss of the tyrosol unit (*m*/*z* 257). Two other peaks (15 and 16) were also present in the trace chromatogram. No differences were observed in their product ion scan spectra.

The literature shows two models of these ligstroside derivatives (models 7 and 9) (*29*, *40*, *41*).

Oleuropein Derivatives. The spectra generated for peaks 8, 9, 17, and 19 gave the deprotonated molecule at *m*/*z* 377, which

Table 2. Possible Models of Derived Secoiridoids

Model	Característics	Base	*m/z*			Bibliography
			Acid Elenolic Derivative[1]	Ligstroside Derivative[2]	Oleuropein Derivative[3]	
1	Closed ring Decarboxilade form		183	303	319	*(12, 19, 26, 29, 39, 44, 46)*
2	Closed ring Carboxilade form		241	361	377	*(19, 23, 26, 30, 42, 44, 46, 50)*
3	Open ring Decarboxilade Dialdehidic form		183	303	319	*(22, 24, 26, 35, 39, 40, 42-44)*
4	Open ring Carboxilade Dialdehidic form		241	361	377	*(26, 29, 43, 44)*
5	Closed ring Decarboxilade Aldehidic form		183	303	319	*(12, 22, 23, 39, 41, 44)*
6	Closed ring Carboxilade Aldehidic form		241	361	377	*(12, 23, 24, 29, 39-41, 43, 44, 51)*
7	Closed ring Carboxilade Hidroxilade form		273	393	409	*(29, 41)*
8	Open ring Decarboxilade Aldehidic form		215	335	351	*(40)*
9	Closed ring Carboxilade form		272	393	409	*(40)*

[1]R = H [2]R=  [3]R= 

demonstrates the presence of oleuropein derivative in carboxylic form (models 2, 4, and 6) (*23*, *24*, *26*, *29*, *30*, *39*−*45*). Product ion scan spectra of *m*/*z* 377 for the four peaks revealed no differences at different collision energies (see **Table 1**), except for peak 19, which shows characteristic product ions at *m*/*z* 333, 301, and 181, which may derive from rearranged fragments. The product ion at *m*/*z* 307 is justified in models 2 and 6 by the loss of a C_4H_6O fragment (*29*). The product ions at *m*/*z* 275 may derive from rearranged fragments. These results are corroborated by Caruso et al. (*29*). The loss of the COOH and

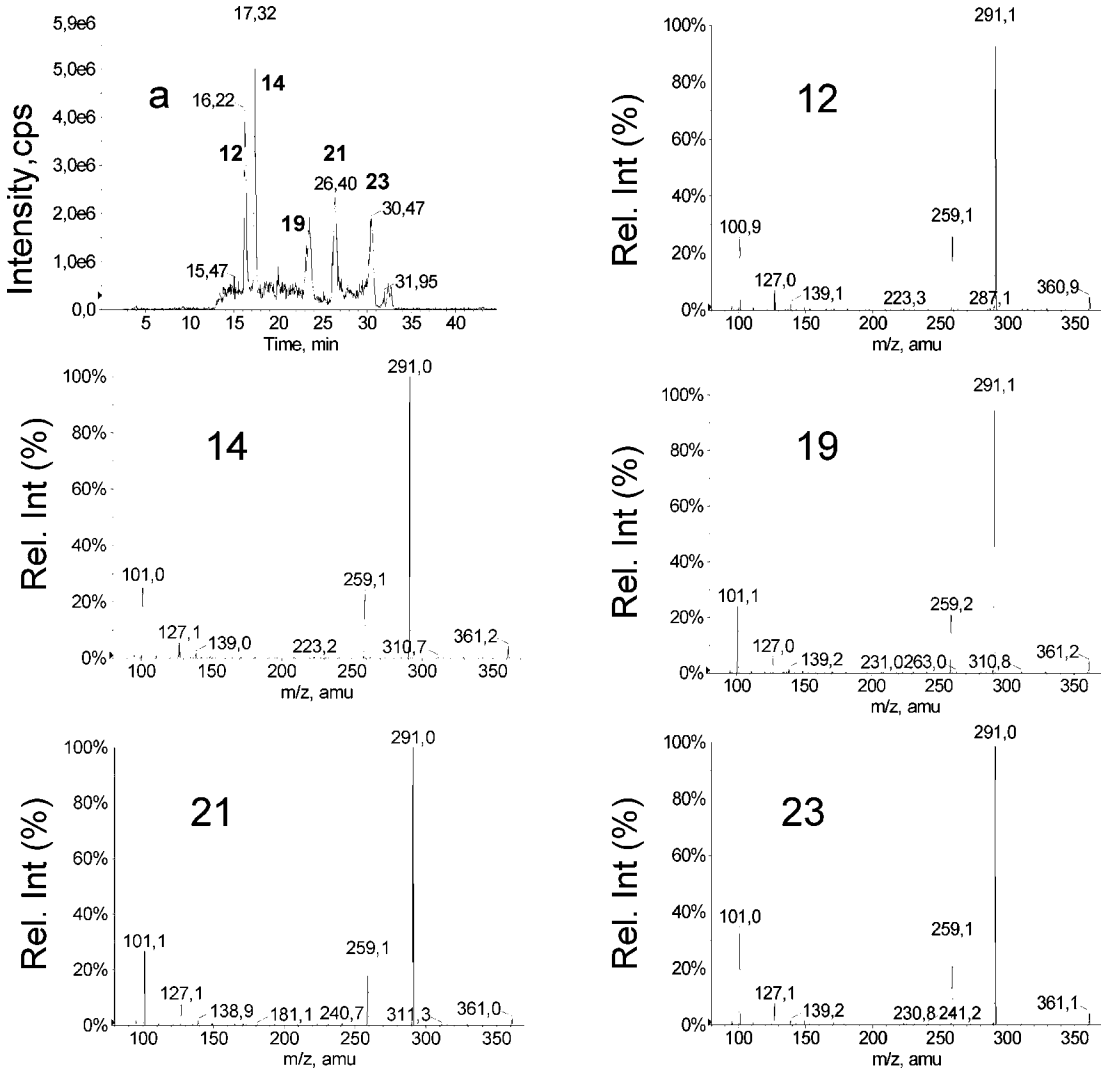


Figure 2. (**a**) Trace chromatogram of *m*/*z* 361 in full-scan mode for the olive oil extract. Mass spectra of five ligstroside derivatives in product ion scan mode of *m*/*z* 361 (12, 14, 19, 21, and 23). HPLC-MS/MS conditions are as described in the text.

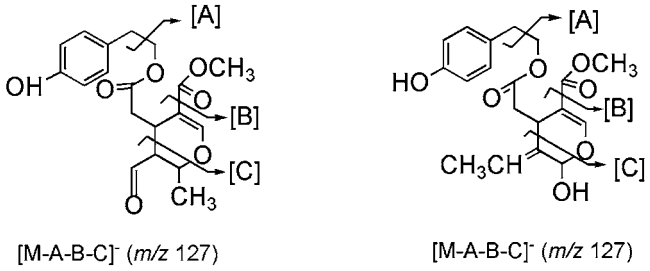


Figure 3. Possible rupture in the 2 and 6 ligstroside model to give the ion molecule at *m*/*z* 127.

the $COOCH_3$ unit of elenolic fragment derivative (*m*/*z* 241) produces a fragment ion at *m*/*z* 139. The ion at *m*/*z* 111 can be justified by $COOCH_3$, COOH, and the aldehyde group loss of the elenolic acid fragment in model 6.

Peak 10 is an oleuropein derivative in decarboxylade form shown by its deprotonated molecule $[M - H]^-$ at *m*/*z* 319 (*12*, *19*, *22*−*24*, *26*, *29*, *39*−*44*, *46*), and by the fact that the product ion scan of *m*/*z* 319 produces an ion at *m*/*z* 153, which demonstrates the existence of the hydroxytyrosol molecule. The bibliography gives three different models of oleuropein derivatives with this mass molecule (models 1, 3, and 5), but in our experience only one derivative showing this *m*/*z* was observed. The molecular ion at *m*/*z* 111 can be explained by the loss of 72 units of mass of the elenolic derivative fragment (*m*/*z* 183) caused by CHO and COOH liberation in aldehyde forms in models 3 and 5.

A compound with a deprotonated molecule at *m*/*z* 365 is present in peak 12. Its spectrum shows other fragment ions: *m*/*z* at 153 indicates the hydroxytyrosol molecule liberation, which means it is probably an oleuropein derivative. To our knowledge, an oleuropein derivative present in olive oil with this *m*/*z* is here described for the first time in this work (**Figure 4**).

Although different collision energies were proved, it was very difficult to differentiate the isoforms, due to their identical spectrum profiles and the identical fractions present.

The presence or absence of aldehyde, carboxyl, and/or methyl groups and the open or closed form of the elenolic acid ring structure indicate the differences between aglycons. Oleuropein and ligstroside aglycons differ from each other, in the existence of a mono or ortho-dihydroxy structure on the phenol ring.

Flavonoids. Peaks 11, 13, and 18, luteolin, apigenin, and methoxyluteolin, respectively, can easily be identified by their full-scan spectrum. They were also corroborated by the spectrum reference and the retention time of the standard and log α.

Elenolic Acid. This compound cannot be considered a phenolic compound, which only corresponds to the secoiridoid

Table 3. Content of Phenolic Compounds

peak	compound	*m/z*	content (μg/mL) oil A	oil B	oil C
1	hydroxytyrosol	153	7.03	63.47	17.87
2	tyrosol	137	2.94	24.38	16.45
3	vanillic acid	167	0.15	0.22	0.85
4	vanillin	151	0.05	0.47	0.04
5	*p*-coumaric acid	193	0.10	0.29	0.34
6	ligstroside derivative	553	18.45	19.84	1.89
7	ligstroside derivative	335	48.80	89.20	23.55
8	oleuropein derivative	377	117.29	202.70	45.92
9	oleuropein derivative	377	17.59	46.74	9.94
10	oleuropein derivative	319	12.22	18.84	30.47
11	luteolin	285	3.97	3.10	6.80
12	ligstroside derivative	361	18.36	28.49	23.77
	oleuropein derivative	365			
13	apigenin	269	0.49	0.73	0.41
14	ligstroside derivative	361	50.00	14.58	20.63
	ligstroside derivative	393			
15	ligstroside derivative	393	4.56	18.74	7.62
16	ligstroside derivative	393	2.01	3.87	2.24
17	oleuropein derivative	377	4.57	5.77	16.89
18	methoxyluteolin	299	0.61	0.61	1.59
19	oleuropein derivative	377	12.98	53.16	27.51
	ligstroside derivative	361			
20	unknown		0.86	0.48	0.72
21	ligstroside derivative	361	4.29	23.45	20.49
22	unknown		0.47	0.63	0.00
23	ligstroside derivative	361	9.81	9.86	41.81
	total		338	630	318

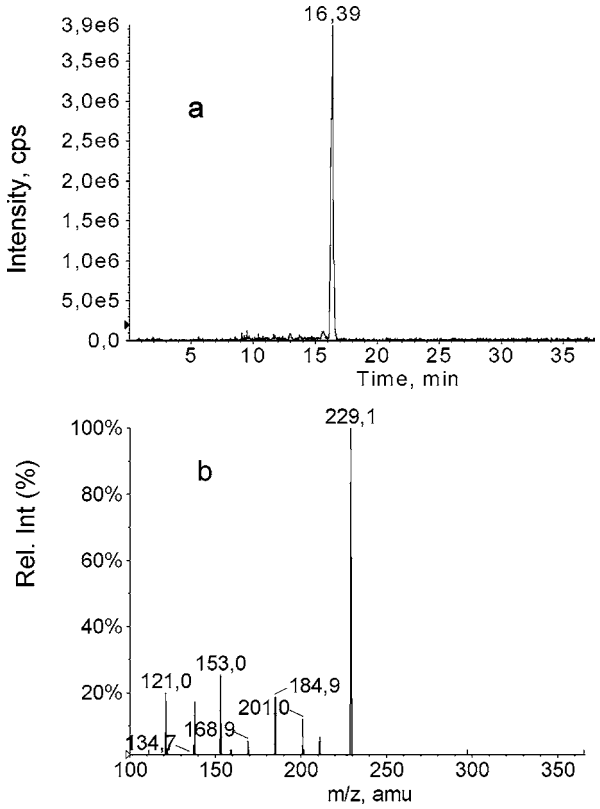


Figure 4. (**a**) Trace chromatogram of *m/z* 365 in full-scan mode for the olive oil extract. (**b**) Mass spectra in product ion scan mode of *m/z* 365. HPLC-MS/MS conditions are as described in the text.

part of oleuropein (*2*). It can only be observed at 240 nm in HPLC-DAD. A very intense peak at minute 10 is observed at this wavelength, and its characteristic ion at *m/z* 241 corresponding to the deprotonated molecule of elenolic acid and *m/z* 139 due to the $COOCH_3$ group loss is clearly present.

Quantification. The quantification of phenolic compounds using HPLC-DAD expressed as caffeic acid, gallic acid, syringic acid, or tyrosol, oleuropein equivalents, or other phenolic compounds has been reported in many papers (*5*, *19*, *21*, *22*, *24*). However, a variation of 18−80% in the total concentration values of phenolic compounds was demonstrated by Pirisi et al. (*24*) using these quantification methods. Here, each phenolic compound was expressed with the respective standard when it was available. When this was not possible, the phenolic compounds were divided and expressed with a representative and available standard compound of each group, on the basis that responses of each group are significantly different from each other. Secoiridoids were expressed as oleuropein; the phenolic alcohols, 3,4-dihydroxyphenyl and 4-hydroxyphenyl were expressed as tyrosol; and unknown flavonoids were expressed as luteolin. Quantification was carried out at the wavelength for the higher absorbency for each selected compound (**Table 3**).

Elenolic acid, a nonphenolic compound, but a derivative, was expressed as oleuropein, and quantification showed it at 287, 502, and 194 μg/mL in oils A, B, and C, respectively.

As reported in previous studies, we found that virgin olive oil contains low amounts of phenyl acids and phenyl alcohols and high concentrations of secoiridoid derivatives such as oleuropein and ligstroside aglycons, which originate from the oleureuropein, dimethyloleuropein, and ligstroside glycosides found in olives (*26*, *27*, *35*, *39*, *47*).

In the sample studied, secoiridoids comprised 77−88% of total phenolic compounds. If hydroxytyrosol and tyrosol are considered, the oleuropein and ligstroside derivatives comprised 87−92% of the total phenolic content in the samples studied.

During the crushing and malaxing processes, hydrolysis of the glycosidic bond occurs and the aglycons pass into the oil. The process also causes partial modification of oleuropein and ligstroside aglycons, which generates isoforms in the elenolic structure, although they conserve the phenol ring (*23*). Some

Table 4. Linearity of Phenolic Compounds Standards and Sensitivity of the HPLC-DAD Method

compound	concentration range (μg/mL)	linear regression	r[a]	LOD[b] (μg/mL)	LOQ[c] (μg/mL)
tyrosol	2–40	$y = 22.01x - 5.08$	0.999	0.28	0.39
vanillic acid	0.05–1.5	$y = 67.61x + 0.68$	0.999	5×10^{-3}	0.04
vanillin	0.5–1	$y = 122.94x + 0.29$	0.999	6×10^{-3}	0.03
p-coumaric acid	0.05–1	$y = 269.41x - 0.17$	0.999	7×10^{-3}	0.02
oleuropein	2–1000	$y = 8.07x - 1.28$	0.999	0.29	0.60
luteolin	0.05–20	$y = 43.73x - 8.89$	0.999	0.25	0.35
apigenin	0.3–15	$y = 101.59x - 28.9$	0.999	0.30	0.35
methoxyluteolin	0.3–15	$y = 110.21x - 14.44$	0.999	0.15	0.19

[a] Correlation coefficients of the regression equation. [b] LOD = limit of detection. [c] LOQ = limit of quantification.

Table 5. Recovery Studies of Phenols[a]

compound	recovery mean (%)	SD[b]
tyrosol	115	3.19
p-coumaric	105	0.99
oleuropein	76	8.64
luteolin	103	4.04

[a] $n = 3$. [b] Standard deviation of recovery studies.

of them are reversible to equilibrium sustenance (*23*, *39*, *40*, *44*).

Method Validation. To assess the validity of the method, validation tests were run. All test parameters were carefully chosen to cover the range of samples and concentrations involved.

Linearity. The linearity of standard curves was expressed in terms of the determination coefficient from plots of the integrated peak area versus concentration of the same standard (μg/mL). These equations were obtained over a wide concentration range in accordance with the levels of these compounds found in the olive samples. Details are given in **Table 4**. The system was linear in all cases ($r > 0.99$).

Precision. The method's precision was satisfactory, with acceptable values under the Horwitz criteria (*48*). Six replicate determinations on the same day and twelve replicate determinations on different days with the same sample were carried out. Relative standard deviations were calculated, with results of coefficients of variation less than 7% in repeatability (intralaboratory precision) and less than 8% in reproducibility (interlaboratory precision).

Sensitivity. LOD and LOQ were studied to check the sensitivity of the methods under the working conditions proposed. Both followed USP criteria (*49*) (**Table 4**). These limits, referring to the concentrations in olive oil needed if they were to be detected and quantified, were of the order of nanograms. The method has excellent sensitivity.

Recovery. To assess the recovery of the proposed method, three samples of refined olive oil without phenolic compounds were spiked with different amounts of tyrosol, *p*-coumaric acid, luteolin, and oleuropein. The samples were submitted to the complete proposed procedure. As is shown in **Table 5**, the mean recoveries were between 76% and 115%.

Conclusions. A simple and reproducible SPE-HPLC-DAD and HPLC-MS/MS method was developed to characterize and quantify the phenolic compounds present in virgin olive oil. In this study, 20 out of 23 compounds detected were characterized through a combination of the HPLC-DAD and HPLC-MS/MS systems. The method proposed is faster, with a very good recovery (76−115%), and low solvent and sample consumption is required. As the method shows good precision, recovery, linearity, and sensitivity, it is suitable for routine analyses of various kinds of olive oil. Compounds such as acids, alcohols, flavonoids, and the various secoiridoids can be detected and quantified. There are a high variety of secoiridoid compounds derived from oleuropein and ligstroside in virgin olive oil, and these secoiridoids make up a high percentage of all phenolic compounds in virgin olive oil. Besides this, in comparison with other investigations, this work recognizes the existence of diverse isomers belonging to the secoiridoids group. As many of them share the mass weight for generated isoforms, further studies are required to look into these keto-elenolic tautomeric forms.

ABBREVIATIONS USED

SPE, solid-phase extraction; HPLC-DAD, high performance liquid chromatography with diode array detector; HPLC-MS/MS, high performance liquid chromatography with double mass spectrometer; MS, mass spectrometry; LOD, limits of detection; LOQ, limits of quantification; ESI, electrospray ionization; APCI, atmospheric pressure chemical ionization; DP, declusterin potential; CE, collision energy; CAD, collision-activated dissociation.

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Supporting Information Available: S-1, hydroxytyrosol fragment; S-2, ligstroside derivative fragments; S-3, oleuropein derivative fragments. This material is available free of charge via the Internet at http://pubs.acs.org.

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