Tentative Identification of Phenolic Compounds in Olive Pomace Extracts Using Liquid Chromatography–Tandem Mass Spectrometry with a Quadrupole–Quadrupole-Time-of-Flight Mass Detector

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Ⓢ Supporting Information

ABSTRACT: The reuse of agronomical residues is a pending goal for sustainable agriculture. Particular residues in olive-oil-producing countries are leaves, wastewater, and olive pomace. Olive leaves and wastewaters have been previously characterized by isolation of the phenolic fraction. However, olive pomace has not yet been qualitatively characterized as a source of phenols. Olive pomace extracts were obtained using superheated solvent extraction using 50:50 (v/v) water/ethanol as a leaching mixture at 160 °C. The extracts were analyzed by liquid chromatography coupled to tandem mass spectrometry using a quadrupole–quadrupole-time-of-flight (QqTOF) hybrid mass analyzer (R = 25 000–45 000). Qualitative analysis was supported upon measurement of accurate masses for precursor and product ions as well as their isotopic distribution. Identification was focused on the main families of phenolic compounds present in extra virgin olive oil. The potential of this residue as a rich source of phenols with antioxidant properties has been proven.

KEYWORDS: Phenolic compounds, olive pomace, superheated liquid extraction, tandem mass spectrometry, QqTOF mass analyzer

■ INTRODUCTION

The olive oil industry produces vast amounts of a semi-solid byproduct after virgin olive oil (VOO) decantation in the two-phase system. The byproduct thus generated is a residue known as olive pomace, also known as alperujo. This is formed by a mixture of the liquid and solid phases (alpechín and orujo, respectively), which were the original residues from the previous three-phase process. Because no water is added during the two-phase process, oil quality is superior to that provided by the three-phase system because the partition equilibrium enables a higher proportion of olive phenols to remain in the oil fraction.[1]

Once VOO is decanted, additional centrifugation steps or a second extraction process (beating and decanting) leads to second-order olive oils characterized by a high wax content. The isolation of these low-quality oils is economically viable; therefore, this strategy is implemented in most olive oil factories.[2] This second extraction step has reduced the interest of olive pomace oil isolated with the aid of organic solvents and heat. Currently, olive pomace is used either as fuel in co-generation plants to produce energy or as organic fertilizer after a composting process.[3] However, the profitability of co-generation plants is still under doubt, whereas the fertilization industry only absorbs a very small proportion of the produced olive pomace; therefore, a frequent option is dumping the residue in controlled areas because of its high polluting organic load.

It is therefore evident that there is a demand for alternatives capable of taking benefits from olive pomace (either before or after extraction of the low-quality oil), which is characterized by a high content of organic substances, such as sugars, tannins, polyalcohols, pectins, lipids, and phenols.[4] However, no exhaustive characterization of any form of this residue has been performed to date. One of the most conflictive fractions of olive pomace corresponds to phenolic compounds.[5] Phenols are the major contributors to the antibacterial and phytotoxic activity of black olive mill residues, which limit their microbial degradability and potential for biogas production.[6] Most phenolic classes present in VOO and olive oil residues are endowed with the features for being free radical scavengers.[5] It is well-known that the antioxidant activity of phenols is based on their capacity, as donors of hydrogen atoms, to neutralize the activity of free radicals.[7] As a general rule, the o-dihydroxyl structure characteristic of hydroxytyrosol and secoiridoid derivatives seems to be responsible for the high antioxidant activity of these phenols, followed by 4-O-monohydroxy compounds (ligstroside and tyrosol) and 3-O-hydroxy-substituted catechols.[8] With these premises, the properties of olive phenols may turn olive pomace into a cheap source of natural antioxidants in concentrations up to 100 times higher than in virgin olive oils.[4] The presence of these compounds in foods is crucial because they reduce oxidative reactions that decrease both the nutritional value and sensory quality.[9]

From a clinical point of view, modest long-term intakes of hydrophilic phenols from olive-tree materials produce a favorable impact on the incidence of cancers and chronic diseases, such as cardiovascular disorders and type II diabetes.[10] For example, hydroxytyrosol, a well-known phenol about which the European Food Safety Authority (EFSA) has recently emitted a very favorable opinion,[11] shows better results in scavenging and/or antioxidant capacity tests than common antioxidants, such as vitamins C and E or 2,6-di-*tert*-butyl-4-methylphenol (BTH).[12] Other olive phenols (e.g., tyrosol,[13] verbascoside,[14] and α-taxifolin[15]) provide health benefits, as demonstrated by *in vitro* activities, in addition to a synergistic effect of their antioxidant capacities when in mixtures.[16] Nevertheless, these studies have shown some limitations when compared to *in vivo* research. Recent studies on the use of apigenin-7-glucoside[17,18] to fight against Alzheimer's[19] and/or liver diseases[20] support the health effects of this compound and constitute a call for continuing the research on olive phenols.

Research carried out thus far for characterization of olive pomace extracts has mainly been focused on panels of target known compounds considered of interest, but global qualitative analysis of these extracts has not yet been made. Therefore, a global profiling of these compounds would be the key for better understanding the biochemical pathways in which they are involved. Similar studies have been reported for olive leaves and wastewater olive mill residues.[21,22] The research reported here was aimed at obtaining a global metabolic profile of phenols from this unexploited raw material. With this purpose, the extracts obtained by ethanol−water mixtures under superheated conditions were the analytical sample subjected to liquid chromatography separation with tandem mass spectrometry detection using a quadrupole−quadrupole-time-of-flight (QqTOF) hybrid detector to take benefits from high mass accuracy.

■ MATERIALS AND METHODS

Samples. Olive pomace samples from different cultivars obtained at different crop dates in the 2009−2010 season were taken directly from the production line of VOO. These pomace samples were homogenized to prepare a pool representative for qualitative analysis. The mixture was dried in an oven at 30 °C for 24 h to eliminate the water content (around 60−80%). After drying, the samples were stored at −20 °C until use.

Reagents. Ethanol was from Panreac (Barcelona, Spain). Liquid chromatography−mass spectrometry (LC−MS)-grade formic acid and acetonitrile were purchased from Scharlab (Barcelona, Spain). Deionized water (18 MΩ cm) from a Millipore Milli-Q water purification system was used to prepare both the water−ethanol extractant mixtures and chromatographic mobile phases. The Folin−Ciocalteu (F−C) reagent, sodium carbonate, and gallic acid were from Sigma (St. Louis, MO).

Apparatus and Instruments. Superheated liquid extractions (SHLEs) were performed by a laboratory-made dynamic extractor[23] consisting of the following units: (a) an extractant supplier, (b) a high-pressure pump (Shimadzu LD-AC10), which propels the extractant through the system, (c) a switching valve placed next to the pump to develop static extractions, (d) a stainless-steel cylindrical extraction chamber (550 × 10 mm inner diameter and 4.3 mL internal volume), where the sample is placed (this chamber is closed at both ends with screws whose caps contain cotton-made filters to ensure that the sample is not carried away by the extractant), (e) a restriction valve to maintain the desired pressure in the system, (f) a cooler made of a stainless-steel tube (1 m length and 0.4 mm inner diameter) and refrigerated with water, and (g) a gas chromatograph oven (Konix,

Cromatix KNK-2000), where the extraction chamber is placed and heated.

The absorbance of the extracts after reaction with the F−C reagent was monitored by a Thermo Spectronic Helios Gamma spectrometer (Waltham, MA). Shaking and centrifugation of the extracts were carried out by means of a MS2 minishaker Vortex (IKA, Germany) and a Mixtasel centrifuge (Selecta, Barcelona, Spain), respectively.

All samples were analyzed by an Agilent 1200 Series LC system (Agilent Technologies, Waldbronn, Germany) coupled to an Agilent 6540 QqTOF hybrid mass spectrometer with a dual electrospray ionization (ESI) source for simultaneous spraying of a mass reference solution that enabled continuous calibration of detected *m/z* ratios.

Superheated Liquid Extraction. A total of 2 g of dried olive pomace was placed into the extraction cell installed into the gas chromatograph oven, and then a relative high flow rate (7 mL/min) was used for 1 min to fill the cell rapidly. To ensure the absence of air inside the extraction cell, the restrictor valve was kept open until the first drop of extractant appeared. At that moment, the restrictor valve was closed, and when the desired pressure was reached, the switching valve was closed, the pump was turned off, and the oven was switched on. During temperature rise (up to 160 °C), the switching valve had to be opened at short intervals to prevent the pressure from surpassing the working value. Once the selected temperature and pressure were reached, static extraction was performed for a preset time (5 min). Finally, the oven was switched off, the chamber was cooled below the boiling point of ethanol, and the switching and restrictor valves were switched to enable clean extractant to flow through the cell and flush out the extract (collected volume of 10 mL).

Determination of Total Phenols by the F−C Method. The total amount of phenolic compounds was quantified by the F−C method using gallic acid as the standard. With this purpose, a calibration curve was run using solutions of 1, 5, 10, 25, and 50 mg/L of this acid ($y = 0.0382x + 0.0296$; $R^2 = 0.9963$). A 0.5 mL aliquot of extract, 10 mL of distilled water, 1 mL of F−C reagent, and 3 mL of Na_2CO_3 (20%, w/v) were mixed, made to 25 mL with distilled water, and heated at 50 °C for 5 min. After heating, the samples were kept at room temperature for 30 min, and finally, the absorbance was monitored at 765 nm against a blank solution containing distilled water, instead of extract. The concentration of phenolic compounds thus obtained was multiplied by the dilution factor of the extract volume and divided by the amount of olive pomace used. The results were expressed as equivalent to micrograms of gallic acid per milliliter of olive pomace extract (μg of GAE/mL).[24]

LC−QqTOF MS/MS Analysis. An injection volume of 5 μL and a flow rate of 0.8 mL/min were used. The mobile phases were 0.1% (v/v) formic acid aqueous solution (phase A) and pure acetonitrile (phase B). Separation of the analytes was performed on an Inertsil ODS-2 C_{18} column (250 × 4.6 mm inner diameter, 5 μm particle, Análisis Vínicos, Tomelloso, Ciudad Real, Spain) at 25 °C. The gradient method was as follows: 96% A for 4 min, from 96 to 0% A in 36 min, and 0% A for 3 min. The dual ESI source operated in negative ionization using the following conditions: nebulizer gas at 35 psi and drying gas flow rate and temperature at 10 L/min and 325 °C, respectively. The capillary voltage was set at 3500 V, while the fragmentor, skimmer, and octapole voltages were fixed at 175, 65, and 750 V, respectively.

The data were acquired in centroid mode in the extended dynamic range (2 GHz). Full scan was carried out at one spectrum per second within the *m/z* range of 100−1000, with subsequent activation of the three most intense precursor ions (only allowed charge, single or double) by MS/MS using a collision energy of 20 eV at one spectrum per second within the *m/z* range of 100−1000. An active exclusion window was programmed after one spectrum and released after 0.75 min to avoid repetitive fragmentation of the most intense precursor ions and, in this way, increase the detection coverage. Before the experiments, the instrument reported mass detection resolution of 25 000 full width at half maximum (fwhm) at *m/z* 112.985 587 and 45 000 fwhm at *m/z* 966.000 725. To ensure the desired mass accuracy of recorded ions, continuous internal calibration was performed during analyses with the use of signals at *m/z* 119.0362 (proton-abstracted

purine) and m/z 966.0007 [formate adduct of hexakis(1H,1H,3H-tetrafluoropropoxy)phosphazine].

Data Processing. Raw data files were converted to m/z data using MassHunter and processed in R statistical language (version 2.15.0, http://www.r-project.org/) using the open-free XCMS (version 1.24, http://metlin.scripps.edu/xcms/index.php) in an automated and unbiased way. This software allows for peak finding, alignment, and peak picking of mass spectral features resulting in a list of m/z precursor ions per retention time with the three MS/MS spectra obtained in each cycle. Parameters for XCMS were taken from the protocol described by Smith et al.[25] using the centWave peak finder method designed for high mass accuracy data.[26] The extraction of molecular features was carried out using a prefilter to take at least two ions in each cycle with intensity above 5000. Briefly, peaks were deconvoluted and aligned across samples using a signal-to-noise threshold of 10, a maximum tolerated m/z deviation of 5 ppm, and a peak width of 10−60 s. The aligned output consisted of accurate mass, retention time, and intensity of each peak. The file from this treatment was created in comma separated value files (.csv) for each sample and exported into the Mass Profiler Professional (MPP) software package (version 2.0, Agilent Technologies, Santa Clara, CA) for further processing. Compound identification was performed using the METLIN Metabolite and MS/MS (http://metlin.scripps.edu/) and PlantCyc (http://plantcyc.org/) databases. The allowed negative precursor ions for identification were formate adducts and deprotonated ions. Dehydratation neutral losses were also allowed.

RESULTS AND DISCUSSION

SHLE of Olive Phenols from Olive Pomace. The use of SHLE for extraction of natural compounds from raw material of
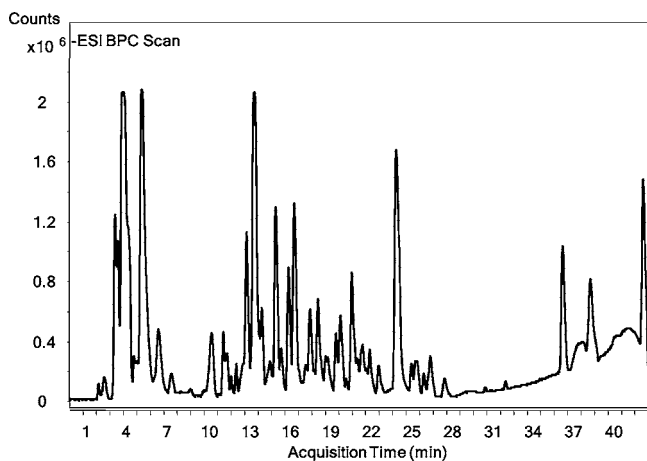

Figure 1. Base peak chromatogram obtained by analysis of olive pomace extract under the optimum working conditions.

plant origin is widely known. SHLE has proven its efficiency for the extraction of natural compounds from different types of vegetables, such as leaves, fruits, roots, or stems.[27] In SHLE, the duality of the extraction temperature and extractant composition plays a crucial role in succeeding in the leaching of target compounds. The extractant composition for isolation of phenolic compounds from vegetal matrices is usually an ethanol/deionized water mixture at ratios typically ranging from 50:50 to 80:20 (v/v) ethanol/water. Japón-Luján et al. used 80:20 ethanol/water for extraction of a panel of representative phenols in olive pomace.[18] In the present study, this extraction protocol was used for a characterization study. Thus, the temperature was set at 160 °C, while the extraction time was programmed for 5 min. Under these conditions, concentrations of phenolic compounds above 500

Table 1. Compounds in Extracts from Olive Identified by LC−QqTOF MS/MS Analysis

	Formula	m/z exp	m/z theo	Error (ppm)	tR	Fragments
Hydroxytyrosol and Tyrosol Derivatives						
Hydroxytyrosol	$C_8H_{10}O_3$	153.0560	153.0557	-1.9601	13.20	123.0450 / 105.0339
Hydroxytyrosol Glucoside	$C_{14}H_{20}O_8$	315.1069	315.1085	5.2046	13.18	123.0446 / 153.0570
Hydroxytyrosol Diglucoside	$C_{20}H_{28}O_{13}$	475.1429	475.1457	5.9140	13.28	153.0567 / 245.0682
Hydroxytyrosol Rhamnoside	$C_{20}H_{34}O_{13}$	481.1943	481.1927	-3.4498	13.35	153.0565 / 265.3271
Tyrosol	$C_8H_{10}O_2$	137.0608	137.0608	0.0000	17.27	119.0505 / 111.0098
Tyrosol Glucoside	$C_{14}H_{20}O_7$	299.1128	299.1136	2.7749	14.56	119.0505 / 137.0603
Iridoids Precursors						
Loganin	$C_{17}H_{26}O_{10}$	389.1468	389.1453	-3.9060	13.67	151.0776 / 113.0248
Loganin Glucoside	$C_{23}H_{38}O_{16}$	569.2113	569.2087	-4.4623	13.42	389.1513 / 313.1332
Loganic Acid	$C_{16}H_{24}O_{10}$	375.1322	375.1297	-6.7976	13.90	151.0776 / 113.0248
Loganic Acid Glucoside	$C_{22}H_{34}O_{15}$	537.1857	537.1860	0.5957	12.00	375.1432 / 179.0575
7-Deoxyloganic Acid	$C_{16}H_{24}O_9$	359.1368	359.1348	-5.6246	15.04	-
Secologanic Acid	$C_{18}H_{26}O_{10}$	401.1477	401.1453	-6.0577	16.42	-
Secologanoside	$C_{25}H_{32}O_{14}$	555.1795	555.1792	-0.5404	18.38	-
Secologanin	$C_{17}H_{24}O_{10}$	387.1268	387.1297	7.4910	13.10	-
Eleanolic Acid	$C_{11}H_{14}O_6$	241.0772	241.0790	7.6324	17.53	-
Oleoside	$C_{16}H_{22}O_{11}$	389.1122	389.1089	-8.3524	15.46	121.0660 / 101.0243 / 209.0477 / 345.1253
Oleoside Glucoside	$C_{22}H_{32}O_{16}$	551.1643	551.1618	-4.6084	14.89	551.1707 / 507.1805 / 209.0480 / 239.0354
Oleoside Diglucoside	$C_{28}H_{42}O_{21}$	713.2205	713.2146	-8.2724	13.50	551.1479 / 507.1585 / 713.2029 / 161.0259
Oleoside Riboside	$C_{20}H_{26}O_{15}$	505.1227	505.1195	-6.2955	16.57	389.1166 / 505.1298 / 345.1256 / 121.0663
Oleoside-11- Methylester	$C_{17}H_{24}O_{11}$	403.1274	403.1246	-6.9457	16.72	101.0244 / 119.0350 / 223.0626 / 179.0707
Oleoside Dimethylester	$C_{18}H_{26}O_{11}$	417.1433	417.1402	7.4316	17.08	-
Secoiridoids and Derivatives						
Oleuropein	$C_{25}H_{32}O_{13}$	539.1776	539.1770	-1.0757	19.95	377.1306 / 307.0874 / 275.0962
10-Hydroxy-Oleuropein	$C_{25}H_{32}O_{14}$	555.1701	555.1719	3.2783	18.31	537.1647 / 376.1114
Oleuropein Aglycone	$C_{22}H_{32}O_{16}$	377.1440	377.1453	3.4469	16.85	123.0446 / 255.0869
Verbascoside	$C_{29}H_{36}O_{15}$	623.1973	623.1981	1.3158	18.48	461.1744 / 161.0254
3,4-DHPEA-EDA	$C_{17}H_{20}O_6$	319.1207	319.1187	-6.1733	22.54	123.0446 / 139.0769
p-HPEA-EDA	$C_{17}H_{20}O_5$	303.1235	303.1238	0.9897	23.32	-
3,4-DHPEA-EA	$C_{19}H_{22}O_8$	377.1266	377.1242	-6.3905	23.95	123.0446 / 255.0869
p-HPEA-EA	$C_{19}H_{22}O_7$	361.1300	361.1293	-1.9384	20.29	-
Oleuropein Derivative 1	$C_{25}H_{36}O_{13}$	543.2107	543.2083	-4.4182	19.38	377.1522
Oleuropein Derivative 2	$C_{24}H_{36}O_{12}$	527.2160	527.2134	-4.9316	20.61	377.1481
Flavonoids						
Rutin	$C_{27}H_{30}O_{16}$	609.1487	609.1461	-4.1698	18.54	301.0391
Apigenin	$C_{15}H_{10}O_5$	465.1432	465.1425	-1.5049	18.91	-
Luteolin	$C_8H_{10}O_6$	285.0420	285.0405	-5.2624	23.92	-
Apigenin Glucoside	$C_{21}H_{20}O_{10}$	431.0999	431.0984	-3.5259	20.35	265.0450
Luteolin Glucoside	$C_{21}H_{20}O_{11}$	447.0970	447.0933	-8.2533	19.27	285.0423
Taxifolin	$C_{15}H_{12}O_7$	303.0527	303.0513	-4.5867	20.68	-
Diosmetin	$C_{16}H_{12}O_6$	299.0584	299.0561	-7.4902	26.53	-
Quercetin	$C_{15}H_{10}O_7$	301.0377	301.0354	-7.6735	24.58	-
Lignans						
Pinoresinol	$C_{20}H_{22}O_6$	357.1345	357.1344	-0.3080	19.47	-
Hydroxypinoresinol	$C_{20}H_{23}O_7$	374.1359	374.1371	3.2074	21.60	-
Acetoxypinoresinol	$C_{22}H_{24}O_8$	415.1400	415.1398	-0.3854	20.10	-
Phenolic Acids						
Shikimic Acid	$C_7H_{10}O_5$	173.0486	173.0495	5.2008	14.69	-
Phenylalanine	$C_9H_{11}NO_2$	164.0721	164.0717	-2.4380	14.88	-
Cinnamic Acid	$C_9H_8O_2$	147.0452	147.0452	0.0000	15.97	147.0459
p-Coumaric Acid	$C_9H_8O_3$	163.0403	163.0401	-1.0427	15.97	119.0503
Caffeic Acid	$C_9H_8O_4$	179.0352	179.0350	-1.1171	17.68	135.0449
Protocatechuic Acid	$C_7H_6O_4$	153.0193	153.0193	0.0000	14.40	109.0295
Vanillic Acid	$C_8H_8O_4$	167.0353	167.0350	-1.9756	13.43	108.0206 / 123.0446
Ferulic Acid	$C_{10}H_{10}O_4$	193.0514	193.0506	-4.1440	16.40	134.0377
Gallic Acid	$C_7H_6O_5$	169.0150	169.0142	-4.7333	15.49	125.0246

μg of GAE/mL were obtained. Figure 1 illustrates the base peak chromatogram (BPC) provided by the extract. As seen, phenolic compounds are mainly eluted in the time window
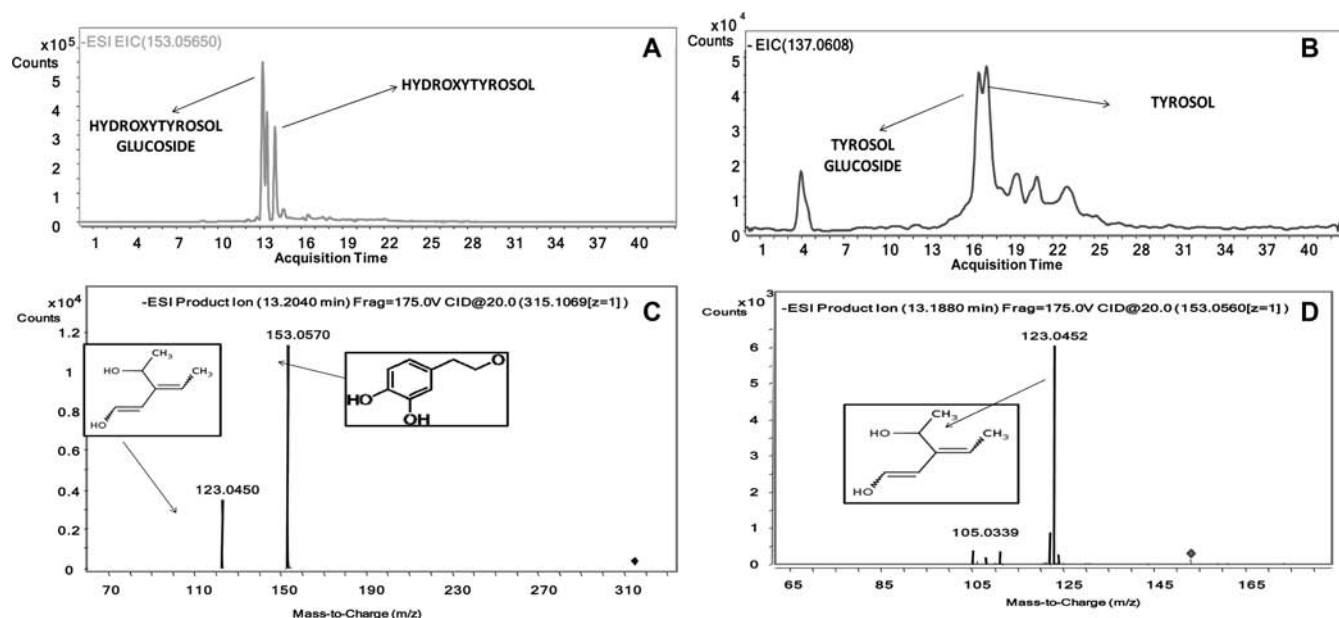
Figure 2. (A) EICs for hydroxytyrosol and tyrosol. (B) MS/MS spectrum for hydroxytyrosol glucoside. (C) MS/MS spectrum for hydroxytyrosol.

from 2 to 28 min, which fits approximately with a concentration of acetonitrile in the chromatographic gradient of 78%. On the other hand, few peaks are detected in the last part of the chromatogram, corresponding to nonpolar compounds, such as α-tocopherol (m/z 429.3759), which eluted at 42.3 min. Once the potential of olive pomace for providing extracts rich in phenolic compounds was defined, characterization of representative families of these compounds was carried out by tandem mass spectrometry in high-accuracy mode. Identification and confirmatory detection was supported on mass accuracy of the precursor ion and representative product ions, the structure of which was elucidated, but also on isotopic distribution of signals detected in full-scan mode. Table 1 lists the identified compounds organized by families (information about theoretical and experimental m/z values as well as characteristic fragment ions are also included).

Hydroxytyrosol and Tyrosol Derivatives. Hydroxytyrosol and tyrosol have been identified as two of the major simple phenols in VOO, which is considered the main source of both compounds in the diet. Both compounds are present in VOO mainly as free forms but also condensed as secoiridoid derivatives.[28] The concentrations of hydroxytyrosol and tyrosol in VOO are usually below 15 μg/g,[29] while in olive pomace, these antioxidants have been determined in concentrations around 106 and 18.5 μg/g, respectively.[30] The concentration of phenols in VOO as well as in olive pomace depends upon genetic factors, such as the cultivar variety, but also other factors, such as olive fruit maturation and agropedoclimatic conditions.[31,32] Glucoside and acetate derivatives have also been found in other *Olea europaea*-derived materials, such as leaves and olive pomace waste after the refining process.[33]

In the case of the olive pomace used in this research (taken directly from the production line after decantation), the extracted ion chromatogram (EIC) for hydroxytyrosol by monitoring the m/z ratio at 153.0560 revealed the presence of several derivatives of this metabolite.[34] The peaks at 13.18 and 13.20 min in Figure 2A were clearly identified by MS/MS fragmentation as hydroxytyrosol glucoside (Figure 2B) and free hydroxytyrosol (Figure 2C). Thus, hydroxytyrosol glucoside

detected by fragmentation of the precursor ion at m/z 315.1069 generated two representative fragments: one of them corresponding to free hydroxytyrosol at m/z 153.0570 by cleavage of the hexose unit and a second one at m/z 123.0450, which was assigned to the structure in Figure 2B, resulting from the loss of the CH_2OH and CHO groups from the hydroxytyrosol original structure. These two fragments are characteristic for identification of any conjugated hydroxytyrosol present in any olive tree material. From a quantitative point of view, the chromatographic signal attributed to hydroxytyrosol glucoside was more intense than that assigned to free hydroxytyrosol.

Concerning the chromatographic peaks eluted at 13.28 and 13.35 min, precursor ions for hydroxytyrosol diglucoside and diramnhoside were detected at m/z 475.1429 and 481.1943, respectively. Identifications were confirmed by the corresponding MS/MS spectra, as illustrated in Figure S1 of the Supporting Information, which shows the cleavage of the sugar units.

Tyrosol was detected in free form but also as a glucoside derivative with m/z 137.0608 and 299.1128 (see Figure 2A). In contrast to hydroxytyrosol, the free form of tyrosol was at the same concentration level as its glucoside derivative. The two MS/MS spectra revealed product ions representative of tyrosol derivatives, such as that at m/z 119.0505 and the representative neutral loss corresponding to the cleavage of the glucoside unit (Figure S2 of the Supporting Information).

Iridoid Precursors. The term iridoid is used, in most cases, to name a wide group of monoterpenes as well as glucoside derivatives, whose structure may be considered as derived from iridane (cis-2-oxabicycle-[4.3.0]-nonane). The secoiridoid compounds, which play a crucial role in the family of olive oil antioxidants, derive from iridoids by opening of the pentacyclic ring. The precursor for biosynthesis of iridoids is mevalonic acid, which leads to different structures by an onward cyclization step from which the iridane skeleton is formed. Taking into account the relevance of this pathway in the synthesis of one of the major phenolic families, as shown in Figure S3 of the Supporting Information, the precursors
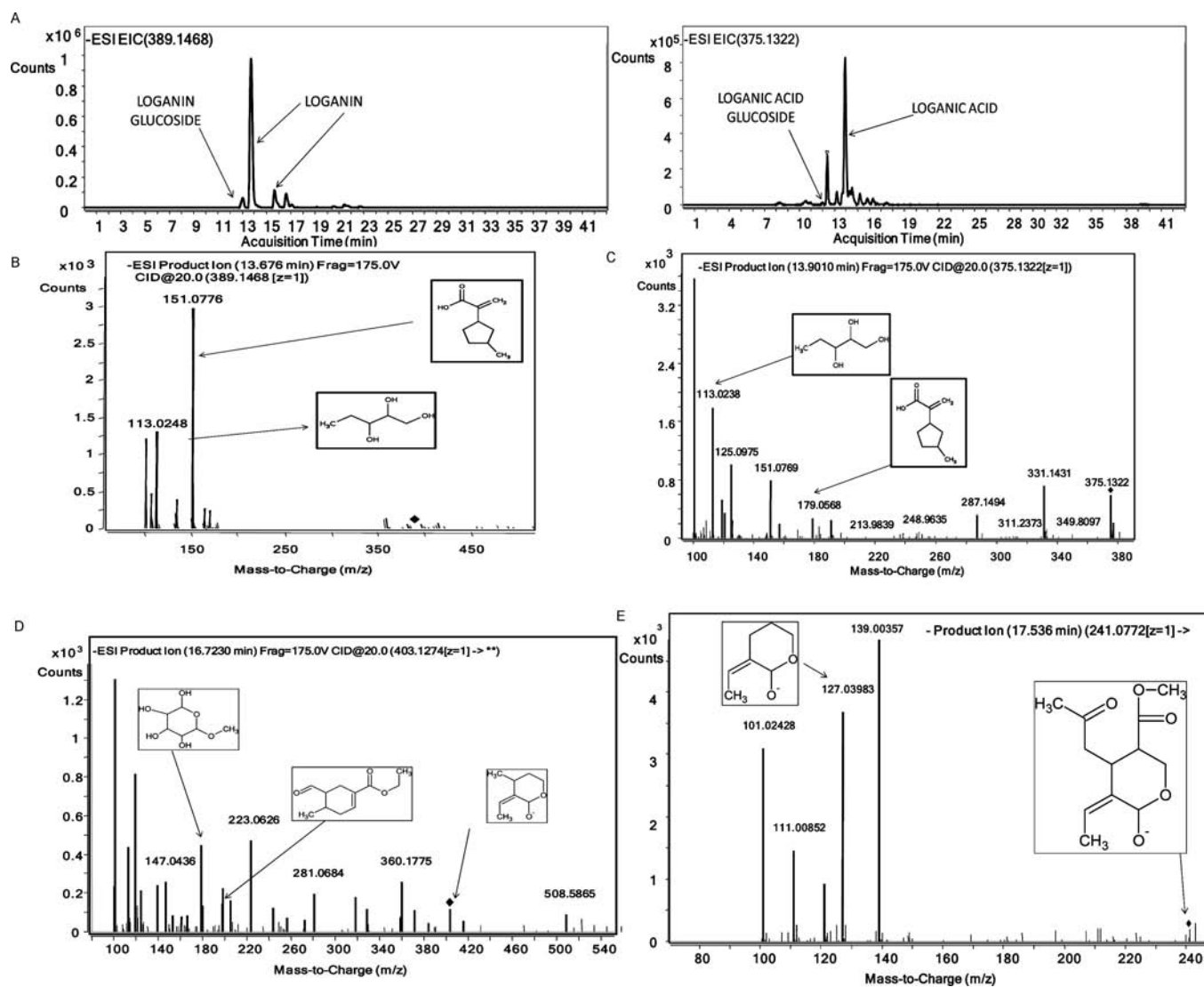
Figure 3. (A) EICs for loganin and loganic acid. (B) MS/MS spectrum for loganin. (C) MS/MS spectrum for loganic acid. (D) MS/MS spectrum for oleoside 11-methylester. (E) MS/MS spectrum for eleanolic acid.

involved in the synthesis of secoriridoids may be present in the extracts from olive pomace. Two of these precursors, loganin and loganic acid, were detected in the SHL extracts from olive pomace together with their glucoside derivatives. Figure 3A shows the EIC of loganin obtained by monitoring its precursor ion at m/z 389.1468, which revealed the presence of loganin eluting at 13.67 min and a derivative form whose precursor ion was detected at m/z 569.2113. This derivative, eluting at 13.42 min, was attributed to loganin glucoside. Tentative identification supported on mass accuracy and isotopic distribution was confirmed by tandem mass spectrometry. The EICs corresponding to loganic acid and its glucoside, at m/z 375.1322 and 537.1857, respectively, can be clearly differentiated at 13.90 and 12.00 min.

Product ions obtained at m/z 151.0776 and 113.0248 were characteristic fragments for identification of loganin and loganic acid (panels B and C of Figure 3), respectively. These fragments correspond to the loss of $C_9H_{11}O_2$ and $C_5H_8O_3$ for loganin and loganic acid, respectively. The glucoside derivatives also generated characteristic fragments at m/z 389.1513 and 375.1432 for loganin and loganic acid conjugates, respectively. These two fragments were obtained by cleavage of the glucose

unit (162 Da) (Figure S4 of the Supporting Information). It is worth mentioning that both loganin and loganic acid were preferentially detected as free forms.

Apart from these precursors, other compounds involved in the different pathways leading to the synthesis of secoiridoids were also detected. These compounds were 7-deoxyloganic acid, secologanic acid, secologanoside, and secologanin, detected at m/z 359.1368, 401.1477, 555.1795, and 387.1268, respectively. Secologanin leads to the synthesis of oleoside-11-methyl ester, which is the immediate precursor of secoiridoids. Oleoside-11-methyl ester is formed by glucosidation of eleanolic acid. Both metabolites were clearly identified in the extracts from olive pomace at retention times of 16.72 and 17.53, respectively, with precursor ions at m/z 403.1274 and 241.0772, respectively. Identification was confirmed for both compounds by MS/MS spectra, as shown in panels D and E of Figure 3. In addition to these identifications, oleoside and its mono- and diglucoside derivatives were also confirmed by MS/MS spectra, which are illustrated in Figure S5 of the Supporting Information. Oleoside was detected by a precursor ion at m/z 389.1122 eluting at 15.4610 min. The MS/MS spectrum presented characteristic fragments at m/z 345.1253 and

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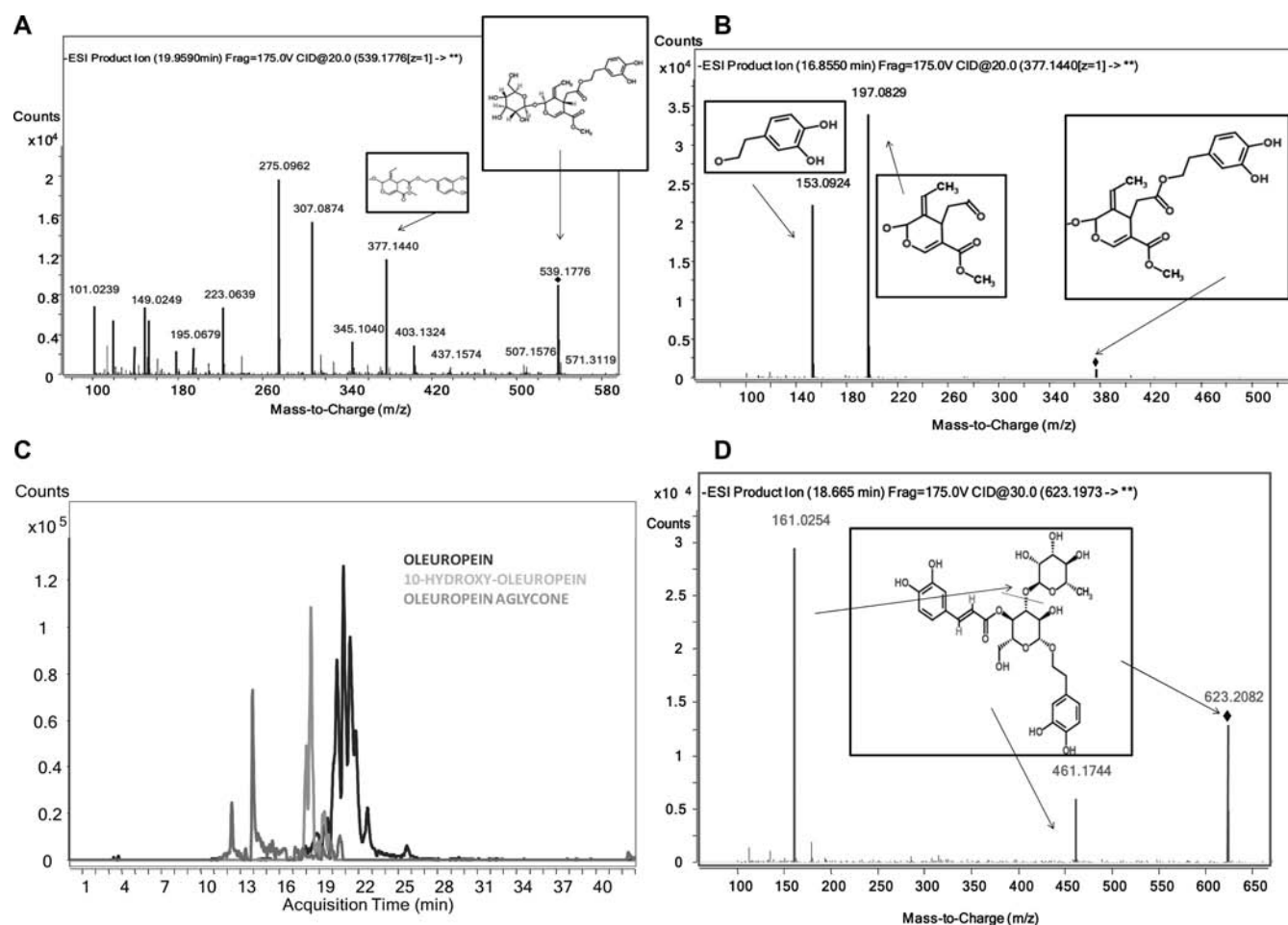
Figure 4. (A) Extracted ion chromatograms for oleuropein and oleuropein aglycone. (B) MS/MS spectrum for oleuropein. (C) MS/MS spectrum for oleuropein aglycone. (D) MS/MS spectrum for verbascoside.

209.0477. The former corresponds to the loss of 44 Da, which can be justified by decarboxylation, while the other fragment can be attributed to the loss of a hexose (180 Da).

Secoiridoids and Derivatives. Secoiridoids are a type of monoterpenes derived from geraniol. The main difference compared to iridoids is the hydrolysis of the cyclopentanopyran ring.[35] Phenolic compounds classified as secoiridoids are characterized by the presence of either eleanolic acid or eleanolic acid derivatives in their molecular structure.[36] Oleuropein, demethyloleuropein, ligstroside, and verbascoside are the most abundant secoiridoid glucosides in olive fruit.[36,37] Several aglycon derivatives of oleuropein (3,4-DHPEA-EA) and ligstroside (p-HPEA-EA) as well as their decarboxymethylated aglycone forms (3,4-DHPEA-EDA for oleuropein and p-HPEA-EDA for ligstroside) have also been found in olive pulp.[38] The concentration of these phenols in VOO and olive fruit strongly depends upon the season period but also agronomical factors.[38]

Among secoiridoids detected in extracts from olive mill waste, oleuropein was one of the most concentrated. This especially well-known compound has been described to possess interesting functional properties, including antioxidant, anti-inflammatory, antiatherogenic, anticancer, and antimicrobial activities, among others.[39] The precursor ion for oleuropein was detected at m/z 539.1776 (Figure 4A). As seen in Figure 4, the fragmentation of this ion generated product ions with m/z 377.1440 and 275.0962, characteristic of the oleuropein structure. The fragmentation scheme of oleuropein enabled

identification of these representative fragments by cleavage of the hexose unit (m/z 377.1440), while the other ion was assigned to a further fragmentation product of the oleuropein aglycone residue. Oleuropein aglycone, detected at 16.85 min (Figure 4B), was fragmented to generate two intense product ions at m/z 153.0924 (corresponding to cleaved hydroxytyrosol) and m/z 197.0829 (fit to the rest of the molecular structure after after decarboxylation). The precursor ion with m/z 319.1207 was associated to 3,4-DHPEA-EDA. The presence of the fragments with m/z 123.0826 and 139.0758, corresponding to a fragment of hydroxytyrosol and the dialdehydic subunit released after hydrolysis of hydroxytyrosol, confirmed this identification.

The BPC of the olive pomace extract showed other predominant chromatographic peaks ascribed to m/z 555.1701 and eluted at 18.31 min (Figure 4C). The main product ion in its MS/MS spectrum was obtained at m/z 537.1647 by the loss of 18 Da, suggesting the structure of a hydroxyl derivative of oleuropein, which is easily dehydrated by MS activation with nitrogen gas. Its aglycone derivative was detected at m/z 376.1114, whose precursor ion reported common product ions to the conjugated secoiridoid, which should be 10-hydroxy-oleuropein.

Two other oleuropein derivatives were detected at m/z 543.2066 and 527.2120 eluting at 19.18 and 20.50 min, respectively (Figure S6 of the Supporting Information). The MS/MS spectra for these two compounds contained ions
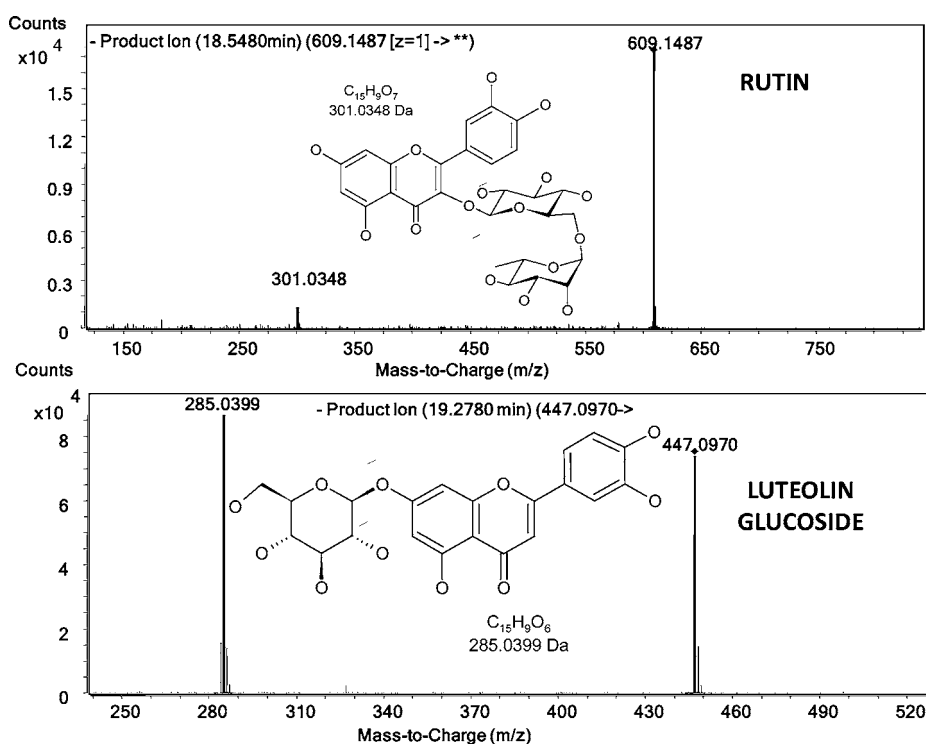
Figure 5. MS/MS spectra for rutin and apigenin-7-glucoside.

common to those provided by oleuropein, particularly the intense ions of oleuropein aglycon at m/z 377.1522, 151.0771, and 101.0240.

The precursor ion at m/z 623.1973, ascribed to verbascoside, was also detected in the olive pomace extracts (Figure 4D). Representative product ions observed in the MS/MS spectrum were in agreement with the fragmentation of this secoiridoid. Thus, the product ion at m/z 461.1744 corresponds to the loss of 162 Da, and m/z 161.0254 corroborated the detection of the glucose cleavage.

Phenols Produced by the General Phenylpropanoid Metabolism. The presence of other simple phenols, such as coumaric acid isomers, ferulic acid, vanillic acid or vanillin, in VOO has been described.[2] These compounds are synthesized in the general phenylpropanoid metabolism that involves the production of 4-coumaric acid from phenylalanine supplied by the shikimate pathway (Figure S7 of the Supporting Information). The analysis reported here revealed the presence of shikimic acid, phenylalanine, cinnamic acid, p-coumaric acid, caffeic acid, protocatechuic acid, vanillic acid, ferulic acid, and gallic acid, as shown Table 1. For example, the precursor ion at m/z 163.0401 was associated to p-coumaric acid. The MS/MS spectrum for this precursor ion presents an intense peak at m/z 119.0502 that corresponds to the loss of a CHO_2 group. This is the same fragmentation occurring to caffeic acid (m/z 179.0352) that results in a great peak at m/z 135.0448. m/z 153.0193 was identified as protocatechuic acid, which has a high peak resulting for the loss of a CHO_2 group at m/z 109.0295. Vanillic acid (m/z 167.0353) presents two high peaks at m/z 123.0778 (loss of the CHO_2 group) and m/z 108.0205 (loss of an additional OH group). Ferulic acid (m/z 193.0514) was also identified thanks to the fragment ion at m/z 134.0377, a result of the loss of the C_2H_5O and OH groups. The last one is gallic acid (m/z 169.0150), whose MS/MS spectrum presents an ion fragment at m/z 125.0246 formed by the loss of a CHO_2

group. Figure S8 of the Supporting Information shows the MS/MS spectra for the identified metabolites.

Lignans are phenylpropane dimers also biosynthesized via the phenylpropanoid pathway, in which pinoresinol lariciresinol reductase catalyzes the last steps of lignin production. The structure of lignans also endows these compounds with antioxidant properties. Three lignan metabolites (viz. pinoresinol, hydroxypinoresinol, and acetoxypinoresinol, eluted at 19, 21, and 20 min, respectively) were also detected in the extracts from olive pomace. Figure S9 of the Supporting Information shows the MS/MS spectra for pinoresinol and acetoxypinoresinol.

Flavonoids, involved in the phenylpropanoid pathway, are abundant in fruits and vegetables. The dietary intake of this phenolic family is considered important for preventing a wide variety of diseases that involve free-radical-mediated damage.[40] Most of the pharmacological effects of flavonoids seem to be associated with their potency as antioxidants.[40] The most relevant flavonoids identified in this research have been rutin, apigenin, luteolin, apigenin glucoside, luteolin glucoside, taxifolin, diosmetin, and quercetin. All tandem mass spectra of flavonoides revealed a similar fragmentation scheme. Rutin was detected at m/z 609.1487, and its MS/MS spectrum presents a peak at m/z 301 because of the loss of glucoside and rhamnoside groups. Apigenin glucoside detected by fragmentation of the precursor ion at m/z 431.0999 generated one representative fragment at m/z 269.0424 by cleavage of the hexose unit. Similarly, luteolin glucoside appeared at m/z 447.0970 and generated a high peak at m/z 285.0422 because of the loss of a hexose unit, as shown in Figure 5, while the fragmentation of aglycone forms of apigenin and luteolin can be deduced from the spectra illustrated in Figure S10 of the Supporting Information.

Attending to the profile and content of phenolic compounds in olive pomace, this residue is an exploitable source for

isolation of phenols. After this step, the residue could be degraded more easily because the antibacterial properties of phenols have been minimized. This study complements previous studies on olive leaves and other mill residues.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

MS/MS spectra for hydroxytyrosol diglucoside and hydroxytyrosol dirhamnoside (Figure S1), MS/MS spectra for tyrosol and tyrosol glucoside (Figure S2), iridoid biosynthetic pathway (Figure S3), MS/MS spectra for loganin glucoside and loganic acid glucoside (Figure S4), MS/MS spectra for oleoside, oleoside glucoside, and oleoside diglucoside (Figure S5), MS/MS spectra for oleuropein derivatives (Figure S6), phenylpropanoid biosynthetic pathway (Figure S7), MS/MS spectra for phenolic acids (Figure S8), MS/MS spectra for pinoresinol and acetoxypinoresinol (Figure S9), and MS/MS spectrum for luteolin (Figure S10). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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