JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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Isolation and Identification of Phenolic Glucosides from Thermally Treated Olive Oil Byproducts

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ABSTRACT: A liquid phase rich in bioactive compounds, such as phenols and sugars, is obtained from olive oil waste by novel thermal treatment. Two groups of fractions with common characteristics were obtained and studied after thermal treatment, acid hydrolysis, and separation by ultrafiltration, chromatography, and finally Superdex Peptide HR. In the first group, which eluted at the same time as oligosaccharides with a low DP (4−2), an oleosidic secoiridoid structure conjugated to a phenolic compound (hydroxytyrosol) was identified as oleuropeinic acid, and three possible structures were detected. In the second group, glucosyl structures formed by hydroxytyrosol and one, two, or three units of glucose or by tyrosol and glucose have been proposed. Verbascoside, a heterosidic ester of caffeic acid, in which hydroxytyrosol is linked to rhamnose−glucose or one of its isomers was also identified. Neutral oligosaccharides bound to a phenol-containing compound could be antioxidant-soluble fibers with bioactive properties.

KEYWORDS: *alperujo, phenol, steam treatment, oligosaccharides, secoiridoids, hydroxytyrosol*

INTRODUCTION

Olive oil production is a very important and traditional activity in the countries of the Mediterranean area, which account for 95% of worldwide olive oil production.[1] The manufacturing process of olive oil generates oil and a byproduct that is a combination of liquid and solid waste, called "alperujo" or "two-phase olive mill waste". This byproduct is a high-humidity residue with the consistency of thick sludge that contains 80% of the olive fruit, including the skin, seed, pulp, and pieces of stones, which is later separated and usually used as solid fuel.[2] In Spain, the annual production of this byproduct is approximately 2.5−6 million tons, depending on the season. Alperujo has many inconvenient and environmental problems due its high organic content and the presence of phytotoxic components, which makes it difficult to use in further bioprocesses.[3] Most of these components, mainly the phenolic compounds, confer the bioactive properties of olive oil; however, during olive oil processing, most of these compounds remain in the alperujo, making it a promising source for substances with high value.[4] Therefore, new strategies are emerging for the utilization of this byproduct, but the main challenges are separating its phases by pretreatments, eliminating the phytotoxic components for bioprocess applications and for bioactive molecule production.

One of the more attractive processes employs thermal pretreatments that allow for recovery of the bioactive compounds and valuable fractions, making it possible to utilize alperujo.[5] Recently, we have developed a simple new process, adaptable to the pomace-processing mill and refining olive oil industry, which allows for easy separation of the solid and liquid phases. In this new, patented[6] steam treatment, an autohydrolytic process occurs and solubilizes part of the alperujo. It also allows the recovery of added-value compounds in the water-soluble fraction, to create a fraction rich in interesting phenols (hydroxytyrosol, 3,4-dihydroxyphenylglycol), sugars, and oligosaccharides,[7,8] leaving behind a solid residue enriched in residual oil with an increased concentration of minor component (sterols, triterpenic acids and alcohols, squalene, and tocopherols) with functional activities.[9]

The cell wall material of olive pulp is mainly composed of cellulose, pectic polysaccharides rich in arabinose, xylans, glucuronoxylans, and xyloglucans, whereas mannans, glycoproteins, enzymes, and phenols occur as minor components.[10−14] The analysis of the phenolic compounds in the olive cell wall mainly showed the presence of *p*-coumaric acid (95%) and small amounts of vanillic acid and *p*-hydroxybenzaldehyde as well as traces of vanillin, protocatechuic acid, and protocatechuic aldehyde. Hydroxytyrosol, tyrosol, or secoiridoid derivatives are very abundant in olive pulp but were not identified in the olive cell wall.[14] However, it has been reported that interaction between polysaccharides and the hydrophilic compounds present in the olive paste may be involved in the loss of the phenolic compounds from the oil during crushing and malaxation.[15] Thermal treatment can provide a large number of bioactive compounds when applied for a short time, including oligosaccharides, that would promote the growth of beneficial bacteria in the large bowel,[16] and phenolics associated with polysaccharides, with potential activities in the food or nutraceutical industry. The production and use of this type of oligosaccharides could enhance the economical viability of the integral process to thermally treat the alperujo.

In this paper, we have characterized the oligosaccharide compounds released from alperujo steam-treated in a bath pilot reactor (100 L capacity) following mild acid hydrolysis according to a previous published procedure.[8] After the

Received: May 22, 2012
Revised: January 11, 2013
Accepted: January 16, 2013
Published: January 16, 2013

Figure 1. Overall scheme of the fractionation process performed to obtain oligosaccharides with a low degree of polymerization.

pretreatments, the sugar fraction was fractionated and isolated, determining a new compound with a common secoiridoid core linked to neutral sugars or a group of compounds with mono- and disaccharides linked to phenolic compounds.

MATERIALS AND METHODS

Raw Material. Olive pomace alperujo (semisolid residue composed of peel, pulp, ground stone, and olive seed) sample was collected directly from the local pomace-processing mill (Oleícola el Tejar, Córdoba, Spain) after a certain storage period and before it was extracted for pomace olive oil. The moisture contents, stone percentages, and oil contents (dry weight) were determined. Alperujo was taken in January, at the halfway mark of the olive oil production season (2009−2010).

Thermal Treatments or Steam Processing of Alperujo. Alperujo was processed in a pilot-scale reactor. The hydrothermal treatment is patented,[6] and it was performed using a steam treatment reactor prototype designed by our research group at the Instituto de la Grasa (Seville, Spain). The reactor has a 100 L capacity stainless steel reservoir that can operate at temperatures between 50 and 190 °C and at a maximum pressure of 1.2 MPa.

The alperujo samples (20 kg) were treated with saturated steam for 15 min at 170 °C. After closing the steam inlet valve, the pressure was blended down to atmospheric at a controlled rate. Then, the wet material was centrifuged at 4700*g* (Comteifa, S.L., Barcelona, Spain) to separate the solids and liquids.

At the end of treatment, an aliquot of the liquid phase (4 L) was concentrated to 1 L by rotary evaporation at 37 °C in a vacuum, and five samples of 200 mL were extracted with an equal volume of ethyl acetate (refluxed at 77 °C) for 5−6 h in a continuous extraction from the heavier liquid (water) to the lighter one (ethyl acetate) to separate the phenolic from the carbohydrate components. The aqueous solution was concentrated and lyophilized.

Adsorption XAD Chromatography. Fractionation and purification of the neutral and pectic oligosaccharides were achieved by adsorption onto an Amberlite XAD-16 resin (Figure 1). Previously, to increase the proportion of oligosaccharides of low molecular weight in the initial lyophilized liquid, a mild chemical hydrolysis with 0.5 N HCl was carried out.[8] Then, 25 mL of the hydrolyzed samples was injected onto a column (35 × 3.0 cm) of Amberlite XAD-16 and eluted at 1.5 mL/min with water (200 mL) and 20% (v/v) methanol/water (200 mL).

The carbohydrates and uronic acid in each eluted fraction were assayed with the anthrone−sulfuric acid colorimetric assay[17] and the *m*-hydroxybiphenyl method,[18] respectively.

Ultrafiltration. Both fractions eluted from Amberlite XAD-16 resin with water and methanol/water were subjected to ultrafiltration through a fiber unit. All volume (200 mL) was ultrafiltered at room temperature using an Amicon 8400 stirred cell (Millipore Corp., Bedford, MA, USA) through a 1000 Da molecular weight cutoff

Table 1. Sugar Composition of Fractions B, C, D, and E Obtained by Elution with Water and Methanol 20% from XAD Chromatography and Ultrafiltration[a]

sugar	B (1000−3000 Da)	C (<1000 Da)	D (1000−3000 Da)	E (<1000 Da)
rhamnose	3.8 ± 0.1[b]	1.7 ± 0.2	3.6 ± 0.3	0.9 ± 0.1
fucose	nd[c]	0.5 ± 0.1	nd	nd
arabinose	8.3 ± 0.5	13.4 ± 0.3	6.1 ± 0.5	2.0 ± 0.2
xylose	4.5 ± 0.2	3.5 ± 0.1	9.8 ± 0.8	3.4 ± 0.1
mannose	17.2 ± 1.5	19.9 ± 1.4	9.9 ± 0.9	8.5 ± 0.8
galactose	10.7 ± 0.7	3.0 ± 0.3	6.6 ± 0.6	1.0 ± 0.1
glucose	55.5 ± 1.7	58.1 ± 2.7	64.0 ± 6.3	84.3 ± 8.3
	6.8[d]	10.5	10.7	12.0

[a]The composition is determined by gas chromatography as alditol acetates. The results are expressed in percentage with respect to the total content. [b]Standard deviation (n = 3). [c]nd, not detected. [d]Percentage of oligosaccharides in each fraction.

membrane (cellulose regenerated). The solutions were washed with water at 35 °C until 500 mL of permeate was collected.

Quantification of Individual Neutral Sugars by Gas Chromatography (CG) after Their Conversion to Alditol Acetates and Analysis of Oligosaccharides. Individual neutral sugars were analyzed from duplicate samples with and without initial TFA hydrolysis (2 N TFA at 121 °C for 1 h) prior to reduction, acetylation, and analysis by GC.[19] Inositol was used as an internal standard. A Hewlett-Packard 5890 series II chromatograph, fitted with a 30 m × 0.25 mm fused silica capillary column (SP-2330 from Supelco, Bellefonte, PA, USA), was employed. The oven temperature program used was as follows: initial, 180 °C, 7 min, raised at 3 °C/min to 220 °C, 15 min. The carrier gas was helium, and it had a flow rate of 1 mL/min. The injector temperature was 250 °C, and the FID temperature was 300 °C. The split ratio was 1/100.

Because oligosaccharides were converted into monosaccharides during the posthydrolysis treatment, the increase in the concentration of monosaccharides caused by posthydrolysis provided a measurement of the concentration of oligosaccharides. All determinations were made in triplicate.

Size Exclusion Chromatography. After separation of the monosaccharides and fractionation of the oligosaccharides by adsorption chromatography, fractions A, B, C, D, and E (Figure 1) were fractionated by size exclusion chromatography on a two-column Superdex Peptide HR 10/30 (30 × 1 cm) (Pharmacia Biotech, Uppsala, Sweden) connected in line to a Jasco LC-Net II/ADC HPLC (Easton, MD, USA). Samples (100 μL) containing 10 mg of oligosaccharides/mL were applied several times onto the column, and they were eluted with 100 mM ammonium acetate buffer (pH 5) at a flow rate of 0.5 mL/min. The peaks were monitored with a Jasco MD-1550 diode array detector (DAD) and a Jasco RI-1530 refraction index detector. Column calibration was performed with a variety of standards; galacturonic acid and trigalacturonic acid (Fluka) were used as standards for the pectic oligosaccharides, and maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose (Sigma) and cellobiose, cellotriose, cellotetraose, cellopentaose, and cellohexaose (Megazyme) were used as standards for the neutral oligosaccharides.

Electrospray Ionization Mass Spectrometry. Compounds collected from the Superdex Peptide column fractions were identified by their mass data collected on a quadrupole mass analyzer (ZMD4, Micromass, Waters Inc., Manchester, UK). Electrospray ionization (ESI) mass spectra were obtained at ionization energies of 50 and 100 eV in negative mode and 50 eV in positive mode. The capillary voltage was 3 kV, the desolvation temperature was 200 °C, the source temperature was 100 °C, and the extractor voltage was 12 V. The flow was maintained at 1 mL/min and in splitless mode (UV detector MS) for each analysis.

A Teknokroma Tracer Extrasil ODS-2 column (250 mm × 4.6 mm, i.d. of 5 μm) was used. The mobile phase was 0.01% trichloroacetic acid in water and acetonitrile utilizing the following gradient over a total run time of 55 min: 95% A initially, 75% A at 30 min, 50% A at 45 min, 0% A at 47 min, 75% A at 50 min, and 95% at 52 min until the run was completed. For MS/MS experiments, the negative ESI tandem mass spectra were obtained on a QTRAP (AB Sciex, Framingham, MA, USA) hybrid tandem mass spectrometer equipped with a nanospray ionization source. The analyses were carried out using a voltage of −4500 V and a collision energy of −30 V. The tandem mass spectra were obtained using Ar as the collision gas.

NMR Spectroscopy. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AMX300 spectrometer operating at 300 and 75 MHz, respectively. Samples collected from Superdex Peptide column fractions at 62−68 min, which coincided with the standard oligosaccharides with DP from 4 to 2 in the calibration sample (first group) and fraction eluted with the monosaccharides standard, after solvent (negative peak) and other eluted after solvent (second group), were examined in acetylated form in CD_3OD and $CDCl_3$ respectively. Chemical shifts (δ) are expressed in parts per million (ppm) values relative to the central solvent peak tetramethylsilane (TMS), $(CH_3)_4Si$ (δ = 0.00 ppm). The experiments were performed at 25 °C.

Acetylation. Fractions were evaporated to dryness, and 20 mg was dissolved in pyridine (2 mL) and cooled in ice bath. Acetic anhydride (2 mL) was added to this solution, and the mixture was allowed to stand overnight at 4 °C. The resulting solution was evaporated to dryness and coevaporated four times with water (2 mL) and toluene (2 mL) to remove the pyridine. The solid was dissolved in 0.5 mL of water.

Sample Preparation. The acetylated samples (0.5 mL) were lyophilized and dissolved in isotopically enriched D_2O and lyophilized to replace exchangeable proton with deuterons. The residue was dissolved in methanol-*d* and chloroform-*d* and transferred to a 5 mm NMR tube.

RESULTS AND DISCUSSION

Extraction and Isolation of Sugars Linked to Phenolics from Alperujo. The conditions for the steam treatment of alperujo and the subsequent mild acid hydrolysis of the solubilized products were described in a previous paper.[8] The overall fractionation procedure is presented in Figure 1.

Four fractions (B, C, D, and E) were obtained after chromatography on Amberlite XAD-16 resin and after ultrafiltration through a 1000 Da molecular weight cutoff membrane. The fractions obtained by elution with H_2O, fractions B and C, were rich in glucose and mannose, and those obtained by elution with 20% MeOH, fractions D and E, contained the highest amount of linked sugars, composed mostly of glucose, xylose, and mannose (Table 1).

These four fractions were further separated through a gel filtration column on Superdex Peptide (Figures 2 and 3) on the basis of their molecular weight, although this resin also

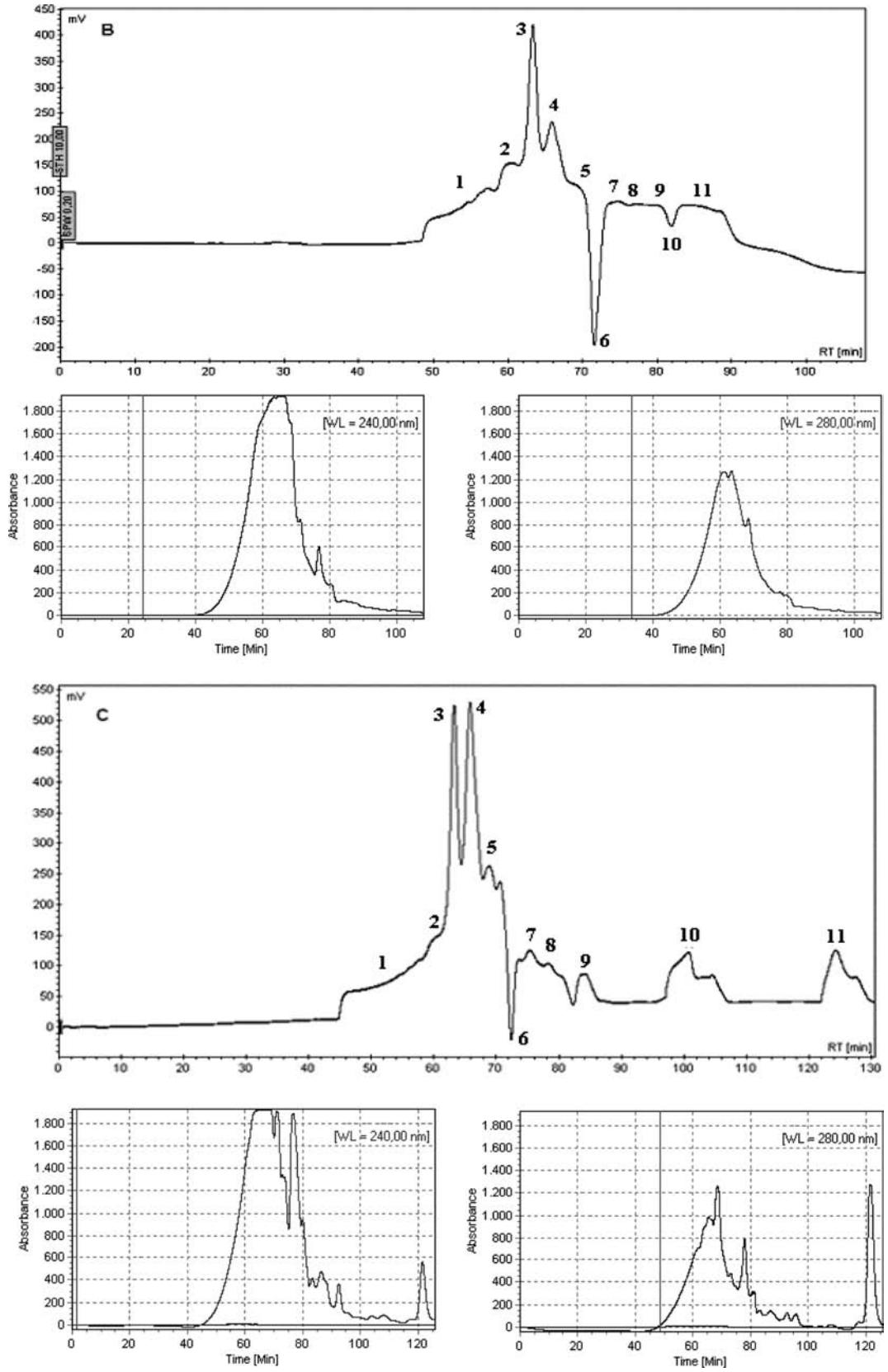

Figure 2. Refractive index elution profiles obtained by size exclusion chromatography with Superdex Peptide of the oligosaccharides obtained in fractions B and C and their ultraviolet absorption chromatograms registered at 240 and 280 nm. The numbers represent each fraction collected.

separates molecules on the basis of their interaction with phenols. Fractions corresponding to peaks of refractive index showed a high UV absorbance. Some of them eluted at the same time as the solvent or after the standard monosaccharide samples. Each of the fractions was pooled and analyzed for its sugar content (Table 2).

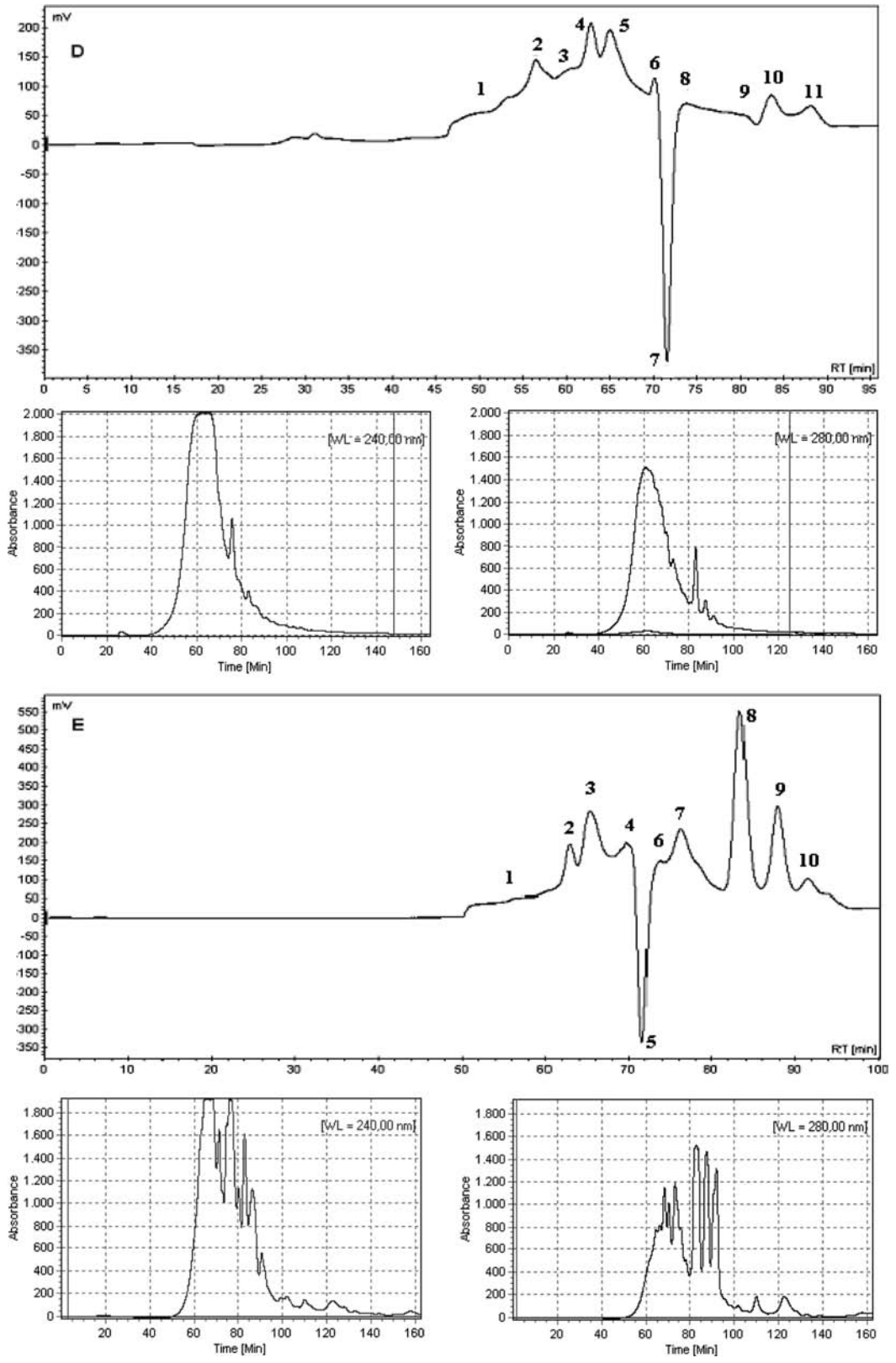

Figure 3. Refractive index elution profiles obtained by size exclusion chromatography with Superdex Peptide of oligosaccharides obtained in fractions D and E and their ultraviolet absorption chromatograms registered at 240 and 280 nm. The numbers represent each fraction collected.

The fractions that eluted at retention times between 48 and 62 min are rich in neutral oligosaccharides and have a low UV absorbance. These fractions correspond to molecules with a degree of polymerization (DP) from 9 to 5 and were previously studied.[8] In this paper, we have studied two other groups of fractions with common characteristics. One of them, which included fractions B2, B3, B4, B5, C3, C4, C5, D4, D5, E2, E3, and E4 (Figures 2 and 3), eluted at retention time in size

dx.doi.org/10.1021/jf303772p | J. Agric. Food Chem. 2013, 61, 1235−1248

Table 2. Composition of the Oligomers of Subfractions Separated by Size Exclusion or Gel Filtration Chromatography from Fractions B, C, D, and E[a]

no.	Rt[b] (min)	DP[c]	rhamnose	fucose	arabinose	xylose	mannose	galactose	glucose
					Fraction B				
3	63	3	nd	nd	3.6 ± 0.1[d]	nd	46.0 ± 2.1	nd	50.3 ± 4.1
4	66	2	nd	nd	nd	nd	38.5 ± 1.8	nd	61.5 ± 3.4
5	69	1	nd	nd	nd	nd		nd	100.0 ± 5.1
					Fraction C				
3	63	3	1.4 ± 0.2	nd	8.7 ± 0.4	2.5	30.6 ± 2.0	1.2 ± 0.1	55.6 ± 2.4
4	66	2	0.5 ± 0.1	nd	7.9 ± 0.3	2.9	33.5 ± 1.2	nd	55.1 ± 1.9
5	68	2−1	1.2 ± 0.1	nd	13.5 ± 0.8	3.2	31 ± 0.9	nd	51.1 ± 2.7
					Fraction D				
4	63	3	2.9 ± 0.2	nd	4.4 ± 0.2	3.2	11.6 ± 0.6	nd	77.9 ± 4.3
5	65	2	2.3 ± 0.1	nd	nd	nd	24.0 ± 1.3	nd	73.7 ± 3.2
6	70	1	nd	nd	nd	nd	26.2 ± 1.7	nd	73.8 ± 1.5
					Fraction E				
2	63	3	nd	nd	7.1 ± 0.5	6.7	11.7 ± 0.5	nd	74.5 ± 3.3
3	65	2	nd	nd	nd	3.5	28.5 ± 0.4	nd	68.0 ± 5.2
4	69	1	nd	nd	3.9 ± 0.3	2.8	33.9 ± 2.6	nd	59.5 ± 3.7

[a]The composition is determined by gas chromatography as alditol acetates. The results are expressed in percentage with respect to the total content. [b]Retention time on HPLC chromatograms. [c]DP values were assigned by comparison with glucose (Rt = 69.5 min) and compounds of a series of cellobiose, cellotriose, cellotetraose, cellopentaose, and cellohexose oligosaccharides (Rt = 66.5, 63.5, 61.1, 59.0, and 56.2 min, respectively). [d]Standard deviation (n = 3).

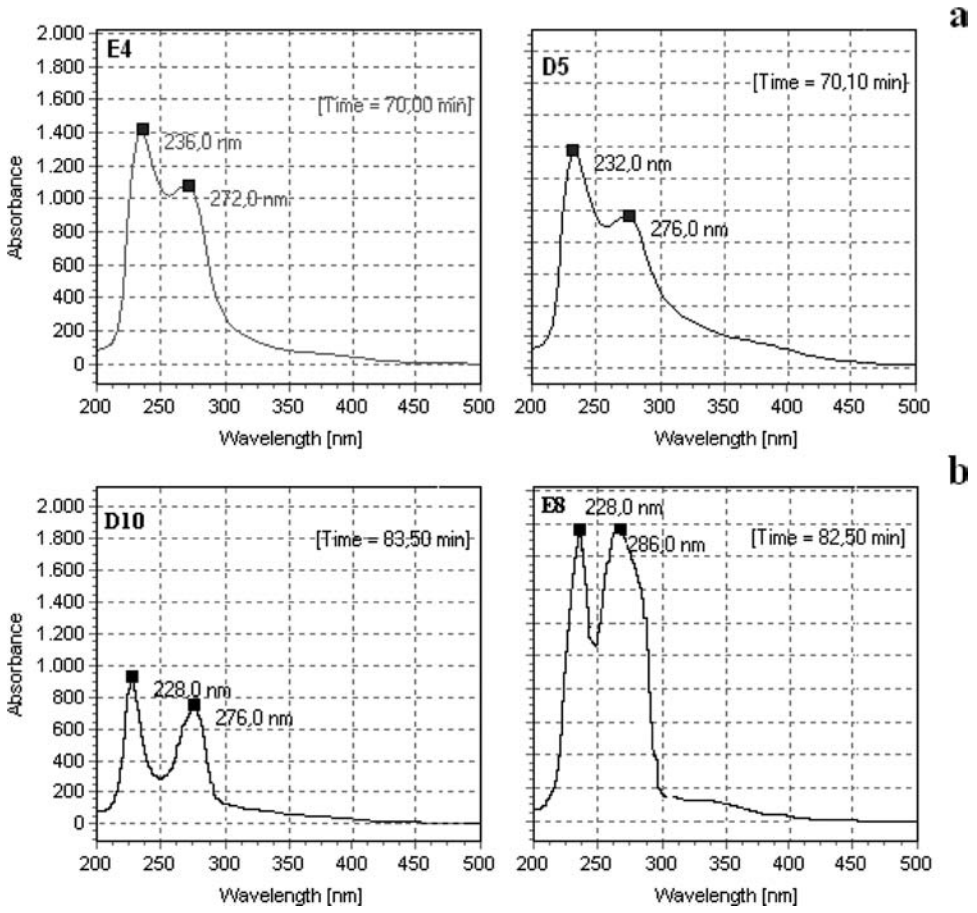

Figure 4. Ultraviolet absorption spectra for (a) fractions E4 and D5 (representative of the first group) and (b) fractions D10 and E8 (representative of the second group). The first group of fractions has an absorption maximum near 240 nm and a shoulder at 268−274 nm. In contrast, the second group of fractions has two absorption maxima, one near 280 nm and a second at 228 nm.

exclusion molecular chromatography from 62 to 68 min, next to standard oligosaccharides with DP from 4 to 2. They had similar absorption spectra with a maximum absorbance near 240 nm and a slight shoulder at 268−274 nm (Figure 4a), signals indicative of secoiridoid glycosides esterified to phenolic compounds.[20,21]

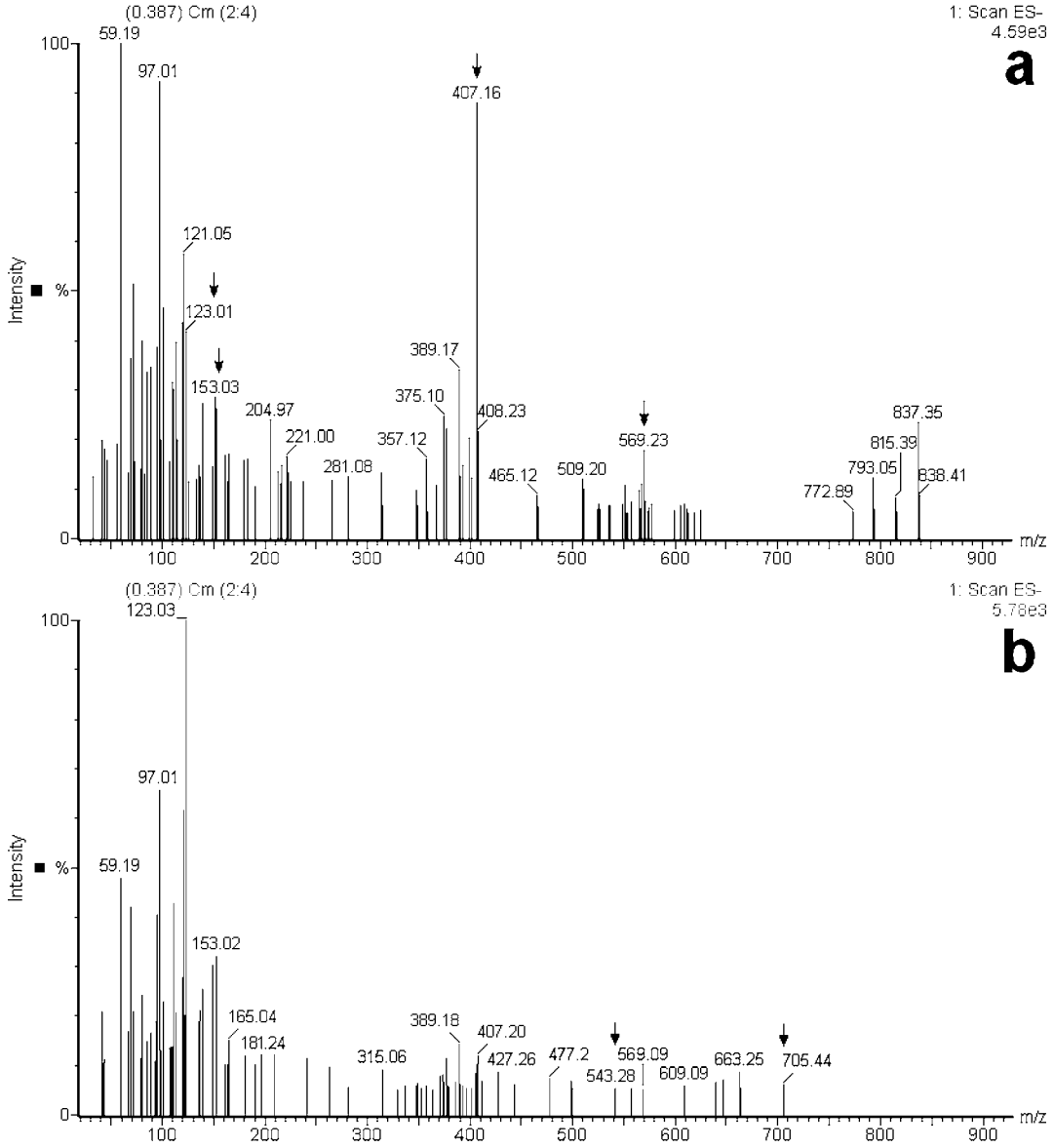

Figure 5. Mass spectra in full scan negative mode of some of the fractions from the first group: (a) characteristic ions of all fractions are observed at m/z 569, 407, 153, and 123 (indicated by the arrows); (b) some fractions contain peaks at m/z 705 and 543.

The second group of fractions included fractions D6, D7, and E5 with a retention time from the Superdex Peptide that coincided with the monosaccharide standard in the calibration sample or with the solvent (negative peak). The second group also included fractions D8, D9, D10, D11, E6, E7, E8, E9, and E10, which eluted after the solvent because of an interaction with the gel. Their absorption spectra differed from the previous group because they showed maximum absorbencies near 280 and 228 nm (Figure 4b).

Characterization of the Two Groups of Fractions. Analysis of the fractions in the first group by electrospray ionization mass spectrometry (ESI-MS) gave a similar pattern of fragmentation, in both positive and negative modes, which suggests they all have a common structure. The mass spectra (Figure 5a) show a predominant ion at m/z 407 that likely corresponds to a deprotonated molecule $[M - H]^-$, and the corresponding sodium $[M + Na]^+$ adduct at m/z 431 was observed in the positive mode (data not shown). Although these ions were previously found in olive fruit,[20,22] their structure is unknown or different from our suggested structure like more polar compounds such as oleosides.[20]

Other species at m/z 153 and 123 that have characteristics of the phenolic hydroxytyrosol[23] were present in all of the mass spectra in negative mode for each fraction. However, the appearance of the peak at m/z 569 was found only in some fractions and is consistent with the existence of one hexose unit, as suggested by the loss of 162 u from m/z 569, and an aglycone unit, as proved by the ions observed at m/z 407. These data together with the absorbance maximum near 240 nm are characteristic of secoiridoid, and the knowledge that olive fruit contains oleuropein (MW = 540) and 10-hydroxyoleuropein (MW = 556)[24,25] suggests that the molecule under investigation might correspond to a glucosyl secoiridoid bearing a phenol unit.

Additionally, peaks at m/z 705 and 543 appear in some of the fractions (Figure 5b). The latter may be due to the loss of hexose (−162 u), and the former could correspond to the presence of a second hydroxytyrosol moiety (+137 u); the

dx.doi.org/10.1021/jf303772p | J. Agric. Food Chem. 2013, 61, 1235−1248

more intense peak at m/z 123 indicates this second phenolic unit. The three proposed structures are shown in Figure 6. The

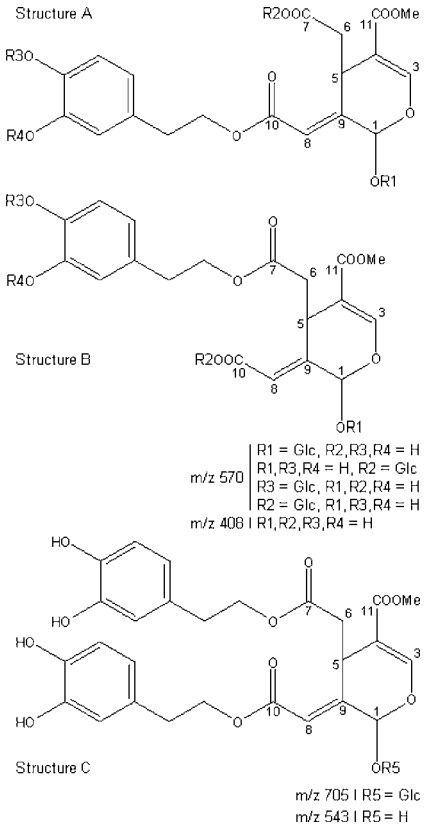

Figure 6. Three proposed structures of the detected compounds in the fractions from the first group. Each contains an oleosidic secoiridoid structure conjugated to the phenolic compound hydroxytyrosol. The secoiridoid ring numeration is also shown.

existence of more than one unit of hexose (these fractions are rich in glucose and mannose), which might be linked to the secoiridoid core (R1) or to the aromatic hydroxyl function of the hydroxytyrosol moiety (R2, R3, and R4) could explain the different behaviors in XAD and gel filtration chromatography. None of the structures proposed (A, B, or C) has been isolated or detected before in olive pulp. However, the molecule called oleuropeinic acid, shown as structure B (R1 = Glc; R2, R3, R4 = H), has been described by Kikuchi and Yamauchi[26] and was found in *Ligustrum japonicum*, which belongs to the Oleaceae family. Its structure was confirmed by ^{1}H and ^{13}C NMR spectroscopy, and its scheme of fragmentation has been proposed in Figure 7. The oleuropeinic acid proposed could be present initially in olive tissues or formed by the thermal treatment, possibly by the oxidation of oleuropein. The presence of this compound in olive tissues could be supported by its presence in other Oleaceae plants and by Menéndez et al.,[22] who have detected, but not identified, the ion at m/z 407 in lipophilic fractions (olive oil). These fractions are rich in oleuropein aglycon and ligstroside aglycon, which have polarity and structure similar to those of the oleuropeinic acid aglycon.

The ^{13}C NMR spectrum of acetylated fraction E2 (Figure 8) exhibited the common features of oleuropeinic acid because the spectroscopic data were consistent with the location of hydroxytyrosol, secoiridoid fraction, and glucose (Table 3). Assignment of the ^{1}H and ^{13}C NMR spectra (Figure 8) was not completely achieved. However, many of the resonances in the spectra were similar to those previously published for oleuropeinic acid.[26]

Analysis of the fraction of the second group, containing fractions D6, D7, D8, D9, E5, E6, and E7, by mass spectrometry gave an $[M + Na]^+$ ion at m/z 501 and the corresponding deprotonated molecule, $[M - H]^-$ at m/z 477, in negative mode (Figure 9). The existence of two hexose units was suggested by the consecutive loss of 162 u from m/z 477,

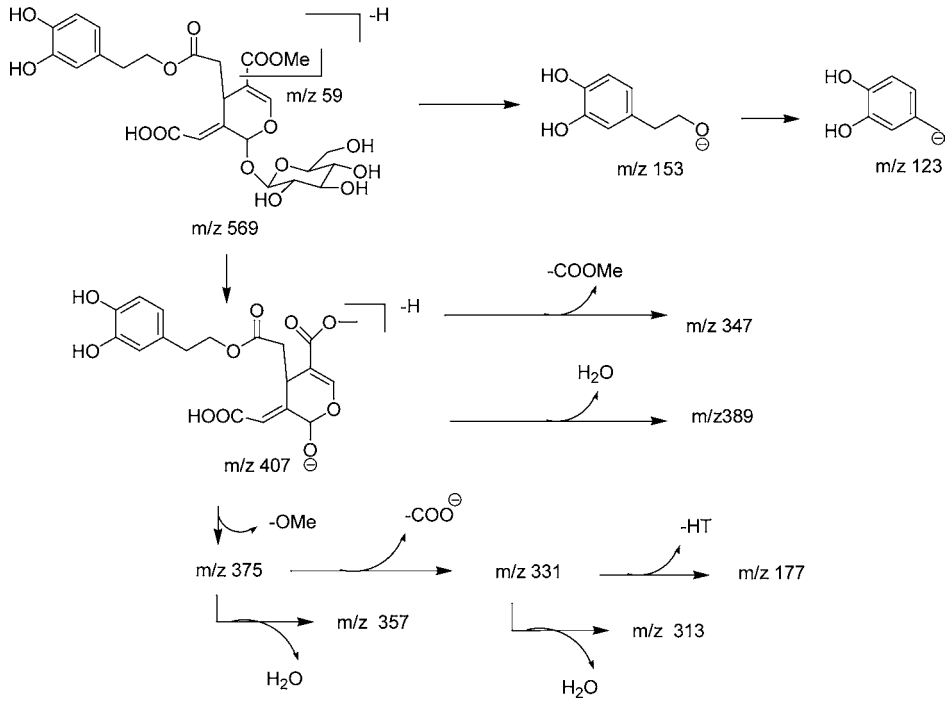

Figure 7. Scheme of fragmentation proposed for the oleuropeinic acid. The signals at m/z 389, 375, 357, 313, and 177 were observed as the MS/MS product spectrum from the ion at m/z at 407 (data not shown).

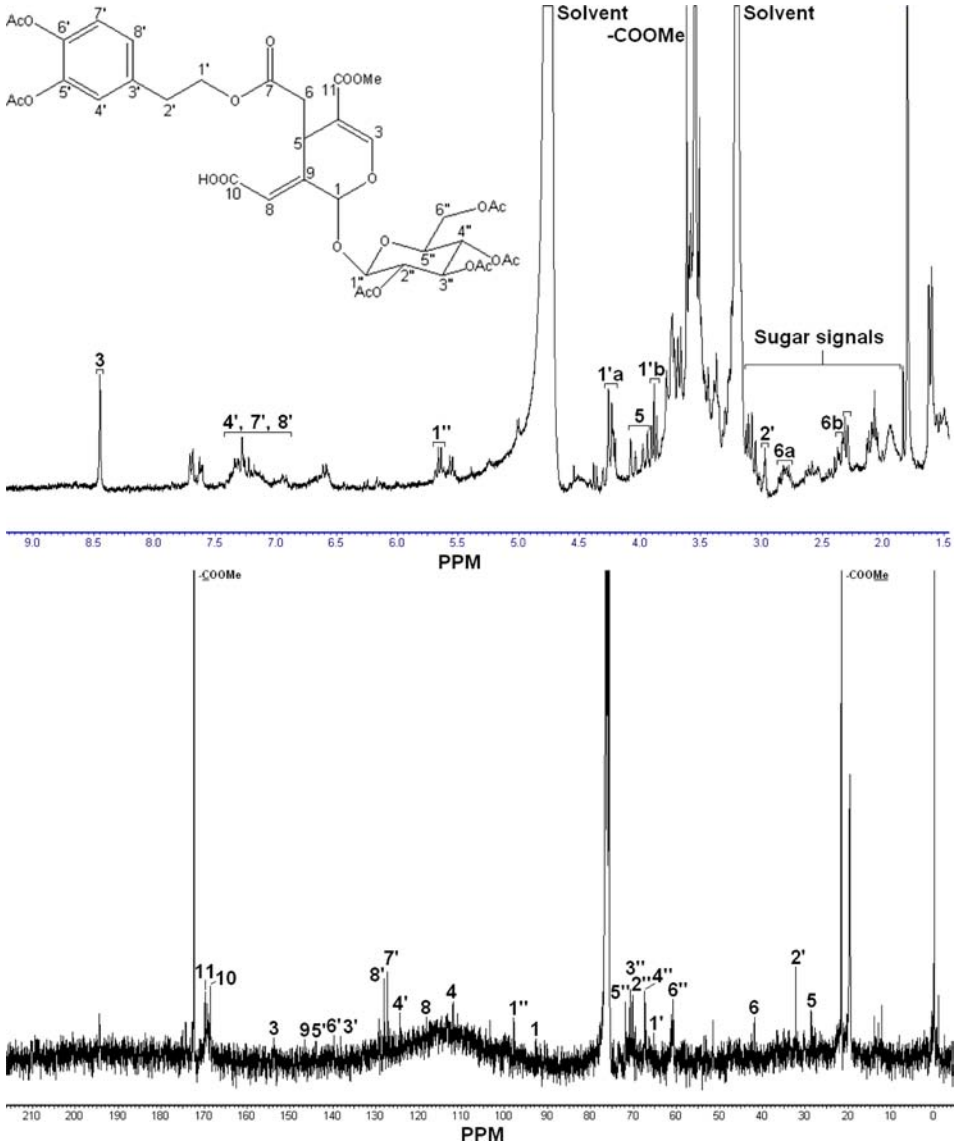

Figure 8. ^{1}H (upper) and ^{13}C (lower) NMR spectra (registered at 300 and 75 MHz, respectively) obtained for fraction E2. Most of the signals are consistent with the structure of oleuropeinic acid.

Table 3. Chemical Shift Data of ^{1}H and ^{13}C NMR Spectra for Fraction E2

	hydroxytyrosol			secoiridoid fraction			glucose	
position	^{1}H	^{13}C	position	^{1}H	^{13}C	position	^{1}H	^{13}C
1′a	4.20[a] (t)[b]	65.1[c]	1		93.1	1″	5.57 (d)	98.7
1′b	3.98 (t)					2″	2−3.2 (m)	70.5
2′	2.95 (t)	33.2	3	8.45 (s)	153.8	3″	2−3.2 (m)	71.4
3′		137.4	4		111.0	4″	2−3.2 (m)	66.6
4′	6.80−7.20 (nr)	124.2	5	4.08 (dd)	28.5	5″	2−3.2 (m)	74.5
5′		143.9	6a	2.81 (nr)	41.9	6″	2−3.2 (m)	61.2
6′		140.1	6b	2.40 (dd)				
7′	6.80−7.20 (nr)	127.0	7		174.1			
8′	6.80−7.20 (nr)	127.8	8		118.1			
			9		146.6			
			10		168.4			
			11	3.65 (s)	170.1			

[a]Chemical shifts at 25 °C are expressed in ppm relative to TMS = 0 in CD_3OD. [b]Multiplicity type is given with parentheses: d, doublet; dd, double doublet; m, multiplet; nr, not resolved; s, singlet; t, triplet. [c]Chemical shifts at 25 °C are relative to TMS = 0 in $CDCl_3$.

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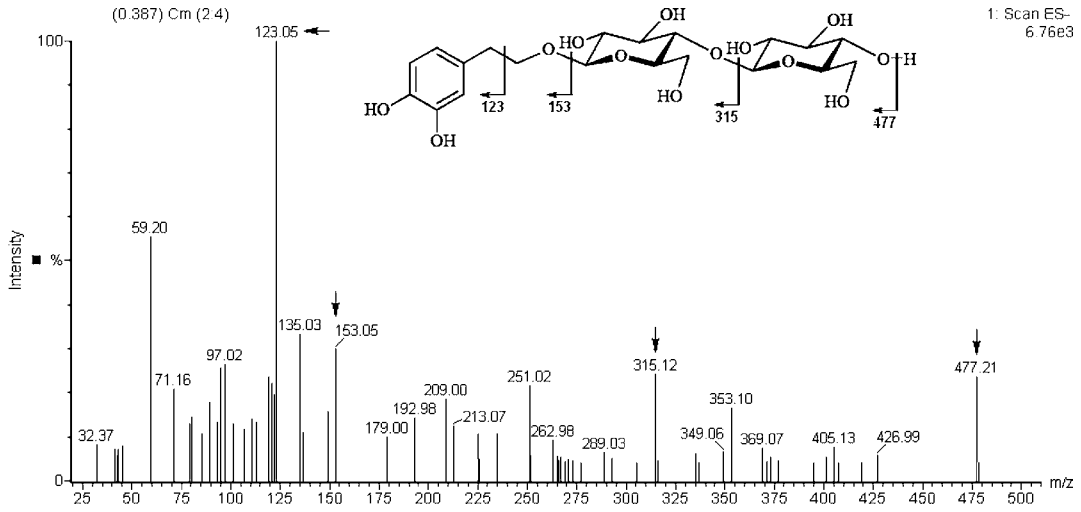

Figure 9. Typical negative mode mass spectrum obtained from fractions of the second group. A proposed diglucosyl structure is depicted in the figure and shows the molecule fragmentation leading to the main fragment ions at *m*/*z* 123, 153, 315, and 477.

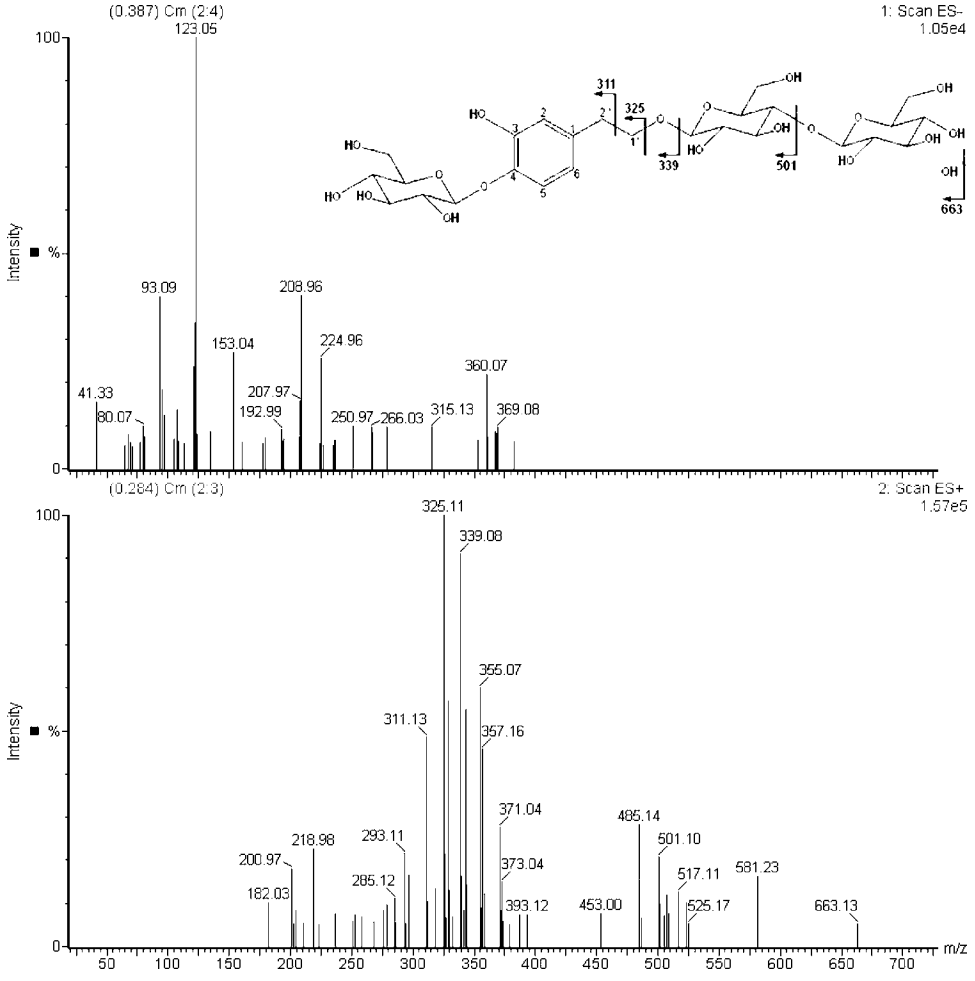

Figure 10. Mass spectra in negative (upper) and positive (lower) ion mode obtained for fraction E8. (Upper) Proposed structure according to the profile of fragmentation observed (all ions represented are sodium adducts).

as observed by the ions at *m*/*z* 315 and 153. The peak at *m*/*z* 123 was indicative of the presence of a hydroxytyrosol moiety, which might be linked to the glucose units through the aliphatic or aromatic hydroxyl functionality, giving rise to the glucoside. One of the proposed diglucosyl structures is shown in Figure 9. To our knowledge, the presence of a 4-diglucoside attached to hydroxytyrosol has only been identified by Vázquez-Roncero et al.[27] in olive fruit by dimensional paper chromatography and spectrophotometric methods. However, Di Donna et al.[28] and Cardoso et al.[29] more recently reported on an oleuropein carrying a diglucoside or a triglucoside moiety on the catechol ring of the olive tissues, which could be the precursor of the

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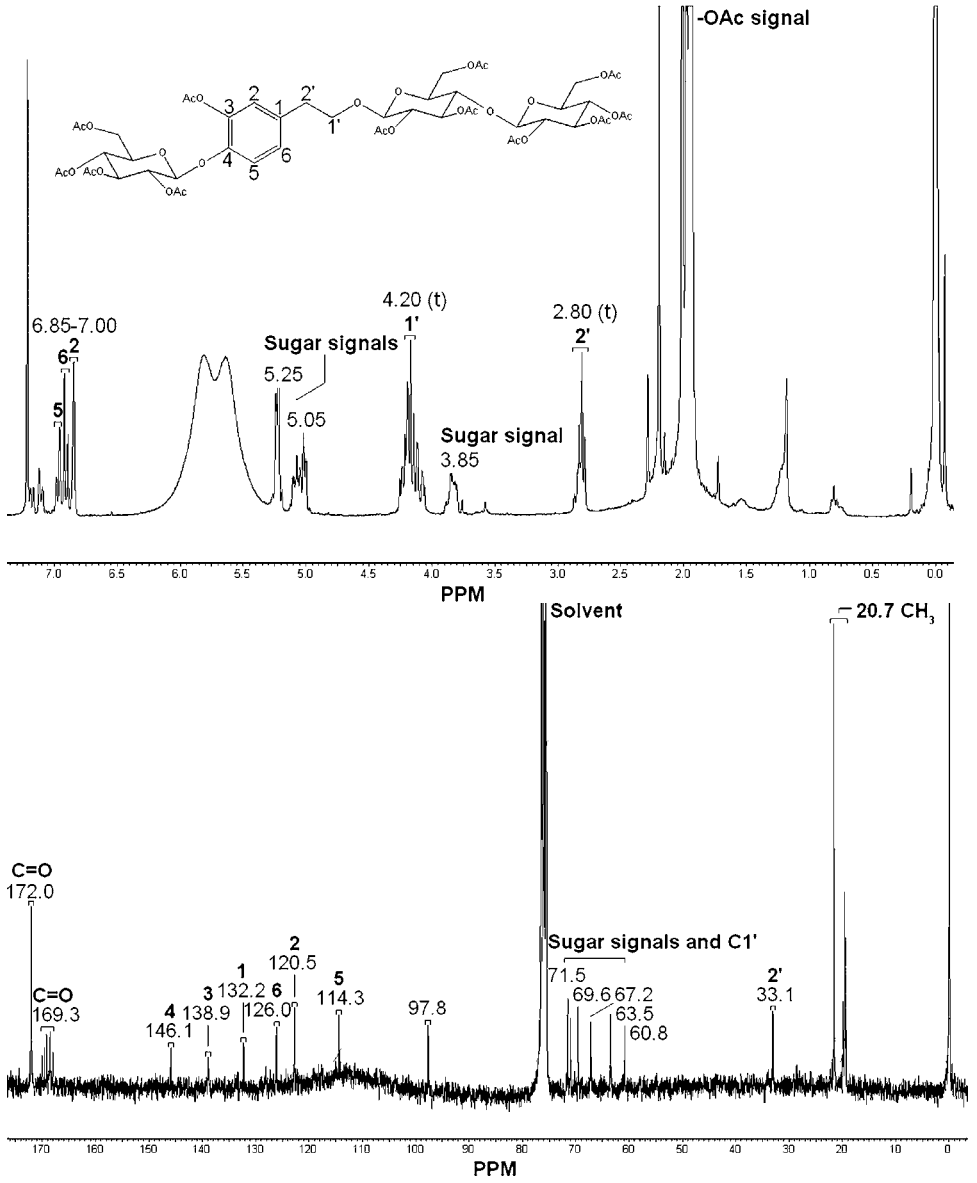

Figure 11. ^{1}H (upper) and ^{13}C (lower) NMR spectra (registered at 300 and 75 MHz, respectively) obtained for the acetylated fraction E8. The chemical shifts are relative to TMS in $CDCl_3$.

molecule identified from the hydrolysates of the steam-treated alperujo.

Another component isolated by Superdex Peptide chromatography was fraction E8. Its mass spectrum showed an $[M + Na]^+$ ion at m/z 663. The molecule's proposed structure and fragmentation are shown in Figure 10 and have never been detected in olive fruit or their byproducts. After the consecutive loss of glucose (fraction E8 contains 99.5% glucose), it was possible to observe the formation of the sodium adduct species at m/z 501 and 339. In the negative mode, the peaks at m/z 315, 153, and 123 are similar to the above molecule and indicate the presence of a glucoside attached to hydroxytyrosol. The resonance signals from the ^{1}H and ^{13}C NMR of the acetylated molecule are in accordance with the proposed structure (Figure 11) of this unknown compound.

The composition of fraction E9 was shown to be a glucoside of tyrosol, also named salidroside. The structure was determined by ESI-MS in both positive and negative modes (Figure 12). The positive spectrum showed the presence of the $[M + Na]^+$ ion predominantly with m/z 323, whereas the negative spectrum showed the ion at m/z 299 attributed to the deprotonated molecule, $[M - H]^-$, together with the characteristic ion at m/z 119. The structure of the acetylated molecule was confirmed by ^{1}H and ^{13}C NMR (data not shown) and coincided with the published data for this compound.[30]

The mass spectra of fractions D11 and E10 (not shown) were consistent with the presence of a glucoside attached to hydroxytyrosol, as determined by the pseudomolecular ion $[M - H]^-$ at m/z 315 and the group of ions at m/z 153, 123, 111, and 95. It is impossible to know if the glucose unit is linked to the hydroxytyrosol moiety through the aliphatic or aromatic hydroxyl functionality. Three isomers have been described in the literature: hydroxytyrosol-4-β-glucoside, hydroxytyrosol-3-β-glucoside, and hydroxytyrosol-1′-β-glucoside. The 4-β is thought to be more abundant in olive pulp, and the 1′-β is more abundant in the waste.[20,31,32]

For fraction D10, two characteristic fragment ions were observed in negative mode, at m/z 461 and 161, and are

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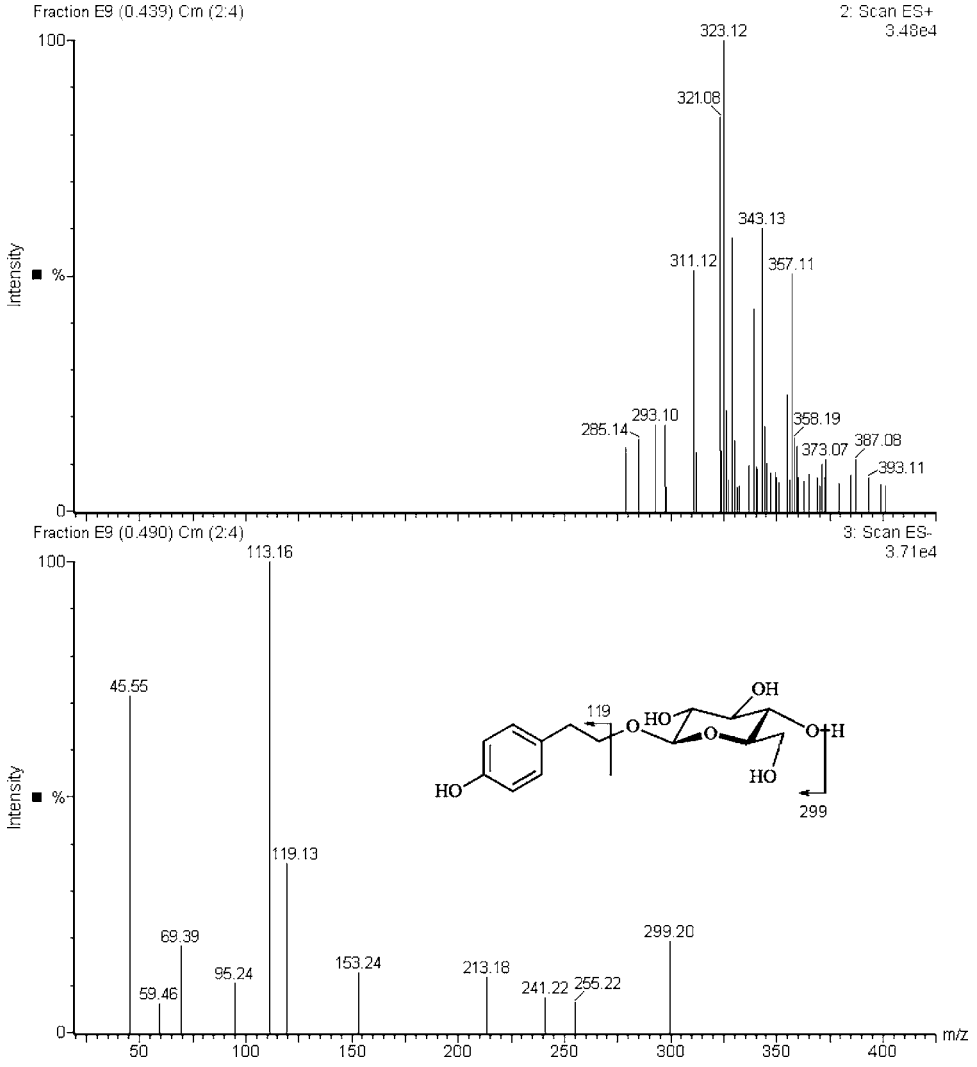

Figure 12. Mass spectra in positive (upper) and negative (lower) mode obtained for fraction E9.

consistent with the structure of verbascoside (Figure 13). However, the pseudomolecular ion, $[M - H]^-$ m/z 623, was not present. On the basis of the fragmentation structure proposed by Ryan et al.,[33] the ion at m/z 461 would appear as a result of the loss of caffeic acid, and the peak at m/z 161 would result from a proton transfer and the formation of an anionic ketene. The loss of caffeic acid and rhamnose yields the ion at m/z 315 and the fragments at m/z 153 and 123, which are characteristic of glucoside attached to hydroxytyrosol, as described above. This result led to the identification of verbascoside or possibly one of its isomers because it is a heterosidic ester of caffeic acid and hydroxytyrosol.

This work is the first report of the isolation, identification, and spectral characterization of several phenolic glycosides, mainly hexose derivatives of phenolic alcohols and secoiridoids, from alperujo that was steam-treated and hydrolyzed with mild acid. After preparative gel filtration chromatography of the hydrolysates, an interesting range of fractions containing secoiridoid, which is closely correlated to oleuropein, was obtained. The results achieved using HPLC followed by mass spectrometry with electrospray ionization and NMR spectroscopy suggested the presence of oleuropeinic acid for the first time in olive tissues. The chemical composition of this compound can be unambiguously assigned; however, the

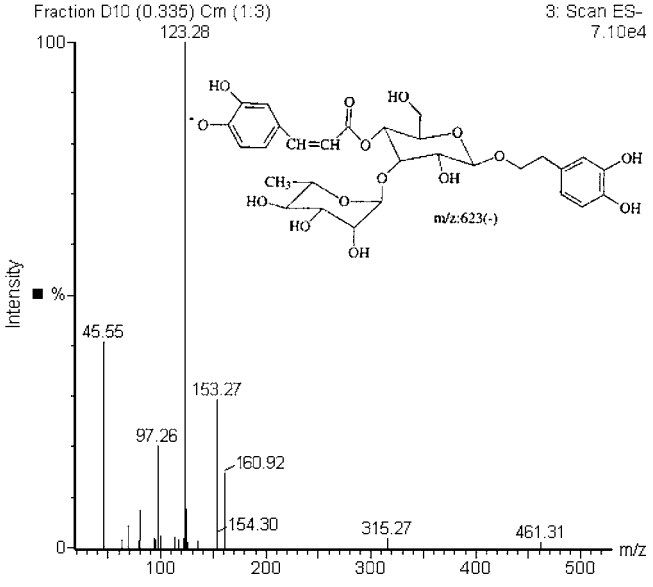

Figure 13. Mass spectrum in negative ion mode for fraction D10 and its proposed chemical structure. Both are consistent with verbascoside.

dx.doi.org/10.1021/jf303772p | J. Agric. Food Chem. 2013, 61, 1235−1248

presence of the ion at m/z 407 in all fractions of the first group, a secoiridoid core linked to one or two hydroxytyrosol moieties with one or two hexose units linked to its aromatic moiety or to the other sugar moiety, makes it possible to speculate on the structure of oleuropeinic acid and several derivatives. Further studies are necessary for the characterization of these compounds.

In addition, there was an indication of the presence of glucosides attached to hydroxytyrosol and tyrosol. This compound was isolated from olive seeds and identified as salidroside. Di- and trisaccharides linked to a hydroxytyrosol moiety have never been found in olive fruits. Another disaccharide known as verbascoside, which contains rhamnose−glucose linked to caffeic acid and hydroxytyrosol, is the main hydroxycinnamic derivative in olive fruit and was also isolated. Recent research indicates that verbascoside is bioaccessible and absorbable.[34] It also presents an antioxidant protective effect on phospholipid membranes and has the ability to modulate plasma antioxidant levels in vivo.[35]

The presence of significant fractions of neutral oligosaccharides, di- and trisaccharides of glucose and mannose, and di- and triglucosides bound to phenolic residues could be considered soluble fibers and antioxidant soluble fibers with bioactive properties assigned to a phenol moiety, respectively. These fractions are not digestible by human enzymes (in the mouth, stomach, pancreas, or small intestine), but they can be fermented by colonic bacteria and may act as prebiotic compounds and be beneficial for gastrointestinal function, especially in terms of selective stimulation of intestinal bacteria growth that contribute to the health and strength of the immune system. During fermentation in the large intestine, phenolic compounds (hydroxytyrosol, tyrosol, or oleuropeinic acid) would be released and might exert the same effects within the colon, such as scavenging free radicals or binding metals. These compounds could then be absorbed and passed into the enterohepatic circulation, thus increasing their nutritional value. The plasma elimination half-life period of the phenols in the organism would be increased, whereas the level of urinary excretion will decrease. However, their effects in vivo still need to be elucidated.

Oleuropein and hydroxytyrosol have been shown to possess a broad range of biological activities, including antimicrobial, hypoglycemic, antiviral, antitumor, anti-inflammatory, neuroprotective, hepatoprotective, and antioxidant activities.[34] Even hydroxytyrosol stimulates mitochondrial function and could act as a potential therapeutic for the prevention and treatment of type 2 diabetes, obesity, and age-related macular degeneration.[36−38] Most of the phenolic compounds isolated, including oleuropeinic acid and the glucosides of hydroxytyrosol and tyrosol, could exhibit biological activities, and we hope that the work presented here will stimulate such studies.

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Funding

We thank the Ministerio de Economía y Competitividad of Spain for financial support (Grant AGL2009-12352), Spain Program JAE-DOC and JAE-PRE (CSIC-FSE) (to G.R.-G. and F.R.-S.), and Junta de Andalucía (Project P08-AGR-03751).

Notes

The authors declare no competing financial interest.

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