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Wastes Generated during the Storage of Extra Virgin Olive Oil as a Natural Source of Phenolic Compounds

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Supporting Information

ABSTRACT: Phenolic compounds in extra virgin olive oil (EVOO) have been associated with beneficial effects for health. Indeed, these compounds exert strong antiproliferative effects on many pathological processes, which has stimulated chemical characterization of the large quantities of wastes generated during olive oil production. In this investigation, the potential of byproducts generated during storage of EVOO as a natural source of antioxidant compounds has been evaluated using solid−liquid and liquid−liquid extraction processes followed by rapid resolution liquid chromatography (RRLC) coupled to electrospray time-of-flight and ion trap mass spectrometry (TOF/IT-MS). These wastes contain polyphenols belonging to different classes such as phenolic acids and alcohols, secoiridoids, lignans, and flavones. The relationship between phenolic and derived compounds has been tentatively established on the basis of proposed degradation pathways. Finally, qualitative and quantitative characterizations of solid and aqueous wastes suggest that these byproducts can be considered an important natural source of phenolic compounds, mainly hydroxytyrosol, tyrosol, decarboxymethyl oleuropein aglycone, and luteolin, which, after suitable purification, could be used as food antioxidants or as ingredients in nutraceutical products due to their interesting technological and pharmaceutical properties.

KEYWORDS: olive oil, storage, byproduct, polyphenols, degradation pathways, antioxidant natural source, RRLC, MS/MS fragmentation

INTRODUCTION

Phenolic compounds are an important class of natural antioxidants. The term "phenolic compound" includes a large number of secondary plant metabolites that differ in chemical structure and reactivity, ranging from simple compounds to highly polymerized molecules. When present in small amounts in food, phenolic compounds are capable of preventing or retarding the oxidation of oils and fats. The presence of these compounds in fats, oils, and lipid-based foods is very important to reduce oxidative reactions that can lead to a decrease in both the nutritional value and sensory quality.[1]

Polyphenols belonging to many chemical classes have been described in extra virgin olive oil (EVOO), and in particular phenolic acids and alcohols, including *p*-coumaric acid, ferulic acid, vanillic acid, vanillin, 3,4-(dihydroxyphenyl)ethanol (hydroxytyrosol), and *p*-hydroxyphenylethanol (tyrosol), have been described. However, the main phenolic compounds are secoiridoid derivatives of oleuropein and ligstroside, such as the decarboxymethylated form of elenolic acid linked to either hydroxytyrosol or tyrosol (oleocanthal).[2−4] The phenolic profile has been used to evaluate the quality of EVOO as well as the presence of these compounds as they differentiate olive oil from other edible vegetable oils as the most hydrophilic phenols in EVOO are not common to other oils or fats.[5]

There are many technological factors that can influence the content of phenolic compounds, and in this regard the effects of the extraction process as well as the changes in minor compounds of EVOO during storage have been evaluated.[6−9]

Furthermore, olive oil production, an agroindustrial activity of vital economic significance for many Mediterranean countries, is associated with the generation of large quantities of wastes. Polyphenolic content has been assessed in these byproducts due to the biological and pharmaceutical interest in olive oil phenolic compounds.[10,11] Indeed, the polyphenolic activity on different cancer cells is also well-known[4,12] and has stimulated research on the profile of phenolic compounds in the different parts of the olive tree, and new methods have been developed to extract these compounds.[13]

Wastewater from olive oil mill wastes is characterized by a high content of phenolic alcohols (mainly hydroxytyrosol and tyrosol).[14] Oleuropein has been described as a major compound in olive leaves and branches, which also contain high concentrations of glucosylated flavones such as luteolin-7-glucoside and apigenin-7-glucoside.[15] Additionally, branches are characterized with an elevated amount of verbascoside, a precursor of hydroxytyrosol.[16,17]

The byproducts generated during the filtration process of EVOO have been also evaluated, and different classes of hydrophilic phenolic compounds are retained in filter aids, including phenolic acids, phenolic alcohols, secoiridoids, lignans, and flavones.[18]

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In the Mediterranean area, olive oil is produced from September to February, and oil is generally stored in the mill until commercialization. During this storage time, both solid and aqueous wastes are generated. The aim of this investigation was to evaluate the wastes generated during the storage of EVOO as a potential source of phenolic compounds. In particular, phenolic compounds and other polar molecules present in both solid and aqueous wastes were identified and quantitated to establish if these byproducts might be a natural source of antioxidant compounds.

MATERIALS AND METHODS

Samples. EVOOs were produced in the San Placido production plant (Oleoestepa S.L., Sevilla, Spain) in September 2009. First, Hojiblanca variety olives (F1) were processed in a continuous industrial plant equipped with a hammer crusher, horizontal malaxator, and two-phase decanter. EVOO obtained with impurities (F8) was stored in a tank without headspace at room temperature in darkness for 9 months. After this time, suspended solids and others materials, which had been deposited by precipitation in the bottom of the tank, generated a mix of solid and aqueous wastes (F9). Afterward, EVOO (F10) was directly transferred from the storage tank to bottling equipment, and wastes were collected. The separation of the two wastes was carried out by decantation and subsequent centrifugation. To obtain representative results and eliminate confounding factors, which could affect the phenolic profile, isolation of this fraction from samples was performed without storage of wastes.

Chemicals and Apparatus. All chemicals were of analytical reagent grade. Methanol and *n*-hexane were purchased from Lab-Scan (Gliwice, Sowinskiego, Poland). Acetic acid was purchased from Fluka, Sigma-Aldrich (Steinheim, Germany), and Lab-Scan, respectively. Solvents were filtered using a solvent filtration apparatus (Supelco, Bellefonte, PA). Double-deionized water with a conductivity of <18.2 MΩ was obtained with a Milli-Q system (Millipore, Bedford, MA). Standards of hydroxytyrosol, tyrosol, vanillin, luteolin, and apigenin were purchased from Sigma-Aldrich (St. Louis, MO), and (+)-pinoresinol was acquired from Arbo Nova (Turku, Finland). Oleuropein was purchased from Extrasynthese (Lyon, France). Stock solutions of phenolic compounds at 1000 mg/L were first prepared by dissolving the appropriate amount of the compound in methanol and then serially diluted to working concentrations.

Extraction Procedure of Phenolic and Other Polar Compounds from Solid and Aqueous Wastes Generated during Storage of EVOO. Isolation of the phenolic fraction from solid waste (5 g) was performed using *n*-hexane, methanol, and water as solvents. The lipophilic fraction of samples was removed with 20 mL of *n*-hexane after shaking for 1 h. The mixture was centrifuged at 13000*g* for 10 min, and the supernatant was eliminated. After this, a comparative study using three different ratios of MeOH/H_2O (100:0, 75:25 and 50:50, v/v) was performed to determine the best solvent to extract the phenolic fraction. To assess this, the sample was soaked for 10 min in an ultrasonic bath with 20 mL of the different solvents indicated above and shaken for 30 min. Samples were centrifuged in the same conditions described above, and supernatants were collected. Each sample was analyzed in triplicate.

The extraction procedure of phenolic compounds from aqueous waste was as follows. To clean samples (10 mL), they were shaken for 1 h with 20 mL of *n*-hexane. In the next step, the samples were centrifuged at 13000*g* for 10 min to remove the nonpolar fraction, and water extracts were collected. This procedure was also carried out in triplicate.

All extracts were stored at −20 °C and filtered through a 0.25 μm filter before chromatographic analysis.

RRLC-DAD-ESI-TOF Analysis. Rapid resolution liquid chromatography (RRLC) coupled to electrospray time-of-flight mass spectrometry (ESI-TOF-MS) was used to characterize the phenolic profile in solid and aqueous wastes using an Agilent 1200-RRLC system (Agilent Technologies, Waldbronn, Germany) equipped with a vacuum degasser, autosampler, binary pump, and diode array detector (DAD). A 150 mm × 4.6 mm i.d., 1.8 μm, Zorbax Eclipse Plus C18 column (Agilent Technologies, Palo Alto, CA) was used for analytical analyses. The flow rate was 0.80 mL/min, and the column temperature was maintained at 25 °C. The mobile phases were water with 0.25% acetic acid as eluent A and methanol as eluent B. The total run time was 27 min using a previously reported multistep linear gradient.[4] The separated compounds were monitored first with a DAD (240 and 280 nm), and then MS was performed using a microTOF instrument (Bruker Daltonik, Bremen, Germany) coupled to a RRLC system (see the Supporting Information). At this stage, the use of a splitter was required for coupling with the MS detector as the flow at the TOF detector had to be 0.2 mL/min to obtain reproducible results and stable spray. The TOF mass spectrometer was equipped with a model G1607A ESI interface (Agilent Technologies) operating in negative ion mode. External mass spectrometer calibration was performed with sodium formiate clusters (5 mM sodium hydroxide in water/2-propanol 1:1 (v/v), with 0.2% of formic acid) in quadratic high-precision calibration (HPC) regression mode.

The calibration solution was injected at the beginning of the run, and all spectra were calibrated prior to polyphenol identification. The optimum values of the source and transfer parameters were set for good sensitivity and reasonable resolution of the mass range for the compounds of interest (m/z 50−1000) to improve ionization performance.[4] The accurate mass data for the molecular ions were processed using the software Data Analysis 3.4 (Bruker Daltonics), which provided a list of possible elemental formulas using the Generate Molecular Formula Editor. The latter uses a CHNO algorithm providing standard functionalities such as minimum/maximum elemental range, electron configuration, and ring-plus double bonds equivalent, as well as a sophisticated comparison of the theoretical with the measured isotopic pattern (Sigma-Value) for increased confidence in the suggested molecular formula. The widely accepted accuracy threshold for confirmation of elemental compositions was established at 5 ppm for most compounds.

Quantitation was carried out by RRLC-ESI-TOF-MS. Eight standard calibration curves of the principal compounds found in samples were prepared using eight commercial standards. All calibration curves showed good linearity.

ESI-IT-MS Analysis. The RRLC system was coupled to a Bruker Daltonics Esquire 2000 ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with the same ESI interface described above. The IT-MS spectrometer was run in the negative ion mode with the capillary voltage set at 3000 V. The IT instrument scanned at the m/z 50−1000 range. The maximum accumulation time and target count values were set at 100 and 10000 ms, respectively. The optimum parameters of the ESI-MS were as follows: dry temperature, 200 °C; drying gas flow, 9 L/min; nebulizing gas pressure, 29 psi. The instrument was controlled by a personal computer running Esquire NT software from Bruker Daltonics.

MS/MS fragmentation obtained by the IT-MS provided additional information, which when combined with UV chromatograms and MS data facilitated the structural identification of phenolic compounds.

Statistical Analysis. Data were analyzed using Origin (version Origin Pro 8 SR0, Northampton, MA) to perform one-way-analysis of variance (ANOVA) at a 95% confidence level ($p \leq 0.05$) to identify significant differences among individual concentrations in phenolic and other polar compounds in extracts using the three different MeOH/H_2O proportions described above to establish the optimum procedure to extract these compounds from solid waste. Individual concentrations of all compounds identified in aqueous waste were also analyzed statistically to evaluate significant differences between both types of wastes generated during storage of EVOO.

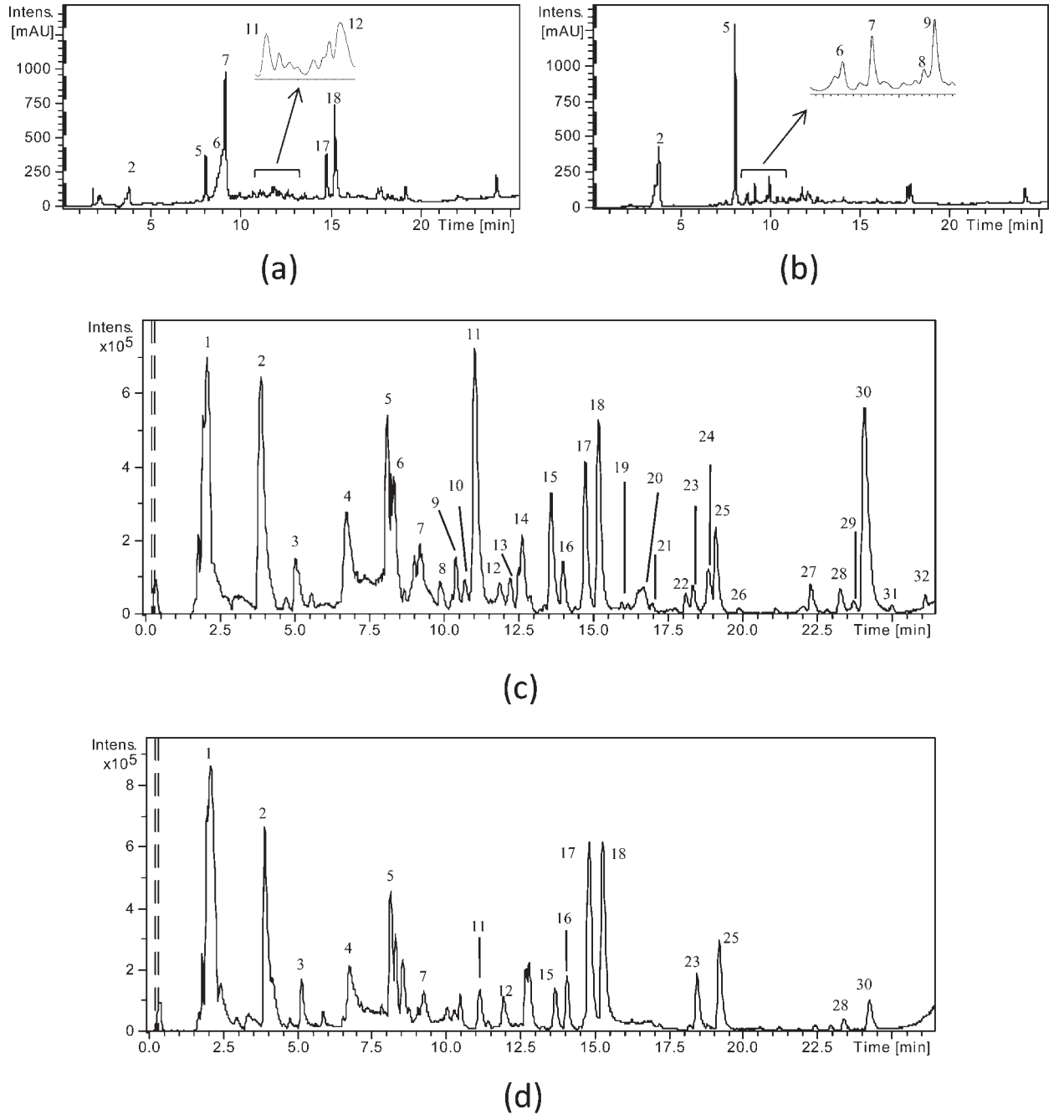

Figure 1. (a, b) HPLC-UV chromatograms detected at 240 and 280 nm of a representative solid waste extract using MeOH/H_2O 75:25, v/v. Base peak chromatograms (BPC) of the same representative solid waste extract (c) and aqueous wastes extract (d) generated during storage of EVOO.

RESULTS AND DISCUSSION

Identification and Quantitation of Phenolic and Other Polar Compounds in Solid and Aqueous Wastes. To our knowledge, this is the first time that these wastes have been characterized using RRLC-DAD-ESI-TOF/IT-MS. Identification of phenolic compounds was carried out by comparing retention times, UV absorbance maxima, MS data, and MS/MS fragmentation patterns from both samples and standards. The remaining compounds for which no commercial standards were available were identified by the interpretation of the information generated by the DAD, TOF analyzer, and IT-MS and the information previously reported (most compounds have been previously described in olive oil).[13,16,19,20]

Figure 1 shows the chromatograms of solid and aqueous waste extracts. The main phenolic compounds, which were identified in byproducts, are included in Table 1. The phenolic compounds vanillin, hydroxytyrosol, tyrosol, dialdehydic form of the decarboxymethyl elenolic acid (a secoiridoid derivative), decarboxymethyl oleuropein aglycone, luteolin, and apigenin as well as quinic acid were identified in both wastes. Additional secoiridoids, lignans, and their derivatives, such as oleuropein aglycone, hydroxylated forms of elenolic acid and decarboxymethyl oleuropein aglycone, and acetoxypinoresinol were found only in solid

Table 1. **Phenolic and Other Polar Compounds Identified in Solid and Aqueous Wastes Generated during the Storage of EVOO by RRLC-DAD-ESI-TOF/IT-MS**

	phenolic compounds					solid waste			aqueous waste				
peak	compound	molecular formula	T_r(min)	λ_{max}	m/z calcd	m/z exptl	error	σ	m/z exptl	error	σ	major fragments ESI negative MS/MS ions	standard
5	hydroxytyrosol	$C_8H_{10}O_3$	8.1	232/280	153.0557	153.0562	2.3	0.0005	153.0572	4.2	0.0024	123	yes
8	tyrosol	$C_8H_{10}O_2$	9.9	276	137.0608	137.0616	4.7	0.0174	137.0608	−0.1	0.0024	93	yes
11	dialdehydic form of decarboxymethyl elenolic acid	$C_9H_{12}O_4$	11.0	230	183.0663	183.0668	−2.7	0.0054	183.0675	−4.5	0.0088	139	no
12	vanillin	$C_8H_8O_3$	11.8	228, 230	151.0401	151.0417	4.3	0.0231	151.0418	3.9	0.0159	123	yes
19	hydroxylated form of elenolic acid	$C_{11}H_{14}O_7$	15.9	280/320	257.0667	257.0654	4.9	0.0347	ND			213	no
20	decarboxymethyl oleuropein aglycone	$C_{17}H_{20}O_6$	16.6	230, 280	319.1187	319.1199	−3.8	0.0136	319.1203	5.0	0.0274	195/183	no
21	hydroxylated form of decarboxymethyl oleuropein aglycone	$C_{17}H_{20}O_7$	16.9	230, 280	335.1136	335.1154	−5.2	0.0431	ND			199	no
26	acetoxypinoresinol	$C_{22}H_{24}O_8$	19.8	236, 280	415.1398	415.1399	0.1	0.0205	ND			151	no
29	oleuropein aglycone or isomer	$C_{19}H_{22}O_8$	23.6	236, 282	377.1242	377.1249	−2.0	0.0320	ND			307, 275	no
30	luteolin	$C_{15}H_{10}O_5$	23.9	254, 348	285.0405	285.0418	−3.7	0.0027	285.0399	2.1	0.0139	175,151	yes
31	oleuropein aglycone or isomer	$C_{19}H_{22}O_8$	23.6	236, 282	377.1242	377.1258	−4.6	0.0177	ND			307, 275	no
32	apigenin	$C_{15}H_{10}O_5$	26.0	268, 338	269.0455	269.0448	2.2	0.0232	269.0460	−1.6	0.0192	225, 151	yes
1	quinic acid	$C_7H_{12}O_6$	2.1		191.0561	191.0572	4.5	0.0073	191.0592	4.9	0.0287	103	yes

waste. Figure 2 shows the structures of the phenolic compounds identified.

The concentrations of phenolic and other polar compounds were determined by RRLC-ESI-TOF-MS, using the area of each individual compound (three replicates) and by interpolation of the corresponding calibration curve. The phenolic compounds vanillin, hydroxytyrosol, tyrosol, luteolin, and apigenin, as well as quinic acid, were quantitated by the calibration curves obtained from their respective commercial standards. The other phenolic compounds, which had no commercial standards, were tentatively quantitated using other compounds with similar structures. Secoiridoid and lignan groups were quantitated using oleuropein and (+)-pinoresinol standards, respectively. Elenolic acid derivatives, which cannot be considered phenolic compounds, were expressed as oleuropein. It should be taken into account that the response of standards may be different from the one of the analytes present in solid and aqueous wastes, and consequently the quantitation of these compounds is an estimation of their actual concentration.

Table 2 summarizes the quantitative results. The phenolic and other polar compound concentrations were determined in three extracts from solid waste obtained with MeOH/H_2O proportions of 100:0, 75:25, and 50:50 (v/v). Each individual concentration was used to carry out one-way analysis of variance at a 95% confidence level ($p \leq 0.05$) to establish the best procedure to extract these compounds from solid waste.

The main components of the phenolic fraction and its derivatives in solid waste extracts were dialdehydic form of decarboxymethyl elenolic acid and hydroxytyrosol. In all extracts, the range of concentrations was from 514 to 601 mg/kg and from 159 to 194 mg/kg for elenolic acid derivatives and phenolic alcohols, respectively. The amount of vanillin, another simple polyphenol, was 149 mg/kg.

Among the secoiridoids, oleuropein aglycone and its hydroxylated and decarboxymethyl derivatives were the most abundant compounds. With regard to MeOH/H_2O proportions, the amount of oleuropein aglycone in the solid waste extract obtained with proportions of 50:50 (v/v) was 2 times lower than in other proportions of MeOH/H_2O. The content of decarboxymethyl oleuropein aglycone in the 75:25 MeOH/H_2O (v/v) extract was significantly higher than in the others. Similarly, the highest quantity of its hydroxylated derivative was obtained using the same procedure.

With regard to lignans and flavones, significant amounts of (+)-acetoxypinoresinol, luteolin, and apigenin were detected. The concentrations of these three compounds in solid waste were higher than previously described in EVOO from Hojiblanca olives.[4]

The best results for all compounds were obtained using the following a MeOH/H_2O proportion of 75:25 (v/v). In reality, the use of aqueous methanol has already been described to provide good selectivity in extracting phenolic compounds from solid residues.[21]

Phenolic and other polar compounds present in aqueous waste extract obtained using the procedure detailed under Materials and Methods were also quantitated (three replicates). Among the polar compounds, quinic acid was the major compound, whereas in the phenolic fraction hydroxytyrosol and tyrosol were the most abundant. The content of hydroxytyrosol in aqueous waste was significantly higher than in the solid waste, and the amount of tyrosol was 6 times higher than in solid waste.

With regard to the amount of flavones, the contents of luteolin and apigenin in aqueous waste were significantly lower than in

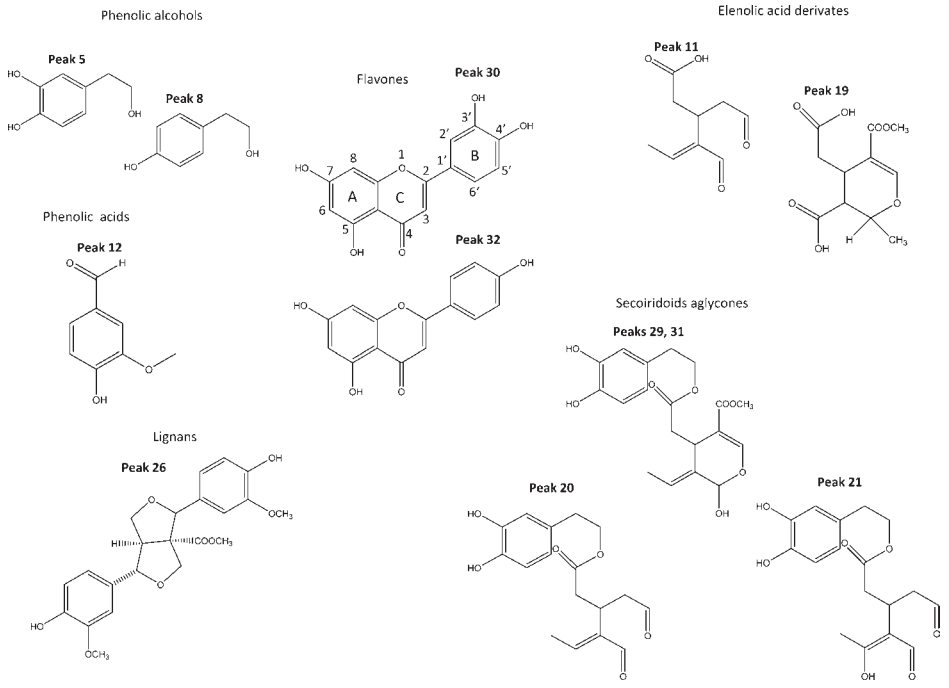

Figure 2. Main phenolic compounds and elenolic acid derivate structures identified in samples. Each compound has been numbered using the elution order obtained by RRLC-ESI-TOF-MS as indicated in Figure1.

Table 2. **Quantitative Results (Value = $X \pm$ SD)**[a]

	solid waste (mg analyte/kg)			
sample	100:0	75:25	50:50	aqueous waste (mg analyte/L)
phenolic compounds/MeOH:H_2O				
hydroxytyrosol	180.916 c ± 11.698	194.673 b ± 3.584	159.312 d ± 14.580	303.069 a ± 4.041
tyrosol	95.942 d ± 4.987	123.940 b ± 3.072	119.226 c ± 2.418	687.841 a ± 9.751
vanillin	139.789 c ± 7.662	149.284 ± 4.703	149.472 b ± 5.258	181.142 a ± 0.535
dialdehydic form of decarboxymethyl elenolic acid	597.161 a ± 1.739	601.269 a ± 1.622	514.916 b ± 3.289	153.844 c ± 1.366
hydroxylated form of elenolic acid	32.387 b ± 1.303	37.590 a ± 2.647	29.690 c ± 1.137	ND
oleuropein aglycone	40.761 b ± 0.329	48.035 a ± 1.254	23.467 c ± 0.253	ND
decarboxymethyl oleuropein aglycone	128.667 b ± 5.403	152.761 a ± 0.456	120.927 c ± 2.239	45.682 d ± 0.711
hydroxylated form of decarboxymethyl oleuropein aglycone	29.272 ab ± 0.235	30.909 a ± 1.027	27.285 b ± 1.350	ND
acetoxypinoresinol	11.364 b ± 0.156	12.220 a ± 0.110	9.324 c ± 0.097	ND
luteolin	145.862 b ± 1.148	147.284 a ± 0.427	79.362 c ± 0.089	11.289 d ± 0.769
apigenin	0.468 a ± 0.002	0.501 a ± 0.001	0.219 b ± 0.007	0.195 b ± 0.004
other polar compounds/MeOH:H_2O				
quinic acid	591.271 b ± 13.303	589.416 b ± 10.418	529.362 c ± 11.671	1484.859 a ± 82.440

[a] Values with the same letter in a row are not significantly different at a 95% confidence level ($p \leq 0.05$).

solid waste. For oleuropein aglycone and its derived compounds, significant amounts of only its decarboxymethylated form were detected in aqueous waste, and its content was less abundant than in the solid extract.

Proposed Derivatives of Phenolic Compounds in Solid and Aqueous Wastes. Table 3 shows the unknown compounds determined by RRLC-ESI-TOF/IT-MS. Among these, several compounds were tentatively identified as products derived from phenolic compounds. Peak 2, with experimental m/z 151 and molecular formula ($C_8H_8O_3$) generated by TOF analyzer, was tentatively proposed as the oxidation product of hydroxytyrosol. This oxidized form, being more polar than its nonoxidized derivative, elutes earlier (3.9 and 8.1 min, respectively). Parts a and b of Figure 1 report the UV chromatograms detected at 240 and 280 nm, respectively. These compounds showed absorbance maxima at 282 nm due to the presence of the *o*-hydroxyphenyl group in these molecules. MS/MS fragmentation carried out by the IT analyzer generated the same fragment at m/z 123 (Figure 3a). This corresponded to the loss of the CH_2OH and CHO groups for hydroxytyrosol and its oxidation product, respectively.

For elenolic acid, different structures have been previously described by basing their differences on the closed or open ring

Table 3. **Unknown and Proposed Derivation Phenolic Compounds Determined by RRLC-ESI-TOF/IT-MS**[a]

	compounds				solid waste			aqueous waste			
peak	proposed compounds	molecular formula	T_r(min)	m/z calcd	m/z exptl	error	σ	m/z exptl	error	σ	major fragments ESI negative MS/MS ions
2	oxidation product of hydroxytyrosol	$C_8H_8O_3$	3.9	151.0401	151.0408	3.3	0.0086	151.00410	3.8	0.0007	123
3	unknown	$C_9H_{14}O_6$	5.0	217.0718	217.0716	0.9	0.0136	217.0725	−4.9	0.0083	173/199
4	unknown	$C_7H_{10}O_4$	6.7	157.0506	151.0517	−4.7	0.0069	157.0522	−6.5	0.0095	69/139
6	hydrated product of the dialdehydic form of decarboxymethyl elenolic acid	$C_9H_{14}O_5$	8.3	201.0768	201.0773	−2.2	0.0025	ND			157
7	hydroxylated product of the dialdehydic form of decarboxymethyl elenolic acid	$C_9H_{12}O_5$	9.2	199.0618	199.0612	−3.1	0.0390	199.0620	−4.0	0.0031	155
9	decarboxylated form of hydroxy elenolic acid	$C_{10}H_{14}O_5$	10.4	213.0768	213.0758	4.8	0.0015	ND			169
10	unknown	$C_{20}H_{24}O_9$	10.7	407. 1348	407.1337	2.7	0.0007	ND			273
13	unknown	$C_{17}H_{18}O_6$	12.2	317.1031	317.1017	4.3	0.0116	ND			287
14	unknown	$C_{19}H_{20}O_8$	12.6	375.1085	375.1083	0.6	0.0136	375.1097	−3.2	0.0270	311/333
15	unknown	$C_9H_{14}O_2$	13.5	153.0921	153.0936	−4.1	0.0019	153.0938	−4.7	0.0156	123/151
16	aldehydic form of decarboxymethyl elenolic acid	$C_{10}H_{16}O_5$	14.0	215.0925	215.0927	−1.0	0.0024	215.0933	−3.6	0.0041	171/197
17	unknown	$C_{11}H_{16}O_6$	14.7	243.0874	243.0876	−0.9	0.0032	243.0884	−4.1	0.0016	123/211
18	unknown	$C_{11}H_{16}O_6$	15.2	243.0874	243.0884	−4.1	0.0041	243.0885	−4.9	0.0063	123/211
22	unknown	$C_{19}H_{20}O_8$	18.0	375.1085	375.1086	−0.2	0.0006	ND			299/343
23	unknown	$C_{12}H_{18}O_6$	18.3	257.1031	257.1029	0.7	0.0223	257.1030	0.1	0.0055	167/211
24	unknown	$C_{19}H_{20}O_8$	18.8	375.1085	375.1094	−2.3	0.0017	ND			299/343
25	unknown	$C_{12}H_{18}O_6$	19.0	257.1031	257.1027	0.7	0.0022	257.1034	−1.2	0.0053	167/211
27	unknown	$C_{10}H_{18}O_3$	22.2	185.1183	185.1177	3.2	0.0041	ND			95/111/139
28	unknown	$C_{12}H_{18}O_6$	23.2	379.1398	379.1416	−4.5	0.0087	379.1379	5.2	0.0102	167/211/243

[a] ND, compound not detected in sample.

and aldehydic or nonaldehydic forms:[22] (1) closed ring carboxylated aldehydic form, (2) closed ring carboxylated hemiacetalic form, and (3) open ring carboxylated dialdehydic form (Figure 3b). Although elenolic acid was not found, other compounds, which were characterized by UV absorbance maxima near 240 nm, typical of elenolic acid and its derivatives, were proposed as hypothetical oxidized and hydrated compounds from different elenolic acid structures.

Figure 3c shows the major negative ESI MS/MS fragment ions. The spectra generated for peaks 6 and 7 yielded deprotonated molecules at m/z 201 and 199, respectively, which could be attributed to derivatives of the dialdehydic form of decarboxymethyl elenolic acid (peak 11). Peak 6 was proposed as its hydrated form. This compound has been reported previously in olive oil wastes.[23] It elutes before the nonhydrated and oxidized forms. Its major fragment ion was at m/z 157. Peak 7, which was proposed as hydroxylated form of peak 11, presented a fragment at m/z 155 corresponding to the same loss of 44 units, which is consistent with the fragmentation pattern of their nonderivative form (acid group decarboxylation).

Peak 9 yielded a deprotonated molecule at m/z 213 and 10.4 min, identified as a decarboxylated form of hydroxyelenolic acid. This compound presented a fragment at m/z 169, whereas the difference represented the loss of another carboxylic group.

Peak 16 had a deprotonated molecule at m/z 215. According literature reports, this compound was assigned to an elenolic acid derivative,[22] which corresponds to an open ring decarboxylated aldehydic form of elenolic acid. The product ion at m/z 171 is also justified by the loss of 44 units. For all of the compounds derived from EA, the loss of 44 units of molecular mass can be justified by the liberation of CO_2.

Relationship between Phenolic Compounds and Their Derivatives. Are These Wastes a Natural Source of the Antioxidant Compounds? Wastes generated during the storage of EVOO contain mainly polyphenols of different molecular masses, which are related to the phenolic composition of oil and its changes during storage. Phenolic acids and alcohols, secoiridoids, lignans, and flavones may undergo modifications due to hydrolysis of complex phenolic compounds, increase in decarboxymethylated secoiridoids, and the appearance of oxidation products of phenolic and derived compounds during the storage of EVOO at an industrial site or after commercialization.[24] Such reactions have the consequence that lypophilic compounds (secoiridoid aglycones) are transformed into hydrophilic substances, which is important for their antioxidant action in micellar systems and absorption in the human body.[25] All of these compounds are found in wastes generated during the storage period.

Figure 4 shows the proposed degradation pathways including the main phenolic compounds identified in aqueous and solid wastes. Different reactions (oxidation, hydrolysis, hydration, and loss of carboxylic and carboxymethyl groups) must be taken into account.

Secoiridoids in their aglyconic forms, the main phenolic compounds in EVOO, are characterized by the presence of elenolic acid or its derivatives linked to phenolic alcohol structures (hydroxytyrosol and tyrosol). Hydrolysis of the ester bond between the phenolic portion and the rest of the molecule may

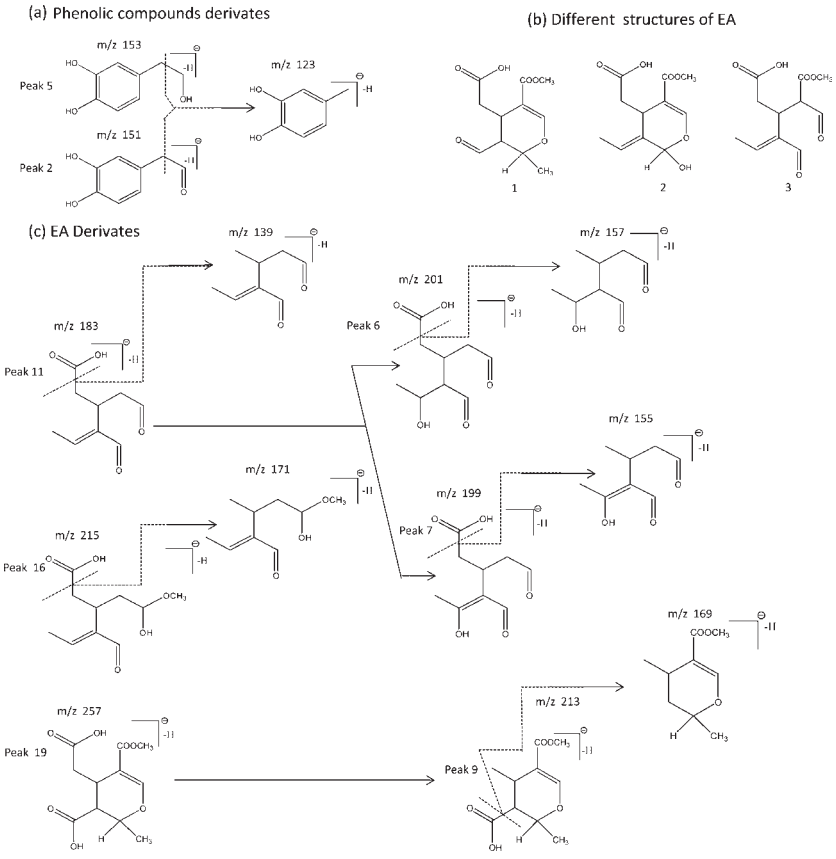

Figure 3. Fragmentation pathways for the proposed phenolic and elenolic acid derivates illustrating the most representative product ions obtained by IT analyzer, which were used for identification purposes: (a) phenolic compounds derivates; (b) different structures of elenolic acid previously described in the literature; (c) elenolic acid derivates.

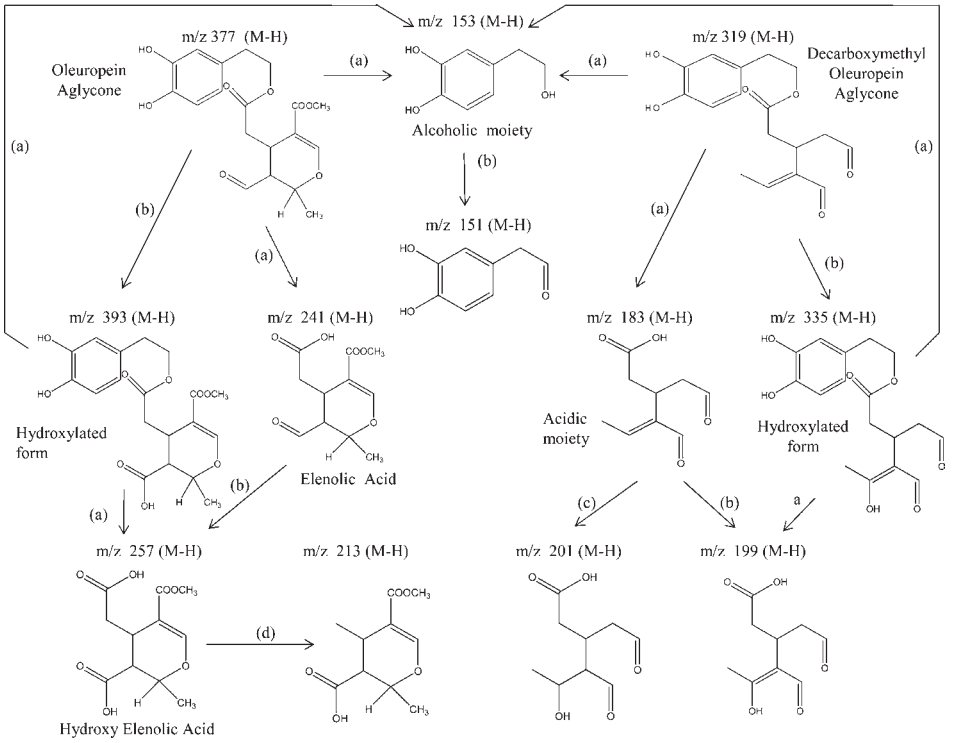

Figure 4. Degradation pathways for the main identified phenols in solid and aqueous wastes: (a) hydrolysis; (b) oxidation; (c) hydratation; (d) loss of the carboxylic group.

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increase the amount of free acidic and aromatic alcoholic moieties derived from these compounds.[8,25,26] Indeed, solid and aqueous wastes were characterized by a high content of simple phenols, mainly hydroxytyrosol, tyrosol, and compounds derived from elenolic acid. The small amounts of these complex phenols detected and the high concentration of hydroxytyrosol can justify the proposed hydrolysis reaction. With regard to tyrosol, its amount could have a dual origin: free phenols present in olive oil plus tyrosol produced due to hydrolysis of ligstroside aglycone and its derivatives. Although these compounds have not been detected in these samples, it should be taken into account that they have been previously described in EVOO[4,25,27] and could be completely hydrolyzed, increasing the amount of tyrosol and elenolic acid derivatives.

As far as elenolic acid is concerned, it was not detected in the present samples. However, several derivatives were identified. The combination of hydrolysis and oxidation could justify the presence of elenolic acid derivatives in olive oil wastes after storage. Oxidation in secoiridoid aglycones involves the acidic portion and not the aromatic alcoholic moiety, which is characterized by the conversion of the aldehydic group of elenolic acid to the carboxylic group. In fact, different oxidized forms according to this conversion have previously been described in EVOO as new compounds (i.e., the hydroxylated form of decarboxymethyl oleuropein aglycone).[4]

The origin of the oxidized free acidic moiety could be either the oxidation of these complex phenols and subsequent hydrolysis of the oxidized forms or hydrolysis previous to the oxidation process. The main complex phenolic compounds identified in the extracts were oleuropein aglycone, decarboxymethyl oleuropein aglycone, and its hydrated form. The hydroxylated form of elenolic acid could be generated by hydrolysis of oleuropein aglycone oxidized form or could arise from the oxidation of elenolic acid. The neoformation of the compound hypothetically proposed as the hydroxylated product of the dialdehydic form of decarboxymethyl elenolic acid (at m/z 199) could be due to the same reactions described above: oxidation of the phenol complex and later hydrolysis of the oxidized form or hydrolysis of decarboxymethyl oleuropein aglycone and subsequent oxidation of the acidic moiety.

Other degradation pathways for elenolic acid derivatives are the loss of the carboxylic group (decarboxylated form of hydroxy elenolic acid) and hydration of the decarboxymethylated dialdehydic form of elenolic acid (compound at m/z 201).

With regard to other phenolic compounds, acetoxypinorexinol was the only lignan detected in these samples. The antioxidant activity of this phenolic molecule is controversial. Several authors have described a strict correlation between the antioxidant activity of EVOO phenols and the amount of lignans, whereas other investigators found no significant relationship between these values.[28−30] Neither the oxidized nor the hydrolyzed forms of lignans were found in solid or aqueous wastes.

Apigenin and luteolin, characterized by the presence of three rings in their structure, were identified in extracts (Figure 2). From an antioxidant activity point of view, the B- and C- rings are the most important sites, whereas the A-ring seems to be less important. The predominant role of the B-ring could be to transfer hydrogen. The $\Delta^{2,3}$ double bond may also contribute to the antioxidant capacity, as it ensures π-electron delocalization between the B- and C-rings, which contributes to the stabilization of radicals formed during oxidation. Furthermore, the 3-OH group has been considered to be important because it contributes to the antioxidant potential. However, Lut (3′,4′,5,7-tetrahydroxyflavone) and Apig (4′,5,7-trihydroxyflavone) are characterized by the absence of this group, and both compounds have shown major stability to oxidation.[31] Indeed, the oxidized forms of Lut and Apig were not found in extracts.

For benzoic and cinnamic acids, different authors have reported the presence of dimers (i.e., *p*-coumaric and ferulic acid dimers) identified as oxidation and degradation products in olive oil mill wastewater[32] and in synthetic mixtures from several commercial standards exposed to different oxidation conditions.[33] Among the phenolic derivatives and unknown compounds, cleavages of cinnamic and benzoic acids have not been identified in the present extracts. This could be because the amounts of these compounds in Hojiblanca variety EVOO are lower than those of other polyphenols.

Finally, among the phenolic compounds identified and quantitated in wastes generated during the storage of EVOO, hydroxytyrosol and secoiridoids, which contain a phenolic alcohol in their structure, have higher antioxidant activity than other phenols including α-tocopherol. The major activity could be linked to their *o*-diphenolic structure. Although the amount of complex secoiridoids in samples is low, the final concentration of derived secoiridoids, mainly decarboxymethyl oleuropein aglycone, is similar to values described in olive oil.[4] Moreover, the amount of hydroxytyrosol is from 30 (solid waste) to 100 (aqueous waste) times higher than the value described in EVOO[4,34,35] due to hydrolysis reactions of secoiridoids containing this alcoholic phenol. Beneficial properties have been reported for hydroxytyrosol in inhibiting the proliferation of cells derived from human colon adenocarcinoma and other pathologies.[36−38]

In this preliminary work, the potential of the wastes generated during storage of Hojiblanca variety EVOO as a source of phenolic compounds has been evaluated. It should be taken into account that the variety chosen could affect the phenolic content of byproduct as it is related to EVOO composition. Phenolic acids and alcohols, secoiridoids, lignans, and flavones were identified and quantitated in both solid and aqueous wastes generated during the storage of EVOO. Different degradation pathways for the main phenols were proposed, and several derived compounds were tentatively identified. Among these, the amount of hydroxytyrosol was much higher than that described in EVOO due to the hydrolysis reactions of secoiridoids containing this alcoholic phenol. It is necessary to consider that wastes generated during storage could represent an interesting natural source of antioxidant phenols and have antiproliferative effects against many pathological processes. Future investigations are warranted to develop purified antioxidant extracts of hydroxytyrosol and other possible active compounds, with the application of more environmentally friendly and selective extraction techniques, such as supercritical fluid extraction and pressurized liquid extraction. The optimal extraction technique should be applied to obtain extracts with applications in food antioxidants as well as the development of nutraceutical products.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Figure S1. Flow diagram of the elaboration process of EVOO. Figure S2. MS spectra of the main phenolic compounds identified in samples. This material is available free of charge via the Internet at http://pubs.acs.org. Table S1. Analytical parameters of the RRLC-ESI-TOF MS methods.

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