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ARTICLE

Tentative Characterization of Novel Phenolic Compounds in Extra Virgin Olive Oils by Rapid-Resolution Liquid Chromatography Coupled with Mass Spectrometry

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Rapid-resolution liquid chromatography (RRLC) coupled with electrospray time-of-flight mass spectrometry (ESI-TOF-MS) and electrospray ion-trap multiple mass spectrometry (ESI-IT-MS^n) has been applied to separate and characterize the phenolic compounds in five Spanish extra virgin olive oils. The phenolic compounds were characterized on the basis of accurate mass data according to studies by TOF-MS, and the fragmentation ions were further confirmed by IT-MS^n whenever possible. Eight compounds, hydroxytyrosol (*m*/*z* 153), tyrosol (*m*/*z* 137), deacetoxy oleuropein aglycon (*m*/*z* 319), luteolin (*m*/*z* 285), pinoresinol (*m*/*z* 357), acetoxypinoresinol (*m*/*z* 415), apigenin (*m*/*z* 269), and deacetoxy ligstroside aglycon (*m*/*z* 303), all normally found in olive oil, were characterized in all five samples. Nevertheless, 30 uncommon phenolic compounds, including 10 ligstroside aglycon isomers (*m*/*z* 361) and two methyl oleuropein aglycon isomers (*m*/*z* 391), were also characterized in some of the five samples, and their fragmentation pathways were successfully elucidated.

KEYWORDS: Extra virgin olive oils; phenolic compounds; rapid-resolution liquid chromatography; mass spectrometry

INTRODUCTION

In recent years, researchers have become increasingly interested in the health properties of olive oil, produced mainly in Mediterranean countries. Extra virgin olive oil is usually extracted directly from the olive fruit (*Olea europaea* L.) by mechanical means and thus contains many phenolic compounds that are usually removed from other vegetable oils during the various stages of refining (*1*). Recent epidemiological studies have proved that the consumption of olive oil rich in phenolic compounds leads to a reduced risk of cardiovascular disease (*2*−*4*), neurodegenerative disease (*5*, *6*), certain types of cancer (*7*−*9*), and HIV-1 infection (*10*, *11*). The main phenolic compounds found in olive oil have been identified by various types of analytical techniques, and their pharmacological effects have been widely reported. Over the past few years, research has been carried out to identify phenolic compounds in monocultivar samples, and in this way, some new ones have been identified by gas chromatography/mass spectrometry (*12*, *13*), capillary electrophoresis/mass spectrometry (*14*), and liquid chromatography/mass spectrometry (*15*−*18*). The identification of phenolic compounds could help to distinguish between different varieties of olive oil and also to simplify controls against its adulteration.

An improvement in chromatographic performance has recently been achieved by the introduction of rapid-resolution liquid chromatography (RRLC). The column in RRLC is normally packed with particle sizes of less than 2 μm and operated at a pressure of up to 600 bar, which results in high resolution and superior peak capacity in a short analysis time (*19*, *20*).

The ongoing development of RRLC coupled with time-of-flight mass spectrometry (TOF-MS) and ion-trap multiple mass spectrometry (IT-MS^n) has opened up a new strategy to approach the online analysis of the composition and structure of the components of complex samples such as plant extracts. Apart from the faster acquisition of data and wider range of mass detection, greater accuracy in mass measurements (routinely below 5 ppm) is another important characteristic of TOF-MS for both qualitative and quantitative analyses (*21*, *22*). TOF-MS permits the rapid and efficient confirmation of the elemental composition of ions when carrying out fragmentation studies and also provides high selectivity in the determination of compounds in complex matrices using the extracted ion chromatogram (EIC) mode when there are overlapping peaks. Furthermore, IT-MS^n can be used to obtain fragmentation ions of structural importance

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Table 1. Microcomponents Detected in Samples OO1−OO5 by RRLC/TOF-MS[a]

no.	time	possible components	*m/z* experimental	tolerance (ppm)	error (ppm)	mσ value	formula	OO1	OO2	OO3	OO4	OO5	refs
1	6.47	hydroxytyrosol	153.0555	10	1.4	6.1	$C_8H_9O_3$	+	+	+	+	+	*18, 26*
2	8.15	tyrosol	137.0605	10	1.9	17.9	$C_8H_9O_2$	+	+	+	+	+	*18, 26*
3	14.53	deacetoxy oleuropein aglycon	319.1177	5	3.2	5.5	$C_{19}H_{17}O_6$	+	+	+	+	+	*18, 26*
4	15.94	luteolin	285.0399	5	1.7	18.9	$C_{15}H_9O_6$	+	+	+	+	+	*18, 27*
5	16.69	pinoresinol	357.1339	5	1.3	12.6	$C_{20}H_{21}O_6$	+	+	+	+	+	*18, 27*
6	17.33	acetoxypinoresinol	415.1381	5	4.0	5.4	$C_{22}H_{23}O_8$	+	+	+	+	+	*18, 27*
7	18.68	apigenin	269.0468	5	−4.7	10.3	$C_{15}H_9O_5$	+	+	+	+	+	*18, 27*
8	17.71	deacetoxy ligstroside aglycon	303.1237	5	0.3	20.9	$C_{17}H_{19}O_5$	+	+	+	+	+	*18, 26*
9	14.12	oleuropein aglycon isomer 1	377.1231	5	2.9	10.3	$C_{19}H_{21}O_8$	+	+	+	+	−	*25, 28*
10	14.78	oleuropein aglycon isomer 2	377.1246	5	0.9	5.7	$C_{19}H_{21}O_8$	+	+	+	+	+	*25, 28*
11	16.22	oleuropein aglycon isomer 3	377.1236	5	4.4	18.9	$C_{19}H_{21}O_8$	+	+	+	+	+	*25, 28*
12	17.41	oleuropein aglycon isomer 4	377.1260	5	−4.8	10.0	$C_{19}H_{21}O_8$	+	+	+	+	−	*25, 28*
13	17.88	oleuropein aglycon isomer 5	377.1253	5	−4.1	10.4	$C_{19}H_{21}O_8$	+	+	+	+	+	*25, 28*
14	18.88	oleuropein aglycon isomer 6	377.1264	10	−3.9	6.1	$C_{19}H_{21}O_8$	+	+	+	+	−	*25, 28*
15	19.05	oleuropein aglycon isomer 7	377.1268	10	−7.0	13.8	$C_{19}H_{21}O_8$	+	+	+	+	+	*25, 28*
16	20.89	oleuropein aglycon isomer 8	377.1271	5	−7.7	4.4	$C_{19}H_{21}O_8$	+	+	+	+	+	*25, 28*
17	21.02	oleuropein aglycon isomer 9	377.1267	5	−6.8	3.6	$C_{19}H_{21}O_8$	+	+	+	+	−	*25, 28*
18	21.59	oleuropein aglycon isomer 10	377.1269	5	−8.1	2.2	$C_{19}H_{21}O_8$	+	+	+	+	+	*25, 28*
19	21.87	oleuropein aglycon isomer 11	377.1259	5	−4.9	5.5	$C_{19}H_{21}O_8$	+	+	+	+	+	*25, 28*
20	7.83	secologanoside or oleoside	389.1076	5	3.5	31.9	$C_{16}H_{21}O_{11}$	+	−	−	+	−	*28, 29*
21	9.00	secologanol	389.1456	5	−0.7	27.5	$C_{17}H_{25}O_{10}$	+	−	−	+	−	*28*
22	13.15	azelaic acid	187.0975	10	0.3	15.7	$C_9H_{15}O_4$	+	+	+	+	+	*30*
23	13.88	deacetoxy 10-hydroxy-oleuropein aglycon	335.1145	10	−2.5	26.5	$C_{17}H_{19}O_7$	+	+	+	+	+	*26*
24	15.72	syringaresinol	417.1530	10	6.0	43	$C_{22}H_{25}O_8$	+	+	+	+	+	*31*
25	16.99	methyl oleuropein aglycon_1	391.1387	5	2.9	26.2	$C_{20}H_{23}O_8$	+	+	+	+	−	
26	19.30	chrysoeriol	299.0577	10	−5.2	27.4	$C_{16}H_{11}O_6$	+	+	+	+	+	*14, 32*
27	23.03	methyl oleuropein aglycon_2	391.1412	5	−3.6	11.0	$C_{20}H_{23}O_8$	+	+	+	−	−	
28	24.39	myristic acid	227.2016	10	0.3	3.9	$C_{14}H_{27}O_2$	+	+	+	+	+	*33*
29	16.47	ligstroside aglycon isomer 1	361.1286	5	1.8	22.6	$C_{19}H_{21}O_7$	+	+	+	+	−	
30	16.64	ligstroside aglycon isomer 2	361.1284	5	2.5	69.3	$C_{19}H_{21}O_7$	+	+	+	+	−	
31	17.48	ligstroside aglycon isomer 3	361.1299	5	−1.6	4.7	$C_{19}H_{21}O_7$	+	+	+	+	+	
32	19.10	ligstroside aglycon isomer 4	361.1312	10	−5.2	22.0	$C_{19}H_{21}O_7$	+	+	+	+	−	
33	20.74	ligstroside aglycon isomer 5	361.1315	10	−6.3	7.7	$C_{19}H_{21}O_7$	+	+	+	+	+	
34	20.99	ligstroside aglycon isomer 6	361.1316	10	−6.6	14.4	$C_{19}H_{21}O_7$	+	+	+	+	−	
35	21.89	ligstroside aglycon isomer 7	361.1305	5	−3.4	17.0	$C_{19}H_{21}O_7$	+	+	+	+	−	
36	22.04	ligstroside aglycon isomer 8	361.1305	5	−3.3	24.5	$C_{19}H_{21}O_7$	+	+	−	−	−	
37	23.33	ligstroside aglycon isomer 9	361.1300	10	−4.7	4.5	$C_{19}H_{21}O_7$	+	+	+	+	+	
38	23.58	ligstroside aglycon isomer 10	361.1289	5	1.0	15.2	$C_{19}H_{21}O_7$	+	+	+	+	+	

[a] Components in the table are described as + (detected) or − (undetected).

when trying to identify target compounds in a highly complex matrix. Here, we describe how we have been able to screen successfully phenolic compounds in extra virgin olive oil extracts by using a combination of RRLC/ESI-TOF-MS and RRLC/ESI-IT-MS^n.

MATERIALS AND METHODS

Plant Materials. The extra virgin olive oils used in this work came from different varieties of olives: Cornezuelo (OO1) (provided by CTAEX), Manzanilla (OO2) (provided by Oleoestepa SCA), Hojiblanca (OO3) (provided by Grupo Hojiblanca), Picual (OO4) (provided by Aceites Maeva SL), and Arbequisur (OO5) (provided by Oleoestepa SCA). These olives were harvested in January 2008 in different regions in Spain (Badajoz, Sevilla, Málaga, and Córdoba), and the oil samples were stored in darkness at 4 °C.

Sample Preparation. The phenolic compounds in extra virgin olive oils were extracted by the method described by Gómez-Caravaca et al. (*23*) with minor modifications. A diol-bonded phase solid-phase extraction cartridge (Bellefonte, PA) was placed in a vacuum elution apparatus and conditioned by passing 10 mL of methanol and then 10 mL of hexane. The oil (60 g) was dissolved in 60 mL of hexane and passed through the column. The solvent went through, leaving the sample in a solid phase. The cartridge was washed with three portions (5 mL) of hexane, which were then discarded to remove the nonpolar fraction of the oil. The sample was finally recovered by passing it through eight portions (5 mL) of methanol and dried in a rotary evaporator under reduced pressure at a temperature of 35 °C. The residue was dissolved with 2 mL of methanol and filtered through a 0.22 μm filter before analysis.

Separation by RRLC. Phenolic compounds in extra virgin olive oils were separated on an Agilent 1200 series RRLC (Agilent Technologies, CA) consisting of a vacuum degasser, an autosampler, and a binary pump equipped with a RP C18 analytical column (4.6 mm × 150 mm, 1.8 μm particle size, Angilent zorbax Eclipse plus). Acidified water (0.5% acetic acid v/v) and acetonitrile were used as the mobile phases A and B, respectively. The mobile phase was programmed as follows: gradient elutin from 5 to 30% B for 10 min, from 30 to 33% for 2 min, from 33 to 38% for 5 min, from 38 to 50% for 3 min, from 50 to 95% for 3 min, and from 95 to 5% for 2 min. Eight minutes was allowed for re-equilibration after each analysis. The flow rate was set at 0.80 mL/min throughout the gradient. The effluent from the RRLC column was split using a T-type phase separator before being introduced into the mass spectrometer (split ratio = 1:3). Thus, the flow entering the ESI-TOF-MS and ESI-IT-MS^n detector was 0.2 mL/min. The column temperature was 25 °C, and the injection volume was 10 μL.

ESI-TOF-MS. The RRLC system was coupled to a micrOTOF (Bruker Daltonics, Bremen, Germany), an orthogonal-accelerated TOF mass spectrometer (oaTOFMS) equipped with an ESI interface. The parameters for analysis were set using the negative ion mode with spectra acquired over a mass range from *m/z* 50 to 1000. The optimum values of the ESI-MS parameters were as follows: capillary voltage, +4.5 kV; drying gas temperature, 190 °C; drying gas flow, 7.0 L/min; and nebulizing gas pressure, 21.7 psi.

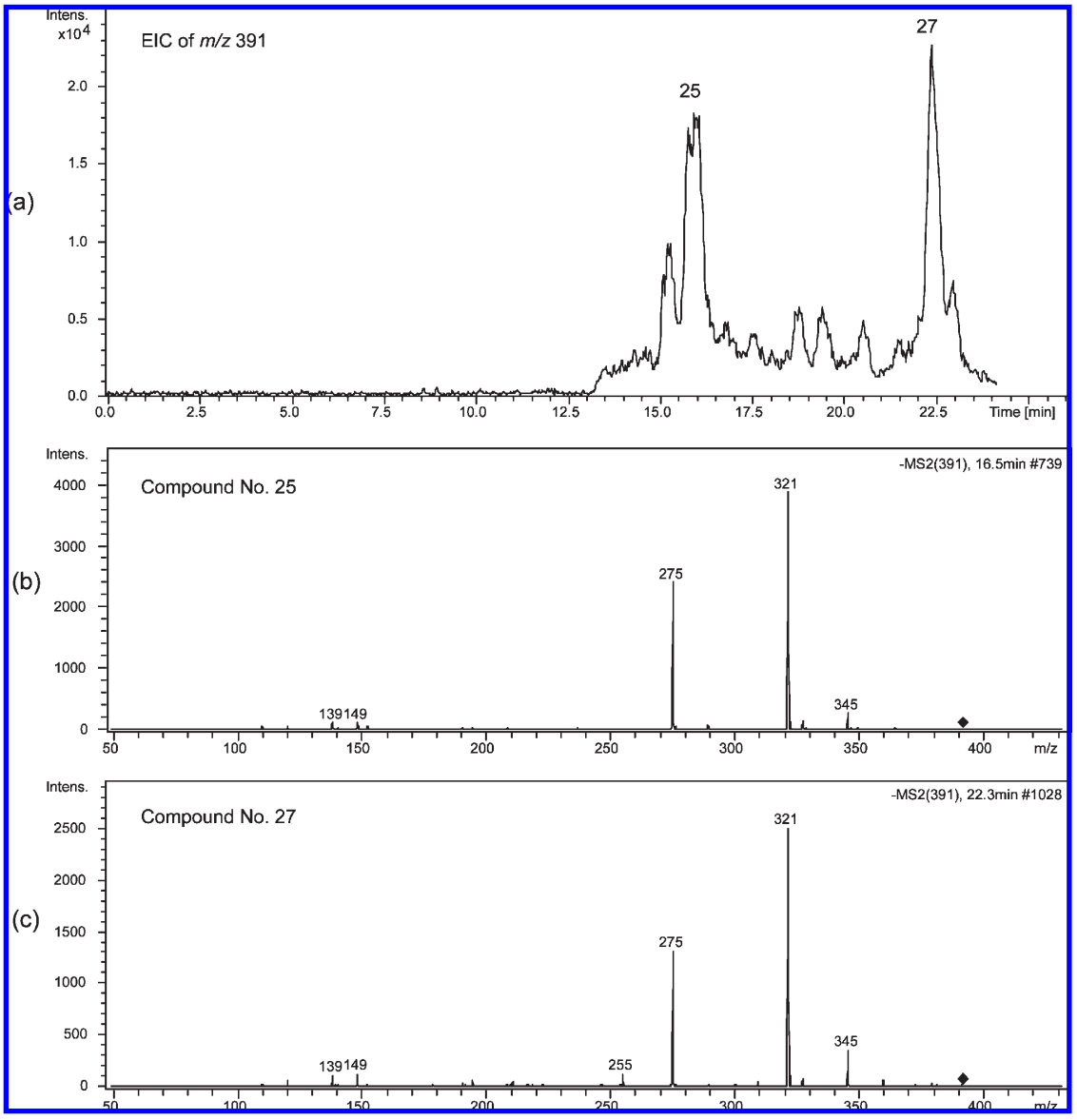

Figure 1. IT-MS spectra of *m*/*z* 391 in sample OO1. (**a**) EIC of *m*/*z* 391 by IT-MS. (**b**) IT-MS^2 spectra of compound **11**. (**c**) IT-MS^2 spectra of compound **20**.

The accurate mass data of the molecular ions were processed with Data Analysis 4.0 (Bruker Daltonics), which provided a list of possible elemental formulas by using the Smart Formula Editor. This editor uses a CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration, and ring-plus double-bond equivalents, as well as a sophisticated comparison of the theoretical and measured isotope patterns (sigma value) for increased reliability in the suggested molecular formula. The widely accepted accuracy threshold for the confirmation of elemental compositions has been established at 5 ppm (*24*). It is important to add that even with very high mass accuracy (< 1 ppm), many chemically possible formulas may be obtained, depending upon the mass regions considered, and thus, high mass accuracy (< 1 ppm) is not in itself enough to exclude sufficient candidates with complex elemental compositions. The use of isotopic abundance patterns as a single further constraint removes more than 95% of false candidates. This orthogonal filter can reduce several thousand candidates down to a small number of molecular formulas.

During the development of the RRLC method, external instruments were calibrated using a Cole Palmer syringe pump (Vernon Hills, IL) connected directly to the interface, and a solution of sodium formate cluster containing 5 mM sodium hydroxide in a sheath liquid of 0.2% formic acid in water/isopropanol 1:1 v/v was injected. Using this method, an exact calibration curve based on numerous cluster masses, each differing by 68 Da ($NaCHO_2$), was obtained. Because of the compensation of temperature drift in the micrOTOF, this external calibration provided accurate mass values of better than 5 ppm for a complete run without the need for a dual sprayer setup for internal mass calibration.

ESI-IT-MS. The same RRLC system was coupled to a Bruker Daltonics Esquire 2000 ion-trap mass spectrometer (Bruker Daltonics) equipped with an electrospray interface (Agilent Technologies, CA) in negative ion mode. The ion trap scanned at 50−1000 *m*/*z* range at 13000 u/s during separation and detection. The maximum accumulation time for the ion trap was set at 200 ms, the target count was set at 20000, and the compound stability was set at 50%. The optimum values of the ESI-MS parameters were as follows: capillary voltage, +3.0 kV; drying gas temperature, 300 °C; drying gas flow, 7.0 L/min; and nebulizing gas pressure, 21.7 psi. The instrument was controlled by Esquire NT software from Bruker Daltonics.

RESULTS AND DISCUSSION

The RRLC/TOF-MS chromatograms of our extra virgin olive oil samples are characterized by predominant peaks, attributable to the presence of isomers of oleuropein aglycon, the main compounds of Spanish extra virgin olive oils, which we have recently reported elsewhere (*25*). We detected the usual predominant

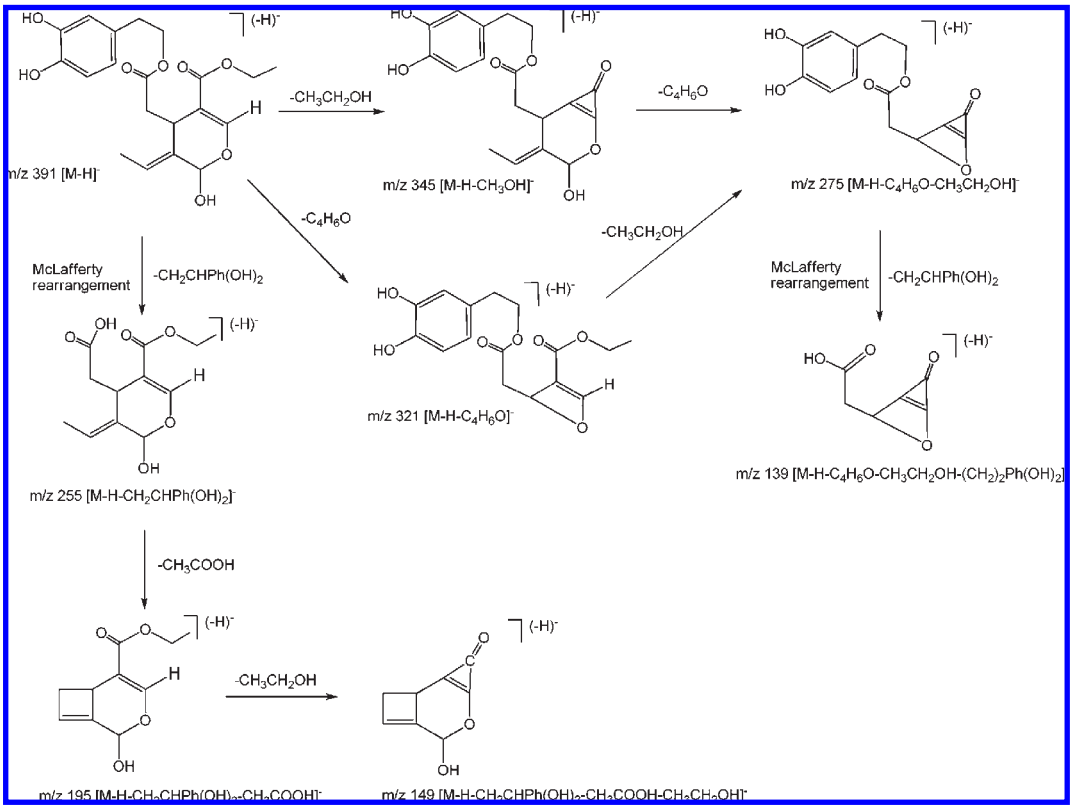

Figure 2. Fragmentation scheme of methyl oleuropein aglycon.

phenolic compounds, such as tyrosol (*m*/*z* 153), hytroxytyrosol (*m*/*z* 137), luteolin (*m*/*z* 285), pinoresinol (*m*/*z* 357), acetoxypinoresinol (*m*/*z* 415), apigenin (*m*/*z* 269), deacetoxy oleuropein aglycone (*m*/*z* 319), deacetoxy ligstroside aglycon (*m*/*z* 303), and oleuropein aglycon isomers (*m*/*z* 377). Nevertheless, there were also a wide variety of phenolic compounds structurally correlated to oleuropein and other secoiridoids, the relatively low percentage of which hinders their detection by conventional LC-UV. In this study, the mass spectra of olive oil extracts determined by RRLC/TOF-MS revealed the presence of more microcomponents (**Table 1**). It is interesting that the formula calculated by TOF-MS for two unknown microcomponents (*m*/*z* 391), eluting at 16.99 (**25**) and 23.03 min (**27**) (see **Figure 1** for their IT-MS spectra), is the same. The two microcomponents have the same molecular ion at *m*/*z* 391 and almost the same fragmentation ions at *m*/*z* 345, 321, 275, 149, and 139, which suggests that they may be oleuropein a glycon derivatives (*28*, *34*, *35*). The loss of one CH_3CH_2OH and one C_4H_6O from *m*/*z* 391 yields the product ions $[M - H - CH_3CH_2OH]^-$ (*m*/*z* 345) and $[M - H - C_4H_6O]^-$ (*m*/*z* 321), respectively, which further form the fragmentation ion $[M - H - C_4H_6O - CH_3CH_2OH]^-$ (*m*/*z* 275). Fragment *m*/*z* 139 is a result of the loss of $CH_2CHPh(OH)_2$ from $[M - H - C_4H_6O - CH_3CH_2OH]^-$ (*m*/*z* 275) by McLafferty rearrangement, while fragment *m*/*z* 149 originates from the consecutive loss of CH_3COOH and CH_3CH_2OH from the rearranged ion $[M - H - CH_2CHPh(OH)_2]^-$ (*m*/*z* 255). This indicated that the two new microcompounds (**25** and **27**) could be identified as isomers of methyl oleuropein aglycon, a structure that has never been reported before in olive oil. **Figure 2** shows the proposed fragmentation scheme for methyl oleuropein aglycon.

Ligstroside aglycon is the hydrolyzed product of ligstroside caused by the hydrolysis of the glycosidic bond, which may produce isomers due to keto−enolic tautomeric equilibrium in olive oil. This phenomenon has been reported in oleuropein (*25*). As expected from the isomerization of the secoiridoid moiety of ligstroside, 10 isomers (compounds **29**−**38** in **Table 1**) at *m*/*z* 361 were detected in all five of our extracts, which is far more complicated than previous reports on ligstroside aglycon (*28*, *35*). Ten isomers (*m*/*z* 361) were detected in samples OO1−OO4, while only four isomers were detected in sample OO5. From the IT-MS^2 spectra of these isomers (see **Figure 3**), we found that *m*/*z* 361, 291, 259, and 139 are the common fragments for all of these isomers, and they are characteristic fragments of ligstroside aglycon, as has been reported (*35*, *36*). Nevertheless, it is still very difficult to assign structures to the 10 compounds because of tiny differences in their fragments.

Ligstroside aglycon readily produces a deprotonated molecular ion $[M - H]^-$ at *m*/*z* 361. The loss of one CH_3OH and one C_4H_6O from *m*/*z* 361 yields the ions $[M - H - CH_3OH]^-$ (*m*/*z* 329) and $[M - H - C_4H_6O]^-$ (*m*/*z* 291), respectively, which further form the fragmentation ion $[M - H - C_4H_6O - CH_3OH]^-$ (*m*/*z* 259). The two fragments (*m*/*z* 241 and *m*/*z* 139) result from the loss of $CH_2CHPhOH$ from $[M - H]^-$ (*m*/*z* 361) and $[M - H - C_4H_6O - CH_3OH]^-$ (*m*/*z* 259) by McLafferty rearrangement, respectively, while the fragmentation ion *m*/*z* 223 originates from the loss of H_2O from the rearranged ion $[M - H - CH_2CHPhOH]^-$ (*m*/*z* 241). The fragmentation pathway of ligstroside aglycon is summarized in **Figure 4**; other isomers of ligstroside aglycon have similar fragmentation pathways with the same characteristic ions. The removal of the glucose causes subsequent transformation of ligstroside aglycon due to keto−enolic tautomeric equilibrium. According to the reported transformation mechanism of oleuropein aglycon (*25*, *28*), the transformations among isomers of ligstroside aglycon should be similar to those among isomers of oleuropein aglycon. **Figure 5** shows all possible transformations among isomers of ligstroside aglycon, where the undee symbols in the molecular structures indicate the existence of *cis*−*trans* isomers. Theoretically,

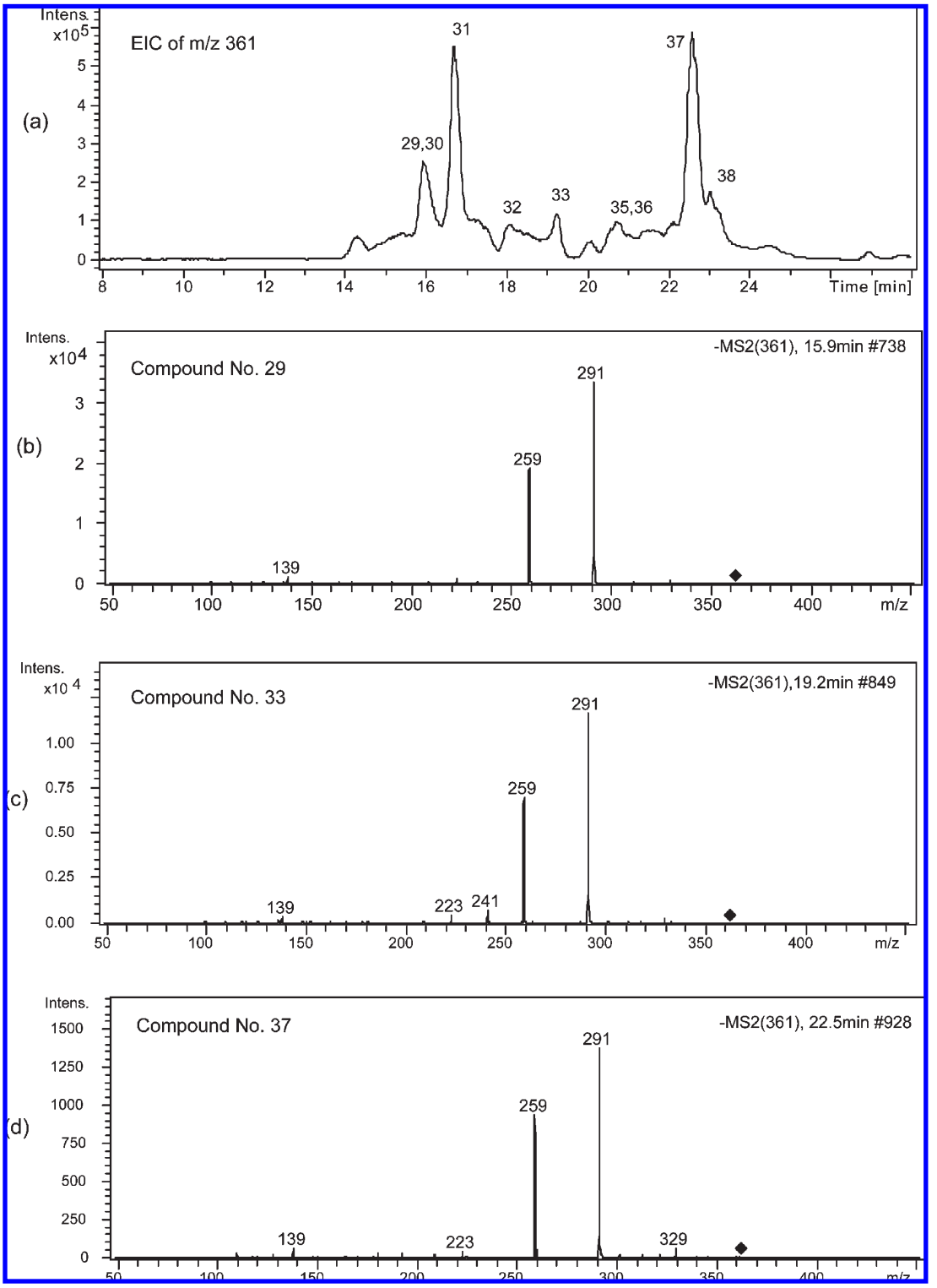

Figure 3. IT-MS spectra of *m/z* 361 in sample OO1. (**a**) EIC of *m/z* 361 by IT-MS. (**b**) IT-MS^2 spectra of compound **29**. (**c**) IT-MS^2 spectra of compound **33**. (**d**) IT-MS^2 spectra of compound **37**.

there are 12 possible isomers of ligstroside aglycon without taking into account *cis*−*trans* isomers (compounds **I**−**XII** in **Figure 5**). The complexity of secondary metabolites in plants must be born in mind, and the occurrence of polyphenols in olive oils may vary according to the strain, growing conditions, and handling processes. Thus, the number of ligstoside aglycon isomers in olive oils could well depend upon the strain in question, which has been shown in the different oils studied in this work.

In summary, we have used a powerful new analytical method to characterize phenolic compounds in extra virgin olive oils deriving from five varieties of olives in Spain based upon a combination of RRLC/TOF-MS and RRLC/IT-MS^n. In this way, we have been able to characterize tentatively 38 phenolic components, including two methyl oleuropein aglycon isomers and 10 ligstroside aglycon isomers. We also successfully elucidated their fragmentation pathways. The detection of new phenolic compounds in olive oil made by coupling RRLC/TOF-MS and RRLC/IT-MS^n provides us

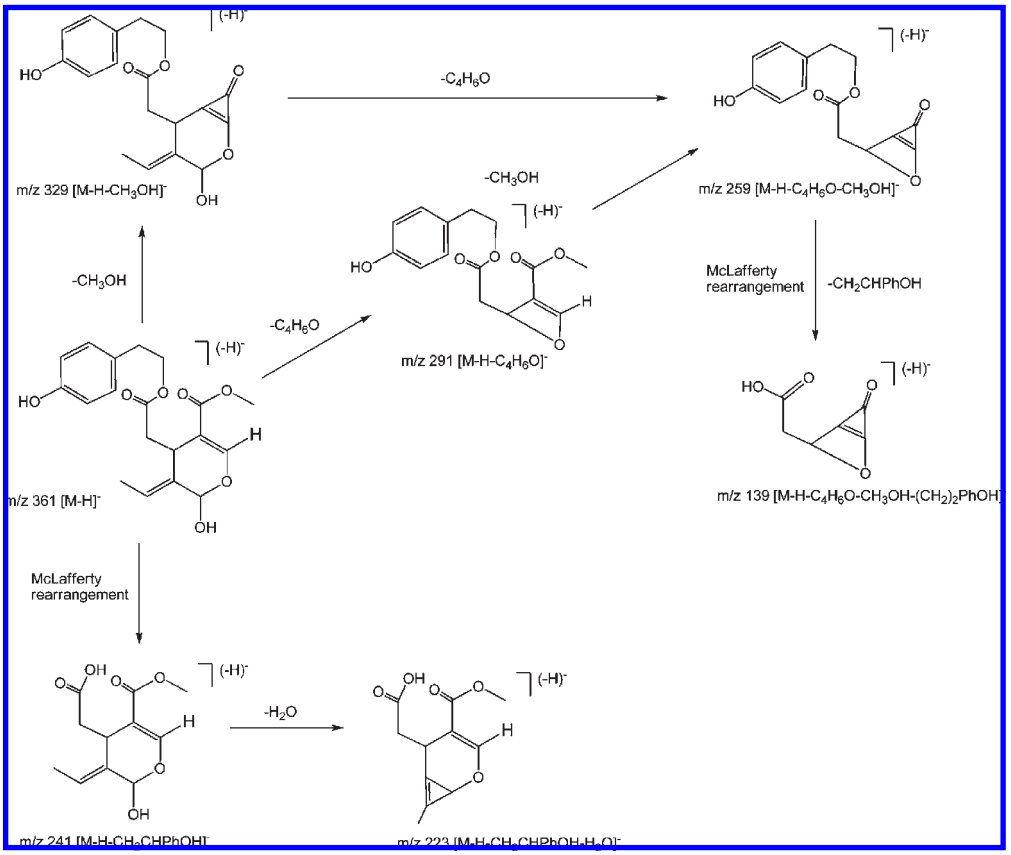

Figure 4. Proposed fragmentation pathway for ligstroside aglycon in IT-MS.

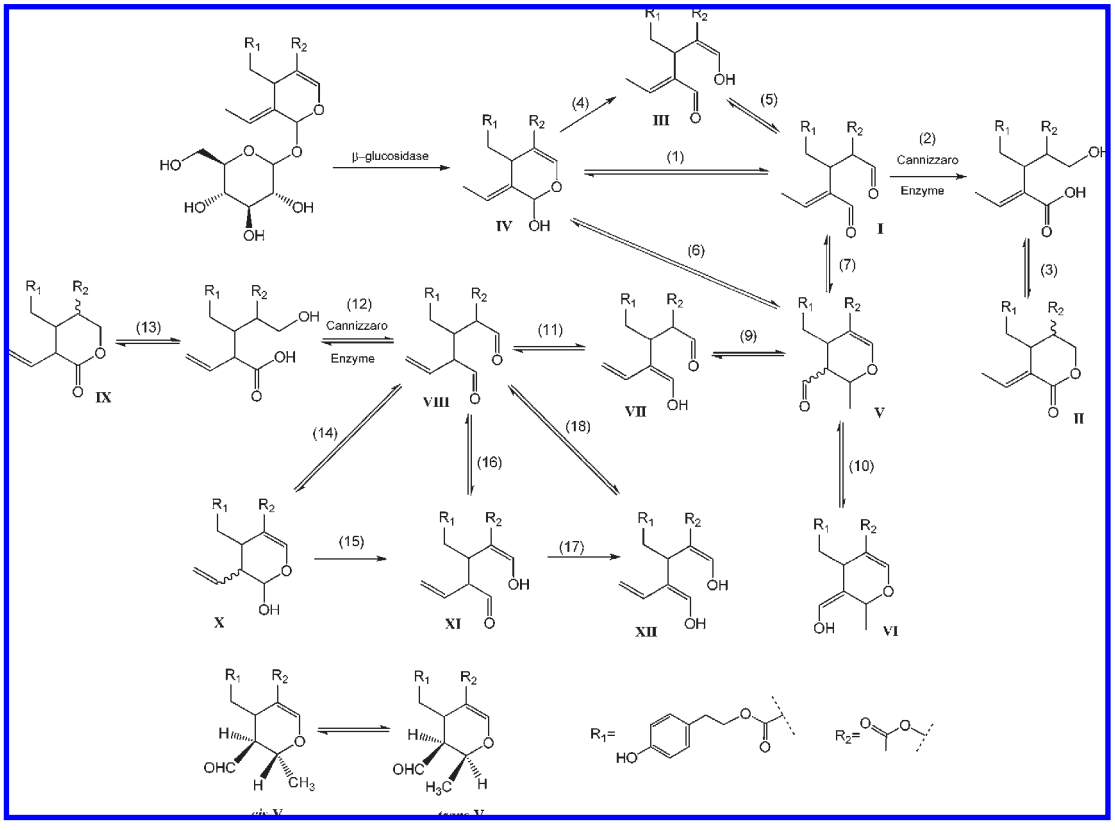

Figure 5. Proposed transformations among isomers of ligstroside aglycon.

with a more complete understanding of the secondary metabolism of this fruit and a more complete assignation of the bioactive components in its oil extracts to help in our ongoing pharmacological tests involving breast cancer.

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