2D-HPLC-CE platform coupled to ESI-TOF-MS to face the characterization of phenolic fraction from olive oil

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ABSTRACT

A two-dimensional HPLC/CE method was developed to separate and characterize more in depth the phenolic fraction of olive oil samples. The method involves the use of semi-preparative high-performance liquid chromatography (C18 column 250 x 10 mm, 5 μm) as a first dimension of separation to isolate phenolic fractions from commercial extra-virgin olive oils (EVOO) and capillary electrophoresis coupled to time- of flight-mass spectrometry (CE-TOF-MS) as a second dimension, to analyze the composition of the isolated fractions. Using this method, a large number of compounds were tentatively identified, some of them by first time, based on the information concerning high mass accuracy and the isotopic pattern provided by TOF-MS analyzer together with the chemical knowledge and the behaviour of the compounds in HPLC and CE. From these results it can be concluded that two dimensional HPLC-CE-MS provides enough resolving power to separate hundreds of compounds from highly complex samples, such as olive oil.

Furthermore, in this paper, the isolated phenolic fractions have been used for two specific applications: quantification of some components of extra virgin olive oil samples in terms of pure fractions, and *in vitro* studies of its anticarcinogenic capacity.

Keywords: Capillary electrophoresis / High performance liquid chromatography / Electrospray-time of flight-mass spectrometry / HPLC-CE-ESI-MS/ Olive oil / Phenolic compounds

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1 Introduction

Extra-virgin olive oil is obtained from the olive fruit (Olea europaea L.) solely by mechanical means, without further treatment other than washing, filtration, decantation, or centrifugation [1]. Its chemical composition consists of major components that represent 98% of the total weight and includes mainly glycerols [2-4], but what makes EVOO unique among other vegetable oils are minor components (about 2% of the total oil weight), specially its high level of phenolic compounds [5,6]. The phenolic fraction of olive oil is a heterogeneous and very complex mixture of compounds. A wide number of phenols have been already identified in olive oil, but even if this fraction has been studied over decades, it is still quite unknown, as many compounds remain unidentified. Simple phenols [7-10], lignans [11,12], flavonoids [13] and secoiridoids [7,14,15] are important well-known categories of phenolic compounds which can be found in olive oil. There is evidence that phenolic compounds from oil have different properties and exert diverse influence on the quality of olive oil [16,17]. One of their most described characteristics is their antioxidant activity [18-22], although *in vitro* studies have demonstrated other biological properties, suggesting beneficial effects on health disease and pathologies prevention such as cancer, obesity, diabetes, or diseases heart, among others [23-26]. In addition, polyphenols are responsible of the exceptional organoleptic characteristics that EVOOs have [27-32].

A huge amount of literature is available on the development of methods for the determination of phenols in olives and VOOs. The most used analytical techniques for the determination of individual phenolic compounds in virgin olive oil are those based on spectrophotometric methods, as well as analytical separation (gas chromatography (GC) [33,34], high-performance liquid chromatography (HPLC) [35-37], and capillary electrophoresis (CE) [9,38,39] coupled to different detectors [40]. However, a considerable number of phenolic compounds have still not been completely characterized and many problems remain to be resolved. One of the reasons lying behind these difficulties is the absence of suitable pure standards, in particular secoiridoid molecules and lignans. Moreover, phenolic fraction of oil is quite heterogeneous and complex and the matrix in which phenols are found (i.e. olive oil) is also rather complicated; these two facts can not facilitate their analysis. Therefore, mono-dimensional (1D) systems are sometimes inadequate to achieve the complete

separation of those compounds and an alternative approach could be to use HPLC with CE-ESI-TOF MS afterwards, i.e. a 2D-system. It has been demonstrated that in instances in which an HPLC method does not provide enough resolution, CE with its flexible experimental conditions should be assayed as a complementary second choice technique. With the CE analyses we added a new dimension of separation (based on completely different principles) after HPLC separation; moreover, MS will separate the analytes depending on the mass/charge ratio. The resolving power of a 2D separation, measured as peak capacity, will be the product of the individual peak capacity in each dimension [41]. Several groups have explored automated, comprehensive multidimensional systems that couple chromatographic techniques (GC-GC, LC-LC, LC-GC) [42] or even chromatographic and electrophoretic separations (LC-CE) on-line [43,44] and off-line [45,46] to carry out different applications, but mainly to separate peptides and protein mixtures.

The aim of this study was to develop a new analytical strategy based on 2D HPLC-CE-ESI-MS to characterize the phenolic profile of olive oil. Fractions from the effluent from the HPLC system were collected and dried, redissolved and afterwards, analyzed by CE-ESI-MS. Apart from being useful to characterize the highly complex phenolic fraction from olive oil more in depth and perhaps to identify tentatively new components; the collected fractions could be an interesting tool used to quantify more properly the phenols present in the mentioned matrix, and to elucidate the contribution of phenols to the positive effects on health attributed to VOO. As far as an appropriate quantification is concerned, it has been previously described that direct comparison between the concentrations of olive oil phenols reported in the literature is quite difficult, as the reported concentrations often greatly differ (sometimes even in orders of magnitude) due to the lack of standards. Moreover, concerning their potential healthy effects, it is still unclear which component or combination of components of olive oil is responsible for this protective effect. The phenolic compounds, both known as unknown, deserve to be analyzed and studied in detail because they are good candidates to explain a substantial part of the benefits of consumption of olive oil, as well as to justify their antioxidant and sensory properties.

2 Materials and methods

2.1 Chemicals and samples

All chemicals were of analytical reagent grade and used as received. Acetonitrile, from Lab-Scan (Dublin, Ireland), and acetic acid, from Panreac (Barcelona, Spain), were used in the mobile phase; whereas ammonium acetate from Panreac (Barcelona, Spain) and ammonium hydroxide from Merck (Darmstadt, Germany) were used for the CE running buffers. Buffers were prepared by weighting the quantity indicated in doubly distilled water and adding ammonium hydroxide to adjust the pH. Water was deionized by using a Milli-Q-system (Millipore, Bedford, MA, USA). 2-propanol of HPLC grade from Lab-Scan (Dublin, Ireland) was used in the sheath liquid, and sodium hydroxide solution (1M) from Panreac (Barcelona, Spain) was used for capillary cleaning procedures before each analysis.

For the extraction of the polar fraction from olive oil, methanol and hexane from Panreac (Barcelona, Spain) were used. Spanish EVOO samples used in the preliminary studies were obtained from a unique variety of olive fruit called Picual, Lechín, Cornicabra, Arbequina, Hojiblanca, and Picudo (January 2006). Two varieties were chosen for being used along the study in order to isolate the different phenolic fractions; they were Picual and Arbequina. The reason for choosing these two varieties were based on the high concentration of phenols and the number of peaks present in their profiles. We prepared a mixture EVOO Picual/ EVOO Arbequina (1/1, v/v) to facilitate the isolation of the phenols.

2.2 Extraction procedure

Several previous publications have described different methods for the extraction of the polar phenolic fraction from the olive oil matrix. In general, we can say that those methods use two basic extraction techniques liquid-liquid (LLE) and solid phase extraction (SPE) with different types and proportions of eluent [47,40]. In a study carried out by Gómez-Caravaca et al. [38], different extraction systems both liquid-liquid and solid phase extraction (C-18, SAX, and DIOL) were compared in order to obtain the best results with regards to the number of compounds extracted and the level of pre-concentration reached. Taking into account the results obtained in that paper, we

chose solid phase extraction (SPE) with Diol-cartridges to isolate the phenolic fraction from extra virgin olive oil.

Briefly, the cartridge was placed in a vacuum elution apparatus and pre-conditioned passing 10 mL of methanol and then 10 mL of hexane. About 60 g of extra virgin olive oil were thoroughly mixed with 60 mL of hexane and carefully loaded onto the pre-conditioned column, leaving the sample on the solid phase. After a wash with hexane (15mL) to remove the non-polar fraction of the oil, the sample was eluted with methanol (40 mL). The methanolic extracts were evaporated to dryness under reduced pressure in a rotary evaporator at 35ºC. The dried residue was then redissolved in 2 mL or 500 µL (depending on its use) of methanol/water (50/50 v/v) and filtered through a 0.25 µm filter before the analysis. We redissolved the residue in 2 mL when the phenolic extract had to be analyzed by analytical HPLC; whilst the residue was dissolved in 500 µL for carrying out the analysis and isolation by using semi-preparative HPLC.

2.3 High performance liquid chromatography analyses

Analyses were carried out operating at room temperature on a System Gold HPLC (Beckman Coulter, Fullerton, CA, USA), including a 126 solvent module, a 168 diode array detector module and a manual sample valve injector with a 20 and 500 µL loop (Rheodyne, Cotati, CA, USA) for analytical or semi-preparative LC, respectively.

The semi-preparative HPLC column used for the isolation of the fractions was a Phenomenex Gemini column, 25 cm x 10 mm filled with C18 reversed-phase packing (5 µm average particle size) and the flow rate was 3 mL/min. However, the analytical HPLC column used for the characterization of the olive oils in the preliminary studies was a C_{18} Gemini column, 5 µm i.d., 25 cm x 3.0 mm (Phenomenex, Torrance, CA, USA), equipped with a pre-column (Phenomenex) filter. The mobile phase flow rate was 0.5 mL/min and the loop was of 20 µL. DAD detector was always set at 240 and 280 nm, because these wavelengths are the most appropriate for the detection of olive oil polyphenols. For most of these compounds, 280 nm is the most specific value of wavelength, although in the case of EA and derivatives, 240 nm would be the optimum value.

The mobile phases consisted of water plus 0.5% acetic acid (Phase A) and acetonitrile (Phase B) and the solvent gradient changed according to the following conditions: from 0 to 30 min, 95% (A): 5% (B) to 80% (A): 20% (B); from 30 to 40 min, 80% (A): 20% (B) to 70% (A): 30% (B); from 40 to 50 min, 70% (A): 30% (B) to 65% (A): 35% (B); from 50 to 60 min, 65% (A): 35% (B) to 50% (A): 50% (B); from 60 to 70 min, 50% (A): 50% (B) to 5% (A): 95% (B); from 70 to 75 min, 5% (A): 95% (B) to 95% (A): 5% (B). This last value was maintained for 5 min, and the run ended. Solvents were filtered using a Solvent Filtration Apparatus 58061 (Supelco, Bellefonte, PA, USA) prior to degassification by ultrasonication. The same chromatographic gradient was used for analytical and semi-preparative separations.

2.4 Capillary electrophoresis analyses

Analyses were performed in a PACE System MDQ (Beckman, Fullerton, CA, USA) coupled to the mass detector using an orthogonal electrospray interface (ESI) from Agilent (see below). The CE instrument was controlled by a personal computer running 32 Karat System Software from Beckman. Bare fused-silica capillaries with 50 μm i.d. and total length 85 cm from Beckman Coulter Inc. (Fullerton, CA, USA) were used. The running buffer was 40mM ammonium acetate at pH 9.5, voltage was set at 25kV and 24 s hydrodynamic injections were made at the anodic end using N_2 at pressure of 0.5 psi.

Before their first use all new capillaries were conditioned by rising with 1 M sodium hydroxide for 10 min followed by a rinse with water for 5 min and then running buffer for 15 min. Capillary conditioning between runs consisted of 2 min with 1 M sodium hydroxide, then 2 min with water and finally 15 min with running buffer. At the end of the day the capillary was rinsed with water for 15 min and dried with air for 5 min.

2.5 Mass spectrometry

MS was performed using the microTOF™ (Bruker Daltonik, Bremen, Germany), equipped with an orthogonal electrospray interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA). MS/MS analyses were made by using a microTOF-Q (Bruker Daltonik, Bremen, Germany). For CE-MS analysis, electrical

contact at the electrospray needle tip was established via a sheath liquid pumped by a syringe pump (74900-00-05, Cole Palmer, Vernon Hills, IL, USA).

The parameters of the mass spectrometer were optimized by direct infusion experiments with extra virgin olive oil extracts, as well as with several of the most important compounds belonging to this polar fraction of olive oil that exist as available commercially standards. We varied the parameters of the mass spectrometer, to achieve good sensitivity with reasonable resolution (5.000-10.000) in the range of masses of interest (50-600 m/z). The optimization of the transfer parameters (radio frequencies and voltages) in the different skimmers, hexapoles and lenses was carried out in the direction of the entry of ions. As a general rule we can say that ions with high m/z values require high voltages to be transmitted.

The mass spectrometer was run in the negative mode and was operated to acquire spectra in the range of 50-600 m/z. The sheath liquid consisted of isopropanol-water (50/50, v/v) pumped at 0.24 mL/h and we used nebulizer gas pressure of 0.5 bar and a dry gas at flow rate of 5 l/min at 180ºC.

The accurate mass data of the molecular ions were processed by DataAnalysis 4.0 software (Bruker Daltonik GmbH) that provides a list of possible elemental formulae by means of the Smart Formula editor, which uses a CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration and ring-plus double bonds equivalents. Besides, an isotopic abundance pattern filter is required to reduce the number of candidates for an appropriate molecular formula (SigmaFit). For this purpose, the Generate Molecular Formula (GMF) tool (Bruker Daltonik GmbH) creates robust statistical models using the masses and intensities of each isotope to do a sophisticated comparison of the theoretical with the measured isotope pattern (SigmaValue^{TM}) for increased confidence in the suggested molecular formula. The smaller the sigma value the better the fit, therefore for routine screening a threshold sigma value of 0.03 is generally considered appropriate.

The calibration of the MS was performed using litium formate clusters by switching the sheath liquid to a solution containing litium hydroxide in the sheath liquid of some formic acid in water:isopropanol 1:1 v/v. Due to the compensation of temperature drift in the MicroTOF, this external calibration provided accurate mass values (better 5 ppm)

for a complete run without the need for a dual sprayer setup for internal mass calibration.

When we had doubts about the identity of some peaks present in the different isolated fractions, we carried out further fragmentation by using the MS/MS fragmentation achieved by high-resolution tandem mass spectrometry (in the microTOF-Q used). MS^2 experiments were performed in the collision cell q on the isotopically pure (^{12}C) peak of the selected precursor ions by keeping the first quadrupole analyzer at 20V relative to ground and operating at unit resolution, and scanning the time-of-flight (TOF) analyzer. The collision energy was set from 15 to 35 eV. All the acquisitions were averaged over 60 scans at a TOF resolving power of approx. 8000.

3. Results and Discussion

3.1 Analysis of polyphenols in different EVOO samples

As a first step in the current work, different varieties of olive oil were studied to make a previous screening and choose the variety (or mix of varieties) that provided the highest number of compounds and amount of all of them.

Extra virgin olive oils of six different varieties of olive fruit-Picual, Lechín de Sevilla, Cornicabra, Arbequina, Hojiblanca and Picudo-were analyzed using HPLC with UV detection (Fig.1) in order to select an olive oil rich in the phenols of interest. The use of an appropriate olive oil or mixture of olive oils for the isolation will allow obtaining higher amount of each compound, requiring less number of injections and therefore, of extractions. All the samples were prepared using the SPE protocol described above and were analyzed with the optima conditions already described in Materials and Method section. All the chromatograms are represented in the same absorbance scale in order to be able to carry out a proper comparison among the different varieties analyzed.

We could observe significant differences concerning the amount of polyphenols present in the different varieties. Picual was characterized because it was the only one where we detected significant amounts of Lig Agl. It was the richest variety in terms of Ol Agl and it had important quantities of simple phenols (HYTY and TY) as well. However, it was one of the varieties with less amount of Ac Pin, as previously reported by Brenes et al. [48]. As far as Lechín de Sevilla is concerned, the most representative compound in

this variety was DOA. Lechín was quite poor in Pin. EVOO Cornicabra was the one with less quantity of simple phenols and Lig Agl. However, it was richer than Lechín, Picudo and Hojiblanca in Ol Agl, but its concentration was much less than in Picual EVOO. Arbequina contained the largest amounts of lignans (Pin and Ac Pin) and it was outstanding the low presence of tyrosol in that variety. Picudo variety was quite rich concerning simple phenols, mostly HYTY, but the concentration of DOA was very low. Hojiblanca showed a chromatographic profile similar to Picudo oil, except from the fact that it was a little richer in simple phenols and also rich in terms of EA (this fact can be observed better at 240 nm).

In general, the varieties very rich in some compounds had small amounts of others, or even lacked them, being very difficult to choose a variety in particular to proceed with the isolation step. Finally, a mixture of two varieties in 50/50 proportion was selected: Picual, with notable concentrations of all compounds and the richest oil in terms of secoiridoids. However, as Ac Pin was almost absent in that oil, we included in the mixture Arbequina EVOO, especially rich in lignans. In that way the two varieties complemented each other and an olive oil rich in the main phenolic compounds could be used for the isolation.

3.2 Bidimensional HPLC-CE analysis of phenols from EVOO

3.2.1 Isolation of phenolic fractions

Once the mixture of olive oils rich in the phenolic compounds of interest was chosen, 17 phenolic fractions corresponding to different peaks in the UV chromatogram at 240 and 280 nm were selected for the isolation (Fig. 2).

Semi-preparative reverse phase-HPLC was used with the same chromatographic conditions as those used in the analytical column, with the only difference of the flow, increased to 3 mL/min and the loop of 500 μL in order to increase the amount of sample injected into the column. The quantity of extract injected into the column was optimized in order to obtain concentrated profiles but with good resolution, and finally an injection of 200 μL was selected as optimum. The isolation of the compounds was carried out from Diol-SPE extracts of the mixture of extra virgin olive oils.

The isolation was done trying to obtain the fractions as pure as possible avoiding other potential interferences. We needed more than 100 injections to get quantities of the isolated fractions that could be weighed. These compounds were manually collected and

kept at -20°C. Once enough quantity was collected it was led to dryness in a rotary evaporator at 35°C and the difference in weight between the empty flask and the flask after the evaporation of the solvent, gave us the amount of solute collected.

3.2.2 Optimization of CE-ESI-TOF MS and CE-ESI-qTOF MS conditions

The fractions isolated with semi-preparative reverse phase-HPLC were analyzed using a complementary technique: capillary electrophoresis (CE) coupled to time-of-flight mass spectrometry (MS-TOF) in order to study their composition. The use of these techniques will improve the characterization of the isolated fractions due to the use of two techniques based on principles of separation completely different. Moreover, the use of TOF detection system can give us an excellent accuracy in the determination of the mass, even allowing the measurement of the correct isotopic distribution pattern which provides valuable additional information for determining the elementary composition.

The effect of different separation parameters on resolution, sensitivity, analysis time, and peak shape was studied using SPE extracts of olive oil samples. Initial electrophoretic conditions were chosen based on parameters previously described in literature [49]. Bare fused-silica capillaries with 50 μm i.d. and 85 cm total length were used and a suitable background electrolyte (BGE) compatible with CE-ESI-MS, ammonium acetate, was chosen. With this buffer different pH values and different concentrations were tested obtaining the best results in terms of resolution, in the shortest analysis time, with 40 mM ammonium acetate at pH 9.5. Based on these conditions the voltage applied was varied between 20 and 30 kV, and we found that in general a voltage of 25 kV shortened the analysis time and also gave good resolution and acceptable current. The injections were made at the anodic end using N_2 pressure of 0.5 psi for 24 s.

Concerning the mass spectrometer, as commented before, the transfer parameters were optimized by direct infusion experiments with EVOO extracts, as well as with several of the most important compounds belonging to this polar fraction of olive oil that exist as available commercially standards. In the CE-ESI-MS coupling, the incompatibility of capillary flow (100 nL/min), with the flow necessary to the formation of a stable electrospray (1-200 μL/min) was solved using an additional liquid isopropanol/water (50/50) at a flow of 0.24 mL/h. The other ESI parameters were chosen according to the

sheath liquid flow of 0.24 mL/h and the most suitable ones were: nebulizer pressure of 0.5 bars, dry gas flow equal to 5 L/min and dry gas temperature 180ºC.

As commented before, MS^2 experiments were performed in the collision cell by keeping the first quadrupole analyzer at 20V relative to ground and operating at unit resolution, and scanning the time-of-flight (TOF) analyzer. Particular attention was paid to the collision energy, since its influence was quite drastic concerning the effective energy applied for the fragmentation of the molecules. It was varied from 15 to 35 eV, checking the fragmentation patterns at diverse values and considering all the MS/MS information generated. All the acquisitions were averaged over 60 scans at a TOF resolving power of approx. 8000.

These CE-ESI-TOF MS and CE-ESI-qTOF MS optimized conditions were used to analyze the 17 different fractions isolated by semi preparative HPLC.

3.2.3 Characterization of the isolated fractions by CE-ESI-TOF MS and CE-ESI-qTOF MS

Fig. 3 shows the extracted ion electropherograms (EIEs) of the different isolated fractions and the compounds tentatively identified are summarized in Table 1, including experimental m/z values, fragments detected by ISCID (Internal source collision induced dissociation) in the TOF that provides information to identify the compounds, fragment achieved after doing MS/MS experiments (in a qTOF instrument), the error and sigma value (comparison of the theoretical with the measured isotope pattern) and a list of possible compounds.

The identification of the compounds was performed by a careful interpretation of the MS spectra combined with information about polarity and electrophoretic mobility provided by the two complementary techniques used. Compounds isolated in the same fraction should have similar polarity and then these compounds are separated taking into account their electrophoretic mobility. Furthermore, ESI-TOF MS analyzer provides information about accurate mass and isotopic pattern that allow obtaining a reduced number of possible elemental compositions that then can be matched against available databases. Some of the possible elemental compositions calculated seem to be not chemically coherent reducing the number of possibilities. With the help of the fragmentation pattern obtained with ISCID, the potential difference between capillary exit and the skimmer, a reliable identification of the compounds is possible, especially

in such cases where other techniques (MS/MS and NMR) can not be utilized due to the low intensity of the analytes. When the intensity of the analytes was enough, MS/MS experiments were carried out to confirm the identification of the compounds. The microTOF-Q offers three dimensions of identification information simultaneously on all results: precise mass, MS/MS and SigmaFit™ isotopic analysis; we took advantage of the three mentioned dimensions to carry out the tentative identification of compounds under study.

Some of the polyphenols mentioned in previous studies were isolated as pure fractions, that was the case of **fraction 1** identified as HYTY, **fraction 2,** TY, **fraction 6**, EA, and **fraction 17**, Lig Agl. These pure fractions were later on used to quantify some compounds in the olive oil extract. Other LC fractions that had been previously assigned, with UV detection, to a single compound, were actually several compounds with similar polarity and therefore with the same retention time.

As the method used for isolating the phenols was a reversed phase LC method, the sixth first fractions contained highly polar compounds. So HYTY, TY and EA appeared as pure substances, whilst in the other fractions we can find a mixture of compounds separated on the basis of their size to charge ratio. In **fraction 3** the main compound (with m/z 195.0663) identified as HYTY-Ac appeared accompanied by another compound with m/z 257.0664, identified by first time as a derivative of elenolic acid: hydroxy elenolic acid. This compound shows up before elenolic acid because of its extra hydroxyl group that increase its polarity; the fragments detected with the same losses as elenolic acid, $[M-H-32]^-$, $[M-H-76]^-$ and $[M-H-102-H_2O]^-$ confirmed its identity. Other compounds with very low intensity have been tentatively identified and can be found in Table 1, such as licodione (m/z 271.0601), a metabolite of flavone pathway and glepidotin C, a phytochemical compound (stilbenoid) previously identified in Glycyrrhiza glepidota with antiviral activity. Last peaks correspond to compounds with carboxylic groups; quinic acid (m/z 191.0567) and succinic acid (m/z 191.0199), with the lowest electrophoretic mobility and therefore high migration time. **Fraction 4** is composed by HYTY-Ac, EA and probably small quantities of other acids, such as xanthoxic (m/z 265.1465) and capric acid (m/z 171.1380), but the most abundant compound with m/z 419.1853 has been identified as a dihydroxymethoxy diprenylisoflavone, an isoflavone with prenyl groups found very commonly in plant extracts. Close to this peak other compound with m/z 401.1729 could correspond to the

loss of a H_2O moiety (-18) from 419 and the closure of the ring resulting in an alpinumisoflavone derivative also with methoxy and prenyl groups. With the techniques used in the current study, we can not predict the positions of the substituents in the chemical structure of these substances. For both compounds the presence of ions fragments at m/z 387 and 369 indicates the loss of 32. A small trace of other flavonoid with prenyl groups was tentatively identified as 8-prenyldihydro-kaempferol-7-glucoside.

In **fractions 5** and **6** the most important compound is EA, although in **fraction 5** other acids in traces like xanthoxic acid, oleuropeic acid, azelaic acid, etc… are also detected. In this fraction a group of different glycosilated flavonoids have also been identified but in very small traces because glycosilated compounds are usually lost during olive oil production and remain in vegetation water and/or solid residues resulting from olive processing.

Fractions from **7** to **17** are mainly lignans and secoiridoids, together with other compounds at very low concentration. In **fraction 7,** different oleuropein derivatives are detected among other compounds. With the highest intensity the DOA (m/z 319.1179) is detected with its main fragment m/z 183 $[M-H-136]^-$. A derivative of this compound, with m/z 335.1137, was found and identified as hydroxy decarboxilated oleuropein aglicone what is in good agreement with the migration time (higher in this case because of its extra hydroxyl group) and with its main fragment m/z 199 with the same loss $[M-H-136]^-$ than DOA. In order to corroborate the results obtained and because of the intensity of the compounds was enough, MS/MS experiments were performed using QTOF (collision energy 17 eV). Figure 4 shows the fragmentation patterns obtained for these two decarboxilated-oleuropein derivatives.

An ion with m/z signal of 409.1135 indicates that two hydroxyl groups have been incorporated into Ol Agl molecule and this compound was identified as dihydroxy oleuropein aglicone. It is eluted in LC before Ol Agl and hydroxy-Ol Agl because of its higher polarity, and in CE, it migrates after DOA due to its high mobility with the introduction of more negative charge. Moreover, it shows the same fragmentation patterns of the Ol Agl with the following fragmentation ions m/z 345, 307, 275, 195, 149, 139, 111 obtained in the MS/MS experiments (see Fig. 5). A compound with m/z 243.0865, very low intensity and with the same fragments as EA $[M-H-32]^-$ and $[M-H-76]^-$ was identified as dihydro-elenolic acid. Besides, other phenolic compounds like methyl caffeate and tetrahydroxy isoflavanone were also found. We found that **Fraction**

8 was mainly composed by DOA (m/z 319.1171) accompanied by other small molecules that can be seen in the table 1. In **fraction 9**, it was possible to identify one of the isomers of hydroxy-oleuropein aglicone (m/z 393.1199) with almost the same fragmentation pattern and small quantities of DOA; but the most interesting fact was that, in this fraction, a MS signal with 319.1141 m/z (the same signal as DOA and the same loss $[M-H-120]^-$ as D-Lig Agl which produces a fragment of 199) was identified as hydroxy decarboxilated-ligustroside aglicone. In figure 4 we can see the fragmentation pattern for this compound that corroborates its identification.

In **Fraction 10** small quantities of Lig Agl, that will accompany the remaining fractions, start to appear due to this compound elutes from the LC column for a long time. We can also find DOA remaining and other compounds.

Fractions 11, **12**, **13** correspond to the collection of lignans. The most important compound in **fraction 11,** with m/z 417.1556, was a lignan identified as syringaresinol. This compound has been recently described by Christophoridou et al. [50] in olive oil studies carried out with LC-SPE-NMR. This lignan was not completely pure, since was eluted with small amounts of other compounds, such as Lig Agl, vanillyl alcohol, trimethoxyhidrocinnamic acid and zinniol.

Fractions 12 and **13** so far identified in UV as Pin and Ac Pin appeared together with a compound of m/z 303.1225 identified as D-Lig Agl with the same fragmentation ion m/z 183 as DOA and small amounts of Lig Agl. Fig. 4 shows the fragmentation of the mentioned compound. **Fraction 14** was identified as 10-hydroxy oleuropein aglycone (m/z 393.1248); that phenolic substance had the same fragmentation ions (m/z 345, 307, 275, 195, 149, 139, 111 obtained in the MS/MS experiments) as Ol Agl and its derivatives (see Fig. 5). In that fraction we found as well small proportions of other secoiridoids like D-Lig Agl (m/z 303.1225), Ol Agl and Lig Agl. A compound with m/z 357.1326 was tentatively identified as a new lignan named matairesinol.

Fraction 15 so far identified as Ol Agl, also contains other compounds with low polarity, as for example a compound of m/z 333.1354 that could be a derivative of Lig Agl $[361-CO]^-$, since it shows some of the same losses [M-H-120] y [M-H-152] and also a fragment with m/z 137 equal to the Lig Agl. That tentative identification has to be still corroborated, that is the reason we do not identify it in Table 1. Moreover, a group of three compounds with very low intensities and similar migration time have been identified as three isoprenylated flavones with O-heterocyclic rings (closed furano and pyrano derivatives) very common in plant extracts. The introduction of these groups

into the flavonoids converts them into more lipophilic substances [51]. It is also possible to find other compounds tentatively identified in Table 1.

Fraction 16 is mainly composed by a mixture of Ol Agl and Lig Agl together with other compounds in small quantities some of them still unidentified and **fraction 17** is pure Lig Agl.

3.3 Application of the isolated and characterized fractions

As commented before, the isolated phenolic fractions offer an important potential because their individual study could help us to understand their importance not only concerning the health beneficial effects of the extra virgin olive oil that contains them, but also regarding its sensory characteristics (bitterness, burning, astrigentness) or its antioxidant capacity. In this paper, the phenolic fractions have been used for two specific applications: quantification of some components of extra virgin olive oil samples in terms of pure fractions, and *in vitro* studies of its anticarcinogenic capacity.

3.3.1 Quantification of some components of extra virgin olive oil samples in terms of pure fractions

When the analyst has no commercially available standards, the three following approaches can be used: to quantify considering an external standard with a similar structure to the compound under study; to use an internal standard added to the extract that we wanted to analyze; or even to take into account external standards (with structure completely different from the analytes under study) in order to compare with other data found in literature [40,47]. In the current work the quantification of several phenols from olive oil was done using the isolated standards in our laboratory (after checking their purity). We could have used every isolated fraction to quantify the different phenols present in oil getting results closer to the real concentrations and avoiding the traditional approaches, but since we figured out that some fractions were not 100% pure, we decided to show here just the results achieved for the quantification in terms of the highly-pure fractions, that is hydroxytyrosol, tyrosol, elenolic acid and ligustroside aglicone. Standard calibration graphs were prepared for each compound by using both UV detection (280nm and 240 nm) (Table 2) and mass spectrometry detection (Table 3). Both tables contain information regarding DL, calibration range and r^2. The calibration plots indicate good correlation between peak areas and analyte

concentrations. We include the calibration curves and the other analytical parameters for Dopac and Ol Gluc (at different wavelengths). They have been widely used to quantify HYTY, TY and HYTY-Ac (Dopac) and lignans and secoiridoids (Ol Glu).

At the moment we used the isolated fractions as standards for quantification, HYTY and TY were commercially available; it is important to highlight that the results achieved by using our standards and those with commercial standards were not statistically significantly different. The quantitative results from HPLC-UV and HPLC-TOF are presented in Table 4 where the units are mg analyte/kg olive oil. We can easily observe the differences found in the quantification results when we compare the values achieved by using the isolated standards or when we use the traditional external ones. The results obtained by using Dopac as external standard to quantify HYTY and TY were quite different from those obtained when isolated standards were used (regardless the detection system used). The same happened when Ol Glu was used in UV to quantify EA and Lig Agl. The greatest differences were found when we used Ol Glu as external standard with TOF because of the unefficient response that this compound shows in mass spectrometry probably due to a poor nebulization.

3.3.2 In vitro studies of its anticarcinogenic capacity

Moreover, some of the isolated fractions were studied to evaluate the anti-proliferative and the pro-apoptotic effects of EVOO phenolic compounds, concluding that oleuropein aglycone is among the first examples of how selected nutrients from an EVOO-rich "Mediterranean diet" directly regulate HER2-driven breast cancer disease [52]. After those findings, we kept working in the same direction and we tested the effects of phenolic fractions from EVOO on the expression of fatty acid synthase (FASN), a key enzyme involved in the anabolic conversion of dietary carbohydrates to fat in mammals protein expression. EVOO lignans and secoiridoids were found to drastically suppress FASN protein expression in HER2 gene-amplified SKBR3 breast cancer cells, revealing that phenolic fractions, directly extracted from EVOO, may induce anti-cancer effects by suppressing the expression of the lipogenic enzyme FASN in HER2-overexpressing breast carcinoma cells, offering a previously unrecognized mechanism for EVOO-related cancer preventive effects [53].

More recently, the ability of EVOO polyphenols to modulate HER2 tyrosine kinase receptor-induced in vitro transformed phenotype in human breast epithelial cells was checked, identifying novel roles for naturally-occurring EVOO-derived polyphenols in

human breast cancer cell growth and HER2-regulated malignant transformation and providing new insights into the molecular mechanisms underlying the protective effects of naturally occurring EVOO biocompounds on breast cancer risk [54].
To the best of our knowledge, isolated fractions from EVOO have been used by first time in these kinds of biomedical studies, proving our point concerning the usefulness of the mentioned fractions for other purposes.

4 Concluding remarks

In the present work a two-dimensional HPLC-CE method has been developed to isolate and characterize different phenolic fractions from EVOO. The use of two complementary techniques with different separation principles allows a more complete characterization of the olive oil profile. The mentioned techniques together with the potential of TOF MS and qTOF MS helped us to find some compounds which have never been described before in this fraction of the olive oil. That opens new possibilities for future works, such as carring out studies with nuclear magnetic resonance (NMR) in order to elucidate the structures of tentatively identified compounds.
Furthermore, the importance of the isolated fractions is remarkable, since most of the compounds can not be purchased as commercial standards and could facilitate their future use to study some characteristics of each individual fraction. In this work some of the isolated fractions have been used to quantify several compounds in the polyphenolic profile of olive oil and to make *in vitro* studies of their anti-cancer properties.

5 Abbreviations used

EVOO, extra-virgin olive oil; **TY**, tyrosol; **HYTY**, hydroxytyrosol; **HYTY-Ac**, 2-(4-hydroxyphenyl)ethyl acetate or hydroxytyrosol acetatet; **10-H-Ol Agl**, 10-hydroxy-oleuropein aglycon; **EA**, elenolic acid; **Pin**, (+)-pinoresinol; **Ac Pin**, (+)-1-acetoxypinoresinol; **H-Pin**, hydroxyl-pinoresinol; **Lig Agl**, ligstroside aglycon; **Ol Agl**, oleuropein aglycon; **D-Lig Agl,** decarboxilated or deacetoxy derivatives of Lig Agl; **DOA,** decarboxilated derivatives of Ol Agl; **RSD**, Relative Standard deviation.

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Caption to figures

Figure 1. Chromatograms of the six different varieties used during the preliminary studies in order to select the most appropriate EVOO to proceed with the isolation collecting several fractions. (a) Picual, (b) Lechín, (c), Cornicabra, (d) Arbequina, (e) Hojiblanca, and (f) Picudo. Chromatographic conditions were those described in Materials and Methods section, the analytical column was a C_{18} Gemini column, 5 μm i.d., 25 cm x 3.0 mm, and the detection was made at 280 nm.

Figure 2. Chromatograms (at 240 and 280 nm) of the mixture of EVOOs selected for carrying out the isolation of several fractions analyzed by semi-preparative HPLC and the different fractions collected (with a number).

Figure 3. Extracted ion electropherograms (EIEs) of the isolated fractions together with information concerning the experimental m/z signal for every compound detected in all the fractions. Well-known phenolic compounds identified in some of the fractions: (1) HYTY, (2) TY, (3) HYTY-Ac, (5) EA, (8) DOA, (12) Pin, (13) Ac-Pin, (14) 10-H-Ol Agl, (16) Ol Agl, and (17) Lig Agl.

Figure 4. MS/MS spectra for (a) 319.1198, (b) 335.1164, (c) 303.1247 and (d) 319.1184 showing, in the structure of each compound, the possible fragmentation patterns.

Figure 5. MS/MS spectra belonging to 377.1244, 393.1464, 409.1495 and 361.1287.

Table 1. m/z experimental, ISCID and MS/MS fragments, formula, error, sigma and possible identification for every compound found in the different fractions.

Fractions	*m/z experimental*	*ISCID Fragments*	*MS/MS fragments*	*Formula (Smart Editor)*	*Error (ppm)*	*Sigma*	*Possible Compounds*
1	153.0557			C8H9O3	0.33	0.0020	HYTY
2	137.0609			C8H9O2	-0.96	0.0065	TY
3	195.0663			C10H11O4	0.01	0.0106	HYTY-Ac
	271.0601*			C15H11O5	4.00	0.0141	Licodione
	297.1511			C19H21O3	5.00	0.0355	Glepidotin C
	191.0567			C7H11O6	-3.30	0.0166	Quinic acid
	257.0664	225,213,181,137	241,225,213,195,181,137	C11H13O7	1.25	0.0057	Hydroxy elenolic acid
	191.0199			C6H7O7	-1.05	0.0026	Succinic acid
4	195.0669			C10H11O4	-3.24	0.0097	HYTY-Ac
	517.1609*			C26H29O11	19.01	0.0829	8-prenyldihydrokaempferol 7-glucosi
	419.1853	387		C26H27O5	2.66	0.0407	Diprenylisoflavone derivative
	401.1729	369		C26H25O4	7.40	0.0224	Alpinumisoflavone derivative
	355.1666*			C14H27O10	-15.71	0.0468	Unknown
	241.0720	209,165,139,127	209,181,171,165,139,127	C11H13O6	-0.91	0.0029	EA
	265.1465*			C15H21O4	-7.31	0.0967	Xanthonic acid
	171.1380*			C10H19O2	5.11	0.0081	Capric acid
5	241.0721	209,165,139,127	209,181,171,165,139,127	C11H13O6	-1.31	0.0038	EA
	265.1471*			C15H21O4	-9.60	0.0248	Xanthonic acid
	183.1001*			C10H15O3	14.10	0.0306	Oleuropeic acid
	257.0668*			C11H13O7	0.65	0.0204	Hydroxy elenolic acid
	187.0971*	169,125		C9H15O4	2.49	0.0074	Azelaic acid
6	241.0711	209,165,139,127	209,181,171,165,139,127	C11H13O6	2.71	0.0365	Elenolic acid
7	319.1179	183	275,249,183,165	C17H19O6	2.59	0.0025	DOA
	193.0513			C10H9O4	-3.39	0.0140	Methyl caffeate
	409.1135	345,275	377,345,327,307,275,241,195,149,139,111	C19H21O10	1.35	0.0059	Dihydroxyoleuropein aglicone
	243.0865*	211,167		C11H15O6	-0.91	0.0132	Dihydroelenolic acid
	335.1137	199	275,249,199,183,165	C17H19O7	-0.09	0.0037	Hydroxy D-oleuropein aglicone
	287.0560			C15H11O6	0.41	0.0092	Tetrahydroxy isoflavanone
8	319.1171	183	275,249,183,165	C17H19O6	4.93	0.0035	DOA
	151.0396			C8H7O3	2.95	0.0331	Vanillin
	227.2007			C14H27O2	4.12	0.0220	Myristic acid
	185.1176			C10H17O3	4.13	0.0113	Oxodecanoic acid
9	393.1199		345,327,321,307,275,213,181,149,139,111	C19H21O9	2.04	0.0120	Hidroxy oleuropein aglicone
	319.1178	183	275,249,183,165	C17H19O6	2.85	0.0190	DOA
	319.1141	199	285,259,233,199,183,165	C17H19O6	11.21	0.0029	Hydroxy D-ligustroside aglicone
	185.1178			C10H17O3	2.74	0.0106	Oxodecanoic acid
	191.0563			C7H11O6	-1.22	0.0177	Quinic acid
10	361.1341			C19H21O7	-4.90	0.0129	Lig Agl
	319.1190	183	275,249,183,165	C17H19O6	-0.97	0.0138	DOA
	153.0550*			C8H9O3	4.71	0.0116	Vanillyl alcohol
	263.1287	219,201,153		C15H19O4	0.56	0.0549	Abscisic acid
	271.0793	227,153		C12H15O7	-1.38	0.0123	Pentamethoxy benzoic acid
11	361.1311			C19H21O7	-5.10	0.0193	Lig Agl
	417.1556			C22H25O8	-0.25	0.0129	Syringaresinol
	153.0557*	123		C8H9O3	3.35	0.0347	Vanillyl alcohol
	239.0928	195,121		C12H15O5	-1.29	0.0207	Trimethoxyhydrocinnamic acid
	265.1453			C15H21O4	-2.79	0.0967	Zinniol
12	361.1293*			C19H21O7	-5.51	0.0329	Lig Agl
	303.1225	183	285,259,233,199,183	C17H19O5	4.36	0.0234	D-Lig Agl
	357.1135			C20H21O6	2.50	0.0064	Pin
13	361.1281*			C19H21O7	-3.32	0.0088	Lig Agl
	303.1225	183	285,259,233,199,183	C17H19O5	4.36	0.0234	D-Lig Agl
	415.1393			C22H23O8	1.37	0.0051	Ac Pin
14	361.1306*			C19H21O7	3.60	0.0125	Lig Agl
	377.1242*			C19H21O8	-4.70	0.0035	Ol Agl
	303.1225	183	285,259,233,199,183	C17H19O5	4.36	0.0234	D-Lig Agl
	357.1326			C20H21O6	-5.01	0.0180	Matairesinol
	327.2164			C18H31O5	4.00	0.0135	2,3-dinor-8-iso prostaglandin F1alpha
	393.1248		377,345,327,321,307,291,275,241,149,139,111	C19H21O9	-14.06	0.0428	10-H-oleuropein aglicone
15	377. 1249	345,307,275	345,327,307,275,239,195,149,139,111	C19H21O8	-1.93	0.0105	Ol Agl
	153.0554	123		C8H9O3	0.90	0.0004	Dimethoxy phenol
	287.2239			C16H31O4	-3.84	0.0047	Dihydroxy palmitic acid
	503.2037*			C30H31O7	7.50	0.0353	Prenylflavones derivative
	471.1784*			C29H27O6	6.20	0.0262	Prenylflavones derivative
	519.2063*			C30H31O8	7.60	0.0449	Prenylflavones derivative
	333.1357	213,181	213,181,137	C18H21O6	-4.03	0.0070	Unknown
	407.1363	363,317		C20H23O9	-3.88	0.0047	Methyl aloeresin
16	361.1311	291,241	291,259,171,127,111,101	C19H21O7	-5.00	0.0144	Lig Agl
	377.1254	345,307,275	345,327,307,275,239,195,149,139,111	C19H21O8	-3.10	0.023	Ol Agl
	329.2331			C18H33O5	0.61	0.0033	Trihydroxy octadecenoic acid
	333.1354	213,181		C18H21O6	-3.80	0.0189	Unknown
	297.1535			C19H21O3	-11.81	0.0484	Ostruthin
	265.1484			C15H21O4	-14.51	0.0408	Vanillic acid, heptyl ester
	173.1189			C9H17O3	-3.10	0.0293	Unknown
17	361.1308	291,241	291,259,171,127,111,101	C19H21O7	-4.76	0.0211	Lig Agl

*compounds in small traces that are not represented in fig. 1.

** In columns for ISCID and MS/MS fragments we do not include decimal figures to contain the size of the table. Anyway, mass accuracy information and isotopic pattern were used to carry out the tentative identification of compounds under study.

-We represent in blue color the main compound in each fraction

Table 2. Analytical parameters for the HPLC-UV method. y=bx±a, where y is the peak area (AU), x is the concentration (μg/ml), a is the y intercept, and r^2 is the correlation coefficient.

Compounds	LD (μg/ml)	Calibration range	Calibration curves	r^2
Hydroxytyrosol	0.032	QL-100	y = 10045.61 x – 6481.98	0.9932
Tyrosol	0.021	QL-100	y = 15368.82 x – 5226.01	0.9933
Elenolic acid[a]	0.019	QL-100	y= 38072.54 x + 349715.80	0.9991
Ligstroside aglicone	0.076	QL-200	y = 4183.30 x + 18142.70	0.9563
Dopac	0.013	QL-50	y = 24868.02 x – 19085.33	0.9990
Oleuropein glucoside	0.045	QL-250	y = 7126.74 x + 22082.33	0.9886
Oleuropein glucoside[a]	0.010	QL-150	y = 34193.45 x + 154568.17	0.9761

[a] 240 nm

LD=$3\sigma_b$/b (σ_b= 106.5668, calculated using 100 data); LQ=$10\sigma_b$/b

Table 3. Analytical parameters for the HPLC-ESI-TOF MS method. y=bx±a, where y is the peak area, x is the concentration (μg/ml), a is the y intercept, and r^2 is the correlation coefficient.

Compounds	LD (μg/ml)	Calibration range	Calibration curves	r^2
Hydroxytyrosol	0.060	QL-100	y = 1312837.35x + 2888083.52	0.9881
Tyrosol	0.094	QL-100	y = 1069088.26x + 576012.73	0.9831
Elenolic acid	1.850	QL-200	y = 176399.71 x + 471557.17	0.9915
Ligstroside aglicone	0.294	QL-200	y = 188216.09x + 2113758.02	0.9513
Dopac	0.078	QL-50	y = 3096341.03x – 9916830.16	0.9878
Ol europein glucoside	5.921	QL-200	y = 5643.60x + 32943.33	0.9607

LD was calculated considering S/N=3

Table 4. Quantitative results achieved by using HPLC-UV and HPLC-ESI-TOF MS methods. (Value= X ± SD) (n=5)

Compounds	***mg analyte/kg olive oil (TOF)***	***mg analyte/kg olive oil (TOF)♣***	***mg analyte/kg olive oil (UV)***	***mg analyte/kg olive oil (UV)♣***
Hydroxytyrosol	15.73 ± 0.97	8.59 ± 0.04	14.34 ± 0.23	6.25 ± 0.95
Tyrosol	9.07 ± 0.52	4.76 ± 0.02	8.90 ± 0.32	5.77 ± 0.20
*Elenolic acid**	64.91 ± 3.93	2123.47 ± 128.82	41.11 ± 0.73	44.03 ± 0.82
Ligstroside aglicone	38.53 ± 3.84	1430.41 ± 128.05	44.77 ± 0.95	26.06 ± 0.56

*240 nm

♣ Quantified in terms of external standards widely used in literature (Dopac (for HYTY, TY and HYTY-Ac) and Oleuropein glucoside (for lignans and secoiridoids)).

Figure 1. Chromatograms of the six different varieties used during the preliminary studies in order to select the most appropriate EVOO to proceed with the isolation collecting several fractions. (A) Picual, (B) Lechı´n, (C), Cornicabra, (D) Arbequina, (E) Hojiblanca and (F) Picudo. Chromatographic conditions were those described in Section 2, the analytical column was a C18 Gemini column, 5 mm id, 25 cm_3.0 mm, and the detection was made at 280 nm.

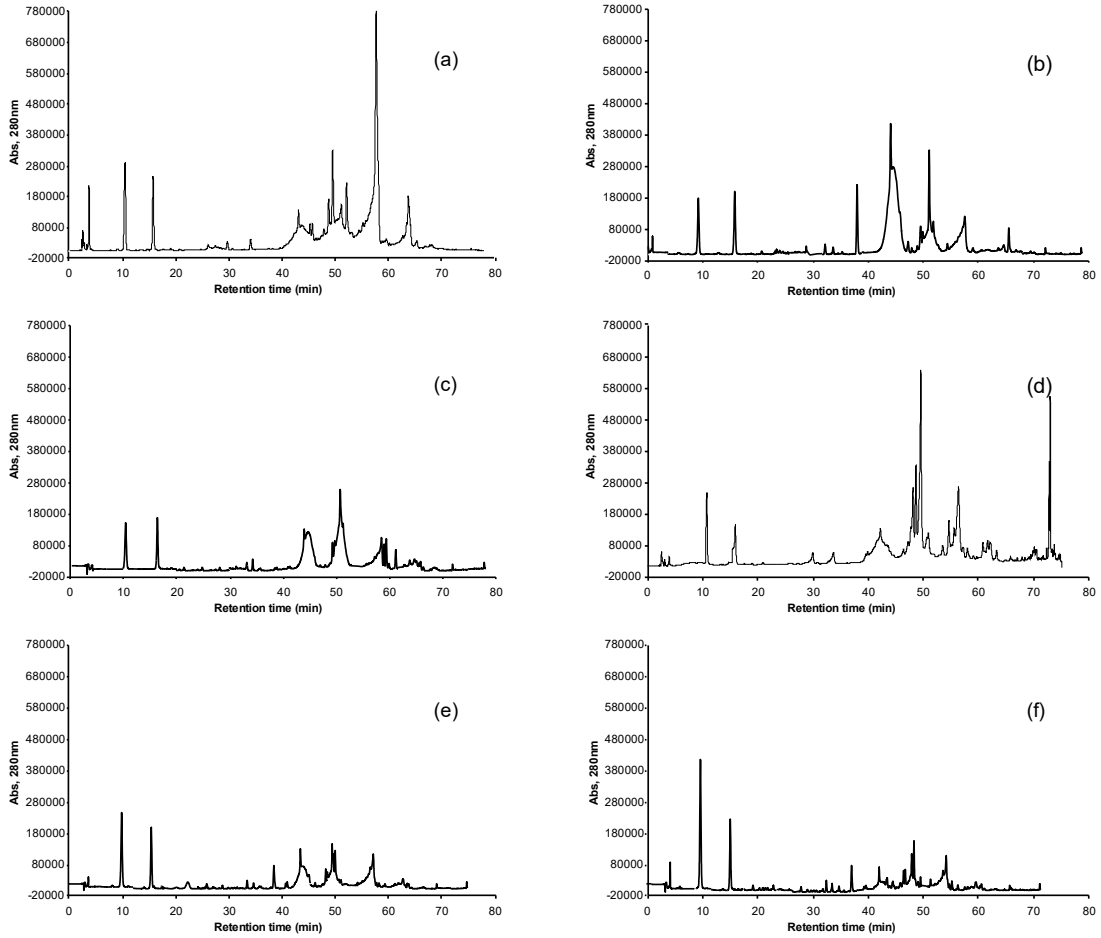

Figure 2. Chromatograms (at 240 and 280 nm) of the mixture of EVOOs selected for carrying out the isolation of several fractions analyzed by semi-preparative HPLC and the different fractions collected (with a number).

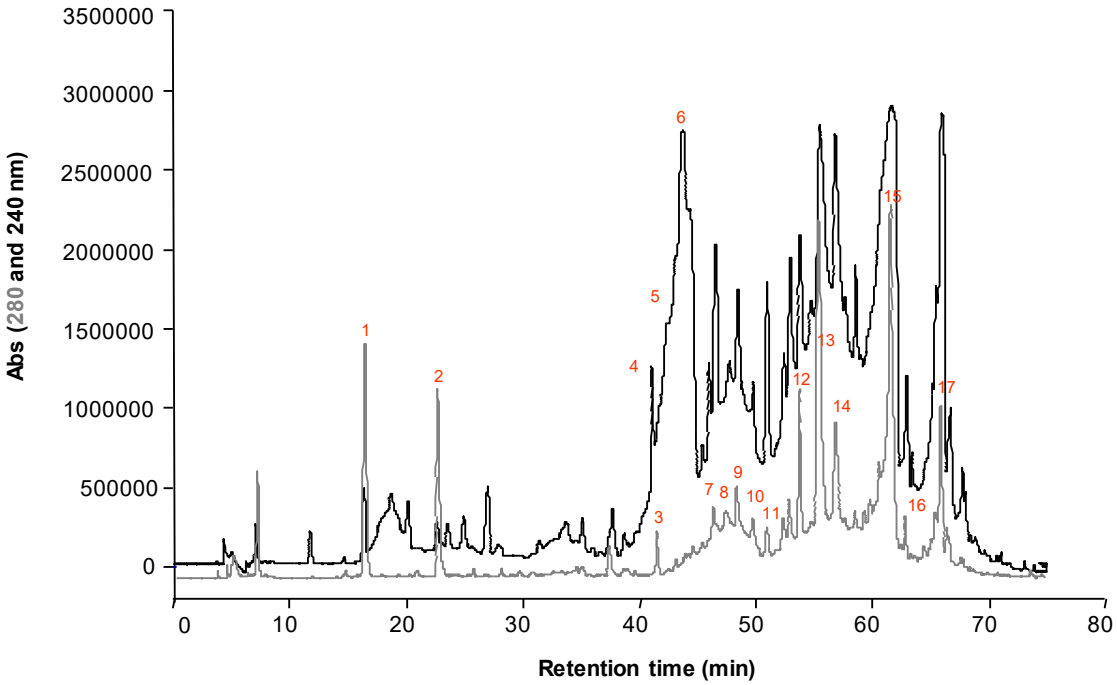

Figure 3. Extracted ion electropherograms of the isolated fractions together with information concerning the experimental *m/z* signal for every compound detected in all the fractions. Well-known phenolic compounds identified in some of the fractions: (1) HYTY, (2) TY, (3) HYTY-Ac, (5) EA, (8) DOA, (12) Pin, (13) Ac-Pin, (14) 10-H-Ol Agl, (16) Ol Agl, and (17) Lig Agl.

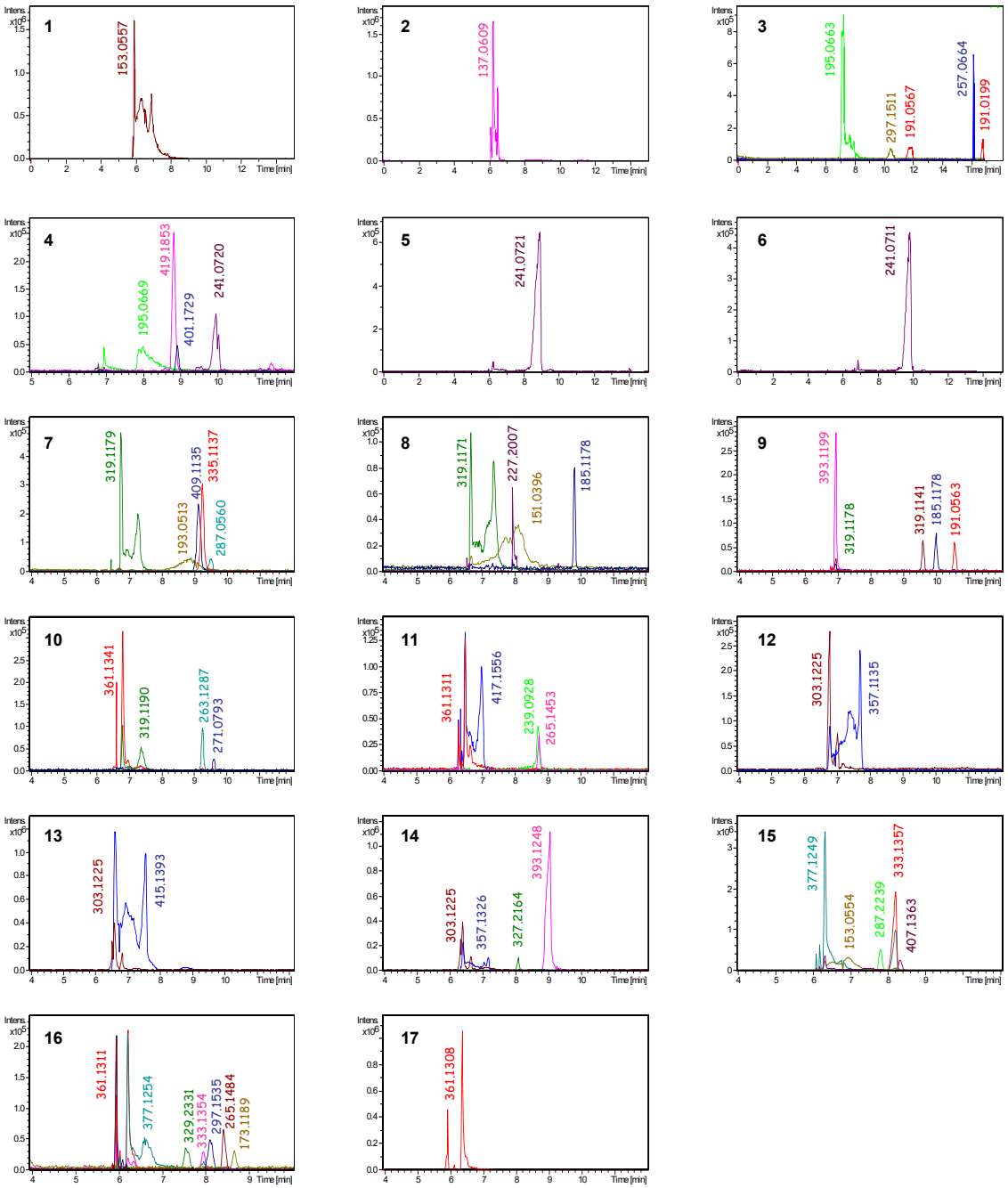

Figure 4. MS/MS spectra for (A) 319.1198, (B) 335.1164, (C) 303.1247 and (D) 319.1184 showing, in the structure of each compound, the possible fragmentation patterns.

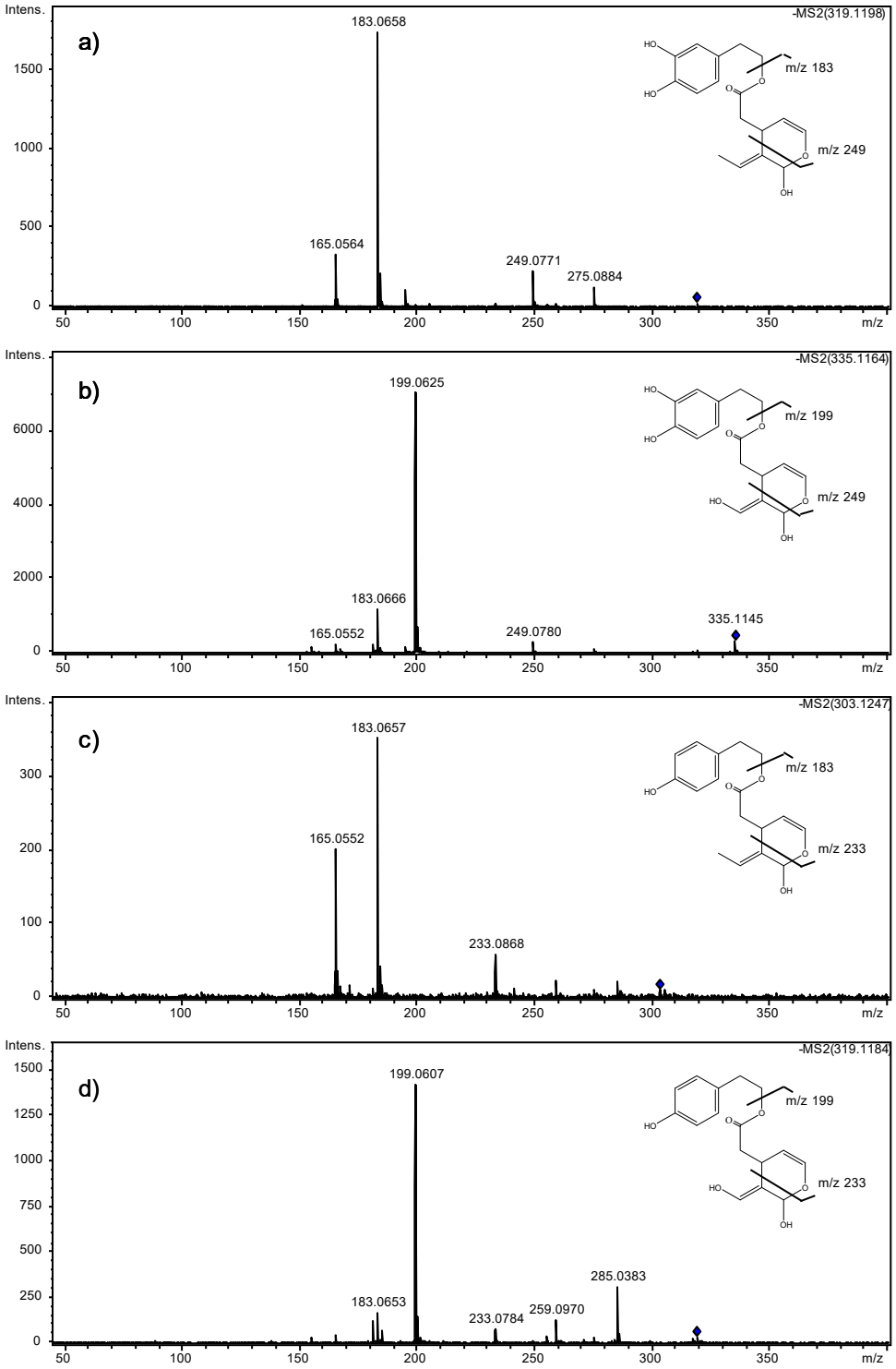

Figure 5. MS/MS spectra belonging to 377.1244, 393.1464, 409.1495 and 361.1287.