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Gas chromatography–atmospheric pressure chemical ionization-time of flight mass spectrometry for profiling of phenolic compounds in extra virgin olive oil

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ABSTRACT

A new analytical approach based on gas chromatography coupled to atmospheric pressure chemical ionization-time of flight mass spectrometry was evaluated for its applicability for the analysis of phenolic compounds from extra-virgin olive oil. Both chromatographic and MS parameters were optimized in order to improve the sensitivity and to maximize the number of phenolic compounds detected. We performed a complete analytical validation of the method with respect to its linearity, sensitivity, precision, accuracy and possible matrix effects. The LODs ranged from 0.13 to 1.05 ppm for the different tested compounds depending on their properties. The RSDs for repeatability test did not exceed 6.07% and the accuracy ranged from 95.4% to 101.5%. To demonstrate the feasibility of our method for analysis of real samples, we analyzed the extracts of three different commercial extra-virgin olive oils. We have identified unequivocally a number of phenolic compounds and obtained quantitative information for 21 of them. In general, our results show that GC–APCI-TOF MS is a flexible platform which can be considered as an interesting tool for screening, structural assignment and quantitative determination of phenolic compounds from virgin olive oil.

1. Introduction

The beneficial effects of the Mediterranean diet on human health such as reducing the risk of atherosclerosis, cardiovascular diseases and certain types of cancer are proven facts [1,2]. The dietary consumption of virgin olive oil (VOO) by Mediterranean populations is believed to play a key role in this health protective phenomenon. Historically, the health protecting properties of VOO have been ascribed to the high proportion of monounsaturated fatty acids. However, the importance of the minor components, such as phenolic compounds, is becoming more and more noticeable [3–5]. This is not surprising as phenols are essential for olive oil resistance to oxidation processes [6,7]. Additionally, the level of these substances is a very important parameter of VOO quality and it largely defines its organoleptic characteristic (flavour, astringency, pungency and bitterness) [8–10]. The phenolic compounds of VOO belong to several classes, such as phenolic acids, phenolic alcohols, flavonoids, hydroxy-isocromans, secoiridoids and lignans [11]. Several factors influence the differences in phenolic compounds composition from one VOO to another: variety of the olive fruit, agricultural techniques used to cultivate the olive fruit, maturity of the olive fruit at harvest time, olive oil extraction, processing, storage methods, etc. [1,11–13]. Consequently, the phenolic content can be an unique characteristic of olive oil and a very important parameter for quality monitoring.

Thus, the structural and quantitative analysis of the individual phenolic compounds present in VOO is an important part in the quality assessment. An analysis of the literature shows that HPLC (used with UV, fluorescence, electrochemical, biosensors, NMR and MS detectors) takes as much as 80% of all described applications of the determination of polyphenols in olive oil; gas chromatography (GC) covers another 15% and the rest is covered by such applications as capillary electrophoresis (CE) [11,14–19]. GC was so far used with FID or MS. So, according to literature, GC is far from being a mainstream method of analyzing of VOOs phenolic compounds. Nevertheless, the results obtained using GC are quite interesting, but the use of GC is less common due to the necessary derivatization and the use of high temperature which could damage the analytes.

The first GC analysis of phenolic compounds in olive oil has been reported more than 30 years ago [20] by Janer del Valle. This report was soon followed by a study where GC was used for authentication purposes, namely for identification of VOOs and refined oils [21,22]. In 1987, Forcadell et al. [23] developed a protocol for the preparation of trimethylsilyl (TMS) derivatives and Solinas [24] showed the feasibility of this approach

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for qualitative/quantitative analysis of the phenolic compounds in VOO of several cultivars at different ripening degrees. With the development of analytical instrumentation and mass spectrometers in particular, the methods of compound identification have improved significantly [25–28]. One of the most recent applications was developed by Ríos et al. [14], when they optimized a solid phase extraction-GC-ion trap MS method for the qualitative evaluation of phenols in VOO and the structural confirmation of oleuropein and ligstroside aglycons and their oxidation products.

The current work is a further attempt to show the feasibility of GC–MS for the analysis of phenolic compounds. However, instead of "classical" GC–MS systems with vacuum stage ionization sources (electron ionization (EI) and chemical ionization (CI)), we evaluated the use GC–MS with a recently developed atmospheric pressure chemical ionization (APCI) source. GC–APCI-MS was introduced in early seventies by Horning [29], but for variety of (mostly technical) reasons has remained an exotic application. The recent explosive development of mass spectrometry instrumentation has created the prerequisites for a reintroduction of GC–APCI-MS [30–32]. The aim of this paper is to carry out an analytical evaluation of a GC–APCI-TOF MS platform to show the benefits of soft ionization source for GC in combination with a high-end time of flight mass analyzer for analyzing phenolic compounds from virgin olive oil. To achieve this purpose we have performed a complete validation of the developed method regarding its linearity, sensitivity, precision, accuracy and possible matrix effects. We demonstrate that GC–APCI-TOF MS could be used not only for screening of samples, but also for detailed structural analysis and quantitative determination of phenolic compounds. Providing a complementary information to the data obtained by LC–MS, CE–MS or/and other GC–MS configurations, this novel platform may contribute significantly to the development of food analysis and food metabolomics fields.

2. Materials and methods

2.1. Chemicals and samples

Only analytical reagent grade chemicals were used for this study. Sinapinic acid, gentisic acid, 4-hydroxyphenylacetic acid, vanillin, vanillic acid, caffeic acid, gallic acid, *trans*-cinnamic acid, protocatechuic acid, *p*-coumaric acid and hydroxytyrosol (HYTY) were purchased from Sigma–Aldrich (St. Louis, MO, USA); syringic acid, *m*-coumaric acid, 4-hydroxybezoic acid, homovanillic acid, ferulic acid, taxifolin and tyrosol (TY) were from Fluka (Buchs, Switzerland); luteolin (Lut) and apigenin (Apig) were from Extrasynthèse (Genay, France); and pinoresinol (Pin) was purchased from Arbo Nova (Turku, Finland). Dopac was purchased from Fluka and was used as internal standard (IS). Secoiridoids are not available as commercial standards, so we isolated them by semi-preparative HPLC (see Section 2.3).

The organic solvents, acetonitrile, methanol, and *n*-hexane, were from Sigma–Aldrich (St. Louis, MO, USA) and acetic acid from Merck (Darmstadt, Germany). Deionized and organic-eliminated water was from the water purifier system (USF^{ELGA} from Purelab Plus, Ransbach-Baumbach, Germany).

N,*O*-bis(Trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (BSTFA + 1% TMCS) and N-methyl-N-trimethylsilyltrifluoroacetamide with 1% trimethylchlorosilane (MSTFA + 1% TMCS) from Pierce (Oud-Beyerland, The Netherlands) were used as derivatization reagents. These reagents were used from freshly opened 1 ml bottles. Methoxyamine hydrochloride was purchased from Supelco.

Spanish extra-VOO samples used in the preliminary studies were obtained from unique varieties of olive fruit named Picual, Arbequina, Cornicabra, Frantoio and Hojiblanca (January 2009). A mixture of two varieties Picual/Arbequina (50/50, v/v) was used as analytical quality control (QC) sample and for the isolation of the different phenolic fractions. The high content of phenols was the reason for the selection of these two varieties as QC and source for isolation of phenolic fractions. For validation purposes we used the mentioned above QC samples and a standard mixture composed by eight phenolic compounds (TY, HYTY, homovanillic acid, *p*-coumaric acid, ferulic acid, Lut, Apig and Pin).

2.2. Solid phase extraction procedure

The isolation of the phenolic fractions from extra-VOO with Diol-cartridges was performed according to the solid phase extraction (SPE) protocol developed by Gómez-Caravaca et al. [33]. Briefly, the Diol cartridge (1 g/6 ml, from Supelco) was placed in a vacuum elution apparatus and pre-conditioned by passing 10 ml of methanol and subsequently 10 ml of hexane. About 60 g of extra-VOO was 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 *n*-hexane (15 ml) to remove the non-polar fraction of the oil, the sample was eluted with methanol (40 ml). The eluents were evaporated to dryness under reduced pressure in a rotary evaporator at 35 °C. The dried residue was then redissolved in 2 ml of methanol.

2.3. HPLC isolation of phenolic compounds

Compounds of lignans and secoiridoids families are neither available as commercial standards nor can be synthesized easily. Therefore they were isolated from extra-VOO samples by semi-preparative scale chromatography. The isolation of the compounds was carried out from the Diol-SPE extracts of the mixture of extra-VOO (Picual/Arbequina) obtained as described in Section 2.2 redissolved in 500 μl of methanol. Two hundred μl of the sample were injected onto the column in order to obtain concentrated profiles with good resolution.

Analyses were carried out 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 500 μl loop (Rheodyne, Cotati, CA, USA). The semi-preparative HPLC C18 column (Phenomenex Gemini, 25 cm × 10 mm, 5 μm average particle size) was used at a flow rate of 3 ml/min. The mobile phases consisted of water with 0.5% acetic acid (phase A) and acetonitrile (phase B). The solvent gradient was programmed as following: 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 until the end of a run.

2.4. Derivatization reaction

The derivatization reaction was carried out by adding 50 μl of BSTFA plus 1% TMCS to the dried sample. The solution was vortexed for 1 min and the trimethylsilylation reaction was performed at room temperature for 30 min. A minimum of 30 min equilibration time was used before the sample injection. The stability of BSTFA-derivatized samples, kept at ambient temperature (20–25 °C), was determined periodically by injecting replicate preparations of the processed samples consecutively for up to 48 h. Peak areas were chosen as parameter for stability evaluation.

A nitrogen flow was used for drying the standard mixture, the extra-VOO extracts and the isolated fractions to complete dryness. Then, the derivatization reagent was added.

2.5. GC–APCI-MaXis MS optima conditions

The GC experiments were performed using an Agilent 7890A GC (Agilent, Palo Alto, USA) equipped with a HP-5-MS column (30 m, 0.25 mm ID, 0.25 (m film thickness). An aliquot of the derivatized samples (1 μl) was applied by splitless injection (injection time 60 s) with a programmable CTC PAL multipurpose-sampler (CTC Analytics AG, Zwingen, Switzerland). Injection programs included sequential washing steps of the 10 μl syringe before and after the injection, and a sample pumping step for removal of small air bubbles. Helium was used as carrier gas and the injector temperature was set 250 °C.

Two temperature gradients were used during the study. Gradient one (run 1) was applied for the exploratory experiments: the column temperature was initially kept at 170 °C for 5 min, then from 170 to 255 °C at 3 °C/min, keeping that value for 1 min and finally from 255 to 310 °C at 2 °C/min and maintaining that temperature for 10 min. A constant flow rate of 0.5 ml/min was used. Using the described chromatographic conditions, the analysis time was about 70 min. Gradient two (run 2) of 50 minutes was used consequently for more routine measurements: the column temperature was initially kept at 160 °C for 5 min, from 160 to 188 °C at 3 °C/min keeping that value for 1 min, from 188 to 241 at 15 °C/min, keeping that value for 1 min, from 241 to 282 °C at 2 °C/min, from 282 °C to 310 °C at 5 °C/min and maintaining that temperature for 5 min. A constant flow rate of 1.0 ml/min was used.

The GC system was coupled to an ultra high resolution time of flight mass spectrometer MaXis (UHR TOF MS, MaXis, Bruker Daltonik, Bremen, Germany) using a multipurpose source equipped with GC transfer line [31]. The parameters of the APCI interface and all the parameters of the MaXis MS detector were optimized using the area of the MS signal for the polyphenols. The GC transfer line to the mass spectrometer was kept at 300 °C. The APCI source and MS were operated in positive mode. The pressure of the nebulizer gas (nitrogen) was set to 2 bar and temperature and flow rate of the dry gas (nitrogen) were 250 °C and 5.00 l/min, respectively. The APCI vaporizer temperature was 450 °C and the voltage of the corona discharge needle was 2000 nA. The mass analyzer was operating within a mass range from 50 to 1000 at spectra rate of 1 Hz. With these conditions a resolving power up to 45,000 was obtained. The instrument was calibrated externally using an APCI calibration tune mix. In addition, an internal calibration using cyclic-siloxanes (a typical background in GC–MS [34]) was used. The SmartFormula™ tool of DataAnalysis package (Bruker Daltonik, Bremen, Germany) was used for the calculation of elemental composition of compounds.

Two different MS/MS modes were used in the study: auto-MS/MS and multiple reaction monitoring (MRM). In the auto MS/MS mode, the spectra were produced by fragmentation of the main detected ions under general collision conditions for all of them. In the MRM mode, the appropriate precursor ion for every compound was selected and fragmented according to its own parameters (collision energy, isolation width, ISCID energy, amplitude). Besides, as the number of compounds studied was quite high and some masses were present throughout the entire chromatogram, we created several segments in our MS method, and in each one we chose the appropriate precursor ions with theirs own isolation and fragmentation conditions. The MS/MS spectra were acquired in eight different elution time windows: 0–9.1, 9.1–20, 20–40.8, 40.8–46.0, 46.0–52.0, 51.0–56.0, 56.0–62.2, and from 62.2 to the end of the run (elution time windows for run 1). Nitrogen was used as collision gas and the collision energy was set from 15 to 35 eV.

2.6. Validation experiments using extra-VOO quality control (QC) samples

2.6.1. Specificity

The specificity of the method was tested by screening analysis of phenolic-free oil samples or blank samples (refined sunflower oil). Refined sunflower oil was only used to evaluate the specificity of our method.

2.6.2. Linearity and sensitivity

The linearity of the detector response was verified with standard solutions at ten different concentration levels over the range from the quantification limit to 250 ppm. Each point of the calibration graph corresponded to the mean value from three independent replicate injections. Calibration curves were obtained for each standard by plotting the standard concentration as a function of the peak area obtained from GC–APCI-MaXis MS analyses. The sensitivity of the analytical procedure was calculated by defining the limits of detection (LOD) and quantification (LOQ) for the individual analytes included in standard solutions according to the IUPAC method [35]. The lowest concentration that could be detected with a reasonable certainty for our analytical procedure (LOD) was considered S/N = 3, whilst LOQ was S/N = 10.

2.6.3. Precision and accuracy

The precision of the analytical procedure described was measured as repeatability and evaluated over the linear dynamic range at three different concentration levels (low (LOQ), medium (intermediate concentration value of the linear calibration range), high (higher concentration value of the linear calibration range)). Spiked quality control (QC) samples were tested in six replicates per concentration and calculated with calibration curves obtained daily. The precision of the analytical procedure was expressed as the relative standard deviation (RSD). The intra- and inter-day repeatability in the peak areas was determined as the RSD obtained for six consecutive injections of each phenol at each concentration value, carried out within the same day and on three different days.

Accuracy was evaluated with separately prepared individual primary stock solutions, mixtures and working solutions of all standards. It was calculated over the linear dynamic range at three different concentration levels, i.e. (low (LOQ), medium (intermediate concentration value of the linear calibration range), high (higher concentration value of the linear calibration range)) by three determinations per concentration on different days. The analyte concentrations were calculated from daily calibration curves and the accuracy was calculated by the ratio of this calculated concentration versus the theoretical (spiked) concentration.

2.6.4. Matrix effects

To evaluate the matrix effect, we compared the MS response of the analytes under study spiked post-extraction with those in a pure solvent solution (in triplicate), calculating the response factors (RF, which is considered to be the ratio between the peak area and the concentration of the analyte) when the analytes were in the presence of the olive oil matrix and in a neat solution. We checked whether significant differences between both values could be found using ANOVA.

3. Results and discussion

3.1. GC–APCI-MaXis MS analysis: preliminary studies

The effects of several parameters such as the concentration of derivatization reagent, reaction time and temperature were

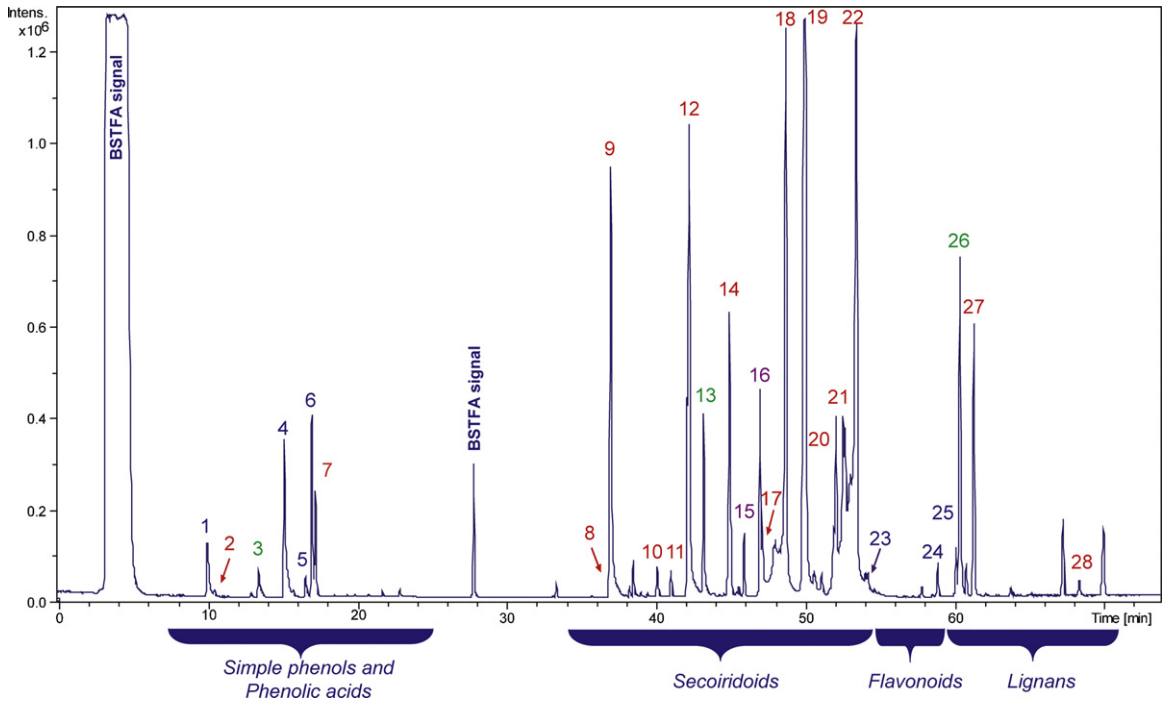

Fig. 1. Base Peak Chromatogram (BPC) of the Diol-SPE extract of a mixture of Arbequina and Picual oils. Elution windows of different phenolic fractions of EVOO are shown. The peaks have been identified by standards (blue), isolated fractions (red), and prior knowledge (literature) (purple). The peaks with considerable intensity which have not been identified are marked in green. *Peak identification*: **1**, Ty-2H + 2TMS; **2**, isolated 4; **3**, *m*/*z* 281.0966/192.9388; **4**, Hyty-3H + 3TMS; **5**, Protocatechuic acid-3H + 3TMS + H; **6**, Dopac-3H + 3TMS + H; **7**, EA-1H + 1TMS + H; **8**, EA-1H + 1TMS + H/isolated 2; **9**, isolated 6 (D-Lig Agl); **10**, isolated 6; **11**, isolated 6; **12**, DOA-2H + 2TMS + H; **13**, *m*/*z* 501.3843/411.3312; **14**, Lig Agl-1H + 1TMS + H; **15**, methyl Ol Agl-2H + 2TMS + H; **16**, H-D-Ol Agl-3H + 3TMS + H; **17**, isolated 6 (Lig Agl); **18**, isolated 6 (Lig Agl); **19**, Ol Agl-2H + 2TMS + H; **20**, isolated 8; **21**, 10 H-Ol Agl-3H + 3TMS + H; **22**, Ol Agl-3H + 3TMS + H; **23**, Apigenin-3H + 3TMS + H; **24**, Luteolin-4H + 4TMS + H; **25**, Pinoresinol-2H + 2TMS + H; **26**, *m*/*z* 397.3825; **27**, acetoxy-pinoresinol-2H + 2TMS + H; **28**, Syringaresinol-2H + 2TMS + H. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

studied. We have achieved the best performance adding 50 μl of BSTFA + 1% TMCS to the dried sample at room temperature and incubation time 1 h. The effect of including an intermediate step of methoxyamination was adequately evaluated and no change in the peak area or stability was observed.

Further, we have optimized the chromatographic and MS (APCI and MaXis) conditions for the maximum coverage, resolution and sensitivity, using the phenolic extracts from extra-VOO. Several varieties of olive oil (Picual, Arbequina, Cornicabra, Frantoio and Hojiblanca) were used during the optimization to ensure the applicability of the presented methodology for the analysis of these compounds in any kind of olive oil. The effect of different GC parameters (gas flow, sample injection and temperature gradient) and mass spectrometry conditions (position of the column in the transfer line, transfer line temperature, flow rate and pressure of nebulizer gas, vaporizer temperature, voltages in the corona and other source and ion transfer settings) were studied, and finally the conditions described in material and methods as run 1 were chosen as optimum. Fig. 1 shows the Base Peak Chromatogram (BPC) of an olive oil extract (mixture Picual/Arbequina) achieved using the optimum GC–APCI-MaXis MS procedure described above. Using these conditions, the analysis time was about 70 min and a clean chromatogram was obtained with high efficiency and good separation of a great number of compounds.

3.2. Identification of the compounds

For the peaks assignment a combination of prior knowledge, commercially available analytical standards and semi-preparative HPLC isolated fractions was used. In addition, MS/MS experiments were carried out to confirm the identification of the compounds.

3.2.1. GC–APCI-MaXis MS analysis of standard mixture

A standard mixture consisting of 22 commercially available phenolic compounds was analyzed under the optimal conditions to understand the signal that each phenolic compound produced in the system APCI-MaXis MS. They have been only studied so far by the classical ionization techniques coupled to GC, mainly with electron ionization which is a rather harsh technique. Table 1 summarizes data for all components of the mixture including their molecular formula, retention time, measured and theoretical *m*/*z*, relative abundance of each *m*/*z* signal present in the MS spectra, error (mDa) and mSigma value. The mass position error remained within 2.1 mDa and high quality Sigma fit values (<20 mSigma) were obtained for all compounds. We have highlighted in bold the prevalent ion which was observed in the MS spectrum.

All the compounds showed the complete silylation of their active hydrogen although they show different "resistance" to the in-source fragmentation process. For example, in such compounds as the flavonoids Lut and Apig, vanillin and phenolic acids with an acidic group in para-position to hydroxyl group (protocatechuic acid, syringic acid and gallic acid), the parent ion remains the most intense ion in the spectra. For other phenolic acids and compounds such as Pin (a lignan) and TY and HYTY (simple phenols), the products of in-source fragmentation appeared to be the most intense ions in the spectra. Simple phenols like TY and HYTY are represented by the radical $[M-XH+XTMS]^{\bullet+}$ instead of the molecular ion $[M-XH+XTMS+H]^{+}$. In general, all the phenolic compounds showed the same losses of 16 and 90, which might correspond to the fragments CH4 and C3H9SiO (trimethylsilyl group with an oxygen), respectively. This last loss (90) yields the prevalent ion in the spectrum of majority of the compounds. In the case of simple phenols a loss of 89 was observed, probably due to the presence of the radicals, as mentioned above. For

Table 1
GC–APCI-TOF MS signals (*m*/*z*) of the set of commercially available standards of the phenolic compounds.

m/*z* experimental	Retention time (minutes)	Predicted molecular composition	*m*/*z* theoretical	Error (mDa)	mSigma value	In-source fragmentation pattern	Compound identity
225.0939	9.4	C11H17O3Si	225.0941	0.2	7.8	209.0992 (7)/197.1001 (39)/166.0457 (21)	Vanillin-1H + 1TMS + H
221.0998 (10)	9.7	C12H17O2Si	221.0992	−0.6	4.1	205.0698 (53)/161.0794 (18)/**131.0507**	*trans*-Cinnamic acid-1H + 1TMS + H
282.1470 (5)	10.0	C14H26O2Si2	282.1466	−0.4	5.1	258.0970 (9)/**193.1061**	Tyrosol-2H + 2TMS
283.1165 (30)	11.3	C13H23O3Si2	283.1180	1.5	5.7	267.0908 (61)/**193.0713**	4-Hydroxybenzoic acid-2H + 2TMS + H
297.1316 (5)	11.6	C14H25O3Si2	297.1337	2.1	4.5	283.0791 (5)/267.0379 (12)/**178.9282**	4-Hydroxyphenylacetic acid-2H + 2TMS + H
313.1287 (46)	14.9	C14H25O4Si2	313.1286	−0.1	3.1	297.1022 (54)/**223.0817**	Vanillic acid-2H + 2TMS + H
370.1809 (10)	15.1	C17H34O3Si3	370.1810	0.1	10.6	**281.1410**/193.0691 (20)	Hydroxyyrosol-3H + 3TMS
327.1426 (5)	15.2	C15H27O4Si2	327.1442	0.1	7.1	281.1401 (20)/**209.1007**/137.0600 (13)	Homovanillic acid-2H + 2TMS + H
371.1563 (39)	15.4	C16H31O4Si3	371.1525	0.2	9.6	355.1248 (10)/**281.1060**/209.0646 (15)	Gentisic acid-3H + 3TMS + H
371.1565	16.6	C16H31O4Si3	371.1525	−1.0	15.1	355.1273 (23)/281.1075 (46)	Protocatechuic acid-3H + 3TMS + H
385.1676 (11)	17.0	C17H33O4Si3	385.1681	0.5	9.4	**267.0719**/172.7891 (7)	Dopac-3H + 3TMS + H
309.1347 (33)	17.9	C15H25O3Si2	309.1337	−1.0	6.7	293.1035 (41)/**219.0852**/172.9581(19)	*m*-Coumaric-2H + 2TMS + H
343.1400	18.8	C15H27O5Si2	343.1392	−0.8	7.1	327.1126 (47)/299.1528 (45)/253.0907 (83)/211.0791 (65)	Syringic acid-2H + 2TMS + H
309.1333 (72)	20.0	C15H25O3Si2	309.1337	−0.4	11.2	293.1069 (42)/**219.0868**/195.0848 (10)	*p*-Coumaric acid-2H + 2TMS + H
459.1860	20.8	C19H39O5Si4	459.1869	0.9	4.6	415.2030 (13)/369.1424 (11)/327.1306 (13)/239.0597 (5)	Gallic acid-4H + 4TMS + H
339.1447 (61)	24.4	C16H27O4Si2	339.1442	−0.5	10.2	323.1124 (24)/**249.0967**/177.0558 (10)	Ferulic acid-2H + 2TMS + H
397.1680 (37)	25.8	C18H33O4Si3	397.1681	0.1	7.2	**307.1232**/172.9582 (22)	Caffeic acid-3H + 3TMS + H
369.1546 (22)	28.7	C17H29O5Si2	369.1548	0.2	9.2	353.1244 (14)/**279.1084**	Sinapinic acid-2H + 2TMS + H
665.2635 (62)	49.9	C30H53O7Si5	665.2632	−0.3	18.3	593.2320 (11)/297.1009 (10)/**225.0608**/172.9581 (32)	Taxifolin-5H + 5TMS
487.1792	54.5	C24H35O5Si3	487.1787	−0.5	9.2	415.1399 (9)/193.0695 (7)	Apigenin-3H + 3TMS + H
575.2142	58.7	C27H43O6Si4	575.2131	−1.1	6.4	503.1744 (9)/281.1007 (10)	Luteolin-4H + 4TMS + H
503.2283 (16)	60.0	C26H39O6Si2	503.2280	−0.3	15.2	**485.2189**/414.1686 (31)/247.1154 (30)	Pinoresinol-2H + 2TMS + H

The prevalent ion in the MS spectrum for each compound highlighted in bold. The relative intensity of the other m/z signals present in the MS spectra of the phenols is shown between brackets (considering the prevalent ion in bold letter as 100%).

the phenolic acids such as 4-hydroxyphenylacetic acid, homovanillic acid and Dopac, with an acetic acid in their structure, the prevalent ion corresponded to the loss of 118 (72 (–C3H9Si) plus 46, which corresponds to Si(CH3)3OH + CO). The flavonoids, Lut and Apig, undergo low fragmentation with a main loss of 72 which corresponds to the trimethylsilyl group (–C3H9Si), and Pin showed the fragment 485 corresponding to a loss of 18. Thus, in-source fragmentation observed practically for all tested standards may have a negative effect reducing the intensity of the parent ions but, the fragmentation patters appear to be compound specific and as such can be efficiently used for the structure confirmation.

3.2.2. Analysis of the isolated phenolic fractions

There are no commercial standards available for all phenolic compounds of extra-VOO. Consequently, a semi-preparative HPLC (see Section 2.3) was used for the isolation of such important compounds as secoiridoids (oleuropein aglycon (Ol Agl), ligstroside aglycon (Lig Agl) and their derivatives) and some lignans ((+)-1-acetoxypinoresinol (Ac Pin) and syringaresinol). In total, nine different fractions were isolated. To obtain reference APCI-MS spectrum for the compounds present in every HPLC isolated fraction, the 9 phenolic fractions were analyzed individually with GC–APCI-TOF MS. Fig. 2 shows the BPC of the phenolic extract from an extra-VOO sample and the 9 isolated fractions (in colour) analyzed using the developed GC method (run 1). A semi-preparative purification provides no 100% pure compounds, therefore a number of peaks were observed in BPCs of each individual fraction. The combination of prior knowledge [36], the superior mass accuracy of TOF mass analyzer and isotopic distribution (SigmaFit) was used for structural assignment of the compounds. Table 2 summarizes the information about each isolated fraction, including the main phenolic compounds identified in each fraction with their retention time, experimental *m*/*z*, molecular formula, mass error and SigmaFit quality value. In-source fragmentation of the parent ions was clearly observed and, in several cases, the fragmentation patterns played an important part in the compound identification. In the mentioned table, we have included some information about the relative intensity of the *m*/*z* signals present in the MS spectra of the phenols. In fraction 1, elenolic acid (EA) and different isomers were identified with the fragments corresponding to a mass loss of 32 and 90. The compound corresponding to the silylation of two active hydrogens (*m*/*z* 387) might correspond to the presence of another isomeric form of EA. Lignans Ac Pin and syringaresinol were identified in fractions 5th and 6th respectively, and in both cases the loss of 18, 89 and 256, as in the case of Pin, was observed. The main secoiridoids (Ol Agl and Lig Agl), their isomers and related compounds (decarboxilated derivatives of Ol Agl (DOA), decarboxilated derivatives of Lig Agl (D-Lig Agl), 10-hydroxy-oleuropein aglycon (10-H Ol Agl)) were identified as well. In general, for the Ol Agl and their derivatives, we observed with a high intensity the fragment 281, and for Lig Agl and derivatives the fragment

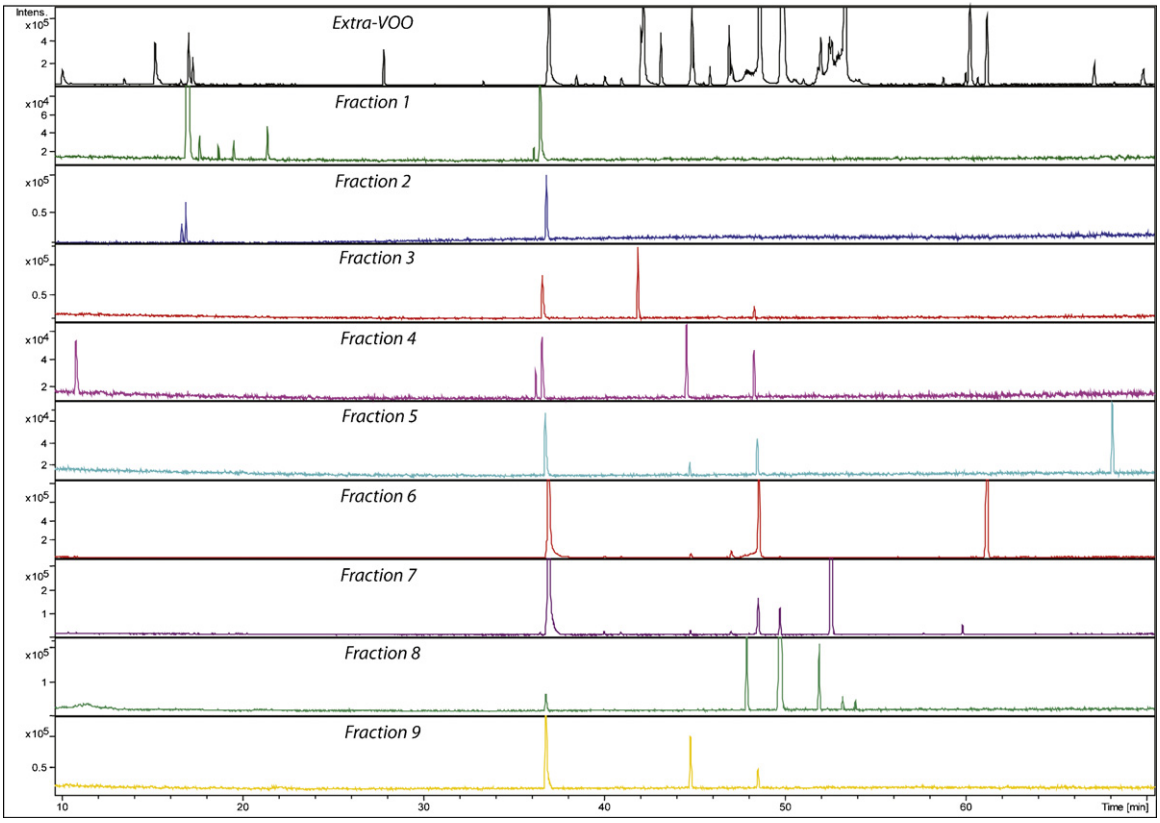

Fig. 2. BPCs of the phenolic extract from an extra-VOO sample and the 9 isolated fractions (in colour) analyzed using GC run 1. The peak with retention time 36.7 min present in every fraction has not been considered since it belongs to BSTFA derivatization reagent.

193. Besides, in most of the compounds a mass loss of 32 and 90 (C3H9SiO) was observed. Different silylated forms were found for Ol Agl (Ol Agl-2H + 2TMS and Ol Agl-3H + 3TMS) and Lig Agl (Lig Agl-H + TMS and Lig Agl-2H + 2TMS) probably due to the presence of different isomeric forms (aldehidic or dialdehidic form of EA).

3.2.3. MS/MS analyses

Assignment of phenolic compounds using reference spectra and prior knowledge is a practical tool for the screening of new products and testing quality of olive oil mixtures. However, unequivocal *de novo* identification of new compounds demands analysis of MS/MS spectra. Two different MS/MS modes were used in the study: auto-MS/MS and MRM. Every peak detected in the profile was isolated and further fragmented after applying the required energy to get a clean MS/MS spectrum. When a compound yields more than one *m*/*z* value, we considered as precursor ions all the different *m*/*z* signals observed in the MS spectrum for making MS/MS analyses. Table 3 includes the *m*/*z* APCI-MaXis MS/MS signals detected for the most relevant phenolic compounds present in the extract of extra-virgin olive oil. The prevalent ion in the MS spectrum for each compound is highlighted in bold. In this way, we were able to unequivocally identify several of phenolic compounds in the GC–APCI-MaXis MS of an extra-VOO.

MS/MS analysis proved to be especially useful for confirmation of the structures of secoiridoids derivatives such as Lig Agl and Ol Agl. In total, we found eight ligstroside derivatives and eleven oleuropein derivatives. Indeed, the intact secoiridoids, such as oleuropein glucoside and ligstroside glucoside, are undetectable in olive oil; due to the high solubility in water they are depleted during olive storage and olive oil extraction. Moreover, they undergo enzymatic hydrolysis first producing Ol Agl and Lig Agl upon removal of the attached glucose moiety and then a number of secoiridoid derivatives upon further molecular transformations via ring opening and rearranged re-closure [37]. Thus, enzymatic hydrolysis may explain the presence of the many isomeric or related forms in this family of compounds and the complexity of the secoiridoids group [38].

When the MS/MS behaviour of compounds such as TY is analyzed, we observe a fragment 109 *m*/*z* of the precursor ion 193 *m*/*z*. The same effect is observed for TY analogues (Lig Agl, for example), with a fragment 109 as prevalent ion in the MS/MS spectra. If we study the fragmentation pattern from HYTY analogues (DOA, Methyl Ol Agl, hydroxy-decarboxilated-oleuropein aglycon (H-D-Ol Agl), 10-hydroxy-Ol Agl), we observe that the main fragment of 193 *m*/*z* comes from a precursor of 281 *m*/*z*. For Ol Agl-related compounds a 118 *m*/*z* fragment would appear to be specific. Keeping that in mind, we might suggest that the fragmentation pattern may reveal whether a compound is a derivative of TY or of HYTY.

Flavonoids demonstrate weak fragmentation and, in general, require higher fragmentation energies. For both flavonoids under study (Apig and Lut), the loss of 16 (CH4) yields the prevalent ion in the MS/MS spectrum.

Lignans, such as Ac Pin and Pin showed in their MS/MS spectra the fragment 209, which is attributable to the stable substituted tropylium ion structure. This ion shows up in the MS/MS spectrum from lignans containing two methoxytrimethylsilyl ether benzylic moieties with either the C-7 or the C-7′ containing one or two hydrogens. The ion *m*/*z* 209 can shift to 239 (as in the case of syringaresinol) with the addition of a second methoxy group to the aromatic rings.

Table 2
m/z signals of the main compounds identified in the isolated phenolic fractions.

Isolated fractions	Retention time (min)	Quasi-molecular ion				In-source fragmentation pattern	Possible compounds
		m/z experimental	Molecular formula	Error (mDa)	mSigma		
1	17.3[a]	315.1253 (3)	C14H23O6Si	0.5	5.1	283.2102 (37)/**225.1742**/173.0415 (9)/139.1161 (15)	EA-H + 1TMS + H
	17.9	315.1267 (3)	C14H23O6Si	0.9	3.8	283.2091 (50)/**225.1728**/173.0415 (20)/139.1193 (16)	EA-H + 1TMS + H (isomer)
	21.7	387.1668 (3)	C17H31O6Si2	1.4	4.8	**355.1296**/297.0831 (30)/265.1965 (12)/223.1753 (74)/173.0413 (29)	EA-2H + 2TMS + 2H
2	16.6	MS signals observed: 429.2267 (4)/**361.2942**/169.1580 (44)					
	16.8[a]	MS signals observed: 481.3377 (31)/**363.2745**/273.2070 (39)/149.1196 (18)					
3	41.8[a]	465.2122 (2)	C23H37O6Si2	0.1	6.2	447.3312 (4)/375.2898 (6)/**281.2498**/209.1942 (82)	DOA-2H + 2TMS + H
	48.3	507. 2284 (2)	C25H39O7Si2	−5.6	4.9	475.3426 (5)/**193.1953**	Lig Agl-2H + 2TMS + H
4	10.8	MS signals observed: **193.1952**					
	36.4	MS signals observed: **249.2699**					
	44.5[a]	MS signal observed: **193.1951**					Lig Agl-related comp
	48.3	507.2185 (2)	C25H39O7Si2	4.3	3.6	475.3422 (5)/**193.1949**	Lig Agl-2H + 2TMS + H
5	44.7	MS signal observed: **193.1952**					Lig Agl-related comp
	48.4	507.2185 (2)	C25H39O7Si2	4.3		475.3429 (5)/**193.1947**	Lig Agl-2H + 2TMS + H
	68.0[a]	563.2445 (2)	C26H39O6Si2	2.5	8.3	545.3963 (7)/527.3779 (2)/474.3342 (3)/**337.2673**/307.2522 (12)/277.2357 (17)	Syringaresinol-2H + 2TMS + H
6	36.9	377.1789 (1)	C20H29O5Si	−1.1	6.8	359.2882/**193.1950**	D-Lig Agl-H + 1TMS + H
	47.0	507. 2257 (2)	C25H39O7Si2	−2.9	6.5	475.3365 (5)/**193.1935**	Lig Agl-2H + 2TMS + H (isomer)
	48.5	507.2232 (2)	C25H39O7Si2	0.4	9.1	475.3359 (5)/**193.1944**	Lig Agl-2H + 2TMS + H
	61.1[a]	561.2330 (2)	C28H41O8Si2	0.4	12.5	543.3710 (1)/501.3554 (30)/483.3417(10)/472.3413 (1)/**305.2331**/275.2165 (48)	Ac Pin-2H + 2TMS
7	36.9	377.1742 (1)	C20H29O5Si	3.6	3.6	359.2935/**193.1950**	D-Lig Agl-H + 1TMS + H
	48.5	507.2232 (2)	C25H39O7Si2	−0.4	15.2	475.3359 (5)/**193.1944**	Lig Agl-2H + 2TMS + H
	49.7	523.2185 (1)	C25H39O8Si2	−0.8	11.1	**281.2492**/225.1673 (7)/209.2006 (7)	Ol Agl-2H + 2TMS + H
	52.5[a]	611.2528 (3)	C28H47O9Si3	0.6	15.6	579.3858 (1)/313.2290 (12)/**281.2497**/209.1933 (4)/173.0419 (6)	10-hydroxy-Ol Agl-3H + 3TMS + H
8	47.8	523.2188 (1)	C25H39O8Si2	−1.1	11.1	313.2261 (9)/**193.1950**/173.0411 (5)	H-Lig Agl-2H + 2TMS + H
	49.7[a]	523.2188 (2)	C25H39O8Si2	−1.1	11.1	**281.2492**/225.1723 (7)/209.1937 (7)	Ol Agl-2H + 2TMS + H
	51.8	521.1987 (40)	C25H37O8Si2	3.4	8.5	**281.2480**/209.1939 (47)	Ol Agl-related comp
	53.1	595.2556 (3)	C28H47O8Si3	−0.8	6.9	563.3841 (4)/**281.2483**	Ol Agl-3H + 3TMS + H
	53.8	625. 3083 (6)	C30H53O8Si3	−4.0	8.5	593.3947 (6)/**281.2483**	Ol Agl-related comp
9	44.7[a]	435.1830	C22H31O7Si	0.4	13.1	**193.1966**	Lig Agl-H + 1TMS + H
	48.5	MS signal observed: 193.1965					Lig Agl-related comp

In bold letter we highlight the prevalent ion in the MS spectrum for each compound. The relative intensity of the other *m/z* signals present in the MS spectra of the phenols is shown between brackets.
[a] Most intense chromatographic peak in the isolated fraction.

Table 3
m/z signals detected of the most relevant phenolic compounds present in the extract of extra-virgin olive oil.

MS signal (when no defined before)	Retention time (minutes)	Parent (precursor) ion isolated and further fragmented	MS/MS fragmentation	Compounds
	10.0	193.1061	144.7203 (18)/126.6961 (12)/**108.6209**	Tyrosol-2H + 2TMS
299.2632/**281.2477**/193.1613	13.4	193.1613	164.8823 (9)/144.7197 (16)/**108.6199**	Unknown
		281.2477	192.9426 (50)/**118.7576**/105.6919 (69)	
		299.2632	266.9440 (10)/**192.9298**	
	15.1	370.1754	267.0739 (5)/**192.9301**/178.8886 (95)	Hydroxyyrosol-3H + 3TMS
		281.2480	192.9304/165.8570 (54)/114.7144 (27)	
	16.6	281.2469	192.9377 (73)/148.7980 (67)/**118.7612**/104.6898 (75)	Protocatechuic acid-3H + 3TMS + H
	17.0	385.1642	**178.8888**/267.0723 (5)	Dopac-3H + 3TMS + H
		267.0716	**178.8913**/148.7747 (81)/108.6205 (17)	
		178.8913	**148.7737**	
	17.3	315.1253	283.0594 (54)/**224.9829**/183.1515 (10)/139.1157 (18)	Elenolic acid-H + 1TMS + H
		224.9829	190.8610 (54)/**164.8771**/118.6764 (41)	
		283.0595	132.8196 (76)/118.6764 (96)/**104.6874**/90.6075 (73)	
192.9661	33.3	192.9661	177.9179 (10)/144.7184 (22)/127.7065 (12)/**108.6197**	
	36.9	192.9667	164.8792 (10)/144.7203 (20)/126.6961 (10)/**108.6209**	D-Lig Agl-related comp
281.0971	38.4	281.0971	192.9426 (30)/118.7612 (63)/**104.6892**	Lig Agl-related comp
192.9717	40.0	192.9717	177.9152 (8)/144.7196 (16)/126.6981 (11)/**108.6201**	Lig Agl-related comp
192.9717	40.9	192.9717	177.9152 (14)/144.7196 (21)/126.6981 (12)/**108.6201**	Lig Agl-related comp
411.3288/**281.0977**/208.9862	42.0	411.3288	**128.7833**/72.4970 (36)	DOA-2H + 2TMS + H
		281.0977	**192.9331**/168.8517 (52)/90.6110 (16)/72.4970 (56)	
		208.9862	190.9405 (79)/**164.8767**/135.7303 (70)/108.6181 (30)	
411.3288/281.0977/208.9862	42.2	411.3288	**128.7833**/72.4970 (36)	DOA-2H + 2TMS + H
		281.0977	**192.9331**/168.8517 (62)/90.6110 (19)/72.4970 (65)	
		208.9862	190.9424 (83)/**164.8771**/135.7294 (68)/108.6234 (40)/90.6110 (10)	
501.3843/**411.4616**	43.1	501.3843	**128.7831**/102.6687 (5)/72.4960 (11)	Unknown
		411.4616	**128.7831**/94.6589 (12)/72.4970 (45)	
	44.8	281.0971	**192.9426**/118.7612 (95)/104.6892 (88)	Lig Agl-H + 1TMS + H
537.2533/**281.0968**	45.8	281.0968	**192.9426**/118.7612 (95)/104.6892 (82)	Methyl Ol Agl-2H + 2TMS + H
553.2491/**281.0975**/192.9678	46.9	553.2491	281.0968 (46)/**192.9296**/122.7343 (5)	H-D-Ol Agl-3H + 3TMS + H
		281.0975	192.9377 (75)/148.7980 (80)/**118.7612**/104.6898 (95)	
		192.9678	177.9152 (12)/144.7196 (17)/126.6981 (11)/**108.6211**	
192.9717/462.2351	47.0	192.9717	177.9152 (12)/144.7196 (17)/126.6981 (11)/**108.6211**	Lig Agl-2H + 2TMS + H
		462.2351	**192.9622**/177.9192 (13)/97.6840 (9)	
	47.9	192.9718	177.9152 (11)/144.7196 (20)/126.6981 (10)/**108.6211**	Lig Agl-2H + 2TMS + H
	48.6	475.1989	**192.9646**/176.9046 (10)/148.8093 (4)/72.4060 (7)	Lig Agl-2H + 2TMS + H
		297.0825	248.9216 (24)/208.9031 (19)/132.8241 (50)/**118.7593**/104.6874 (78)/90.6075 (31)/72.4970 (12)	
		192.9665	177.9152 (8)/144.7196 (18)/126.6981 (10)/**108.6211**	

Table 3 *(Continued)*

MS signal (when no defined before)	Retention time (min)	Parent (precursor) ion isolated and further fragmented	MS fragments	Compounds
	49.8	523.2188	281.2492 (36)/225.1733 (3)/**193.1593**	Ol Agl-2H + 2TMS + H
		281.4481	**192.9304**/165.8570 (60)/114.7144 (25)/90.6075 (11)/72.4970 (48)	
550.2678/**281.0973**	51.8	550.2678	281.0971 (75)/**192.9302**/177.9207 (16)/165.0849 (9)/97.6855 (9)	Ol Agl-related comp
		281.0973	192.9377 (48)/148.7980 (83)/**118.7612**/104.6898 (80)	
521.2068/**281.0879**/ 208.9912	51.9	521.2068	281.0971 (8)/**192.9302**	Ol Agl-related comp
		281.0879	192.9377 (23)/148.7980 (75)/**118.7612**/104.6898 (95)	
		208.9912	190.9457 (68)/178.9249 (46)/**164.8781**/108.6224 (21)	
	52.5	313.0876	142.8366 (27)/132.8210 (52)/**118.7623**/104.6888 (56)/90.6088 (15)	10-hydroxy-Ol Agl-3H + 3TMS + H
		281.0981	192.9377 (90)/148.7980 (64)/**118.7612**/104.6898 (70)/90.6075 (30)/72.4970 (55)	
	53.3	595.2540	281.0975 (78)/**192.9300**	Ol Agl-3H + 3TMS + H
		563.2299	281.0960 (42)/**192.9300**	
		297.0929	266.9500 (21)/224.9536 (23)/192.9335 (41)/**118.7611**/104.6895 (67)/90.6055 (21)	
		281.0978	**192.9377**/148.7980 (31)/118.7612 (24)/104.6898 (42)	
	53.9	281.0975	192.9649 (47)/178.9214 (58)/154.8671 (90)/**118.7611**/104.6895 (90)/90.6055 (29)	Ol Agl-related comp
	54.0	281.0975	192.9649 (68)/178.9214 (52)/154.8671 (90)/**118.7611**/104.6895 (95)/90.6055 (25)	Ol Agl-related comp
	54.5	487.1792	**471.1498**/399.1083 (30)/415.1399 (10)/193.0695 (10)	Apigenin-3H + 3TMS + H
	58.7	575.2142	**559.3394**/487.2883 (5)/297.2119 (4)	Luteolin-4H + 4TMS + H
		503.3205	431.2735 (28)/415.2367 (29)/225.1546 (38)/**191.1419**	
	59.9	503.2274	367.2070 (35)/**354.1982**/179.1393 (96)	Pinoresinol-2H + 2TMS + H
		485.3569	414.3040 (10)/384.1199 (88)/289.2369 (3)/259.0503 (35)/**208.9846**/178.8874 (30)/128.7817 (61)	
		414.1698	354.0687 (90)/146.8805 (45)/**118.7618**	
		247.0525	201.9547 (53)/177.9131 (82)/**164.8770**/127.7674 (42)	
397.3832/160.9290	60.2	397.3832	160.9312 (46)/**146.8805**/132.8342 (58)/118.7614 (74)/104.6907 (56)	Unknown
		160.9290	127.7686 (73)/**114.7141**/90.6062 (50)	
	61.1	501.2143	275.0644 (14)/247.0429 (15)/**222.9837**/208.9865 (31)	Ac Pin-2H + 2TMS
		305.0929	228.9791 (74)/**216.9663**/202.9288 (38)/114.7153 (95)/72.4960 (21)	
		276.0701	216.9666 (73)/202.9288 (47)/**114.7153**/72.4960 (19)	
		259.0576	**228.9806**/202.9288 (31)/198.8934 (38)/104.6901 (10)	
	68.0	503.2283	**337.2673**/238.9865 (10)	Syringaresinol-2H + 2TMS + H

In bold letter we highlight the prevalent ion in the MS spectrum for each compound. The relative intensity of the other *m*/*z* signals present in the MS spectra of the phenols is shown between brackets.

In conclusion, the combination of analytical standards, MS and MS/MS analysis of semi-preparative fractions and the prior knowledge gives us the possibility to perform a detailed assignment of phenolic compounds in our test sample (a mixture of Arbequina and Picual oils). Fig. 1 showed the BPC of the Diol-SPE extract obtained from a mixture of Arbequina and Picual oils; the approach used for the assignment of a particular structure is colour coded: commercial standards – blue, phenolic fractions – red and prior knowledge – purple. Analysis of the chromatogram reveals a clear pattern of the migration for the phenolic compounds of different families: simple phenols and phenolic acids reappear first, followed by secoiridoids, flavonoids and lignans.

After achieving the identification of 28 compounds in the profile and define the elution areas of each family of phenols, we re-optimized the GC method in terms of flow rate and temperature gradient in order to improve chromatographic resolution in the "areas of interest" and to reduce the total analysis time. The method described in Materials and Methods as "run 2" was used for further experiments. The total analysis time with the modified conditions was reduced to 50 min, whilst maintaining good resolution and efficiency. To carry out the validation of our method and for the application of the method to the analysis of different extra-VOOs, we used run 2.

Table 4
Analytical parameters of the GC–APCI-MaXis MS method.

Analytes	LOD (ppm)	Ions used quantification	LOQ (ppm)	Linearity ((g/ml)	Calibration curves[a]	r^2	Repeat. Intra-day[b]	Repeat. Inter-day[b]	Accuracy[c]	Response factor (neat solution)[d,e]	Response factor (with matrix)[d,e]
Tyrosol	0.40	193.1061	1.33	LOQ-100	$y = 29{,}080x - 13{,}5585$	0.987	1.53	4.16	97.7	25.1	24.0
Hydroxytyrosol	0.21	281.1410	0.70	LOQ-50	$y = 5809x - 6690$	0.994	1.61	4.10	95.4	5.8	5.5
Homovanillic acid	1.01	209.1007	3.37	LOQ-100	$y = 4650x - 17{,}309$	0.994	1.30	3.76	97.3	4.0	3.8
p-Coumaric acid	0.19	219.0868	0.63	LOQ-100	$y = 6649x - 13{,}911$	0.994	0.89	3.67	99.1	6.6	6.9
Ferulic acid	0.19	249.0967	0.63	LOQ-100	$y = 7313x - 33{,}576$	0.985	0.73	6.01	98.6	7.3	7.2
Luteolin	1.05	575.2142	0.32	LOQ-50	$y = 22{,}182x - 14{,}950$	0.993	1.77	6.07	101.5	22.7	23.7
Apigenin	0.63	487.1792	2.10	LOQ-100	$y = 3223x - 24{,}031$	0.998	1.45	4.74	100.5	3.2	3.0
Pinoresinol	0.13	485.2189	0.43	LOQ-100	$y = 2657x - 18{,}999$	0.993	1.03	5.01	98.2	2.6	2.5

[a] A (peak area) $= a + b \times C$ (ppm) for ten points ($n = 5$).
[b] RSDs values (%) for peak areas corresponding to each compound; measured from three injections of each analyte within the same day (intra-) and on three different days (inter-).
[c] The accuracy of the assay is the closeness of the test value obtained to the nominal value. It is calculated by determining trueness and precision (%Recovery, %RSD).
[d] RF was defined as the ratio between the peak area and the concentration of the analyte. Peak area values were multiplied by 10^{-3} to express the RF values with less significant figures.
[e] Coefficient of variation (%) was in every cases lower than 4.8%.

3.3. Validation experiments: analytical parameters of the method

The stability of derivatized samples is an important factor for large scale food metabolomics studies. To address this issue, we kept derivatized samples in 1.5 ml screw capped vials (with inserted micro-vials) at room temperature and performed analysis at equal time intervals between 0 and 48 h. Data proved to be rather consistent from 0 to 35 h. However, data collected at later time points demonstrated a steadily increasing variability. Thus, if a technical solution of the derivatization problem, such as for example, on-line sample processing is not available, material should be processed within the first 24 h to avoid any possible risk of derivatization-depended variability.

The specificity of the developed method was tested by analysis of blank oil samples or phenolic-free oil samples (refined sunflower oil) and no significant chromatographic interference around the retention times of the analytes was observed.

Calibration curves were obtained for each standard by plotting the peak areas as a function of the concentration. The parameters of the calibration functions: LOD, LOQ, linearity, calibration range, correlation coefficient, repeatability and accuracy have been summarized in Table 4. Several factors have influenced the selection of compounds for the calculation of the calibration curves and validation experiments: (a) availability of the analytical standards; (b) the presence of a given compound in extra-VOO samples and (c) an attempt to keep the selection as diverse as possible. Those standards of phenolic compounds used in the preliminary studies which were not present in the analyzed samples of extra-VOOs were not included in the final selection of analytes, which includes: TY, HYTY, homovanillic acid, *p*-coumaric acid, ferulic acid, Lut, Apig, and Pin. In order to calculate the calibration functions and LODs we took the EIC of the most intense ion in the mass spectrum for each selected compound. If the compound was represented by more than one silylated form, the one with higher linearity in the calibration range was used for calculation of analytical parameters. For example, in the case of tyrosol, for quantitation we used the m/z signal 193.1061; for homovanillic acid, we used m/z 209.1007; for *p*-coumaric, we used m/z 309.1333; for ferulic acid, m/z 249.0967; for luteolin, m/z 575.2142; for apigenin, we used m/z 487.1792; and for pinoresinol, m/z 485.2189. All calibration curves showed good linearity ($r^2 > 0.985$) for the selected concentration range. LODs were found to be within the range between 0.13 and 1.05 ppm, for Pin and Lut, respectively. The intra- and inter-day repeatability in the peak areas was determined as the RSD obtained for six consecutive injections of the analytical QC sample spiked with each phenol at an intermediate concentration value of the calibration curve, carried out within the same day and on three different days. Acceptable levels of precision were obtained for the developed method in terms of repeatability since in all cases RSDs calculated were lower than 6.07%. The accuracy ranged from 95.4% to 101.5%.

As described above, to evaluate the matrix effect, RFs of the 8 phenols when the analytes were in the presence of the olive oil matrix and in a neat solution were determined. No statistical differences in peak area and response factors were observed for any of the analytes under study.

3.4. Application of the method to the analysis of different extra-VOOs

Finally, to demonstrate the feasibility of our method for analysis of real samples we have analyzed the extracts of three different commercial extra-VOOs: (a) a mixture of Arbequina and Picual, (b) Frantoio, and (c) Hojiblanca. All samples were analyzed in triplicate ($n = 3$). The representative chromatograms are shown in Fig. 3a. To facilitate visual comparison, the intensity scale was kept the same in all cases. Already visual inspection of BPCs shows significant

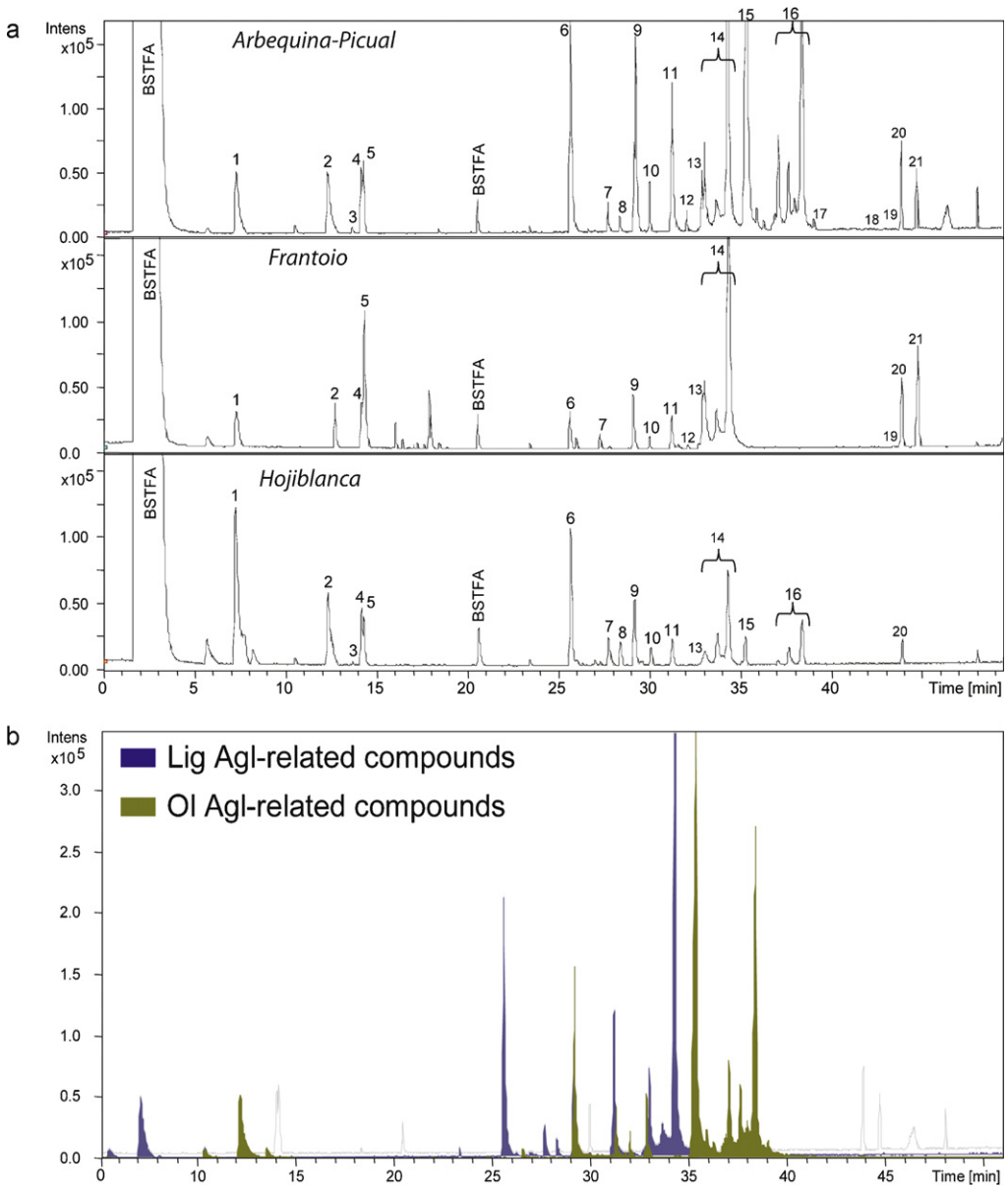

Fig. 3. Polyphenolic profiles (BPCs) of the three extra-VOOs (run 2). (A) A mixture of Arbequina and Picual extra-VOOs, Frantoio extra-VOO and Hojiblanca extra-VOO Auchan. (B) Extracted ion chromatograms (EICs) of *m/z* 281.2480 and 193.1944, which facilitate the study of the Ol Agl-derivatives and Lig Agl-derivatives or related compounds. *Peak identification*: **1**, Ty-2H + 2TMS; **2**, Hyty-3H + 3TMS; **3**, Protocatechuic acid-3H + 3TMS + H; **4**, Dopac-3H + 3TMS + H; **5**, EA-1H + 1TMS + H; **6**, D-Lig Agl; **7**, compound present in isolated fraction 6 (Lig Agl-related comp); **8**, compound present in isolated fraction 6 (Lig Agl-related comp); **9**, DOA-2H + 2TMS + H; **10**, *m/z* 501.3843/411.3312; **11**, Lig Agl-1H + 1TMS + H; **12**, methyl Ol Agl-2H + 2TMS + H; **13**, H-D-Ol Agl-3H + 3TMS + H; **14**, Lig Agl-2H + 2TMS + H; **15**, Ol Agl-2H + 2TMS + H; **16**, Ol Agl-3H + 3TMS + H and related comp; **17**, Apigenin-3H + 3TMS + H; **18**, Luteolin-4H + 4TMS + H; **19**, Pinoresinol-2H + 2TMS + H; **20**, 397.3825; **21**, acetoxy-pinoresinol-2H + 2TMS + H.

differences between the samples. The quantitative data summarized in Table 5 provide a numeric expression of the differences found for the different products. Since standards for complex phenols and elenolic acid are not available, in the table we included their quantification in terms of other commercial standards (TY and HYTY, respectively). Using TY and HYTY for the quantification of ligstroside- or oleuropein-analogues is quite common, since those compounds contain TY and HYTY in their structure.

Our results show that Frantoio extra-VOO has the lowest phenolic content; it has the lowest concentration of simple phenols (tyrosol and hydroxytyrosol) and secoiridoids. The content of flavonoids, on the contrary, is comparable with Hojiblanca extra-VOO. As far as Ac Pin content is concerned, Frantoio is the richest oil.

Levels of simple phenols found in Hojiblanca extra-VOO appeared to be higher than in the other two samples. The mix of Picual and Arbequina, however, shows a high content of secoiridoids (Ol Agl, Lig Agl and their derivatives). The levels of decarboxilated forms of Ol Agl and Lig Agl (DOA and D-Lig Agl) in Picual-Arbequina mix were found to be 12.76 and 6.55 mg/kg, respectively, whilst in the other samples they were present at much lower concentrations. The comparison of Ol Agl (35.3 min) concentrations revealed even stronger differences between the samples: 46.04 mg/kg for Picual-Arbequina, 2.27 mg/kg for Hojiblanca and below detection limits for Frantoio. A similar trend was observed for another isomer of Ol Agl (38.3 min). In general, the content of Ol Agl- and Lig Agl-derivatives in olive oils could be estimated quickly from APCI–GC data using EICs, 281.2481, and 193.1944, respectively. Fig. 3b shows an example of such analysis, which appears to be useful in the future for making a quick estimation of oleuropein- and ligstroside-analogues amount.

Table 5
Quantitative overview of phenolic compounds in the extra-VOO.

Analyte	t_r (min) (run 2)	Picual-Arbequina oil		Frantoio oil		Hojiblanca oil	
Tyrosol[a]	7.2	3.33		1.67		7.21	
Hydroxytyrosol[b]	12.3	8.31		2.42		9.32	
Protocatechuic acid[c]	13.6	0.25		n.d.		0.21	
Dopac	14.1	Internal standard (IS)					
		Area	mg/kg	Area	mg/kg	Area	mg/kg
Elenolic acid[b,d]	14.2	356,567	4.42	936,997	11.62	228,073	2.82
D-Lig Agl[a,d]	25.6	1,460,407	6.55	182,971	0.82	821,396	3.68
Lig Agl-related comp[a,d]	27.6	103,272	0.46	68,637	0.30	203,280	0.91
Lig Agl-related comp[a,d]	28.3	49,646	0.22	n.d.	n.d.	148,478	0.67
DOA[b,d]	29.2	1,029,476	12.76	276,124	3.42	422,605	5.24
501.3843/411.3312[b,d]	30.0	167,040	2.07	43,682	0.54	92,022	1.14
Lig Agl[a,d]	31.2	830,890	3.72	172,235	0.77	151,760	0.68
Methyl Ol Agl[b,d]	32.0	56,047	0.69	16,797	0.21	n.d.	n.d.
H-D-Ol Agl[b,d]	32.8	211,872	2.63	219,061	2.72	33,328	0.41
Lig Agl[a,d]	34.2	3,907,685	17.52	3,962,249	17.76	1,334,142	5.98
Ol Agl[b,d]	35.3	3,713,714	46.04	n.d.	n.d.	183,453	2.27
Ol Agl-related comp[b,d]	37.0	540,608	6.70	n.d.	n.d.	25,417	0.32
10-H-Ol Agl[b,d]	37.6	429,370	5.32	n.d.	n.d.	124,260	1.54
Ol Agl[b,d]	38.3	2,209,565	27.40	n.d.	n.d.	304,794	3.78
Apigenin[e]	39.2	0.35		0.19		0.20	
Luteolin[f]	42.9	1.65		n.d.		n.d.	
Pinoresinol[g]	43.8	3.25		0.75		0.54	
Ac Pin[g]	44.6	19.37		25.45		n.d.	

Data given in mg/kg or area (when pure standards were not available); $n = 3$ (value shown = mean value). RSD in all the cases $\leq 5\%$.
Recoveries described by Gómez-Caravaca et al. [33] were applied for quantitative calculations.
n.d.: non detected.
[a] Quantified with the calibration curve of tyrosol.
[b] Quantified with the calibration curve of hydroxytyrosol.
[c] Quantified with the calibration curve of protocatechuic acid.
[d] Semi-quantitative information (mean value of area of the compound). Pure standards were not available.
[e] Quantified with the calibration curve of apigenin.
[f] Quantified with the calibration curve of luteolin.
[g] Quantified with the calibration curve of pinoresinol.

4. Conclusions

Here, we demonstrate for the first time the applicability of gas chromatography with atmospheric pressure ionization source (GC–APCI-MaXis-MS) for the qualitative and quantitative analysis of the phenolic compounds present in extra-VOO samples. A combination of prior knowledge, commercially available standards and semi-preparative HPLC isolated standards, supported by intrinsic qualities of the UHR-TOF mass analyzer (operating in MS, auto-MS/MS and MRM modes), gave us the opportunity to perform detailed analysis of phenolic profiles of the extra-VOOs. Moreover, a complete validation of the method was carried out considering the specificity, linearity, sensitivity, precision, accuracy and matrix effects. Thus, GC with the soft atmospheric pressure ionization source and UHR-TOF mass analyzer may offer new complementary information in addition to the methods widely used so far to analyze dietary phenolic compounds.

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