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ENHANCED PROFILE CHARACTERIZATION OF VIRGIN OLIVE OIL MINOR POLAR COMPOUND EXTRACTS BY COMPREHENSIVE TWO-DIMENSIONAL GAS CHROMATOGRAPHY WITH TIME-OF-FLIGHT MASS SPECTROMETRIC DETECTION

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Abstract: The Minor Polar Compounds (MPC) (free fatty acids, acylglycerols, aliphatic alcohols, sterols, triterpenic acids and phenolic compounds) of virgin olive oil (VOO) play not only an important role for health and sensory properties (strong antioxidants and radical scavengers) but have also been used as natural chemical markers to characterize the quality of VOO. The complexity of the MPC fraction has precluded its detailed characterization for better elucidation of potential key trace compounds (regardless the chemical class) that can be useful in assessing the authenticity or other quality claims in a ‚foodomic' approach. Reversed-phase HPLC with UV-Vis diode array detection (DAD) is challenged in the determination of the entire MPC profile because it is limited to compounds that absorb at selected wavelengths and is thus not suitable for free fatty acids, mono and diacylglycerols, and triterpenic acids. The use of a two-dimensional gas chromatograph/time-of-flight mass spectrometry (GC×GC/TOF-MS) approach was investigated in this work, in which the number of compounds (as TMS derivatives) were identified through the MS library. The GC×GC/TOF MS approach yielded a greater number of analytes to be profiled than the other instrumental methods used in this work. A novel approach to obtain a reliable screening of MPCs by GC×GC is also discussed.

Key words: extra virgin olive oil, minor polar components, bidimensional chromatography (GC×GC), mass spectrometry, foodomics

Introduction

Virgin olive oil (VOO) is a high-quality food product from a nutritional standpoint and is, therefore, one of the main components of the Mediterranean diet which has been recently included in the representative list of the Intangible Cultural Heritage of Humanity by UNESCO [1]. The healthy value of VOOs is not exclusively correlated to its high oleic acid content and the high monounsaturated/saturated fatty acid ratio but also to the high amount of natural antioxidants that are part of its minor polar components (MPCs). Even though the MPCs constitute a rather small fraction of the total composition of VOOs (1-2%) they are of great significance because they contain a rich array of natural products (over 250 compounds) that include free fatty acids, mono and diacylglycerols, aliphatic alcohols, sterols, and phenolic compounds [2] that are considered amongst the most powerful antioxidants. These compounds are peculiar of VOO because the latter is obtained solely by mechanical means, without refining operations. Biophenolics determine the higher stability of VOO with respect to other plant oils because they are the main cause of the resistance of triglycerides towards auto-oxidation reactions [3–10], and they play an important role in the prevention of the degenerative chronic pathology because most of them exert an important scavenging activity [11–17]. Moreover, MPCs strongly influence the sensory characteristics of olive fruits and the derivative products (oil and preserves). In particular, in VOO, the secoiridoids determine the peculiar sensations of bitter and pungent taste that are respectively associated to oleuropein and/or ligstroside aglycon [18] and to the presence of the dialdehydic form of decarboxymethyl ligstroside-aglycon [19].

Given the great influence of MPCs on the nutritional, sensory and health impact of VOO and particularly on high quality Extra Virgin Olive Oil (EVOO), and EVOO with protected designation of origin (PDO), their qualitative/quantitative characterization has been of great interest to food chemists for a few decades. The qualitative/quantitative characterization is commonly conducted by means of reversed-phase high performance liquid chromatography (RP-HPLC) coupled with a spectrophotometric detector, usually a diode array detector (DAD). This is in fact the eligible technique for non volatile and UV absorbing compounds such as polyphenols (they usually show a maximum absorption at around 280 nm). However, the chromatographic profiles are sometimes difficult to interpret for the presence of a large number of congeners belonging to the group of secoiridoids, with similar absorption spectra in the ultraviolet and similar characteristics of polarity leading to peak coelution. Moreover, HPLC-DAD allows the display of only a small fraction of the total MPCs, excluding a significant number of compounds that either do not adsorb or adsorb poorly in the UV-VIS range (free fatty acids, mono and di-acylglycerols, carbohydrates, triterpenic acids). In an effort to increase the number of analyzed compounds, MPCs analysis is sometimes carried out by using mass spectrometry as a detection system. Mass spectrometry is a powerful tool for the identification of MPCs because it offers the possibility to detect many interferences in the VOO extract obtained prior to chromatographic analysis, HPLC or GC [20]. HPLC-MS-MS with soft ionization, as for example electrospray ionization (ESI), is really powerful because it allows the attribution of the molecular weight with primary ionization and then the structural formula through experiments in tandem mode.

Even if GC is not the most suitable technique for the determination of thermally labile compounds, its main advantage is the possibility of detecting several compounds which have a low absorbance in the UV-VIS spectrum (glycerides, triterpenic acids or even sugars). However, it should be emphasized that the less volatile compounds, such as flavonoids (e.g., luteolin) can be hardly detected by single column GC due to the presence of interfering peaks at high retention times (and thus high GC oven temperature) [20].

In the last decade, the use of comprehensive multidimensional gas chromatography (GC×GC) has been shown as a very useful approach in order to obtain a high-resolution separation and advanced fingerprinting of complex samples [21–23]. In comprehensive GC×GC, the sample is subjected to two different stationary phases in series. The second column is employed to provide further separation of compounds which elute from the first column, resulting in a more powerful separation power than that obtained with conventional one-dimensional (1-D) GC. The separation carried out by the second stationary phase is fast enough (5-10 s) to allow the interrupted introduction, in the second column, of the peaks eluting from the first column.

GC×GC has been applied to the analysis of fatty acid composition of food or biological products [24, 25]. De Geus et al. [26] have determined the fatty acid profile of virgin olive oil with GC×GC for the first time.

Since the bidimensional chromatographic profile needs an unmistakable peak identification due to its complexity, GC×GC is generally coupled on-line with mass-spectrometry.

The goal of the present research was to delineate and to compare the chromatographic profiles of MPC's extracts originated from four samples of EVOO. The chromatograms were obtained by means of four hyphenated chromatographic techniques (HPLC-DAD; HPLC-MS; GC-MS; GC×GC-MS) in order to establish the potential and/or the limits of each of them and to evaluate the possibility of profiling a higher number of compounds by GC×GC.

Materials and methods:
Samples

Four extra virgin olive oils were characterized. Two of them (FL_1 and FL_2) were a 1:1 blend of Frantoio and Leccino cultivars grown in the Marche region (Italy). The other two samples (AT_1 and AT_2) were monovarietal oils and derived from Ascolana Tenera, a cultivar diffused in the Marche region.

All four virgin olive oil samples were obtained by using a hammer crusher and a dual phase decanter centrifugation. After processing, the oils were immediately bottled and stored at room temperature in darkness.

Reference compounds

Standard MPCs were obtained from Sigma Aldrich (Milan, Italy). They were benzoic acid (1), cinnamic acid (2), ferulic acid (3), vanillic acid (4), palmitic acid (5), stearic acid (6), syringaldehyde (7), syringic acid (8), vanillin (9), p-coumaric acid (10), 2-hydroxybenzoic acid (11) and 4-hydroxybenzoic acid (12). Four solutions containing different compounds (1st solution: 1, 4 and 11; 2nd solution: 2, 3 and 12; 3rd: 8, 9 and 10; 4th: 4, 6 and 7) were separately prepared in methanol at a concentration of 1 mg/mL.

Extraction of the minor polar compounds

The MPC extracts of virgin olive oil were obtained by following the procedure of Montedoro et al. [27]. Aliquots of oil (5 g) were added to 10 mL of a methanol/water solution (80:20 by vol.) in a 50-mL centrifuge tube and homogenized for 2 min. The mixture was centrifuged for 5 min at 2500 g.

The hydroalcoholic phase was collected and the oil phase was re-extracted twice with 10 mL of the methanol/water solution. The hydroalcoholic fractions were combined and washed with n-hexane (40 mL) to remove residual oil, and then concentrated and dried under vacuum at 35°C. Finally, the dried extracts were dissolved in 1 mL of methanol.

RP-HPLC/DAD analysis

Prior to injection, the methanolic MPC extracts were filtered through a membrane of regenerated cellulose (0,2 μm). The phenolic compounds were separated with a HPLC ternary pump (mod. 9010, Varian) coupled with a diode array detector (DAD) (Varian Prostar 330). A Chrompack (Middleburg, The Netherlands) 25 cm × 4.6 mm i.d. column packed with Chromospher C18 (5 μm particle size) was used. The mobile phase A was acetic acid (2%) in water (HPLC grade), and the mobile phase B was pure methanol. Separation was achieved with an elution gradient starting at 95% of mobile phase A and maintained in this condition for 2 minutes, decreasing to 75% in the next 22 min; after other 16 min, phase A was decreased to 60% and subsequently, at 58 min of run the mobile phase B reached 100% and was held for 12 min; after another 5 min, phase A was increased to 95%, the initial condition.

The column was left overnight in methanol in order to prevent damage due to the strong acidic medium. The HPLC flow was 800 μLmin^{-1}, and the injection loop was 20 μl. The analyses were carried out at room temperature. The DAD monitored a wavelength range from 220 nm to 600 nm. The HPLC phenolic profile was displayed at 280 nm and the data were acquired by using the Varian Star 6.3 software.

Phenolic compounds were identified on the basis of their retention times and their UV absorption profiles according to a previous study [20].

RP-HPLC/TIS-MS analysis

A HPLC system coupled on-line to an LCQ ion-trap mass spectrometer (Finnigan, San José, CA, USA) equipped with an electrospray ionization source was used. The HPLC effluent was split, and 0.3 mL/min entered the MS through a steel ionization needle set at 4,5 kV, and a heated capillary set to 200°C. The sheath gas flow was approximately 20 arbitrary units. The molecular mass peak from the HPLC effluent was detected by using negative ion full-scan ESI-MS. Mass resolution was 0.6 Da at half peak height and isolation width ±0.3 Da. Tandem mass (MS^2) experiments were carried out with a relative collision energy of 30-40%. The mass acquisition range was 50-1000 amu. The column, the mobile phases and their flow rate were the same used for the HPLC-DAD experiment.

The second experiment was carried out by using a HPLC/MS system from Shimadzu (Kyoto, Japan). It was equipped with a LC-20AD Pump, a Sil-20AC Autosampler with a 1 μl loop injector and a CTO-20AC column oven. A Phenomenex Gemini column, C18, 3 μm, 100 × 2.1 mm i.d. was used; during the run it was heated to 50°C.

The mobile phase was 4 mM ammonium formate with 0.1% formic acid in water (solvent A) and 0.1% formic in methanol (solvent B). The elution started at 5% of eluent B for 1 minute, then was linearly increased at 95% of eluent B in six minutes and it was held for other 6 minutes (13 min of run). Finally, after other 4 minutes (17 min of run) phase B was decreased to 5%, the initial condition. The flow-rate was 0.4 mL min^{-1}. An ABI Sciex 5500 MS triple quadrupole with Turbo IonSpray (TIS) interface operating in negative mode was used. The Turbo spray temperature was set at 400°C, and the ion spray voltage was 4500 V. The scan rate was 2000 Da/s and the mass acquisition range was 50-550 amu. The compounds were identified by injecting the standard solutions with negative ionization.

GC/TOF-MS and GC×GC/TOF-MS analysis

Before GC analysis, the methanolic MPC extract was silylated. An aliquot (50 μl) of each methanolic extract was dried with a gentle N_2 flow. The residue was reconstituted with 50 μl of pyridine and then derivatized with other 50 μl of bis(trimethylsilyl)trifluoracetamide (BSTFA) at a temperature of 70°C for 60 minutes. The injection volume for both GC/TOF-MS and GC×GC/TOF-MS was 1 μl.

The GC/TOF-MS and the GC×GC/TOF-MS systems consisted of an Agilent 7890A gas chromatograph coupled to a Pegasus 4D GC×GC (LECO Corporation, St. Joseph, MI, USA) equipped with a quad-jet thermal modulator and a time-of-flight mass spectrometer. The carrier gas was helium (99.9999% purity) at a constant flow rate of 1 mL min^{-1}. Nitrogen, compressed air and liquid nitrogen were used for the operation of modulator. All gases were purchased from Air Gas (Atlanta, GA).

The GC×GC column set used consisted of a 30-m first dimension column with a 5% phenyl-substituted, 95% methylpolysiloxane stationary phase (0.25 mm internal diameter, 0.25 μm stationary phase thickness) joined, by a press-connection, to a 2-m second dimension column with a 35% diphenyl, 65% dimethyl polysiloxane stationary phase (0.10 mm internal diameter and 0.1 μm stationary film thickness). Both columns were from Restek Corporation (Bellefonte, PA, USA) and are commercially known as Rtx-5MS and Rtx-35, respectively. The columns were heated independently. The initial temperature of the first dimension

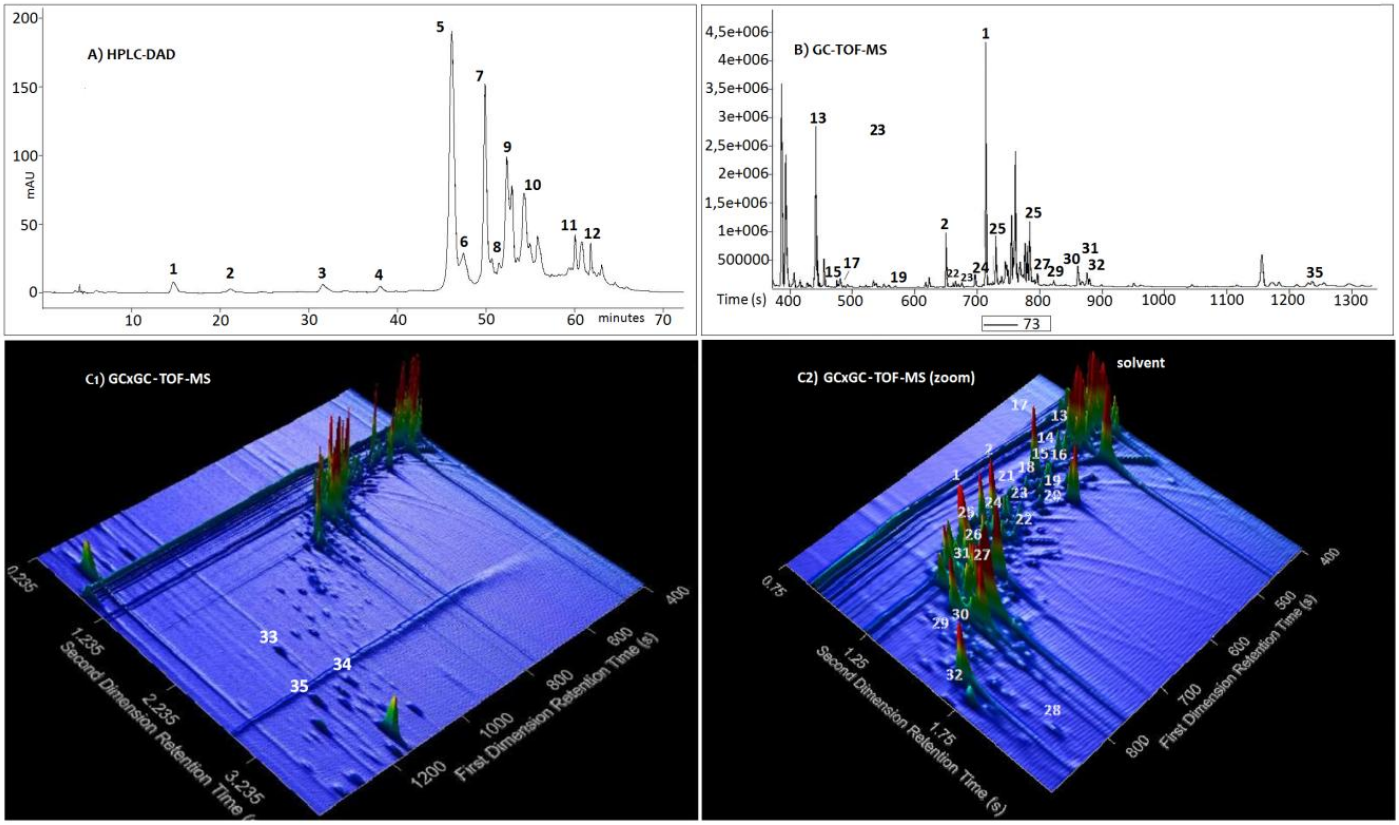


Fig. 1: Chromatographic profiles of the MPCs of the AT_1 oil. A, HPLC-DAD trace (280 nm); B, GC/TOF-MS (m/z 73); C1, GC×GC/TOF-MS (total ion current); C2, GC×GC/TOF-MS (zoom in of the region between 400 s and 900 s of C)

column was 40°C and was held for 0.5 min. The temperature program provided a heating of 20°C min^{-1} until reaching the final temperature of 270°C, and it was held for 18 min for a total run time of 30 min. The temperature program of the second dimension column was as follows: the run started at 45°C and was held at that temperature for 0.5 min. A temperature ramp of 20°C/min was used up to the final temperature of 275°C. The quad-jet modulator was used with a 4 s modulation period (0.4 s hot pulses and 1.6 s cold pulses) and a 30°C offset relative to the secondary oven. The sample inlet was heated to 250°C, and the samples were injected with a split ratio of 20:1. The time-of-flight MS transfer line was held at 280°C, and the ionization chamber was held at 200°C. Electron impact ionization was conducted with an energy of 70 eV and the detector voltage was 1700 V. The mass range of analysis was between 25 and 1000 amu, and the data acquisition rate was 200 averaged spectra/s.

The column used for the one-dimensional analysis and its temperature program were the same used, as first column, for the GC×GC analysis.

Retention indices (RI) were calculated by using external alkane standards solutions (C5-C40) which were injected in both GC and GC×GC. Data files were collected and stored on the Pegasus 4D instrument. The GC and GC×GC data were processed by using the ChromaTof 4.21 software (LECO Corporation); the resulting peaks were identified by using the NIST 08 Mass Spectral Library.

Results and discussions

Figure 1 shows the 4 chromatograms relative to the oil sample AT_1 obtained respectively by means of single dimension chromatographic techniques, i.e. HPLC/DAD (A) and GC/TOF-MS (B), and bidimensional GC×GC/TOF-MS (C1 and C2).

In Fig. 1, the reported compounds (numbered from 1 to 35, as in Table 1) were those MPCs detected in all 4 olive oil samples. Only hydroxytyrosol (1) and tyrosol (2) could be detected by using all 3 techniques. The characterization of the oils by each of the chromatographic techniques is discussed below.

HPLC/DAD analysis

The HPLC-based analyses were performed to verify that the extraction of the MPCs was consistent with previous studies, so that the fraction analysed by GC techniques could be validated. With RP-HPLC/DAD, the elution order

Table 1: Identified MPCs by the four chromatographic techniques.

Identified compounds	HPLC-DAD[(1)]	HPLC-ESI/MS[(2)]	HPLC-TIS/MS[(2)]	GC/TOF-MS[(2)]	GCxGC/TOF-MS[(2)]
Hydroxytyrosol (in GC in form of TMS derivate) **(1)**	**230/280**	**153**	-	**370**	
Tyrosol (in GC in form of TMS derivate) **(2)**	**230/276**	**137**	-	**282**	
Vanillic acid **(3)**	**260/291**	**167**	**167**	-	-
Hydroxytyrosol acetate (3,4-DHPEA-AC) **(4)**	**230/280**	**195**	-	-	-
Dialdehydic form of decarboxymethyl oleuropein aglycon **(5)**	**235/280**	**319**	-	-	-
Dialdehydic form of oleuropein aglycon **(6)**	**235/280**	**377**	-	-	-
Dialdehydic form of decarboxymethyl ligstroside aglycon **(7)**	**235/275**	**303**	-	-	-
Dialdehydic form of ligstroside aglycon **(8)**	**235/275**	**361**	-	-	-
Aldehydic form of oleuropein aglycon **(9)**	**235/280**	**377**	-	-	-
Aldehydic form of ligstroside aglycon **(10)**	**235/275**	**377**	-	-	-
Luteolin **(11)**	**254/348**	**285**	-	-	-
Apigenin **(12)**	**230/268/335**	**269**	-	-	-
Propanoic acid, 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester **(13)**	-	-	-	**234**	
Propanoic acid, 3-[(trimethylsilyl)oxy]-, trimethylsilyl ester **(14)**	-	-	-	-	**234**
Benzoic acid (in GC in form of trimethylsilyl ester) **(15)**	-	-	**122**	**194**	
Octanoic acid, trimethylsilyl ester **(16)**	-	-	-	-	**216**
Trimethylsilyl ether of glycerol **(17)**	-	-	-	**323**	
Propanoic acid, 2,3-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester **(18)**	-	-	-	-	**322**
Nonanoic acid, trimethylsilyl ester **(19)**	-	-	-	**230**	
Decanoic acid, trimethylsilyl ester **(20)**	-	-	-	-	**244**
Benzoic acid, 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester **(21)**	-	-	-	-	**282**
Cinnamic acid (in GC in form of trimethylsilyl ester) **(22)**	-	-	**148**	**220**	
Benzoic acid, 4-[(trimethylsilyl)oxy]-, trimethylsilyl ester **(23)**	-	-	-	**282**	
Dodecanoic acid, trimethylsilyl ester **(24)**	-	-	-	**272**	
Glucopyranose, pentakis-O-trimethylsilyl- **(25)**	-	-	-	**541**	
(4-Hydroxy-3-methoxyphenyl)ethylene glycol tris(trimethylsilyl) ether **(26)**	-	-	-	-	**400**
Scopoletin, trimethylsilyl ether **(28)**	-	-	-	-	**264**
Heptadecanoic acid, trimethylsilyl ester **(29)**	-	-	-	**343**	
Oleic acid, trimethylsilyl ester **(30)**	-	-	-	**355**	
α-Linolenic acid, trimethylsilyl ester **(31)**	-	-	-	**351**	
Stearic acid (in GC in form of trimethylsilyl ester) **(32)**	-	-	**284**	**256**	
2-Monopalmitin trimethylsilyl ether **(33)**	-	-	-	-	**475**
Docosanoic acid, trimethylsilyl ester **(34)**	-	-	-	**413**	
1-Monooleoylglycerol trimethylsilyl ether **(35)**	-	-	-	**501**	
Ferulic acid **(36)**	-	-	**194**	-	-
Syringaldehyde **(37)**	-	-	**182**	-	-
Syringic acid **(38)**	-	-	**198**	-	-
p-Coumaric acid **(39)**	-	-	**164**	-	-
4-Hydroxybenzoic acid **(40)**	-	-	**138**	-	-
Vanillin **(41)**	-	-	**151**	-	-

[1] maximum wavelengths recorded (nm); [2] $[M-H]^-$(m/z); [3] MW of the TMS derivates (m/z)

of MPCs is by decreasing polarity and increasing weak interactions between stationary phase and analytes. So, more polar compounds such as the simple phenols (1 to 4) were eluted in the first 40 minutes of the run. MPCs with higher molecular weight (and thus higher weak interactions with the C18 phase) and less polar character were eluted within 70 min. Twelve compounds were identified by using HPLC/DAD. The identification was achieved by observing the absorption spectra in the UV region and confirmed by the spectral data. Of these, 4 belonged to the group of simple phenols (hydroxytyrosol, tyrosol, vanillic acid, and hydroxytyrosol acetate), 6 compounds were secoiridoids (dialdehydic form of decarboxymethyl oleuropein aglycon, dialdehydic form of oleuropein aglycon, dialdehydic form of decarboxymethyl ligstroside aglycon, dialdehydic form of ligstroside aglycon, aldehydic form of oleuropein aglycone, aldehydic form of ligstroside aglycone), and two compounds were flavones (luteolin and apigenin). The compounds iden-

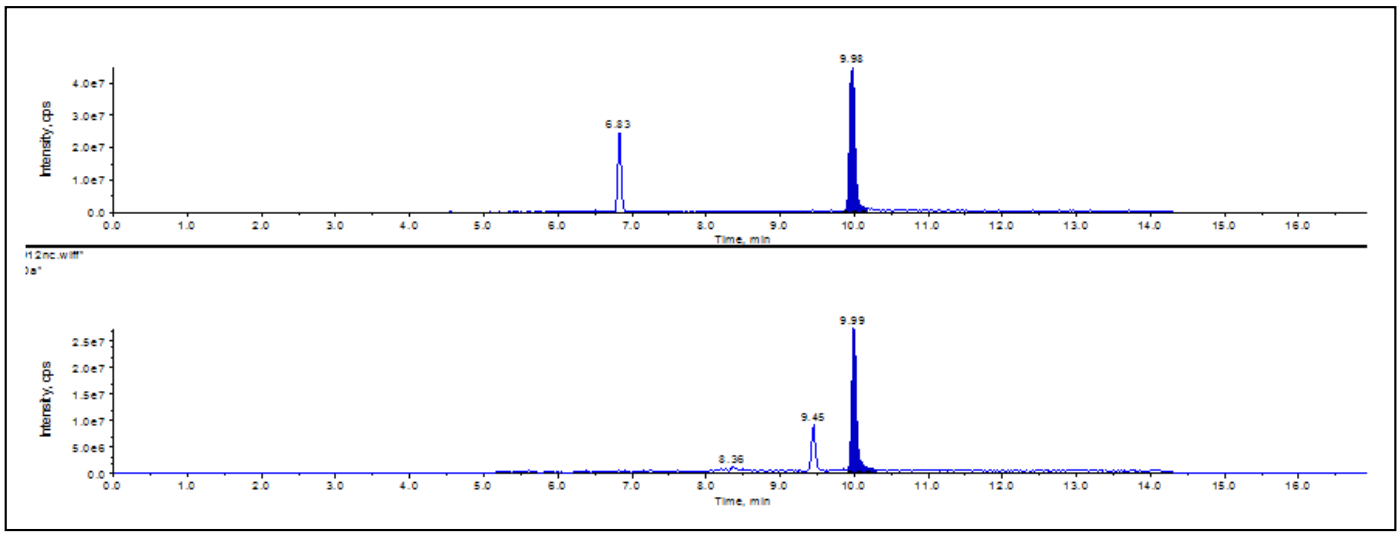


Fig. 2: Single ion current (m/z 283) corresponding to stearic acid in a standard solution (A) and in the oil sample AT_1 (B).

tified in all the samples are listed in Table 1, where also the maximum wavelength recorded for each compound was reported.

HPLC/TIS-MS analysis

Coupling HPLC with Turbo Ion Spray (TIS) soft ionization was not efficient in the determination of chromatographic profiles with sufficient resolution; the recognition of the compounds and the interpretation of the mass spectra was then difficult due to interfering analytes all along the chromatographic trace. Only the injection of standard compounds was successful. They were identified by their pseudomolecular ion especially in negative mode. Figure 2 shows the results obtained by searching the mass corresponding to negative ionization of stearic acid (m/z 283 amu) in the four samples.

HPLC/ESI-MS analysis

HPLC/ESI-MS was used to confirm the presence of phenolic compounds detected by HPLC/DAD. The mass spectra of hydroxytyrosol (3,4-dihydroxyphenylethanol) obtained by negative ionization provided a deprotonated molecule (m/z 153); a fragment at m/z 123 was detected in MS-MS mode; it was linked to the to the loss of the methoxy group [(M-H-CH_2)$^-$]. Tyrosol (4-hydroxyphenylethanol) was identified by the presence of the deprotonated molecule at m/z 137. In negative mode, vanillic acid originated a spectra characterized by a deprotonated molecule [M-H] $^-$ (m/z 167); the ion fragment (m/z 123) was due to the loss of CO_2. Hydroxytyrosol acetate was identified through the deprotonated molecule [M-H]$^-$ at m/z 195 and by the ion fragment at m/z 135, generated by the loss of acetic acid. The dialdehydic form of decarboxymethyl oleuropein aglycon was identified by the mass spectra characterized by the deprotonated molecule [M-H]$^-$ at m/z 319, through a daughter fragment (m/z 301) due to the loss of water and by a fragment ion of decarboxymethylated elenolic acid (m/z 195). Dialdehydic form of oleuropein aglycon was detected by the presence of the pseudomolecolar ion (m/z 377) and, in tandem mode, through the presence of a fragment ion at m/z 345 (loss of methanol) and of other ions, of which one was at m/z 307 (loss of CH_3-CH=CH-CHO) and another one was at m/z 275 (simultaneous loss of CH_3-CH =CH-CHO and methanol). The dialdehydic form of decarboxymethyl ligstroside aglycon and dialdehydic ligstroside aglycon were identified respectively through the detection of the deprotonated molecules at m/z 303 and at m/z 361. An ion fragment at m/z 285 due to the loss of a molecule of water; another at m/z 179 due to the loss of the fragment $C_7H_8O_2$, and another due to the loss of tyrosol (m/z 165) permitted to confirm the presence of dialdehydic form of decarboxymethyl ligstroside aglycon. Dialdehydic ligstroside aglycon was instead confirmed by the presence of the ion fragment at m/z 329 (due to the loss of methanol) and through the presence of the ion fragments at m/z 291 (loss of CH_3-CH=CH-CHO) and at m/z 259 (due to losses either of methanol and CH_3-CH=CH-CHO). Aldehydic form of oleuropein aglycone and aldehydic form of ligstroside aglycone were identified through the pseudomolecular ion at m/z 377 e 361 respectively. Since the mass spectra of the two compounds were similar, the distinction of the two different aldehydic form was possible comparing the retention time of these compounds [28]. The recognition of luteolin was permitted by the detection of the pseudomo-

lecular ion at m/z 285 and by an ion fragment at m/z 257 (loss of carbon monoxide). Finally, apigenin was identified by the presence of the deprotonated molecule $[M\text{-}H]^-$ at m/z 269.

GC/TOF-MS and GC×GC/TOF-MS analysis

The GC/TOF-MS and GC×GC/TOF-MS profile of the samples (within the time range 400 s - 1400 s) showed an average number of 630 and 6500 compounds, respectively. Thus, the bidimensional technique allowed the detection of about 10 times more compounds than monodimensional chromatography. For this reason, deconvolution was applied in order to identify the entire profile and exclude artifacts or interfering peaks.

After the deconvolution process, the trimethylsilyl derivatives of MPCs present in all 4 samples and with the same retention time were selected just by using the worksheet function ‚sort & filter'. After filtering, the total average number of silylated compounds was reduced to 58 and 285, for GC/TOF-MS and GC×GC/TOF-MS, respectively (Table 2).

Table 2: Number of total identified peaks by GC/TOF-MS and GC×GC/TOF-MS.

Sample	GC/TOF-MS		GCxGC/TOF-MS	
	Total identified peaks	TMS derivatives	Total identified peaks	TMS derivatives
AT_1	729	62	6448	279
AT_2	664	58	6465	255
FL_1	484	51	6613	291
FL_2	645	63	6445	314

The TMS derivatives present in all the 4 olive oil samples (common TMS derivatives) were 15 and 25 for monodimensional and bidimensional chromatography, respectively (Table 1).

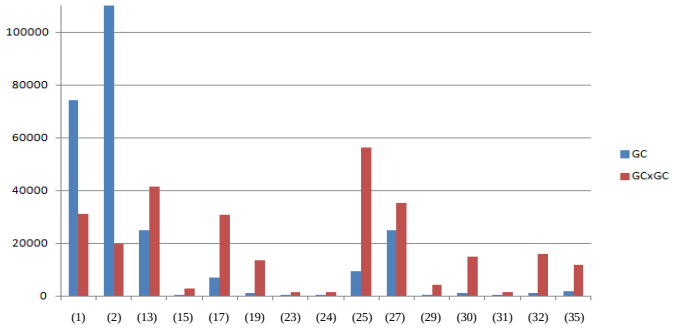
For these compounds, the retention index (RI) was then calculated according to the following formula:

$$RI = 100 \times [n + (N - n)\frac{t_{r(unknown)} - t_{r(n)}}{t_{r(N)} - t_{r(n)}}]$$

where:

RI = Kovats retention index,
n = the number of carbon atoms in the smaller n-alkane,
N = the number of carbon atoms in the larger n-alkane,
t_r = retention time.

The retention index of MPC was obtained by comparing the RT with that of a series of alkanes (C5-C40) injected in both GC and GC×GC. The calculated RI for each compound was compared with that contained in the NIST database and reported in Table 3. They were similar to what reported in previous work using the same stationary phases. Fatty acids, glycerides, organic acids and phenolics were identified. The identified phenolic compounds were tyrosol and hydroxytyrosol and trans-cinnamic acid. Benzoic acid was also identified.



(1) Hydroxytyrosol diTMS
(2) 4-Hydroxyphenylethanol, di-TMS (Tyrosol, diTMS)
(13) Propanoic acid, 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester
(15) Benzoic acid trimethylsilyl ester
(17) Trimethylsilyl ether of glycerol
(19) Nonanoic acid, trimethylsilyl ester
(23) Benzoic acid, 4-[(trimethylsilyl)oxy]-, trimethylsilyl ester
(24) Dodecanoic acid, trimethylsilyl ester
(25) Glucopyranose, pentakis-O-trimethylsilyl-
(27) Hexadecanoic acid, trimethylsilyl ester
(29) Heptadecanoic acid, trimethylsilyl ester
(30) Oleic acid, trimethylsilyl ester
(31) α-Linolenic acid, trimethylsilyl ester
(32) Octadecanoic acid, trimethylsilyl ester
(35) 1-Monooleoylglycerol trimethylsilyl ether

Fig. 3: Signal to noise ratio (S/N) of the common compounds detected either by GC and by GC×GC, calculated as average value of four samples.

For the 15 MPCs detected in all the 4 samples of EVOO either by GC and by GC×GC, the signal to noise ratio (S/N) was calculated as the average value of the four different injections (Fig. 3). Only for tyrosol and hydroxytyrosol, S/N was lower in GC×GC with respect to GC, confirming the increased power and major resolution capacity of this technique.

Conclusions

The HPLC/DAD was an effective and fast method to identify the profile of phenolics, flavonoids and secoiridoids of the oils, allowing for the identification of twelve different compounds with typical absorption in the UV region. However this technique was not able to detect minor polar compounds different from phenolics, such as fatty acids, glycerides and sugars.

With HPLC/ESI-MS experiments carried out by coupling mass spectrometry of the second order as a detection system it was possible to provide information, confirming the profile of phenolics, flavonoids and secoiridoids detected with HPLC-DAD. The HPLC/TIS-MS experiments were useful only when standard compounds were used for the identification.

The gas chromatographic techniques (such as GC/TOF-MS and GC×GC/TOF-MS) are the elective analytical procedure for volatile and semivolatile analytes; they involve a previous silylation procedure which slows down the analysis rate. However, unlike HPLC-DAD, they were successful

Table 3: Retention time and RI of the compounds detected with both GC and GC×GC.

Name	GC		GCxGC			RI NIST
	Rt1	RI(GC)	Rt1 (1st dimension)	Rt2 (2nd dimension)	RI GCxGC	
Propanoic acid, 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester **(13)**	44.19	1054	464	1.116	1086	1057; 1086
Propanoic acid, 3-[(trimethylsilyl)oxy]-, trimethylsilyl ester **(14)**	-	-	500	1.17	1159	1151
Benzoic acid trimethylsilyl ester **(15)**	528.625	1238	544	1.21	1267	1242; 1267
Octanoic acid, trimethylsilyl ester **(16)**	-	-	544	1.18	1267	1262
Trimethylsilyl ether of glycerol **(17)**	534	1254	548	1.15	1278	1266
Propanoic acid, 2,3-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester **(18)**	-	-	572	1.18	1340	1336
Nonanoic acid, trimethylsilyl ester **(19)**	569.25	1348	580	1.19	1360	1355
Decanoic acid, trimethylsilyl ester **(20)**	-	-	616	1.2	1452	1450
Benzoic acid, 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester **(21)**	-	-	640	1.24	1518	1519
trans-Cinnamic acid, trimethylsilyl ester **(22)**	-	-	648	1.285	1542	1546
4-Hydroxyphenylethanol, di-TMS (Tyrosol, diTMS) **(2)**	648.325	1563	656	1.23	1565	1566
Benzoic acid, 4-[(trimethylsilyl)oxy]-, trimethylsilyl ester **(23)**	669.275	1624	672	1.265	1611	1625; 1621
Dodecanoic acid, trimethylsilyl ester **(24)**	673.45	1637	676	1.22	1623	1651
Hydroxytyrosol diTMS **(1)** cas number: 68595-80-2*	714.15	1776	716	1.24	1750	n.f.
Glucopyranose, pentakis-O-trimethylsilyl- **(25)**	755.025	1913	756	1.126	1911	1913
4-Hydroxy-3-methoxyphenyl)ethylene glycol tris(trimethylsilyl) ether **(26)**	-	-	736	1.25	1833	1850
Hexadecanoic acid, trimethylsilyl ester **(27)**	796.3	2042	796	1.43	2025	2047
Scopoletin, trimethylsilyl ether **(28)**	-	-	812	1.92	2076	2068
Heptadecanoic acid, trimethylsilyl ester **(29)**	830.95	2141	832	1.535	2133	2148
Oleic acid, trimethylsilyl ester **(30)**	861.525	2219	860	1.68	2210	2212
α-Linolenic acid, trimethylsilyl ester **(31)**	863.75	2225	864	1.705	2220	2218
Octadecanoic acid, trimethylsilyl ester **(32)**	869.95	2239	868	1.73	2227	2250
2-Monopalmitin trimethylsilyl ether **(33)**	-	-	1044	2.238	2550	2576
Docosanoic acid, trimethylsilyl ester **(34)**	-	-	1112	2.67	2636	2644
1-Monooleoylglycerol trimethylsilyl ether **(35)**	1255.65	2767	1244	3.345	2764	2784

The RI of Hydroxytyrosol-diTMS was not present on NIST database.

in the determination of compounds with poor absorption in the UV region such as fatty acids, glycerides and sugars. The number of the identified phenolic compounds was lower than HPLC/DAD and HPLC/ESI-MS.

In GC×GC/TOF-MS, the number of compounds (as TMS derivatives) identified through the MS library was about 5 times higher than those obtained by the GC/MS method due to the higher resolution power of bidimensional techniques. This confirms the strong effectiveness of GC×GC as a separative technique due to its high resolution power. This technique is very promising in a ‚foodomic' approach; however, the enormous data amount produced with a GC×GC/TOF-MS trace can be a hindrance for a rapid evaluation of the results.

The new proposed method based on the ‚sort and filter' approach represents a valid tool to obtain reliable analytical information rapidly and without the aid of standard compounds. It is based on three screening criteria (or phases):

1. processing of the raw chromatograms and peak filtering to remove peaks that contain no relevant information,
2. verification of standards and known compounds in the peak table, and
3. determination of the identity for new compounds.

By using these criteria, we used the total set of pertinent peaks for profile comparisons of the different types of EVOOs. With this approach, the analytical fingerprint of a selected extract of different virgin oil samples (regardless of the nature of the chemical class) can be used in order to assess the quality and the authenticity of a protected designation (PDO) or other quality labels (organic products, etc.) and thus prevent commercial fraud.

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