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Bioactive Constituents, Metabolites, and Functions

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1 **Insights into Analysis of Phenolic Secoiridoids in Extra Virgin Olive Oil**

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ABSTRACT: Extra virgin olive oils (EVOOs) containing more than 5 mg/20 g of tyrosol, hydroxytyrosol and their secoiridoids can be recognized with the health claim related to the protection of blood lipids from oxidative stress. Therefore, a reliable, accurate and standardized analytical procedure is needed to determine these markers of EVOO quality. In order to overcome the limitations of current methods, a detailed investigation of sample preparation and chromatographic conditions was performed by UHPLC-UV-HRMS. The use of C18 fused-core column and a non-acidified gradient elution provided single sharp peaks for oleocanthal and oleacein, allowing their reliable quantitation in UV profile. Positive and negative UHPLC-HRMS/MS characterization of methanolic extracts revealed the presence of dimethyl acetal, methyl hemiacetal and monohydrate derivatives of all secoiridoids. These artefacts were formed in aqueous methanol, usually employed to extract and analyse the EVOO phenols, making the HPLC profiles more complex and the measurements less accurate and reproducible. Acetonitrile proved to be a suitable solvent to avoid the formation of secoiridoid dimethyl acetals and methyl hemiacetals, and to efficiently extract EVOO bioactive phenols. Finally, the phenolic contents of Italian EVOO samples were determined by UHPLC-UV analysis of acetonitrile extracts before (direct method) and after acid hydrolysis (indirect method). The results indicated that the use of tyrosol and hydroxytyrosol as reference standards allowed more accurate quantitative data to be obtained. Direct and indirect methods provided comparable levels of EVOO phenols, highlighting the usefulness of acid hydrolysis in routine analyses. The improved procedure defines the most reliable conditions to provide an analytical method with suitable accuracy and repeatability in the analysis of healthy and functional EVOO phenols.

KEYWORDS: extra virgin olive oil; phenolic secoiridoids; positive and negative-UHPLC-HRMS analysis; artefacts.

INTRODUCTION

Extra virgin olive oil (EVOO) has nutritional, technological, sensory characteristics and health-protecting activities that make it a unique ingredient of the Mediterranean diet. These properties are strictly related to its high content of oleic acid and hydrophilic phenolic compounds.[1–5] In particular, the EVOO quality is mainly affected by the content of hydroxytyrosol (3,4-dihydroxyphenylethyl alcohol, 3,4-DHPEA, **1**), tyrosol (4-hydroxyphenethyl alcohol, *p*-HPEA, **2**), and their esterified derivatives with elenoic acid, known as secoiridoids (Figure 1).[1–6] These compounds are the most complex, abundant and typical family in the EVOO polar fraction.[1–5] The most abundant secoiridoids in EVOO are the monoaldehydic forms of oleuropein (3,4-DHPEA-EA, **3**) and ligstroside (*p*-HPEA-EA, **4**) aglycones, and the dialdehydic forms of their decarboxymethylated derivatives, oleacein (3,4-DHPEA-EDA, **5**) and oleocanthal (*p*-HPEA-EDA, **6**) (Figure 1).[7,8] Secoiridoids act as the main natural antioxidants of EVOO,[1,9] and they are the main contributors to EVOO organoleptic characteristics (bitter and pungent attributes).[9] Moreover, they are the most studied and best-known components in terms of health-protecting activities.[5,10–12] The content of **1**, **2**, and their secoiridoid derivatives in EVOO have been correlated in humans with the increase in the antioxidant content of LDL and a nutrigenomic effect, modulating the expression of atherosclerosis-related genes towards a protective mode.[5,13]

Actually, the regulation EU n.432/2012 permits acknowledgement of the health claim “Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress” for EVOOs containing at least 5 mg of hydroxytyrosol and its derivatives (e.g. oleuropein complex and tyrosol) per 20 g of olive oil.[14] This is a very important tool with a significant impact on the field of olive oil marketing and labeling. The possibility of adopting a label with the health claim based on the content of bioactive phenols would be useful to effectively signal both the “healthiest” and the “highest quality” EVOOs.[15] However, the olive oil industry has not taken advantage of this opportunity. The main concerns involve the lack of clarity in determining the bioactive compounds and the analytical protocol to apply the claim.

Beyond the phenol content, the determination of phenolic profile (amount and type of the individual components) of EVOO is of great interest in olive oil manufacturing process, as the phenolic composition depends on various factors, e.g. olive cultivar and maturity stage, climatic and agronomic conditions, the pre-processing, processing, and post-processing procedures of EVOO production.[15] Hence, reliable and accurate analytical methods are needed to characterize the complex EVOO phenolic pattern, to develop complete compositional databases and to obtain more accurate intake data of healthy and functional EVOO phenols.

Many methods have been developed to characterize the complex phenolic pattern of EVOOs, mainly based on HPLC separation followed by UV/Vis or mass spectrometry (MS) detection, and quantitative NMR.[1,2,7,8,16] In 2011, the International Olive Council (IOC) proposed a standard procedure based on the HPLC-DAD analysis of the methanolic extract where all phenols are quantitated as *p*-HPEA (**2**) equivalents.[17] However, the determination of EVOO phenols by HPLC is rather difficult, mainly due to chromatographic resolution (co-elutions, broadened peaks, isomeric forms of secoiridoids), peak identification problems and to the lack of standards for a reliable quantitative analysis.[18]

In addition, several limitations of HPLC methods in the determination of oleocanthal (**6**) and oleacein (**5**) have been recently identified.[7,19] These dialdehydic compounds react immediately with methanol and/or water to give mixtures of hemiacetals or acetals.[7] This interaction can occur during either extraction and/or chromatographic steps, since these protic solvents are commonly used for the determination of EVOO phenols. Consequently, some peaks in HPLC-UV or LC-MS chromatograms may correspond to artefacts produced by that type of reaction, making the HPLC profiles much more complex and less reproducible. Also, in quantitative methods based on selective MS/MS fragmentation of aldehydic secoiridoid forms, the hemiacetal and acetal artefacts are not measured, leading to questionable conclusions about the reliability of such measurements.

Based on these complications of current analytical methods and the urgent need to facilitate the EVOO phenols determination for their potentiality as markers of the EVOO quality, in the present

research a comprehensive re-evaluation of EVOO phenolic profile, focusing on secoiridoids, was performed. To gain insight into secoiridoid characterization, different extraction and chromatographic conditions were studied by UHPLC-UV-HRMS with negative and positive electrospray ionization modes. The main aims were therefore: i) to enhance the chromatographic resolution; ii) to investigate the formation of hemiacetals and acetals from secoiridoids during the sample preparation; and iii) to define the most reliable conditions to provide an analytical method with suitable accuracy and repeatability.

MATERIALS AND METHODS

Reagents and Standards. Analytical-grade methanol (MeOH), acetonitrile (MeCN) *n*-hexane, caffeine (volumetric internal standard, IS), and MS-grade acetic acid, ammonium formate, ammonium acetate and formic acid (HCOOH), were obtained from Sigma-Aldrich (Milan, Italy). Ultrapure water (18 MΩ) was prepared by a Milli-Q purification system (Millipore, BedfordMA). MS-grade MeCN and water were supplied by Romil (Cambridge, UK).

Reference standards (≥98% HPLC grade) of hydroxytyrosol (3,4-DHPEA, **1**), tyrosol (*p*-HPEA, **2**) and pinoresinol were purchased from Extrasynthase (Lyon, France). Oleacein (3,4-DHPEA-EDA, **5**) and oleocanthal (*p*-HPEA-EDA, **6**) were provided by PhytoLab GmbH (Vestenbergsgreuth, Germany). Standard stock solutions of **1** and **2** (1 mg/mL) were prepared in acetonitrile and stored at 4 ºC. Diluted solutions and standard mixtures were prepared in MeCN/H_2O 2:8, v/v.

Extra Virgin Olive Oil Samples. Eleven Italian EVOOs (O1-11) of the season 2016–2017 were collected from supermarkets and Calabrian farmers and stored away from light. Six EVOO samples (O5-10) are protected denomination of origin (PDO) oils from various Italian regions.

Isolation of EVOO Phenolic Compounds by Liquid-Liquid Extraction. EVOO phenolic compounds were extracted following the IOC method,[17] with some modifications. A 1.5 g ± 0.03 aliquot of EVOO sample was weighed in a 15 mL conical tube and extracted with 4.5 mL of MeOH/H_2O (8:2, v/v) or MeCN/H_2O (8:2, v/v). The mixture was shaken by electronic shaker for 1 min and the extraction was performed with the aid of an ultrasonic bath for 15 min. Then, the two phases were separated by centrifugation at 13000 rpm for 10 min, and the hydrophilic phase was evaporated to dryness under vacuum at 30 °C in a rotary evaporator. The residue was dissolved with 1 mL of MeOH/H_2O (3:7, v/v) or MeCN/H_2O (2:8, v/v) containing IS (final concentration 10 μg/mL). *n*-Hexane (1 mL) was added to the solution to wash away any remaining oil. The tube was

centrifuged for phase separation, before the chromatographic analysis. Each EVOO sample was extracted in triplicate.

Acid Hydrolysis. To determine the total amount of free and linked **1** and **2**in EVOO phenolic extracts, the acid hydrolysis procedure of Mulinacci et al.[20] was used with slight modifications. Briefly, 200 µL of extract was added with 200 µL of H_2SO_4 1.0 M. The samples were maintained at 80 °C for 2 h in a thermostat, then the reaction was stopped by freezing the tubes in an ice bath. Finally, the samples were diluted with 200 µL of NaOH 2 M, and IS was added at the final concentration of 10 µg/mL before UHPLC-UV analyses. The hydrolysis procedure was carried out in duplicate for each phenolic extract (triplicates) and then the hydrolysates were combined for UHPLC-UV analyses.

Preliminary experiments were performed to verify the chemical stability of **1** and **2** and the completeness of the ester linkage hydrolysis (monitoring the hydrolysis of extract up to 4 h by UHPLC-UV) under hydrolysis conditions.

UHPLC-HRMS Analyses. Chromatographic analyses were performed using a Platin Blue UHPLC system (Knauer, Labservice Analytica, Bologna, Italy), consisting of two ultra high-pressure pumps, an autosampler, a column temperature manager, coupled to a LTQ OrbiTrap XL mass spectrometer (Thermo Fisher Scientific, Milan, Italy). A 100 x 2.1 mm I.D., 2.6 µm column, Kinetex C18, (Phenomenex, Bologna, Italy) was used at a flow rate of 400 µL/min and at the temperature of 25 °C. The mobile phase was a binary gradient of water (A) and MeCN (B). The gradient elution program is as follows: 0-6 min, 2% B; 6-10 min, 2-23% B; 10-15 min, 23% B; 15-22, 23-50 % B; 22-27 min, 50-98% B. After each injection (5 µL), cleaning (98% B, 6 min) and re-equilibration of the column (6 min) were performed.

The mass spectrometer, equipped with ESI source, was operated in negative and positive ionization modes. High purity nitrogen (N_2) was used as sheath gas (50 arbitrary units) and auxiliary gas (25

arbitrary units). High purity helium (He) was used as collision gas. Optimized mass spectrometer parameters in negative ionization mode were as follows: source voltage 2.5 kV, capillary voltage –25 V, tube lens voltage –110 V. Optimized conditions in positive ionization mode were: source voltage 3.5 kV, capillary voltage 48 V, tube lens voltage 65 V. In both modes, capillary temperature was 250 °C. Mass spectra were acquired by full range acquisition covering *m/z* 130–800. For fragmentation study, a data dependent scan was performed and the normalized collision energy of the collision-induced dissociation (CID) cell was set at 35 eV and the isolation width of precursor ions was set at 2.0. The resolving power was 100000 and 30000 for the full mass and the data dependent MS scan, respectively. Compounds were characterized according to the corresponding HRMS spectra, accurate masses, characteristic fragmentations, and retention times. Xcalibur software (version 2.2) was used for instrument control, data acquisition and data analysis.

UHPLC-UV Analyses. The UHPLC-UV analyses were performed using a Dionex Ultimate 3000 UHPLC system (Thermo Fisher Scientific) constituted of an Ultimate 3000 RS Pump, an Ultimate 3000 RS autosampler, an Ultimate 3000 RS column compartment and Ultimate 3000 RS variable wavelength detector. The chromatographic conditions were the same as those used for UHPLC–HRMS analysis, and the UV chromatograms were recorded at 254, 278 and 350 nm. For quantitative determination of 3,4-DHPEA (**1**) and *p*-HPEA (**2**) and secoiridoids in EVOO phenolic extracts and hydrolysates, the wavelength of 278 nm was used. **1** and **2** were employed as reference standards, and six concentration levels were prepared diluting with water appropriate volumes of stock solutions (1 mg/mL, MeCN). IS was added to each level at the concentration of 10 μg/mL. Linearity of calibration curves were evaluated in the concentration ranges of 5-200 μg/mL, and triplicate injections for each level. UV peak area ratios (*p*-HPEA/IS and 3,4-DHPEA/IS) were plotted against the corresponding standard concentrations (μg/mL).

The regression curves were tested with the analysis of variance (ANOVA) and linear model was found appropriate over the tested concentration ranges (**1**, $y = 0.0166 x – 0.1130$; $R^2 = 0.9991$; **2**, y

172 = 0.0204 x – 0.023; R^2 = 0.9993). For reference compounds (**1** and **2**), the limits of detection (LOD)
173 and of quantification (LOQ) were calculated by extrapolation of the concentrations giving a signal-
174 to-noise ratio (S/N) of 3 and 10, respectively, from a linear regression (S/N versus concentration):
175 for **1**, LOD = 0.5 μg/mL and LOQ = 1.0 μg/mL; for **2**, LOD = 1.4 μg/mL and LOQ = 2.5 μg/mL.
176 The accuracy of the LLE methods (aqueous methanol and acetonitrile) was estimated by recovery
177 experiments, adding known amounts of **1** and **2** to a seed oil sample that did not contain the
178 molecules studied. Both LLE solvents showed exhaustive extraction of **1** and **2** (98-106%).
179 3,4-DHPEA secoiridoids (**3**) isomers, **5**, **7** isomers and **9**) and *p*-HPEA secoiridoids (**4**) isomers, **6**,
180 **8** isomers and **10**) were quantitated using the calibration curves of **1** and **2**, respectively. Secoiridoid
181 levels, were finally expressed as mg/20 g of oil.
182
183 **Statistical Analyses.** Data were expressed as mean ± standard deviation of triplicates. The data
184 were statistically analysed using the statistical software Statgraphics Centurion XVI Version 16.1
185 (Statistical Graphics, Rockville, MD). Statistically significant differences in the quantitative data of
186 EVOO samples were evaluated by a multiple sample comparison procedure (ANOVA, Multiple
187 Range Tests). Correlations between pair of variables (3.4-DHPEA and *p*-HPEA contents by direct
188 and indirect methods) were assessed using Pearson's correlation coefficients (Multiple-Variable
189 Analysis procedure, confidence level of 95%).

RESULTS AND DISCUSSION

UHPLC Conditions. Currently, the analysis of EVOO phenolic compounds in LLE or SPE extracts employ reversed-phase HPLC. The most useful solvents are acidified water, and acetonitrile, methanol or methanol/acetonitrile.[1,2,16,17]

In the present study, in order to enhance chromatographic resolution of EVOO extracts several C18 columns were tested, obtaining the best results with a C18 fused-core column, which provides high column efficiency with short analysis time (results not shown), according to literature data.[21,22] In the selection of the mobile phase, various gradients between water and MeCN, and different modifier were tested. MeOH was excluded as organic solvent to avoid the conversion of secoiridoid aldehydic forms (**3**-**6**) to acetal and hemiacetal derivatives.[7,19] UHPLC profiles of different chromatographic conditions were recorded both with UV and HRMS detectors. Figure 2 shows the negative HRMS extracted ion chromatograms (EICs) corresponding to the main phenolic secoiridoids (**3**-**6**) of a EVOO sample processed by IOC procedure (extracted and reconstituted in aqueous MeOH) and analysed by UHPLC with (black lines) or without acid (red lines). In Figures 3A and B are reported the corresponding UV profiles (278 nm). As can be seen, the best resolution and peak shapes were obtained with water/MeCN gradient without acid modifier (Figure 2, red lines). In acid conditions (similar profiles for 0.05 and 0.1%, v/v, of formic and acetic acids), **5** (peak 10) and **6** (peak 16) gave broadened peaks (Figures 2A and B, black lines) and produced chromatographic humps and co-elutions in the UV profile (Figure 3A), reducing the precision and the accuracy of the measurements. This fact is most likely due to equilibrium of isomeric forms of these compounds promoted in the acid conditions.[22] When 2 mM ammonium formate (or acetate) were used for elution, additional peaks were observed in UHPLC profiles. These were identified by HRMS as Schiff bases generated from reaction between secoiridoid aldehyde carbonyls and ammonium ion (results not shown). Thus, a gradient of water and MeCN without modifier was selected for UHPLC analysis of EVOO phenols. This gradient resulted in much more effective

separation of EVOO extracts, and **5** and **6** were separated into single sharp peaks (Figures 1A and B, red lines), as previously reported,[23] allowing their reliable quantitation in UV profile (Figure 2B).

UHPLC-HRMS/MS characterization of EVOO phenolic secoiridoids. To date, LC-MS has been widely accepted as the main tool in identification and structural characterization of EVOO phenolic compounds. Usually, they are detected in the negative ionization mode related to a greater sensitivity,[1,2] nevertheless the PI mode appears to be more diagnostic for EVOO phenolic secoiridoids.[1,2,24]

In this respect, IOC extracts of two EVOO samples were analysed by UHPLC-HRMS/MS, with positive and negative ionization modes, to provide a comprehensive characterization of phenolic secoiridoids. The accurate masses, molecular formulae, typical product ions of EVOO secoiridois, as well as of other typical EVOO phenolic compounds, are listed in Tables 1 and 2.

EVOO Phenolic Secoiridoids. Different isomers of **3** (peaks 15, 17, 18 and 21, $C_{19}H_{22}O_8$) and **4** (peaks 19, 20 and 22-24, $C_{19}H_{22}O_7$) were tentatively characterized according to mass (Tables 1 and 2) and literature data.[21] **5** (peak 10, $C_{17}H_{20}O_6$) and **6** (peak 16, $C_{17}H_{20}O_5$) were identified by HRMS, literature data[21] and comparison with reference standards. The presence of **3** ($C_{19}H_{22}O_8$) and **4** ($C_{19}H_{22}O_7$) isomers, usually reported by LC-MS methods,[21,22] is justified by the elenolic acid ring opening and following equilibria between aldehydic groups, and by the presence of many oleuroside derivatives.[25]

Phenolic secoiridoids **3**-**6** followed in positive ionization mode all the same fragmentation pattern depending on the phenol moiety esterified (Table 2): their HRMS/MS spectra ($[M+H]^+$ ions) displayed the diagnostic product ions $[CH_2CH_2Ph(OH)_2]^+$ (*m/z* 137.0597), for hydroxytyrosol-secoiridoids **3** and **5** (peaks 10, 15, 17, 18 and 21), and $[CH_2CH_2PhOH]^+$ (*m/z* 121.0648), tyrosol-secoiridoids **4** and **6** (peaks 16, 19, 20 and 22-24), given by a McLafferty-type rearrangement of the ester function.[24] Also, the fragmentation of corresponding $[M+Na]^+$ ions produced "marker" product ions of hydroxytyrosol ($C_8H_8O_2Na^+$ at *m/z* 159.0417 for **3** and **5**) and tyrosol ($C_8H_8ONa^+$ at *m/z* 143.0467 for **4** and **6**) moieties (Table 2). As these product ions in positive MS/MS spectra

corresponded to the phenolic moiety of EVOO secoiridoids, they were not affected by secoiridoid isomerization or hemiacetal and acetal formation. Thus, they were suitable diagnostic ions for the characterization of hydroxytyrosol-and tyrosol-secoiridoid derivatives. Unlike, in negative ionization mode the product ions of **3**-**6** (Table 2) did not allow to distinguish directly the phenyl ethyl alcohol of EVOO secoiridoids, as previously reported.[21] Only **5** and **6** showed product ions related to phenolic part of structures ($[CH_2COOCH_2CH_2Ph(OH)_2]^-$ at *m/z* 195.0659 and $[CH_2COOCH_2CH_2Ph(OH)]^-$ at *m/z* 179.0710, respectively) (Table 2).[21,26]

Hemiacetal/Acetal and Monohydrate Derivatives of EVOO Phenolic Secoiridoids. In addition to phenolic secoiridoids **3**-**6** (peaks 10 and 15-24), positive and negative EICs of **3**-**6** (Figures 2 and 4) of IOC extracts (aqueous MeOH) showed some mismatched peaks (peaks 3-9, 11, 14 and a-d), which turned out to be extraction artefacts following a careful analysis of HRMS/MS spectra. In particular, the assigned molecular formulae of base peaks in positive and negative HRMS spectra of peaks 3-9, 11, 14 and a-d were different. In their positive HRMS spectra, the base peaks corresponded to $[M+Na]^+$ ions, as observed for secoiridoids **3**-**6** (Table 1). In negative ionization mode, instead, $[M-H-H_2O]^-$ and $[M-H-CH_4O]^-$ ions were observed as base peaks of 3-9, 11, and 14 peaks on one hand and a-d peaks on the other, respectively (Table 1). The molecular formulae, assigned by positive and negative HRMS complementary data of peaks 3-9, 11, 14 and a-d, were also confirmed by accurate masses of $[M+H]^+$/$[M-H]^-$ ions present in spectra with low abundances (Table 1). These data strongly suggest that these peaks correspond to methyl-hemiacetals (**a**-**d**) and monohydrate forms (**7**-**10**) of secoiridoids **3**-**6** (Figure 1). Positive HRMS/MS spectra supported further the structures tentatively assigned to these secoiridoid derivatives. In fact, fragmentation of $[M+Na]^+$ ions produced always the product ions due to the loss of H_2O from **7**-**10** and CH_4O from **a**-**d** (Table 2). Likewise, dimethyl acetals of **3**-**6** (**e**-**h**) were also identified in IOC extracts (aqueous MeOH) (Figure 1). In the case of secoiridoids **5** and **6**, the identity of artefacts **9**, **10**, **a** and **c** was proved analyzing by UHPLC-HRMS the reference standards of **5** and **6** in aqueous methanol.

Methyl-hemiacetals, dimethyl-acetals and monohydrate forms of **5** and **6** were previously observed in EVOO by NMR[7] and LC-MS analysis.[19] Isomer peaks of methyl-hemiacetals of **5** (**a)** and **6** (**c**) (Figure 4A and B, red lines) detected in this study correspond probably to two isomers identified by NMR study of Karkoula et al.[7] In the case of **3** and **4**, their methyl hemiacetals (**b** and **d**), dimethyl acetals (**g** and **f**) and monohydrate forms (**7** and **8**) are reported here for the first time.

These UHPLC-HRMS/MS results highlight the ability of the positive ionization mode to provide more diagnostic and complete data in the characterization of EVOO phenolic secoiridoids. In addition to affording structurally significant product ions to identify the phenolic moiety ($C_8H_8O_nNa^+$ and $C_8H_9O_n^+$ ions in MS/MS spectra), positive ionization mode also allows identification of hemiacetal/acetal derivatives and monohydrate forms of EVOO secoiridoids **3**-**6** through $[M+Na]^+$ ions. Conversely, in negative HRMS spectra the latter showed $[M-CH_4O-H]^-$ or $[M-H_2O-H]^-$ ions with masses and molecular formulae coincident to $[M-H]^-$ ions of the corresponding aldehydic forms (**3**-**6**) (Table 1). Not even the negative MS/MS spectra of **7**-**10** and **a**-**d** (Table 2) displayed useful data to differentiate hemiacetal/monohydrate derivatives from related aldehydic forms. These results suggest that the ions detected at *m/z* 319, 303, 377 and 361 by negative LC-MS methods could correspond to both phenolic secoiridoids (**3**-**6**) and their hemiacetal/monohydrate derivatives (**a**-**d** and **7**-**10**). Since negative LC-MS has been usually employed in the identification and quantitative analysis of phenolic secoiridoids in EVOO extracts, false positives can be easily detected in presence of H_2O and MeOH, making the reliability of previous results more or less questionable. Thus, in LC-MS methods the positive and negative modes should be considered complementary for a correct identification and characterization of EVOO phenolic compounds.

Formation of Hemiacetal/Acetal in Sample Preparation Procedure. As shown in the UHPLC-UV profile of EVOO (Figure 3B), methyl-hemiacetal (**a**-**d**) and monohydrate **7**-**10** (peaks 3-9, 11, 14) derivatives of secoiridoids were well detectable peaks that contributed significantly to the

phenolic content. Many of them were partially co-eluted with other components, ruining the separation efficiency and the precision and accuracy of quantitative results. In particular, the complete co-elution of two methyl-hemiacetals of **4** (**d**) with pinoresinol (**12**) and acetoxy-pinoresinol (**13**) did not allow their correct quantitation by UHPLC-UV. Also **5** showed a poor chromatographic resolution due to the presence of a methyl-hemiacetal of **6** (**c**).

According to these results, the IOC sample preparation procedure was investigated in more detail, in order to prevent the formation of hemiacetal/acetal and monohydrate derivatives and, consequently, to simplify the EVOO chromatographic profile. For this purpose, two different EVOO samples were processed by the IOC procedure, replacing MeOH with MeCN in two main sample preparation steps that could potentially promote the formation of artefacts: the extraction and dissolution of extracts for UHPLC analysis.

When MeCN was employed in both IOC steps, the hemiacetal and acetal derivatives were not detected by UHPLC-UV-HRMS analysis (Figure 3C; blue lines in Figure 2; red lines in Figure 4). Also, UHPLC-UV profile of methanolic IOC extracts reconstituted with aqueous MeCN did not reveal the hemiacetal/acetal peaks, indicating that the evaporation of MeOH restored the aldehydic forms of EVOO secoiridoids. This was also observed for **5** and **6** in previous NMR experiments.[7] Regarding the monohydrate forms **7**-**10**, the peaks 3-9, 11, 14 were always observed (Figures 2-4), since the water is necessary in the sample dissolution and chromatographic eluents of reversed-phase HPLC analyses.

The above findings demonstrate that hemiacetal/acetal and monohydrate derivatives of EVOO secoiridoids were formed mainly in aqueous methanolic solution by the spontaneous reaction of secoiridoids with MeOH and H_2O. In HPLC methods this mixture is usually employed to dissolve the EVOO phenolic extracts and these derivatives most likely were present in previous measurements reported in the literature. Thus, the use of MeCN is strongly recommended to avoid the formation of methyl hemiacetal (**a**-**d**) and dimethyl acetals (**e**-**h**) of EVOO secoiridoids. Besides, the UHPLC profile of EVOO extract in aqueous MeCN (Figure 3C) showed a less

318 complex composition than to the same extract in aqueous MeOH (Figure 3B), making easier and
319 more accurate the quantitative measurements.
320 The conversion of hemiacetal/acetal in aldehydic forms after the evaporation of MeOH was not
321 studied in detail, because the UHPLC-HRMS analysis of methanolic IOC extracts reconstituted
322 with aqueous MeCN still showed methyl hemiacetals at very low levels. Hence, the use of MeCN as
323 extraction solvent in the IOC procedure was quantitatively evaluated in order to eliminate MeOH in
324 all steps of sample preparation. Two EVOOs with different quali-quantitative profile of secoiridoids
325 (O-1 and O-2) were extracted by IOC procedure using MeOH/H_2O and MeCN/H_2O. All extracts
326 were reconstituted with aqueous MeCN. The quantitative data (analyte/IS area ratios) of MeCN
327 extracts were normalized to the methanolic IOC extract to determine the extraction efficiency of
328 tested solvent. As can be seen in Figure 5, MeCN/H_2O showed extraction efficiency comparable to
329 MeOH/H_2O, and no significant difference ($p > 0.05$) was observed between two EVOO samples.
330 These results proved that MeCN is a suitable solvent to extract phenolic secoiridoids from EVOO
331 with the IOC procedure, avoiding the formation of artefacts (dimethyl acetals and methyl
332 hemiacetals).
333
334 **Quantitative Analysis and Application to Commercial EVOOs.** Once the reliability of MeCN in
335 the analysis of EVOO phenolic secoiridoids by IOC procedure and UHPLC-UV had been proved,
336 the determination of the secoiridoid contents in eleven Italian EVOO samples was carried out
337 replacing MeOH with MeCN in preparation sample steps.
338 Currently, there is no standardized analytical method for the accurate quantitative analysis of
339 phenolic secoiridoids in EVOO, mainly due to the lack of available reference standards, in
340 particular secoiridoids and lignans.[16,27] A quantitative protocol was proposed by IOC which
341 expressed the total EVOO phenols as *p*-HPEA (**2**) equivalents, using **2** response factor against
342 syringic acid at 280 nm for all compounds detected in UV chromatograms.[17] Nevertheless, standard
343 equivalent units do not directly reflect the real content of EVOO phenolic compounds, since each

344 phenolic compound give a different response under the UV detection.[16,18,27] Another possible way
345 to determine the phenolic content in EVOO is the acid hydrolysis of all the linked forms (phenolic
346 secoiridoids) followed by the quantitative analysis of their total free forms (**1** and **2**) by LC-
347 UV.[18,20,27] This method provides a rapid, simple and suitable tool to quantitate EVOO bioactive
348 phenols and, consequently, to support the health claim. In addition, the acid hydrolysis prevents
349 misinterpretation of the results, due to the change in profile of phenolic compounds during
350 storage.[4,18]
351 On the basis these evidences, the content of **1** and **2**and their secoiridoids in Italian EVOO samples
352 (O1-O11) was determined by UHPLC-UV analysis both in MeCN-IOC extracts (direct method) and
353 in acid hydrolysates (indirect method). Moreover, **1** and **2** calibration curves were employed to
354 express the quantitative results. Italian EVOO samples revealed different EVOO phenolic profiles
355 (amount and type of the individual components), indicating the usefulness of the direct method in
356 olive oil manufacturing process to characterize the EVOOs and to obtain complete compositional
357 databases.
358 Table 3 summarizes the amounts of **1** and **2** derivatives and their total levels in EVOO samples,
359 obtained with direct and indirect methods and calculated as 3,4-DHPEA (**1**) and/or *p*-HPEA (**2**)
360 equivalents. The correlations between the quantitative data of direct and indirect methods were
361 calculated by Pearson's coefficient (r), considering the levels of **1** obtained after acid hydrolysis
362 versus the sum of **1** and all secoiridoids containing the 3,4-DHPEA moiety. Similarly, the
363 correlations were evaluated for **2**and the total EVOO phenol contents (Table 3). Very strong
364 correlations between the two quantitative methods were observed ($r > 0.916$). Moreover, no
365 statistically differences ($p > 0.05$) were observed between the **1** and **2** concentrations determined by
366 both methods and calculated using the respective calibration curves. These findings indicate that the
367 determination of healthy and functional EVOO phenols by the indirect method can play a useful
368 role in routine quality control.

With respect to the reference standard, the use of **2** calibration curve for quantitative analysis of EVOO phenols resulted in higher levels ($p < 0.05$) in comparison to those obtained using **1**as reference standard (Table 3). O4 and O11 samples satisfied the health claim (≥ 5 mg/20 g oil) only when the levels were expressed in *p*-HPEA (**2**) equivalents. Thus, the use of both reference standards is strongly recommended to furnish more accurate quantitative data. In this case (Table 3), direct and indirect methods provided statistically comparable levels of total EVOO phenols ($p > 0.05$). These results are in agreement with those previously reported[27] and underline the need to have a simple, reproducible, and indisputable quantitative protocol. Further studies are underway to establish the reference standards and/or correction factors for the accurate determination of EVOO bioactive phenols.

In summary, the comprehensive investigation of chromatographic and IOC procedure conditions permitted identifying and overcoming some limitations (chromatographic resolution, peak identification and formation of secoiridoid artefacts) of the methods currently employed in the analysis of EVOO phenolic secoiridoids. The detailed UHPLC-UV-HRMS analysis revealed the presence of a large number of peaks in the chromatographic profiles of IOC extracts, characterized in depth for the first time in this study, corresponding to dimethyl acetal (**e**-**h**), methyl hemiacetal (**a**-**d**) and monohydrate (**7**-**10**) derivatives of **3**-**6** produced from the spontaneous reaction of with protic solvents (water and methanol). In particular, only positive ionization mode allowed recognition of the hemiacetal/acetal derivatives and monohydrate forms of secoiridoids, whereas in negative ionization mode these compounds can be easily mistaken with the corresponding aldehydic forms of EVOO secoiridoids. These findings emphasize the ability of positive ionization mode to provide more diagnostic and complete data, and raise questions as to the reliability of previous results obtained using negative LC-MS in the identification and quantitative analysis of phenolic secoiridoids in EVOO extracts. The study of sample preparation procedure demonstrated that the formation of hemiacetal/acetal and monohydrate derivatives occurs in aqueous methanolic solution. The use of MeCN in the IOC procedure (extraction and dissolution solvents) prevents the formation

of dimethyl acetals (**e-h**) and methyl hemiacetals (**a-d**) of EVOO secoiridoids, and, consequently, permits simplification of the EVOO chromatographic profile. Thus replacement of MeOH with MeCN is strongly recommended to obtain more accurate and reproducible measurements.

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Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

HRMS/MS spectra ($[M+H]^+$, $[M+Na]^+$ and $[M-H]^-$ ions) of EVOO phenolic secoiridoids and hemiacetal/acetal derivatives and monohydrate forms.

Positive and negative HRMS extracted ion chromatograms ($[M+Na]^+$ and $[M-H]^-$ ions) of dimethyl acetals (**e**-**h**) of secoiridoids **3**-**6** in EVOO sample processed by IOC using aqueous MeOH.

UHPLC-UV profiles and phenolic secoiridoid contents of 11 Italian EVOO samples.

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503

504 **FIGURE CAPTIONS**

505

506 **Figure 1.** Chemical structures of phenolic secoiridoids of extra virgin olive oil.

507

508 Different isomers of compounds **3**, **4**, **7**-**10**, **a**-**d** were observed in the UHPLC profile of EVOOs.

509

510 **Figure 2.** Negative HRMS extracted ion chromatograms of (A) 3,4-DHPEA-EDA (**5**), (B) *p*-
511 HPEA-EDA (**6**), (C) 3,4-DHPEA-EA (**3**) isomers and (D) *p*-HPEA-EA (**4**) isomers in a EVOO
512 aqueous MeOH extract analysed with (black lines) or without (red lines) acidified gradient, and a
513 EVOO aqueous MeCN extract (blue lines).

514

515 Mass tolerance 5 ppm; peak numbering is according to Table 1; * corresponding to oxygenated *p*-
516 HPEA-EDA (**6**).[21]

517

518 **Figure 2.** UHPLC-UV profiles (278 nm) of a EVOO aqueous MeOH extract analysed (A) with or
519 (B) without acidified gradient, and a (C) EVOO aqueous MeCN extract.

520

521 Peak numbering is according to Table 1. EVOO sample is O2.

522

523 **Figure 3.** Positive HRMS extracted ion chromatograms of Na adducts of (A) 3,4-DHPEA-EDA (**5**),
524 (B) *p*-HPEA-EDA (**6**), (C) 3,4-DHPEA-EA (**3**) isomers and (D) *p*-HPEA-EA (**4**) isomers (black
525 lines) and corresponding monohydrate (blue lines) and methyl hemiacetal (red lines) derivatives in
526 EVOO sample processed by IOC using aqueous MeOH (left) and aqueous MeCN (right).

527

528 Mass tolerance 5 ppm; peak numbering is according to Table 1; * corresponding to oxygenated *p*-
529 HPEA-EDA.[21]

530

531 **Figure 4.** Extraction efficiency of EVOO secoiridoids achieved by the IOC procedure involving
532 aqueous MeCN as extraction solvent. Experiments performed on two EVOO samples (O1 and O2)
533 with different quali-quantitative profiles.

534

535 Values are means of three replicates ± standard deviation (SD). No significant differences amongst
536 EVOO samples ($p > 0.05$).

Table 1. UHPLC-HRMS Data of Phenolic Compounds Detected in Extra Virgin Olive Oils.

Peak No.	t_R (min) [a]	Compound [b]	Molecular formula	Positive HRMS			Negative HRMS		
				m/z [c]	ppm	Diagnostic ion	*m/z* [c]	ppm	Diagnostic ion
1		3,4-DHPEA (**1**) [b]	$C_8H_{10}O_3$	177.0524	0.8	$[M+Na]^+$	153.0557	6.8	$[M-H]^-$
				155.0703	0.1	$[M+H]^+$			
2		p-HPEA (**2**) [b]	$C_8H_{10}O_2$	-			137.0610	8.6	$[M-H]^-$
3	12.0	monohydrate 3,4-DHPEA-EDA (**9**)	$C_{17}H_{22}O_7$	**361.1255**	-0.8	$[M+Na]^+$	**319.1180**	1.2	$[M-H_2O-H]^-$
							337.1285	1.0	$[M-H]^-$
4	12.4	monohydrate 3,4-DHPEA-EA (**7**)	$C_{19}H_{24}O_9$	**419.1307**	-1.7	$[M+Na]^+$	**377.1231**	0.0	$[M-H_2O-H]^-$
				397.1486	1.7	$[M+H]^+$	395.1334	0.6	$[M-H]^-$
5	12.5	monohydrate 3,4-DHPEA-EA (**7**)	$C_{19}H_{24}O_9$	**419.1304**	-1.9	$[M+Na]^+$	**377.1231**	0.0	$[M-H_2O-H]^-$
				397.1489	-1.1	$[M+H]^+$	395.1334	0.7	$[M-H]^-$
6	13.0	monohydrate *p*-HPEA-EDA (**10**)	$C_{17}H_{22}O_6$	**345.1303**	-1.6	$[M+Na]^+$	**303.1230**	1.0	$[M-H_2O-H]^-$
							321.1336	0.9	$[M-H]^-$
a1	13.1	methyl hemiacetal 3,4-DHPEA-EDA (**a**)	$C_{18}H_{24}O_7$	**375.1411**	-1.0	$[M+Na]^+$	**319.1180**	1.3	$[M-CH_4O-H]^-$
							351.1442	0.9	$[M-H]^-$
a2	13.3	methyl hemiacetal 3,4-DHPEA-EDA (**a**)	$C_{18}H_{24}O_7$	**375.1408**	-1.5	$[M+Na]^+$	**319.1179**	0.8	$[M-CH_4O-H]^-$
							351.1440	0.5	$[M-H]^-$
b1	13.5	methyl hemiacetal 3,4-DHPEA-EA (**b**)	$C_{20}H_{26}O_9$	**433.1462**	-0.7	$[M+Na]^+$	**377.1230**	-0.2	$M-CH_4O-H]^-$
				411.1649	-0.2	$[M+H]^+$	409.1487	-1.4	$[M-H]^-$
7	13.7	monohydrate *p*-HPEA-EA (**8**)	$C_{19}H_{24}O_8$	**403.1360**	-0.9	$[M+Na]^+$	**361.1283**	0.4	$[M-H_2O-H]^-$
							379.1385	0.6	$[M-H]^-$
8	13.9	monohydrate *p*-HPEA-EA (**8**)	$C_{19}H_{24}O_8$	**403.1359**	-1.0	$[M+Na]^+$	**361.1282**	1.9	$[M-H_2O-H]^-$
							379.1390	0.7	$[M-H]^-$
b2	14.1	methyl hemiacetal 3,4-DHPEA-EA (**b**)	$C_{20}H_{26}O_9$	**433.1460**	-2.1	$[M+Na]^+$	**377.1231**	-0.1	$M-CH_4O-H]^-$
							409.1489	-1.0	$[M-H]^-$
9	14.7	monohydrate 3,4-DHPEA-EA (**7**)	$C_{19}H_{24}O_9$	**419.1314**	0.3	$[M+Na]^+$	**377.1231**	-0.1	$[[M-H_2O-H]^-$
							395.1333	-0.9	$[M-H]^-$
c1	14.9	methyl hemiacetal *p*-HPEA-EDA (**c**)	$C_{18}H_{24}O_6$	**359.1463**	-0.4	$[M+Na]^+$	**303.1229**	0.8	$M-CH_4O-H]^-$
							335.1485	-1.1	$[M-H]^-$
10	15.0	3,4-DHPEA-EDA (**5**) [b]	$C_{17}H_{20}O_6$	**343.1147**	-1.4	$[M+Na]^+$	319.1178	0.6	$[M-H]^-$
				321.1329	-0.1	$[M+H]^+$			
c2	15.2	methyl hemiacetal *p*-HPEA-EDA (**c**)	$C_{18}H_{24}O_6$	**359.1461**	-1.1	$[M+Na]^+$	**303.1229**	0.7	$M-CH_4O-H]^-$
							335.1488	-0.8	$[M-H]^-$
11	15.8	monohydrate *p*-HPEA-EA (**8**)	$C_{19}H_{24}O_8$	**403.1357**	-1.5	$[M+Na]^+$	**361.1287**	0.7	$[M-H_2O-H]^-$
				381.1540	-0.9	$[M+H]^+$	379.1388	0.3	$[M-H]^-$
d1	16.2	methyl hemiacetal *p*-HPEA-EA (**d**)	$C_{20}H_{26}O_8$	**417.1513**	-1.5	$[M+Na]^+$	**361.1283**	0.2	$M-CH_4O-H]^-$
							393.1545	0.2	$[M-H]^-$

12	16.2	pinoresinol [b]	$C_{20}H_{22}O_6$	-			357.1336	1.0	$[M-H]^-$
d2	17.2	methyl hemiacetal *p*-HPEA-EA (**d**)	$C_{20}H_{26}O_8$	**417.1514**	-1.5	$[M+Na]^+$	**361.1282**	0.2	$M-CH_4O-H]^-$
							393.1547	0.8	$[M-H]^-$
13	17.2	acetoxy-pinoresinol	$C_{22}H_{24}O_8$	439.1362	-0.4	$[M+Na]^+$	**415.1391**	0.8	$[M-H]^-$
14	17.6	monohydrate *p*-HPEA-EA (**8**)	$C_{19}H_{24}O_8$	**403.1361**	-0.5	$[M+Na]^+$	**361.1282**	0.2	$[M-H_2O-H]^-$
				381.1546	0.5	$[M+H]^+$	379.1381	-1.8	$[M-H]^-$
15	17.9	3,4-DHPEA-EA (**3**)	$C_{19}H_{22}O_8$	**401.1205**	-0.6	$[M+Na]^+$	**377.1231**	0.0	$[M-H]^-$
				379.1386	-0.4	$[M+H]^+$			
b3	18.1	methyl hemiacetal 3,4-DHPEA-EA (**b**)	$C_{20}H_{26}O_9$	**433.1464**	-1.1	$[M+Na]^+$	**377.1231**	0.0	$M-CH_4O-H]^-$
				411.1650	-0.0	$[M+H]^+$	409.1490	-0.7	$[M-H]^-$
b4	18.4	methyl hemiacetal 3,4-DHPEA-EA (**b**)	$C_{20}H_{26}O_9$	**433.1462**	-1.5	$[M+Na]^+$	**377.1230**	-0.1	$M-CH_4O-H]^-$
				411.1646	-0.8	$[M+H]^+$	409.1491	-0.6	$[M-H]^-$
16	18.6	*p*-HPEA-EDA (**6**) [b]	$C_{17}H_{20}O_5$	**327.1200**	-1.0	$[M+Na]^+$	**303.1230**	1.0	$[M-H]^-$
				305.1281	-0.8	$[M+H]^+$			
b5	18.9	methyl hemiacetal 3,4-DHPEA-EA (**b**)	$C_{20}H_{26}O_9$	**433.1465**	-0.9	$[M+Na]^+$	**377.1229**	-0.5	$M-CH_4O-H]^-$
				411.1649	-0.1	$[M+H]^+$	409.1487	-1.5	$[M-H]^-$
b6	19.0	methyl hemiacetal 3,4-DHPEA-EA (**b**)	$C_{20}H_{26}O_9$	**433.1465**	-0.8	$[M+Na]^+$	**377.1229**	-0.4	$M-CH_4O-H]^-$
				411.1649	-0.0	$[M+H]^+$	409.1495	0.6	$[M-H]^-$
17	19.3	3,4-DHPEA-EA (**3**)	$C_{19}H_{22}O_8$	**401.1202**	-1.1	$[M+Na]^+$	**377.1231**	0.1	$[M-H]^-$
				379.1386	-0.4	$[M+H]^+$			
18	19.8	3,4-DHPEA-EA (**3**)	$C_{19}H_{22}O_8$	**401.1201**	-1.3	$[M+Na]^+$	**377.1232**	0.2	$[M-H]^-$
				379.1386	-0.2	$[M+H]^+$			
19	19.9	*p*-HPEA-EA (**4**)	$C_{19}H_{22}O_7$	**385.1256**	-0.3	$[M+Na]^+$	**361.1286**	1.0	$[M-H]^-$
				363.1435	-1.0	$[M+H]^+$			
20	20.1	*p*-HPEA-EA (**4**)	$C_{19}H_{22}O_7$	**385.1254**	-0.8	$[M+Na]^+$	**361.1284**	0.6	$[M-H]^-$
				363.1437	-0.4	$[M+H]^+$			
21	20.4	3,4-DHPEA-EA (**3**)	$C_{19}H_{22}O_8$	**401.1202**	-1.0	$[M+Na]^+$	**377.1230**	-0.4	$[M-H]^-$
				379.1387	-0.1	$[M+H]^+$			
d3	20.5	methyl hemiacetal *p*-HPEA-EA (**d**)	$C_{20}H_{26}O_8$	**417.1515**	-1.0	$[M+Na]^+$	**361.1283**	0.3	$M-CH_4O-H]^-$
				395.1698	-0.5	$[M+H]^+$	393.1541	-0.8	$[M-H]^-$
22	21.4	*p*-HPEA-EA (**4**)	$C_{19}H_{22}O_7$	**385.1254**	-1.0	$[M+Na]^+$	**361.1285**	0.8	$[M-H]^-$
				363.1436	-0.5	$[M+H]^+$			
23	21.5	*p*-HPEA-EA (**4**)	$C_{19}H_{22}O_7$	**385.1254**	-0.8	$[M+Na]^+$	**361.1282**	0.1	$[M-H]^-$
				363.1435	-0.7	$[M+H]^+$			
24	21.7	*p*-HPEA-EA (**4**)	$C_{19}H_{22}O_7$	**385.1251**	-1.7	$[M+Na]^+$	**361.1283**	0.4	$[M-H]^-$
				363.1434	-1.0	$[M+H]^+$			

[a] UHPLC-UV retention times; base peaks in bold; [b] compared with reference standards; [c] in bold the base peak of HRMS spectrum.

Table 2. Diagnostic Product Ions in Positive and Negative HRMS/MS Spectra of EVOO Phenolic Secoiridoids (3-6) and Related Methyl-Hemiacetals (a-d) and Monohydrate Forms (7-10).

Compound	Positive HRMS/MS (*m/z*, diagnostic ions) [a]	Negative HRMS/MS (*m/z*, diagnostic ions) [a]
3 [b]	$\underline{[M+Na]^+}$: 369.0947 $[M-CH_4O+Na]^+$; 331.0789 $[M-C_4H_6O+Na]^+$; 265.0684 $[EA+Na]^+$; 159.0417 $[C_8H_8O_2+Na]^+$ $\underline{[M+H]^+}$: 347.1111 $[M-CH_4O]^+$; 243.0854 $[EA+H]^+$; 225.0747 $[EA-H_2O+H]^+$; 137.0589 $[C_8H_9O_2]^+$	$\underline{[M-H]^-}$: 345.0971 $[M-CH_4O]^-$; 307.0814 $[M-C_4H_6O]^-$; 275.0915 $[M-C_4H_6O_3]^-$
4 [b]	$\underline{[M+Na]^+}$: 353.1000 $[M-CH_4O+Na]^+$; 315.0842 $[M-C_4H_6O+Na]^+$; 265.0684 $[EA+Na]^+$; 143.0468 $[C_8H_8O+Na]^+$ $\underline{[M+H]^+}$: 331.1164 $[M-CH_4O]^+$; 225.0747 $[EA-H_2O+H]^+$; 121.0641 $[C_8H_9O]^+$	$\underline{[M-H]^-}$: 291.0866 $[M-C_4H_6O]^-$; 259.0967 $[M-C_4H_6O_3]^-$
5	$\underline{[M+Na]^+}$: 207.0631 $[M-C_8H_8O_2+Na]^+$; 159.0417 $[C_8H_8O_2+Na]^+$ $\underline{[M+H]^+}$:303.1217 $[M-H_2O]^+$; 137.0591 $[C_8H_9O_2]^+$	$\underline{[M-H]^-}$: 195.0659 [CH_2CO-3,4-DHPEA]$^-$; 165.0553 [M−H−3,4-DHPEA]$^-$
6	$\underline{[M+Na]^+}$: 207.0631 $[M-C_8H_8O+Na]^+$; 143.0467 $[C_8H_8O+Na]^+$ $\underline{[M+H]^+}$: 287.1269 $[M-H_2O]^+$; 121.0642 $[C_8H_9O]^+$	$\underline{[M-H]^-}$: 179.0710 [CH_2CO-*p*-HPEA]$^-$; 165.0553 [M−H−*p*-HPEA]$^-$
7 [b]	$\underline{[M+Na]^+}$: 401.1200 $[M-H_2O+Na]^+$; 373.1254 $[M-H_2O-CO+Na]^+$	
8 [b]	$\underline{[M+Na]^+}$: 385.1257 $[M-H_2O+Na]^+$; 357.1307 $[M-H_2O-CO+Na]^+$	
9	$\underline{[M+Na]^+}$: 343.1151 $[M-H_2O+Na]^+$	
10	$\underline{[M+Na]^+}$: 327.1201 $[M-H_2O+Na]^+$	
a [b]	$\underline{[M+Na]^+}$: 357.1307 $[M-H_2O+Na]^+$; 343.1149 $[M-CH_4O+Na]^+$	
b (peaks b1, b2) [b]	$\underline{[M+Na]^+}$: 401.1202 $[M-CH_4O+Na]^+$; 373.1253 $[M-CH_4O-CO+Na]^+$	
b (peaks b3-6) [b]	$\underline{[M+Na]^+}$: 401.1200 $[M-CH_4O+Na]^+$	
c [b]	$\underline{[M+Na]^+}$: 327.1198 $[M-CH_4O+Na]^+$	
d (peaks d1, d2) [b]	$\underline{[M+Na]^+}$: 385.1253 $[M-CH_4O+Na]^+$; 357.1304 $[M-CH_4O-CO+Na]^+$	
d (peak d3)	$\underline{[M+Na]^+}$: 385.1252 $[M-CH_4O+Na]^+$	

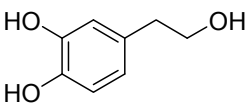
[a] HRMS/MS spectra with molecular formulae and ppm are reported in supplementary material; [b] no differences in fragmentation patterns of isomers; EA: elenolic acid.

Table 3. Concentrations of 3,4-DHPEA and *p*-HPEA Derivatives and their Total Levels in Italian EVOOs, Obtained with Direct (3,4-DHPEA, p-HPEA and Total Moieties) and Indirect (Hydrolized 3,4-DHPEA, p-HPEA and Total) Methods, Calculated as 3,4-DHPEA (1) and/or p-HPEA (2) Equivalents.

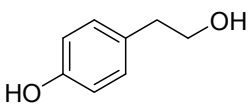
Sample	O1	O2	O3	O4	O5	O6	O7	O8	O9	O10	O11	
	mg/20 g oil (3,4-DHPEA (**1**) equivalents) [a]											r [d]
3,4-DHPEA moieties [e]	2.0	1.4	1.4	1.7	1.1	1.5	1.6	1.5	1.2	1.2	1.9	
Hydrolysed 3,4-DHPEA	*2.5*	*1.5*	*1.6*	*1.7*	*0.9*	*1.7*	*1.3*	*1.5*	*1.2*	*1.1*	*2.3*	0.916**
p-HPEA moieties [f]	2.9	1.7	1.4	2.5	1.4	2.0	1.8	1.6	2.4	1.6	2.6	
Hydrolysed p-HPEA	*2.8*	*1.7*	*1.2*	*2.2*	*1.0*	*1.8*	*1.1*	*1.1*	*1.9*	*1.3*	*2.2*	0.939**
Total moieties [g]	5.0	3.1	2.8	4.1	2.5	3.5	3.4	3.1	3.6	2.8	4.5	
Hydrolysed Total	*5.4*	*3.3*	*2.9*	*3.9*	*1.9*	*3.5*	*2.4*	*2.6*	*3.1*	*2.4*	*4.6*	0.934**
	mg/20 g oil (p-HPEA (**2**) equivalents) [b]											r [d]
3,4-DHPEA moieties [e]	2.7	1.5	1.5	2.0	1.0	1.7	2.0	1.7	1.2	1.1	2.5	
Hydrolysed 3,4-DHPEA	*4.1*	*2.3*	*2.5*	*2.6*	*1.3*	*2.6*	*1.9*	*2.3*	*1.7*	*1.5*	*3.7*	0.916**
p-HPEA moieties [f]	4.0	1.9	1.4	3.2	1.3	2.4	2.0	1.8	3.1	1.7	3.3	
Hydrolysed p-HPEA	*4.6*	*2.3*	*1.7*	*3.5*	*1.5*	*2.8*	*1.6*	*1.5*	*2.9*	*1.9*	*3.5*	0.939**
Total moieties [g]	6.7	3.4	2.9	5.2	2.3	4.1	4.0	3.5	4.3	2.9	5.8	
Hydrolysed Total	*8.6*	*4.6*	*4.2*	*6.2*	*2.7*	*5.4*	*3.6*	*3.8*	*4.7*	*3.4*	*7.2*	0.934**
	mg/20 g oil (3,4-DHPEA (**1**) and p-HPEA (**2**) equivalents) [c]											r [d]
Total moieties [g]	6.0	3.3	2.8	4.8	2.4	3.9	3.7	3.3	4.2	2.9	5.3	
Hydrolysed Total	*7.1*	*3.8*	*3.3*	*5.2*	*2.4*	*4.5*	*3.0*	*3.1*	*4.1*	*3.0*	*5.9*	0.937**

Coefficient of variation (CV) < 11%; ** denote p values < 0.001; [a] 3,4-DHPEA (**1**), *p*-HPEA (**2**) and related secoiridoids were quantified as 3,4-DHPEA (**1**) equivalents; [b] 3,4-DHPEA (**1**), *p*-HPEA (**2**) and related secoiridoids were quantified as *p*-HPEA (**2**) equivalents; [c] 3,4-DHPEA (**1**), *p*-HPEA (**2**) and related secoiridoids were quantified using respective external calibration curves; [d] Pearson's coefficients between indirect and direct methods; [e] sum of 3,4-DHPEA (**1**) and secoiridoids containing 3,4-DHPEA moiety in the MeCN-IOC extracts; [f] sum of *p*-HPEA (**2**) and secoiridoids containing *p*-HPEA moiety in the MeCN-IOC extracts; [g] sum of 3,4-DHPEA (**1**), p-HPEA (**2**) and secoiridoids in the MeCN-IOC extracts.

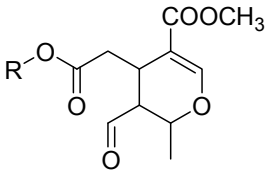
Figure 1.



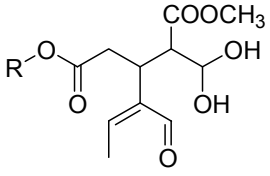
1 3,4-DHPEA (R_1)



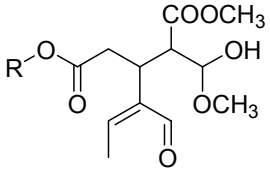
2 *p*-HPEA (R_2)



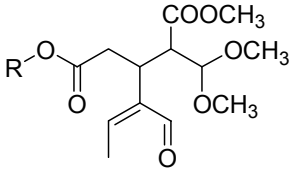
3 3,4-DHPEA-EA (R_1)
4 *p*-HPEA-EA (R_2)



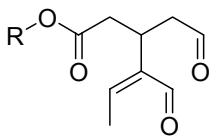
7 monohydrate-**3** (R_1)
8 monohydrate-**4** (R_2)



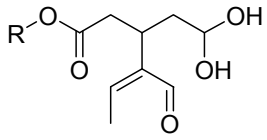
b methyl hemiacetal-**3** (R_1)
d methyl hemiacetal-**4** (R_2)



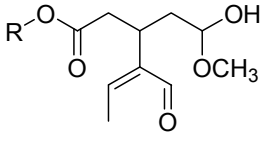
g dimethyl acetal-**3** (R_1)
h dimethyl acetal-**4** (R_2)



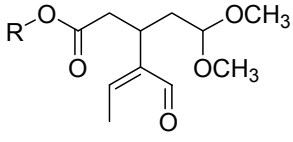
5 3,4-DHPEA-EDA (R_1)
6 *p*-HPEA-EDA (R_2)



9 monohydrate-**5** (R_1)
10 monohydrate-**6** (R_2)



a methyl hemiacetal-**5** (R_1)
c methyl hemiacetal-**6** (R_2)



e dimethyl acetal-**5** (R_1)
f dimethyl acetal-**6** (R_2)

Figure 2.

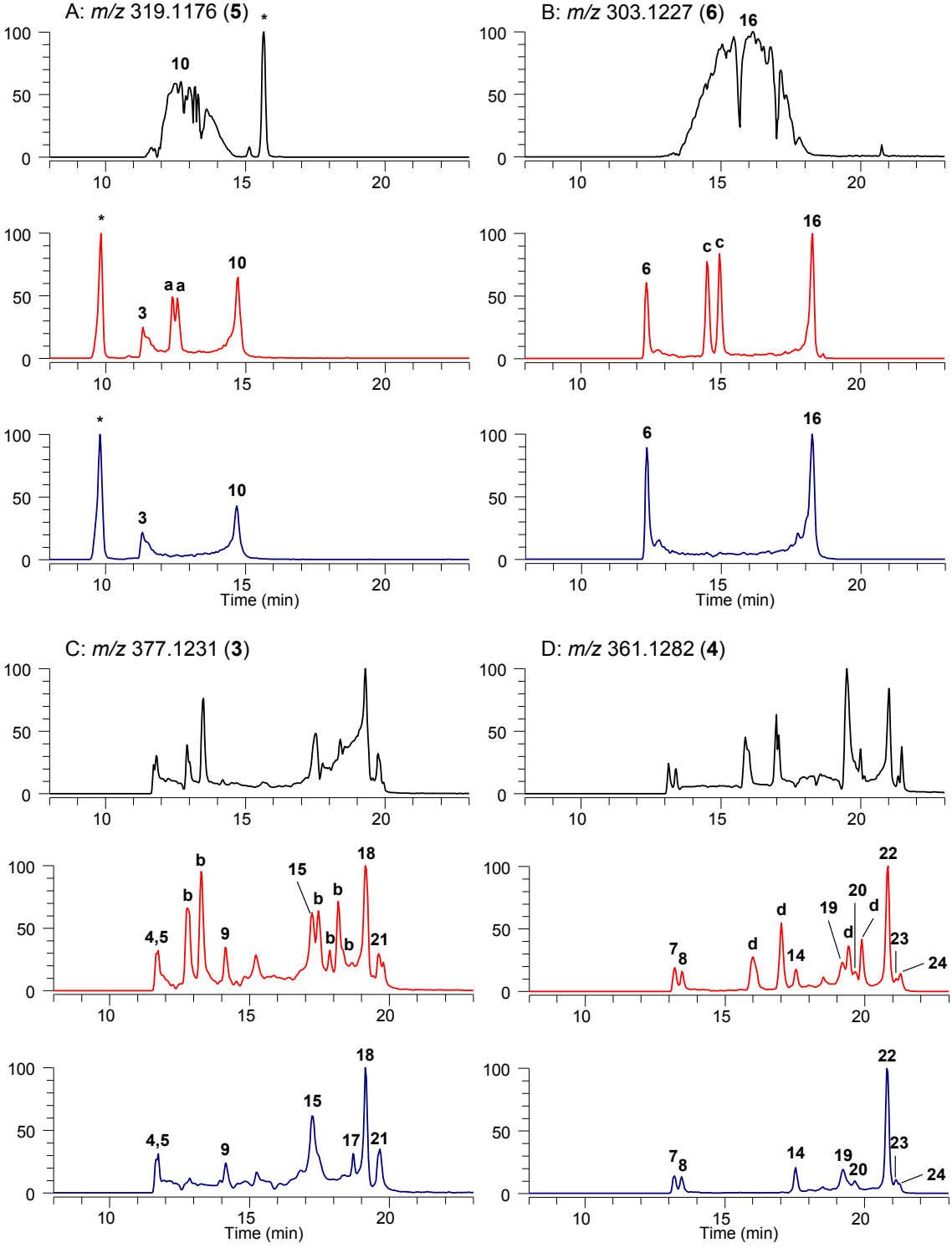
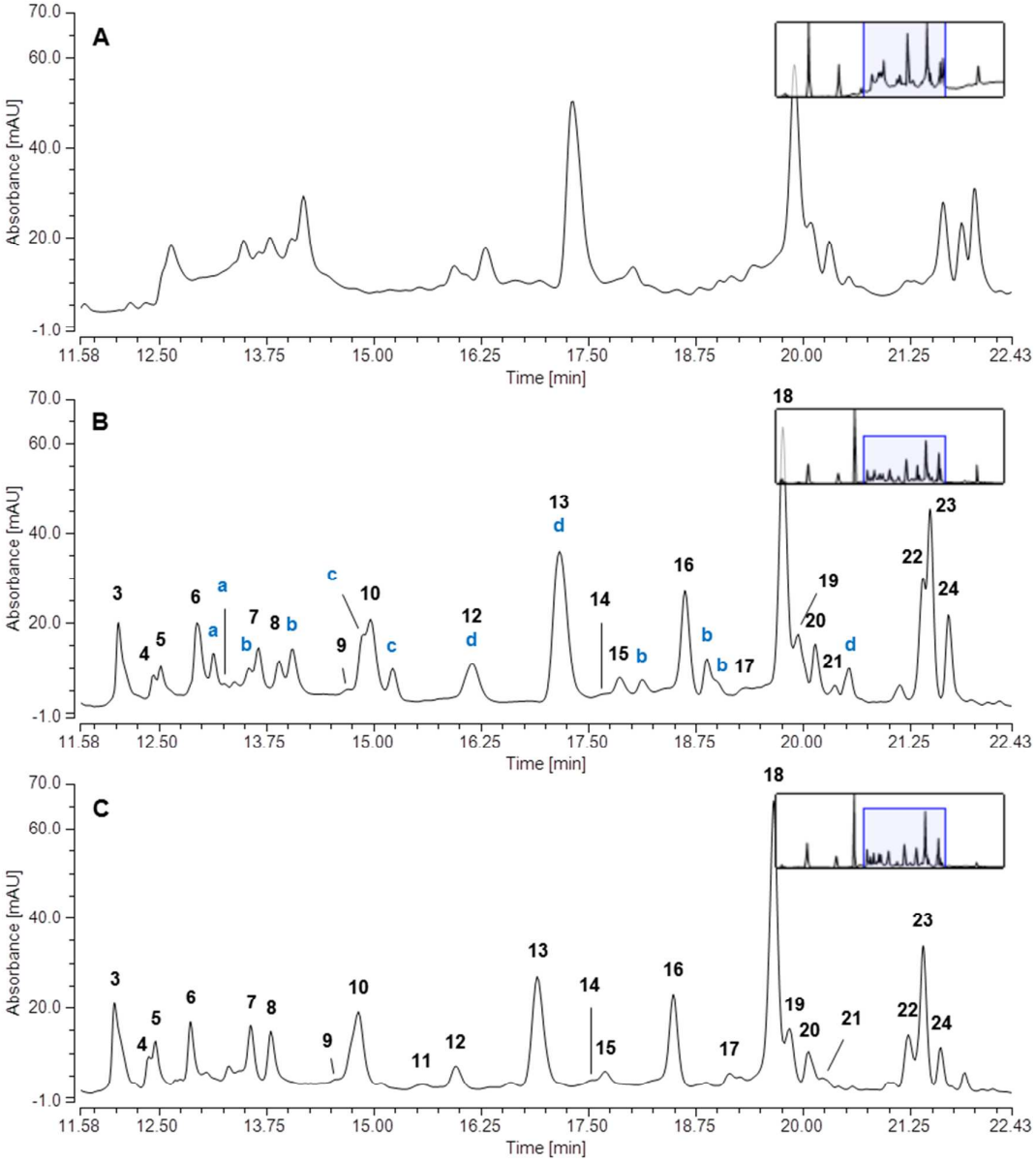

A: m/z 319.1176 (5)
B: m/z 303.1227 (6)
C: m/z 377.1231 (3)
D: m/z 361.1282 (4)
Time (min)
Time (min)
Time (min)
Time (min)

Figure 3.


A
B
C
Absorbance [mAU]
Time [min]
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60.0
40.0
20.0
-1.0
11.58
12.50
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15.00
16.25
17.50
18.75
20.00
21.25
22.43
3
4
5
6
a
7
8
b
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c
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d
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Figure 4.

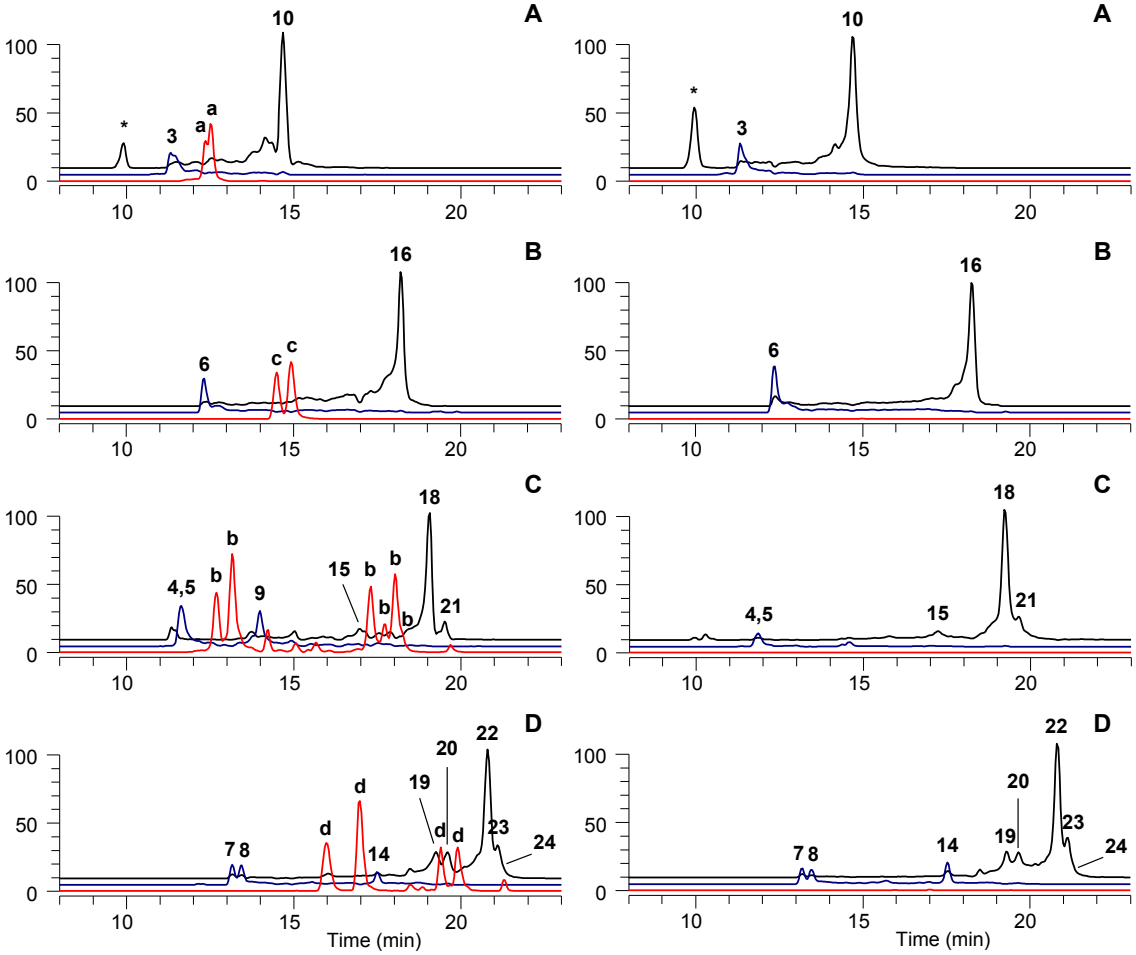
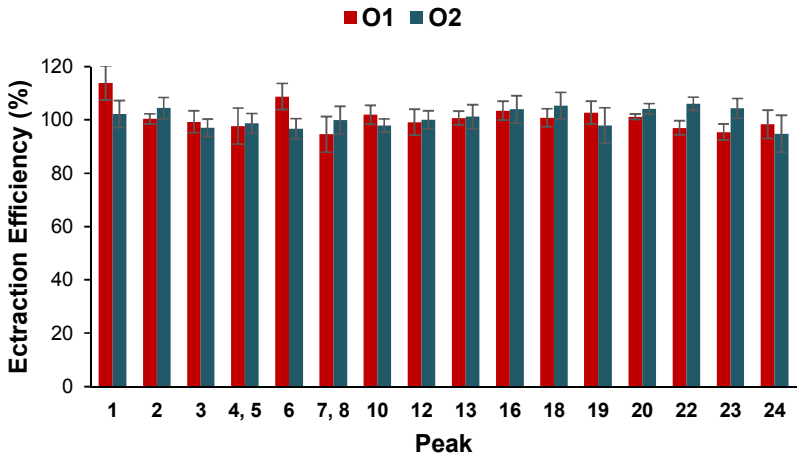


Figure 5.


O1
O2
Ectraction Efficiency (%)
120
100
80
60
40
20
0
1
2
3
4, 5
6
7, 8
10
12
13
16
18
19
20
22
23
24
Peak

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